ULTRASTRUCTURAL AUTORADIOGRAPHIC ANALYSIS OF THE INTERACTIONS OF BOTULINUM NEUROTOXINS WITH NERVE TERMINALS

by

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A dissertation submitted for the degree of Doctor of Philosophy of the University of London and the Diploma of Imperial College

August, 1984
TO MY PARENTS

Carol and Malcolm

with thanks for everything
ABBREVIATIONS

ACh  Acetylcholine
AChe  Acetylcholinesterase
ADP  Adenosine diphosphate
ATP  Adenosine triphosphate
BoNT  Botulinum neurotoxin
BSA  Bovine Serum Albumin
BuTX  Bungarotoxin
ChAT  Choline acetyltransferase
CNS  Central Nervous System
EGTA  Ethyleneglycol-bis-(β-aminoethyl ether)N,N\(^1\)-tetra-acetic acid
epps  End plate potential
ER  Endoplasmic reticulum
FCS  Foetal calf serum
\(^{125}\)I-BoNT  \(^{125}\)I-radio-iodinated botulinum neurotoxin
\(^{125}\)I-BSA  \(^{125}\)I-radio-iodinated bovine serum albumin
K\(_d\)  Dissociation constant
LEMS  Lambert-Eaton myasthenic syndrome
mepps  Miniature end plate potential
M\(_r\)  Relative molecular weight
MLD\(_{50}\)  Mouse lethal dose \(_{50}\)
NANA  N-acetylneuraminic acid
PBS  Phosphate buffered saline
QAE  Diethyl-(2-hydroxypropyl) aminoethyl
Q\(_{10}\)  Temperature coefficient (\(10\)°C)
SDS  Sodium dodecyl sulphate
sub mepp  Subminiature end plate potential
CONTENTS

Abbreviations...........................................................................(iii)
Table of Contents......................................................................(iv)
List of Tables...........................................................................(xiii)
List of Figures..........................................................................(xiv)
Acknowledgments......................................................................(xx)
Abstract...................................................................................(xxiii)

Chapter 1  General Introduction ...........................................1
1.1 The neuromuscular junction .............................................2
1.2 Neurotransmission ........................................................9
1.3 Neurotransmitter release .................................................9
1.3.1 The vesicular hypothesis ..........................................10
1.3.2 Non-vesicular release of neurotransmitter .....................12
1.4 Botulism .......................................................................16
1.4.1 Purification and radio-iodination of the neurotoxic
     moiety of botulinum toxin .........................................18
1.4.2 Structure and function of the neurotoxin molecule ............19
1.4.3 Target sites for botulinum neurotoxin ............................22
1.4.4 Pharmacological action of botulinum neurotoxin ..........25
1.5 Endocytosis .....................................................................28
1.6 Autoradiography ..........................................................38
1.7 The present study .......................................................44

Chapter II  Materials and Methods ......................................45
A. The neuromuscular junction .............................................46
   I. In vitro studies .........................................................46
2.1 Dissections .....................................................................46
2.2 Incubations ...............................................................46
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>Fixation of tissue and location of endplate regions</td>
<td>47</td>
</tr>
<tr>
<td>2.4</td>
<td>Dehydration, infiltration and embedding</td>
<td>50</td>
</tr>
<tr>
<td>2.5</td>
<td>Light-microscope autoradiography</td>
<td>51</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Sectioning</td>
<td>51</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Dipping</td>
<td>53</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Developing</td>
<td>53</td>
</tr>
<tr>
<td>2.6</td>
<td>Electron microscope autoradiography</td>
<td>54</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Sectioning</td>
<td>54</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Dipping</td>
<td>54</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Exposure</td>
<td>56</td>
</tr>
<tr>
<td>2.6.4</td>
<td>Developing</td>
<td>56</td>
</tr>
<tr>
<td>2.6.5</td>
<td>Stripping</td>
<td>56</td>
</tr>
<tr>
<td>2.6.6</td>
<td>Staining</td>
<td>57</td>
</tr>
<tr>
<td>2.6.7</td>
<td>Viewing in the electron microscope</td>
<td>57</td>
</tr>
<tr>
<td>II.</td>
<td>In vivo studies</td>
<td>60</td>
</tr>
<tr>
<td>2.7</td>
<td>Tissue preparation</td>
<td>60</td>
</tr>
<tr>
<td>B.</td>
<td>The central nervous system</td>
<td>60</td>
</tr>
<tr>
<td>2.8</td>
<td>Cryostat - sectioning of brain tissue</td>
<td>60</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Perfusion of the animal</td>
<td>60</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Dissection, mounting and freezing</td>
<td>60</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Cryostat sectioning, mounting and hardening</td>
<td>61</td>
</tr>
<tr>
<td>2.8.4</td>
<td>Preparation of slides for use of $^{3}$H-ultrofim, exposure and development</td>
<td>61</td>
</tr>
<tr>
<td>2.8.4.1</td>
<td>Exposure</td>
<td>62</td>
</tr>
<tr>
<td>2.8.4.2</td>
<td>Development</td>
<td>62</td>
</tr>
<tr>
<td>2.8.4.3</td>
<td>Histological staining of brain sections</td>
<td>62</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>2.9</td>
<td>Electron-microscope autoradiography using unfixed brain tissue</td>
<td>62</td>
</tr>
<tr>
<td>2.9.1</td>
<td>Dissection of the cerebellum and preparation of cerebellar slices</td>
<td>62</td>
</tr>
<tr>
<td>2.9.2</td>
<td>Incubations</td>
<td>67</td>
</tr>
<tr>
<td>2.9.3</td>
<td>Fixation and osmication</td>
<td>67</td>
</tr>
<tr>
<td>2.9.4</td>
<td>Electron-microscope autoradiography</td>
<td>67</td>
</tr>
<tr>
<td>C.</td>
<td>Synaptosomes and cell lines</td>
<td>68</td>
</tr>
<tr>
<td>2.10.1</td>
<td>Preparation</td>
<td>68</td>
</tr>
<tr>
<td>2.10.2</td>
<td>Processing for electron-microscope autoradiography</td>
<td>69</td>
</tr>
<tr>
<td>2.11</td>
<td>Cell lines</td>
<td>69</td>
</tr>
<tr>
<td>2.11.1</td>
<td>Cell lines tested for ability to bind $^{125}$I-BoNT type A</td>
<td>69</td>
</tr>
<tr>
<td>2.11.2</td>
<td>Preparation of cells for electron-microscope autoradiography</td>
<td>69</td>
</tr>
<tr>
<td>D.</td>
<td>$^{125}$I-Radiolabelling of proteins</td>
<td>70</td>
</tr>
<tr>
<td>2.12.1</td>
<td>$^{125}$I-Iodination of BoNT type A for autoradiographic studies</td>
<td>70</td>
</tr>
<tr>
<td>2.12.2</td>
<td>$^{125}$I-Iodination of BoNT type B for autoradiographic studies</td>
<td>71</td>
</tr>
<tr>
<td>2.12.3</td>
<td>$^{125}$I-Iodination of bovine serum albumin for efficiency determinations in autoradiography</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Chapter III</td>
<td>Localisation of acceptor sites for BoNT types A and B at the murine neuromuscular junction</td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>75</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and methods</td>
<td>78</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Studies at the light microscope level</td>
<td>78</td>
</tr>
</tbody>
</table>
3.2.2 Studies at the ultrastructural level .........................79
  3.2.2.1 Interaction of $^{125}$I-BoNT types A and B
      with the neuromuscular junction ......................79
  3.2.2.2 Effect of nerve stimulation on the distribution of
      toxin molecules at the murine motor nerve terminal ...80
  3.2.2.3 Interaction of BoNT types A and B at the
      motor nerve terminal ....................................83

3.3. Results ....................................................... 84
  Part A Localisation of $^{125}$I-BoNT type A in a murine
      nerve-muscle preparation ................................84
    3.3.1 In vitro and in vivo localisation of $^{125}$I-BoNT type A .. ....84
    3.3.2 In vitro and in vivo localisation of $^{125}$I-BoNT type A
        at the ultrastructural level ..............................87
    3.3.3 Localisation of the acceptor sites for $^{125}$I-BoNT type A
        at the motor nerve terminal ............................95
    3.3.4 In vitro localisation of $^{125}$I-BoNT type A in a
        nerve-muscle preparation from newborn rats ............98
    3.3.5 Localisation of $^{125}$I-BoNT type A in a preparation
        stimulated electrically until transmission was blocked ......104
  Part B Localisation of $^{125}$I-BoNT type B in a murine
      nerve-muscle preparation ..................................107
    3.3.6 In vitro localisation of $^{125}$I-BoNT type B under
        physiological conditions and in the presence of metabolic
        inhibitors ..................................................107
    Part C Interaction of types A and B BoNT ..................110
    3.3.7 Effect of BoNT type B on the binding of $^{125}$I-BoNT type A
        at the murine motor nerve terminal ....................110
3.3.8 Effect of BoNT type A on the binding of $^{125}$I-BoNT type B at the murine motor nerve terminal

3.4 Discussion

3.4.1 Interaction of $^{125}$I-BoNT type A with the murine neuromuscular junction: a light microscope autoradiographic study

3.4.2 $^{125}$I-BoNT at the motor nerve terminal: an ultrastructural study of binding and internalisation

3.4.3 Possible mechanism of toxin uptake

3.4.4 Retrograde intra-axonal transport of $^{125}$I-BoNT type A in vivo

3.4.5 Interaction of BoNT types A and B at the murine motor nerve terminal

Chapter IV Characteristics of binding and internalisation of $^{125}$I-BoNT type A and quantitation of acceptor sites for $^{125}$I-BoNT types A and B at the murine neuromuscular junction

4.1 Introduction

4.2 Materials and methods

4.2.1 General methodology for studying BoNT binding and internalisation

4.2.2 Development and analysis of results

4.2.3 Determination of percent efficiency in electron-microscope autoradiography using $^{125}$I

4.2.4 Resolution in electron-microscope autoradiography using $^{125}$I

4.2.5 Quantitation of $^{125}$I-BoNT types A and B binding sites at the murine motor nerve terminal
Chapter V  Nature of the botulinum neurotoxin binding and internalisation steps at the murine motor nerve terminal ..................................................189

5.1  Introduction ...............................................190

5.2  Materials and Methods ......................................196

5.3  Results ..................................................197

   Part A: Binding ............................................197

   5.3.1 Effect of the large subunit of BoNT type A on the binding of intact $^{125}$I-BoNT type A ....................197

   5.3.2 Effect of neuraminidase on the binding and internalisation of $^{125}$I-BoNT at the motor nerve terminal .........198

   5.3.3 Effect of tetanus toxin on the binding and internalisation of $^{125}$I-BoNT ........................................205

   5.3.4 Binding of $^{125}$I-BoNT to diaphragms from mice treated with IgG from myasthenic (Lambert-Eaton syndrome) patients ..........210

   Part B: Internalisation ....................................214

   5.3.5 Effect of the absence of Ca$^{++}$ on the internalisation and binding of $^{125}$I-BoNT at the motor nerve terminal ...............214

   5.3.6 Effect of lysosomotropic agents on the internalisation of $^{125}$I-BoNT at the murine nerve terminal .....219

   5.3.7 Effect of cytochalasin B on the uptake of $^{125}$I-BoNT into the nerve terminal ..................................224

   5.3.8 Effect of temperature and antitoxin antibodies on the internalisation of $^{125}$I-BoNT at motor nerve terminals ..........227

5.4  Discussion .................................................231

   5.4.1 The binding step .......................................231

   5.4.2 The internalisation step ................................238
4.3. Results .....................................................142

Part A: Characterisation of the binding and internalisation of \(^{125}\text{I-BoNT type A at the murine neuromuscular junction and concentration dependence of binding of }^{125}\text{I-BoNT type B} \) ...............................................142

4.3.1 Concentration dependence of \(^{125}\text{I-BoNT type A binding and internalisation} \) .........................................142

4.3.2 Time course of binding and internalisation of \(^{125}\text{I-BoNT type A} \) ............................................146

4.3.3 Effect of temperature on the binding and internalisation of \(^{125}\text{I-BoNT at the motor nerve terminal} \) ....................152

4.3.4 Concentration dependence of binding of \(^{125}\text{I-BoNT type B in the presence of sodium azide} \) .....................154

Part B: Density of acceptor sites for \(^{125}\text{I-BoNT types A and B} \) ................................................................157

4.3.5 Calibration of the autoradiographic technique: Determination of the efficiency and resolution of electron-microscope autoradiography using \(^{125}\text{I} \) ...............................................157

4.3.6 Density of \(^{125}\text{I-BoNT type A binding sites} \) .................................................169

4.3.7 Quantitation of \(^{125}\text{I-BoNT type B binding sites} \) ..................................................176

4.4 Discussion ..................................................176

4.4.1 Concentration dependence and time course of toxin binding to the motor nerve terminal ........................................179

4.4.2 Concentration dependence and time course of the internalisation step .........................................................181

4.4.3 Effects of temperature on binding and internalisation .......185

4.4.4 Density of acceptor sites for \(^{125}\text{I-BoNT types A and B} \) ........185
Chapter VI Interaction of $^{125}$I-BoNT with nerve terminals in the central and peripheral nervous systems of different species ........................................249

6.1 Introduction ........................................................................250

6.2 Materials and Methods ..................................................253

Part A: The Central Nervous System .......................................253

6.2.1 Acceptors for BoNT types A and B in rat brain ..............253

6.2.1.1 Effect of paraformaldehyde on the binding of $^{125}$I-BoNT type A to synaptosomal membranes ...............253

6.2.1.2 Binding of $^{125}$I-BoNT types A and B to cryostat sections of rat brain .................................................254

6.2.2 Ultrastructural localisation of $^{125}$I-BoNT type A in rat cerebellum in vitro .........................................................255

6.2.3 Binding of $^{125}$I-BoNT type A to rat cerebrocortical synaptosomes in the presence and absence of sodium azide ...256

Part B: The Peripheral Nervous System ....................................257

6.2.4 Cholinergic nerve terminals in non-mammalian tissue:
binding of $^{125}$I-BoNT type A to electroplaques from Electrophorus electricus and to the frog neuromuscular junction ................................................................. 257

6.2.5 Adrenergic, muscarinic and inhibitory nonadrenergic-noncholinergic nerve terminals: binding of $^{125}$I-BoNT BoNT type A to nerve endings in the mouse vas deferens and ileum .................................................................258

Part C: Neuroblastoma and Phoeochromocytoma (PC12)
cell lines ..............................................................................259

6.2.6 Binding of $^{125}$I-BoNT type A to neuroblastoma and to phoeochromocytoma (PC12) cell lines ..........................259
6.3 Results ........................................................................................................................................260

6.3.1 Acceptors for $^{125}$I-BoNT in the central nervous system

of the rat ........................................................................................................................................260

6.3.1.1 Effect of tissue fixation on the binding of

$^{125}$I-BoNT to brain synaptosomal membranes ..................260

6.3.1.2 Binding of $^{125}$I-BoNT types A and B to different

areas of the rat forebrain and cerebellum .........................260

6.3.2 Ultrastructural localisation of $^{125}$I-BoNT type A in

slices of rat cerebellum ..................................................269

6.3.3 Binding of $^{125}$I-BoNT type A to rat cerebrocortical

synaptosomes ........................................................................276

Part B: The Peripheral Nervous System .........................276

6.3.4 Cholinergic nerve terminals in non-mammalian tissue ...276

6.3.5 Adrenergic, cholinergic muscarinic and nonadrenergic-

noncholinergic nerve terminals ........................................281

6.3.6 Binding of $^{125}$I-BoNT type A to neuroblastoma and

pheochromocytoma (PC12) cell lines .................................288

6.4 Discussion ....................................................................................................................................289

6.4.1 BoNT as a marker for cholinergic nerve terminals .........289

6.4.2 Why is BoNT ineffective at certain synapses? ..........295

Chapter VII Conclusions and Perspectives ......................297

7.1 The site of action of botulinum neurotoxin ..................298

7.2 The mechanism of action of botulinum neurotoxin ........302

7.2.1 The binding step .........................................................304

7.2.2 The internalisation step .............................................307

7.2.3 The lytic step ..........................................................320

References ....................................................................................................................................325
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Distribution of silver grains at the motor nerve terminal under different conditions</td>
<td>91</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Interactions of types A and B botulinum neurotoxins</td>
<td>119</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Concentration dependence of binding of $^{125}\text{I}-\text{BoNT}$ type A</td>
<td>143</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Time course of binding and internalisation of $^{125}\text{I-BoNT}$ type A</td>
<td>147</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Concentration dependence of binding of $^{125}\text{I-BoNT}$ type B in the presence of sodium azide</td>
<td>155</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Density of acceptors obtained with different concentrations of $^{125}\text{I-BoNT}$ type A</td>
<td>174</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Quantitation of $^{125}\text{I-BoNT}$ type B acceptor sites at the murine motor nerve terminal</td>
<td>178</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Effects of various agents on the binding and internalisation of $^{125}\text{I-BoNT}$ (type A) at the murine nerve terminal</td>
<td>199</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Effect of various agents on the internalisation of $^{125}\text{I-BoNT}$ at the nerve terminal</td>
<td>215</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Effect of treatment with paraformaldehyde on the acceptors for $^{125}\text{I-BoNT}$ type A on synaptosomal membranes</td>
<td>261</td>
</tr>
<tr>
<td>Fig. 1.1</td>
<td>Diagrammatic representation of the mammalian neuromuscular junction</td>
<td>3</td>
</tr>
<tr>
<td>Fig. 1.2</td>
<td>Motor nerves and the neuromuscular junction</td>
<td>4</td>
</tr>
<tr>
<td>Fig. 1.3</td>
<td>Structure of the synaptic membrane</td>
<td>8</td>
</tr>
<tr>
<td>Fig. 1.4</td>
<td>Generalised structure of botulinum toxin</td>
<td>21</td>
</tr>
<tr>
<td>Fig. 1.5</td>
<td>Probable pathways of nicking and activation of botulinum neurotoxin</td>
<td>23</td>
</tr>
<tr>
<td>Fig. 1.6</td>
<td>Schematic diagram of the three types of endocytosis</td>
<td>29</td>
</tr>
<tr>
<td>Fig. 1.7</td>
<td>Acceptor-mediated endocytosis</td>
<td>31</td>
</tr>
<tr>
<td>Fig. 2.1</td>
<td>Preparation of sections for light-microscope autoradiography</td>
<td>52</td>
</tr>
<tr>
<td>Fig. 2.2</td>
<td>Preparation of sections for electron-microscope autoradiography</td>
<td>55</td>
</tr>
<tr>
<td>Fig. 2.3</td>
<td>Procedure for electron-microscope autoradiography</td>
<td>58</td>
</tr>
<tr>
<td>Fig. 2.4</td>
<td>Thickness measurement using folds in the tissue sections</td>
<td>59</td>
</tr>
<tr>
<td>Fig. 2.5</td>
<td>Cutter used for preparation of cerebellar slices</td>
<td>63</td>
</tr>
<tr>
<td>Fig. 2.6</td>
<td>Guide for cutting cerebellar slices</td>
<td>65</td>
</tr>
<tr>
<td>Fig. 2.7</td>
<td>Preparation of surface slices from cerebellar Vermis</td>
<td>66</td>
</tr>
<tr>
<td>Fig. 2.8</td>
<td>Separation of $^{125}$I-BSA from free $^{125}$I-iodine by gel filtration</td>
<td>73</td>
</tr>
<tr>
<td>Fig. 3.1</td>
<td>Procedure for dissection of a murine phrenic nerve-hemidiaphragm preparation</td>
<td>80</td>
</tr>
<tr>
<td>Fig. 3.2</td>
<td>Set-up for electrical stimulation of a nerve-muscle preparation</td>
<td>82</td>
</tr>
<tr>
<td>Fig. 3.3</td>
<td>Localisation <em>in vitro</em> of $^{125}$I-BoNT type A target sites in mouse diaphragm muscle by light-microscope autoradiography</td>
<td>85</td>
</tr>
</tbody>
</table>
Fig. 3.4  Localisation of $^{125}$I-BoNT type A target sites in tissue stained to detect acetylcholinesterase at the neuromuscular junction ........................................ 86

Fig. 3.5  Localisation in vivo of the target sites for $^{125}$I-BoNT type A in mouse diaphragm muscle by light-microscope autoradiography ........................................ 88

Fig. 3.6  Localisation of $^{125}$I-BoNT type A target sites in vivo in tissue stained to detect acetylcholinesterase at the neuromuscular junction ............. 89

Fig. 3.7  Interaction of $^{125}$I-BoNT type A with the murine motor nerve terminal ........................................ 90

Fig. 3.8  Binding of $^{125}$I-BoNT type A to the membrane of intracellular vesicular structures ........................................ 92

Fig. 3.9  Association of $^{125}$I-BoNT type A with the plasma membrane of unmyelinated axons ........................................ 92

Fig. 3.10  Interaction of $^{125}$I-BoNT type A with motor nerve terminals after in vivo administration of radiolabelled toxin to mice ........................................ 93

Fig. 3.11  Localisation of acceptor sites for $^{125}$I-BoNT type A at the murine motor nerve terminal .......................... 96

Fig. 3.12  Saturable acceptors for $^{125}$I-BoNT type A on the membrane of unmyelinated axons ........................................ 99

Fig. 3.13  Binding of $^{125}$I-BoNT type A to the murine motor nerve terminal in the presence of dinitrophenol .......... 100

Fig. 3.14  Interaction of $^{125}$I-BoNT type A with developing motor nerve terminals in newborn rats ..................... 101

Fig. 3.15  Localisation of $^{125}$I-BoNT in a preparation stimulated electrically until transmission was blocked ........................ 105
<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.16</td>
<td>Interaction of $^{125}\text{I-BoNT type B}$ with the murine motor nerve terminal</td>
<td>108</td>
</tr>
<tr>
<td>3.17</td>
<td>Interaction of $^{125}\text{I-BoNT type B}$ with unmyelinated axons</td>
<td>111</td>
</tr>
<tr>
<td>3.18</td>
<td>Retrograde intra-axonal transport of $^{125}\text{I-BoNT type B}$</td>
<td>112</td>
</tr>
<tr>
<td>3.19</td>
<td>Acceptor sites for $^{125}\text{I-BoNT type B}$ at the murine motor nerve terminal</td>
<td>113</td>
</tr>
<tr>
<td>3.20</td>
<td>Acceptor sites for $^{125}\text{I-BoNT type B}$ on the plasma membrane of unmyelinated axons</td>
<td>115</td>
</tr>
<tr>
<td>3.21</td>
<td>Interaction of $^{125}\text{I-BoNT type A}$ with motor nerve terminals pretreated with native $\text{BoNT type B}$</td>
<td>116</td>
</tr>
<tr>
<td>3.22</td>
<td>Interaction of $^{125}\text{I-BoNT type A}$ with motor nerve terminals pretreated with native $\text{BoNT type A}$</td>
<td>120</td>
</tr>
<tr>
<td>4.1</td>
<td>Concentration dependence of binding of $^{125}\text{I-BoNT type A}$ in the presence and absence of Na azide</td>
<td>145</td>
</tr>
<tr>
<td>4.2</td>
<td>Time course of binding of $^{125}\text{I-BoNT type A}$ in the presence and absence of Na azide</td>
<td>150</td>
</tr>
<tr>
<td>4.3</td>
<td>Effect of temperature on the binding and internalisation of $^{125}\text{I-BoNT type A}$ at the motor nerve terminal</td>
<td>153</td>
</tr>
<tr>
<td>4.4</td>
<td>Concentration dependence of binding of $^{125}\text{I-BoNT type B}$ in the presence of Na azide</td>
<td>156</td>
</tr>
<tr>
<td>4.5</td>
<td>Grain production as a function of exposure time</td>
<td>158</td>
</tr>
<tr>
<td>4.6</td>
<td>Grain density as a function of exposure time and radiation dose</td>
<td>159</td>
</tr>
<tr>
<td>4.7</td>
<td>Efficiency of the autoradiographic procedure and emulsion developer combination used as a function of exposure time and radiation dose</td>
<td>163</td>
</tr>
</tbody>
</table>
Fig. 4.8 Histogram of experimental grain density distribution around a radioactive line source: determination of the HD value .............................................167

Fig. 4.9 Time course of grain development .............................................170

Fig. 4.10 Grain density on the nerve terminal membrane as a function of exposure time .............................................172

Fig. 5.1 Effect of the large subunit of BoNT type A on the binding and internalisation of intact $^{125}$I-BoNT ......201

Fig. 5.2 Effect of neuraminidase on the binding and internalisation of $^{125}$I-BoNT at the murine motor nerve terminal: a qualitative analysis .........................203

Fig. 5.3 Effect of neuraminidase on the binding and internalisation of $^{125}$I-BoNT type A at the motor nerve terminal: a quantitative analysis .........................206

Fig. 5.4 Effect of tetanus toxin on the binding and internalisation of $^{125}$I-BoNT .................................208

Fig. 5.5 Binding of $^{125}$I-BoNT to diaphragms from myasthenic (Lambert-Eaton syndrome passively transferred) mice: qualitative analysis by light-microscope autoradiography ..........................................................212

Fig. 5.6 Binding and internalisation of $^{125}$I-BoNT type A at the motor nerve terminal of mice with myasthenic syndrome (passively transferred): a quantitative, ultrastructural study ..........................................................213

Fig. 5.7 Effect of Ca$^{++}$ deprivation on the internalisation and binding of $^{125}$I-BoNT type A at the motor nerve terminal ..........................................................217
Fig. 5.8  Effect of lysosomotropic agents on the internalisation of $^{125}$I-BoNT at the murine motor nerve terminal ........................................... 220

Fig. 5.9  Retrograde intra-axonal transport in the presence of lysosomotropic agents ........................................... 225

Fig. 5.10 Effect of cytochalasin B on the internalisation of $^{125}$I-BoNT at the motor nerve terminal ......................... 226

Fig. 5.11 Effect of temperature and antitoxin antibodies on the internalisation of $^{125}$I-BoNT at the motor nerve terminal ........................................... 228

Fig. 6.1  Specific binding of $^{125}$I-BoNT to cryostat sections of rat forebrain: caudate nucleus, putamen and neocortex ........................................... 263

Fig. 6.2  Specific binding of $^{125}$I-BoNT type A in the rat hippocampal formation ......................... 265

Fig. 6.3  Binding of $^{125}$I-BoNT types A and B to areas of the rat cerebellum ........................................... 270

Fig. 6.4  The neurons of the cerebellar cortex ........................................... 273

Fig. 6.5  Localisation of $^{125}$I-BoNT type A binding in the cerebellum ........................................... 274

Fig. 6.6  Interaction of $^{125}$I-BoNT type A with rat brain synaptosomes ........................................... 277

Fig. 6.7  Interaction of $^{125}$I-BoNT type A with synaptosomes in the presence of Na Azide ......................... 278

Fig. 6.8  Interaction of $^{125}$I-BoNT type A with the electric organ of Electrophorus electricus ........................................... 280

Fig. 6.9  Binding and internalisation of $^{125}$I-BoNT type A at the frog neuromuscular junction ......................... 282
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 6.10</td>
<td>Binding of $^{125}$I-BoNT type A to adrenergic nerve terminals in the vas deferens of the mouse</td>
<td>284</td>
</tr>
<tr>
<td>Fig. 6.11</td>
<td>Interaction of $^{125}$I-BoNT type A with nerve endings in the mouse ileum</td>
<td>285</td>
</tr>
<tr>
<td>Fig. 7.1</td>
<td>Structures involved in the process of acceptor-mediated endocytosis</td>
<td>308</td>
</tr>
<tr>
<td>Fig. 7.2</td>
<td>Model for the translocation of secretory proteins across the membrane of the endoplasmic reticulum</td>
<td>315</td>
</tr>
<tr>
<td>Fig. 7.3</td>
<td>Hypothetical scheme of the transfer of diphtheria toxin fragment A across membranes</td>
<td>316</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Oliver Dolly for making it possible for me to carry out this research, for his guidance and for many helpful discussions; Professor Barnard for allowing me to join his department; Richard Williams for his constant friendship, support and encouragement throughout my years at Imperial college and for providing such excellent BoNT preparations; John Cavanagh for teaching me all I know about electron-microscope autoradiography, for all his help and endless patience, for his encouragement and friendship and for the beautiful artwork he did for this thesis, all of which would be so hard to repay; Steve Bunn for his kindness in helping me with the light microscope work and unravelling some of the 'mysteries' of the brain; Liz Ashton for her patience and excellence in typing this thesis; Dave Green for all those BoNT injections ('); Glyn Millhouse for his helpful advice on the photography; Josephine Lai for putting up with such an erratic flatmate and for her friendship; Adrian Black for more than I could possibly express or begin to repay; my family for always being there when I needed them and for their loving support and encouragement; and finally all those wonderful people (Liz Allen, Nandita Ray, Luke Newman, Colin Butter, Bill Randall, Tony Ashton, Annegret Pelchen-Matthews, Anne Maisey, Simon Lande... see Fig. I) who helped make my time at Imperial so special.
Fig. 1
Botulinum neurotoxin (BoNT), which occurs in 7 immunologically different forms produced by toxigenic strains of Clostridium botulinum, is responsible for the neuroparalytic condition known as botulism. BoNT is the most toxic substance known and acts by specifically and irreversibly inhibiting the release of acetylcholine from peripheral nerves; thus it is an invaluable probe for investigating the molecular mechanisms of neurotransmitter release. In this study, the techniques of light- and electron- microscope autoradiography were used to visualise the interaction of types A and B BoNT, radiolabelled to high specific radioactivity (450-3000 Ci/mmol), with the mammalian neuromuscular junction (chapter 3). In both cases, silver grains were observed both on and within the nerve terminal membrane but Schwann cells, muscle cells, myelin, blood vessels and collagen were totally devoid of grains. The interaction of $^{125}$I-BoNT types A and B with the nerve terminal was saturable as it could be prevented by excess native BoNT. The observed internalisation could be inhibited by azide, dinitrophenol or lowering the temperature to 4°C; it is thus energy- and temperature- dependent. Under these conditions, specific and saturable acceptors for both toxin types were demonstrated on all unmyelinated areas of the nerve terminal; the average density of these acceptors was found to be 150 and 630/$\mu$m$^2$ membrane for type A and B BoNT, respectively (chapter 4). The majority of sites for each toxin type were found to be distinct, although saturating concentrations of type A BoNT could inhibit the binding of type B by 25%.

Conditions were devised for studying concentration dependence, time course, and temperature sensitivity of both the binding and internalisation of radiolabelled toxin (Chapter 4). BoNT type A was shown to
interact with the nerve membrane through its larger subunit, and the nature of the binding site was investigated using agents such as neuraminidase, tetanus toxin and myasthenic syndrome antiserum. From the observed effects of compounds known to interfere with the process of acceptor-mediated endocytosis, e.g. lysosomotropic agents, cytochalasin B, and antitoxin antibodies, a model for the uptake of $^{125}$I-BoNT type A into the nerve terminal was proposed (chapter 5).

Finally, the interaction of $^{125}$I-BoNT type A with different nerve endings in the peripheral and central nervous systems was studied in an attempt to explain the lack of toxicity of BoNT at some of these synapses (chapter 6). Possible steps in the mechanism of action of the toxin at cholinergic and other synapses are discussed (chapter 7).
1.1 THE NEUROMUSCULAR JUNCTION.

Much of the detailed knowledge of the process of synaptic transmission has been acquired through studies of the vertebrate neuromuscular junction, in which acetylcholine (ACh) is the excitatory neurotransmitter (Fatt and Katz, 1952; del Castillo and Katz, 1954; Heuser and Reese, 1973). Skeletal muscle is innervated by motor neurons whose cell bodies are situated in the ventral horn of the spinal cord and axons traverse the ventral roots to terminate in motor end-plates on the muscle fibres. The aggregate of fibres (usually several hundred) supplied by a single neuron is called a 'motor unit'. Each peripheral nerve fibre is surrounded by a myelin sheath (see Figs. 1.1, 1.2), interrupted, at regular intervals, by the nodes of Ranvier. The myelin sheath is composed of concentric layers derived from the plasma membrane of myelin-forming Schwann cells (Geren, 1954) and is surrounded by the neurolemmal sheath, which consists of the nucleated cytoplasmic portion of the Schwann cells (Figs. 1.1, 1.2). Outside the neurolemmal sheath is a basal lamina which is enclosed by a delicate endoneurial sheath of connective tissue. At the motor end-plate, the endoneurium is continuous with the endomysium (or connective tissue of the muscle fibre), forming a tent over the myoneurial junction.

In the region of innervation, the surface of the muscle fibre contains a specialised zone of modified sarcolemma called the soleplate, characterised by extensive infolding of the plasma membrane (Figs. 1.1, 1.2). As the nerve fibre approaches this specialised region, it loses its myelin sheath (at the terminal node of Ranvier) (Figs. 1.1, 1.2). The terminal branches of the motor nerve are surrounded loosely by the neurolemmal sheath and the axonal membrane is, therefore, exposed to the external environment.

The unmyelinated nerve branchlet divides extensively and the endings, or synaptic boutons, make contact with the muscle fibre
As the motor axon (a) approaches the muscle fibre, terminal branches emerge from the myelin sheath (m) at the final node of Ranvier (r) and expand to form synaptic boutons (sb) with crowds of synaptic vesicles (v), 40-50nm in diameter, numerous mitochondria (mi) and tubules of endoplasmic reticulum. The nerve terminals occupy a depression, the synaptic gutter, indenting the surface of the muscle fibre (mf). In this region, the sarcolemma, or muscle plasma membrane, is thrown into folds, the post-junctional folds (pf) which constitute the secondary synaptic cleft. At the crests of the folds (where ACh receptors are clustered) the sarcolemma is separated from the membrane of the nerve terminal by the primary synaptic cleft (sc), about 20-60nm wide. Each terminal region is covered by a Schwann cell, the myelinated axon is enclosed by the endoneurium (e), the muscle cell by the endomysium (en) and the terminal axon branches by the neurolemmal sheath (n) (adapted from Zacks, 1969).
Fig. 1.2 Motor nerves and the neuromuscular junction.

a. Transverse section through a peripheral nerve. Each nerve fibre (n) is enclosed by a myelin sheath (m) composed of concentric layers derived from the plasma membrane of Schwann cells. The myelin sheath is surrounded by the neurolemma (n), the nucleated cytoplasmic portion of the Schwann cells, which is enclosed by a basal lamina (b). The endoneurium (e) immediately surrounds the Schwann cells and is composed of all the connective tissue elements lying inside the perineurium (p) which, in turn, separately encloses each fascicle of nerve fibres.

b. Longitudinal section through an unmyelinated terminal branchlet of the motor nerve (t). It is enclosed by delicate processes of the neurolemmal sheath (n) and lies in close proximity to a muscle fibre (mf).

c. The neuromuscular junction. Nerve terminals (t) enclosed by Schwann cell processes (s) lie in gutter-like depressions in the muscle cell (m). Note the postsynaptic folds (pf), synaptic vesicles and nucleus of the muscle fibre (n).
occupying gutter-like depressions in the sarcolemma (Figs. 1.1, 1.2). The motor end-plate is typically 30-100μm long and 20-60μm wide, and is usually located midway along the muscle fibre. Between the crests of the postsynaptic folds and the nerve terminal (presynaptic) membrane is a gap, approximately 20-60nm wide, known as the primary synaptic cleft (Figs. 1.1, 1.2). A single basal lamina is present in this interval and follows the contours of the sarcolemma, thus entering and lining the postjunctional folds (known as the secondary synaptic cleft or subneural apparatus).

The nerve ending can be recognised by a dense aggregation of vesicles, mitochondria, neurotubules, neurofilaments, and tubules of smooth endoplasmic reticulum (Fig. 1.2). The numerous synaptic vesicles, 40-50nm in diameter, are characteristic of virtually all chemically-transmitting synapses (Elfvin, 1976) and are thought to be packages of neurotransmitter. At the motor nerve terminal they contain acetylcholine (de Robertis et al., 1963; Whittaker et al., 1964). Some of these vesicles, instead of being randomly distributed, are lined up in a double row along narrow transverse bars of electron-dense material attached to the presynaptic membrane. This presynaptic region is known as the active zone (Fig. 1.3), and is thought to be the site where vesicles fuse with the plasmalemma and ACh is released (Heuser et al., 1974; Peper et al., 1974) (See Section 1.3.1). Released ACh diffuses across the primary synaptic cleft and interacts with specific receptors (nicotinic) on the crests of the postsynaptic folds of the muscle cell. The neuromuscular junction is, therefore, a specialised region responsible for communicating signals from nerve to muscle; the possible mechanisms involved in transmission of the signal are discussed in the following section.
Three dimensional view of pre- and postsynaptic membranes with active zones and immediately adjacent rows of synaptic vesicles. The plasma membranes are split to illustrate the structures observed upon freeze-fracturing. The cytoplasmic half of the presynaptic membrane at the active zone shows on its fracture face protruding particles whose counterparts are seen as pits on the fracture face of the outer membrane leaflet. Vesicles which fuse with the presynaptic membrane give rise to characteristic protrusions and pores in the fracture faces. The fractured postsynaptic membrane in the region of the folds shows a high concentration of particles on the cytoplasmic leaflet. The particles are probably ACh receptors (adapted from Kuffler and Nicholls, 1976).
1.2 NEUROTRANSMISSION

The nervous system is a highly complex network of interconnecting cells communicating with one another by means of electrical signals. As these signals, or action potentials, are propagated down the nerve, transient changes in the permeability for Na\(^+\) and later K\(^+\) ions occur in sequential areas of the membrane (Hodgkin and Huxley, 1952a,b). Depolarizing and repolarizing currents are thus achieved, and the action potential is conducted in a regenerative manner to the terminal (Hodgkin, 1939). As it invades the nerve terminal, it causes the transient opening of voltage-dependent calcium channels. A brief influx of Ca\(^{++}\) follows, which rapidly stimulates release of transmitter into the synaptic cleft (Katz and Miledi, 1965; Baker et al., 1971; Baker, 1976). This in turn causes postsynaptic changes in conductance and regeneration of the action potential is achieved. In this way, messages are relayed rapidly and efficiently within the nervous system.

The majority of synapses rely on chemical means of impulse propagation (Dale, 1953; Kuffler, 1948). Although a wealth of information exists on the anatomy and physiology of the nervous system, knowledge of the molecular events involved in neurotransmitter release is very limited. The nature of this process remains one of the most elusive aspects of neurobiology and is the subject of much controversy amongst investigators.

1.3 NEUROTRANSMITTER RELEASE

Biochemical, anatomical and electrophysiological observations made over several decades have led to the formulation of two major hypotheses on the nature of the release of neurotransmitter from nerve terminals: the vesicular hypothesis and the non-vesicular (gating, operator, or vesigate mechanisms) hypothesis.
1.3.1 The vesicular hypothesis.

This hypothesis, first proposed by del Castillo and Katz (1956, 1957), is based on two observations: the quantal nature of ACh release at the neuromuscular junction (Fatt and Katz, 1952) and the discovery of vesicles in presynaptic nerve terminals (Palay and Palade, 1954; de Robertis and Bennett, 1954). Katz and co-workers (1966; 1969) demonstrated that ACh is released in discrete packages of $10^3$ to $10^4$ molecules known as quanta. When quanta are released singly, small fluctuations (miniature end-plate potentials, mepps) occur in the resting potential of the postsynaptic membrane (Fatt and Katz, 1952); these mepps constitute non-stimulated quantal release. Del Castillo and Katz (1957) proposed that mepps are the result of release of the contents of individual synaptic vesicles; this suggestion was then strengthened by subcellular fractionation studies on nervous tissue carried out by de Robertis et al. (1963) and Whittaker et al. (1964) which showed that ACh is localised within synaptic vesicles. The following sequence of events is thought to occur following Ca** uptake into the nerve terminal: vesicles, packed with transmitter, migrate to specialised zones on the presynaptic membrane (active zones), fuse with the plasmalemma and discharge their contents into the synaptic cleft by the process of exocytosis (Eccles, 1964). The synaptic membrane thus becomes an integral part of the presynaptic membrane as demonstrated by expansion of its surface area during nerve stimulation (Ceccarelli et al., 1972; 1973; Heuser and Reese, 1973). Bittner and Kennedy (1970) suggested that new synaptic vesicles are formed by recycling of the plasma membrane and evidence for this came from the ultrastructural studies of Ceccarelli et al., (1972) and Heuser and Reese (1973). By using horseradish peroxidase as a tracer in stimulation experiments, these workers showed that membrane is retrieved by the formation of coated vesicles, which lose their coats and coalesce to form cisternae,
from which the supply of synaptic vesicles is re-established. Moreover, calculations by Heuser and Reese (1973) indicated that the loss of synaptic vesicle membrane is equal to the amount of membrane incorporated into the cisternae plus the amount by which the nerve terminal increases, since it becomes irregular as a result of stimulation. Nerve impulses result in the release of large numbers (a few hundred for each impulse) of quanta which generate the end-plate potential (epp). This causes the depolarisation of the postsynaptic membrane and a message is thus relayed to the postsynaptic cell.

Further evidence for the vesicle hypothesis includes the observed reduction in vesicle number following intense nerve stimulation (Ceccarelli et al., 1972; Zimmermann and Whittaker, 1974). A dramatic example of this is seen in response to black widow spider venom which causes bursts of quantal release in the presence of Ca^{++} (del Castillo and Pumplin, 1975). Morphological results support the conclusions of electrophysiological experiments by showing that prolonged exposure to the toxin causes severe depletion of vesicles in the nerve terminal. In addition, neuronal exocytotic events were demonstrated in freeze-fractured preparations of the frog neuromuscular junction (Heuser et al., 1974). These workers showed that the cytoplasmic face of the presynaptic membrane possesses a series of transverse ridges, demarcated by double rows of intramembraneous particles which are thought to be Ca^{++} channels. These ridges are elevated towards the muscle fold opposite and during nerve stimulation are paralleled on either side by many discrete plasmalemmal deformations, like dimples or craters. These dimples, which are also seen in secretory cells, are thought to be synaptic vesicles fusing with the plasma membrane; they are never observed at the resting nerve terminal (Heuser, 1976).
This attractive exocytotic release hypothesis is now widely accepted as an anatomical phenomenon, but remains an obscure process at the biochemical level that has been challenged by a number of workers (Tauc, 1979; 1982; Marchbanks, 1979) as a general model for neurotransmission.

1.3.2 Non-vesicular release of neurotransmitter.

A number of criteria must be satisfied if the vesicular hypothesis is to be accepted as the mechanism for neurotransmitter release (at the neuromuscular junction) (Tauc, 1982): at rest, each synaptic vesicle should contain one quantum of ACh and this should be responsible for one mepp detected in the postsynaptic cell; a relationship should exist between the number of filled vesicles and the postsynaptic response; vesicle exocytosis should occur before the appearance of the mepp; vesicular ACh should be released preferentially and directly; and other compounds in the vesicle should be released with ACh in a stoichiometric ratio. Over the last few years, evidence has accumulated to suggest that a number of these criteria are not satisfied, and this seriously questions the validity of the exocytotic hypothesis. It is now known that ACh is found not only in vesicles but also in the cytoplasm of nerve terminals (Birks, 1974; Israel and Dunant, 1975). Moreover, choline acetyltransferase (the enzyme responsible for synthesis of ACh) is not found in vesicles (Marchbanks and Israel, 1972); ACh synthesis occurs in the cytoplasm (Fonnum, 1967) and, by use of radioactive precursors, it has been shown that in many tissues (e.g. sympathetic ganglion, electric organ of Torpedo, rat diaphragm) newly synthesised ACh is released preferentially on stimulation (Collier, 1969; Potter, 1970; Dunant et al., 1972; Israel and Dunant, 1975). In addition, Tauc et al. (1974) showed that when acetylcholinesterase (AChE) was injected into cholinergic neurons in the buccal ganglion of Aplysia, the
vesicles were not destroyed but neurotransmitter release was inhibited completely. It has also been shown that when the nerve is stimulated physiologically, the level of acetylcholine within synaptic vesicles remains unchanged (Dunant et al., 1972; Israel and Dunant, 1975). Collectively, these results suggest that cytoplasmic, and not vesicular, ACh is responsible for neurotransmission.

Birks (1974) showed that when the sympathetic ganglion was stimulated for 20 minutes, the number of vesicles decreased by half but, because of the active synthesis in the cytosol, the amount of ACh in the ganglion did not change. It is clear from the findings in this experiment and others (Dunant et al., 1974) that, although there seems to be general agreement that vesicle number is reduced following intense stimulation of synapses (Ceccarelli et al., 1972; Heuser and Reese, 1974; Zimmermann and Whittaker, 1974), there is not a simple correlation between the number of vesicles and the amount of ACh released. The situation is even more complicated when the vesicular content of ACh is considered. Zimmermann and Whittaker (1974) found that, after having applied a strong tetanic stimulation to the Torpedo electric organ, the vesicle population was re-established after 4 hours, the electrical response in 5 hours, and the vesicular ACh in 3 days. In order to explain this observation, these workers proposed a model in which only a small fraction of the vesicles effectively participates in release. This fraction, VP₂, was isolated (Zimmermann and Whittaker, 1977) and found to consist of vesicles associated with membrane fragments. In addition, ACh radioactive precursor was found to accumulate in this fraction on stimulation (Suszkiew et al., 1978). Dunant and Israel (1979), however, denied the existence of specific active vesicles, and argued that the labelled ACh in the VP₂ fraction can be attributed to the 'survival' of cytoplasmic ACh which was associated with membranes heavier than the synaptic vesicles. In
fact, comparison of the results obtained by the two groups shows that during stimulation vesicular ACh remained unchanged, while cytoplasmic and VP2-bound ACh were modified identically. In addition, Large and Rang (1978) showed that the false transmitter acetylmonoethylcholine is incorporated evenly throughout all the quanta available for release and is not preferentially taken up into a population of active vesicles. In terms of the vesicular hypothesis, one would expect an intermediate stage during which new vesicles, preferentially filled with the false transmitter, would be released together with older ones containing ACh.

The exocytotic events demonstrated by freeze-fracture were rarely seen under physiological conditions. Manipulations that promote large Ca++ fluxes (e.g. 4-aminopyridine) and elevate end-plate responses were required to see pits surrounding the active zones (Heuser, 1977). Heuser argued that these pits resulted from the exocytosis of transmitter; however, transmitter release appears to have occurred before the pits appeared (Tauc, 1979; 1982; Cooper and Meyer, 1984). These pits could reflect sites of non-exocytotic events, such as cation channels (Cooper and Meyer, 1984). Likewise, the vesicles containing horseradish peroxidase in tracer studies (Heuser and Reese, 1973) may not be related to the release of ACh (Marchbanks, 1979). The fact that vesicles can be charged by an extracellular marker, indicates that they could be organelles responsible for clearing the nerve terminals of undesirable ions (e.g. Ca++; Tauc, 1982) and catabolites. In addition, unlike ACh release, the stimulated release of protein markers taken up into vesicles is not Ca++-dependent (Marchbanks, 1977), suggesting that the two processes are indeed independent. Vesicles contain not only ACh but also ATP (Dowdall et al., 1974); however, no release of ATP was observed in stimulated sympathetic ganglia (Kato et al., 1974) or Torpedo synaptosomes (Michaelson, 1978). Vesicles are also found at electronic synapses (Bennett et al., 1967) and there are
transmitters for which it has not been possible to demonstrate a vesicular localisation. Collectively, these results strongly suggest that synaptic vesicles may be involved in a process(es) not directly concerned with the release of neurotransmitter.

There are also difficulties in explaining vesicle fusion with the plasmalemma. One aspect concerns the repulsive forces between phospholipid membranes (Parsegian, 1977); to overcome this barrier to membrane fusion would take about a millisecond whereas the time elapsed between Ca\(^{++}\) entry and the start of the postsynaptic potential is in the order of 200 microseconds (Llinas, 1977). Also, exocytosis would require structural rearrangement of considerable amounts of membrane which seems very uneconomical. Finally, the half-life of the protein components of the presynaptic membrane and vesicles is about 10 days (Von Hungen et al., 1968) and this is inconsistent with rapid membrane turnover. Improvements in recording techniques allowed the discovery of small mepps which are about 10-20% of the size of the normal mepp; this is also difficult to reconcile with the vesicular hypothesis. Collectively, these findings suggest that although there is overwhelming evidence for synaptic vesicle exocytosis, the relevance of this process to neurotransmitter release remains to be ascertained.

Other possible mechanisms have been proposed; the simplest alternative would be a gate hypothesis (Marchbanks, 1978) but this has several drawbacks as it could not be reconciled with the non-electrogenic nature of quantal release, the constancy of mepp amplitude whichever polarisation is imposed on the terminal, or the fact that modification of the cytoplasmic concentration of ACh is not reflected in the size of a quantum. An operator mechanism was proposed by Israel et al. (1979) which involves a macromolecule organised like an ACh molecule forming a triple tetramer able to bind 480 molecules of ACh, enough to account for a small mepp. Ten such molecules operating
synchronously would result in a classic quantum and such structures would occupy only a small area of the total membrane surface (1/μm²).

Finally, Tauc (1979; 1982) has proposed a 'vesigate' mechanism which combines some properties attributed to vesicles (such as packaging of ACh) and others to a gate (membrane mechanism). These vesigates would be macro-molecular structures with high affinity binding sites for ACh exposed to the cytosol. Extrusion of ACh would be the result of a conformational change, or 'collapse', of the loaded vesigates, produced by activation of contractile protein molecules.

It is clear that many questions remain unanswered and new approaches are required to explain this complex mechanism found at nerve terminals.

1.4 BOTULISM.

A classic approach to unravelling mechanisms in biological systems involves the selective inhibition or alteration of a key step in the pathway being examined, followed by thorough investigation of the nature of the block that was caused. Neurobiology has greatly benefitted from this approach; for example, the acetylcholine receptor has been studied using the snake toxin α-bungarotoxin which binds specifically and with high affinity to ACh receptors causing neuromuscular paralysis (for review see Dolly and Barnard, 1984), the voltage-sensitive sodium permeability mechanism was investigated using the selective inhibitor tetrodotoxin extracted from the ovaries of puffer fish (Moore et al., 1967), and the potassium conductance mechanism was studied using its selective blocker tetraethylammonium (Armstrong and Hille, 1972).

Botulinum neurotoxin, the most toxic substance known, specifically and irreversibly inhibits neurotransmission at peripheral cholinergic synapses (for review, see Simpson, 1981). It acts by depressing neurogenic release of ACh at the neuromuscular junction and other peripheral
cholinergic synapses, and may thus prove useful as a probe for elucidation of components involved directly or indirectly in the process of neurotransmitter release. The toxin is produced by toxigenic forms of the anaerobic, gram-positive bacterium *Clostridium botulinum* in seven (Simpson, in press) antigenically different, but structurally related forms (A, B, C, D, E, F, G) which are responsible for the condition known as botulism. The word botulism is derived from the latin 'botulus' meaning sausage, a common source of the syndrome in the nineteenth century. The toxin can be acquired in three ways: by ingestion of food containing the toxin (foodborne botulism), by formation of toxin *in vivo* in a wound (wound botulism) and, in infants, by production of the toxin during growth of the organism in the intestine (infant botulism) (Feldman *et al.*, 1981). The latter may prove to be the most common form of botulism and is thought to be responsible for a number of cases of sudden infant death syndrome (Arnon *et al.*, 1978). Human cases are almost always due to types A, B or E; types C and D may not be absorbed by the human gastrointestinal tract (cf. Cherington, 1981). Home-canned or home-processed foods account for most of the foodborne outbreaks of botulism (Feldman *et al.*, 1981) as the conditions are ideal for toxin production. Although botulinum toxin is heat-labile and is thus often destroyed by cooking, the spores of *Clostridium botulinum* are heat-resistant and, when they are present in food, actively multiplying bacilli produce the toxin under anaerobic conditions at a pH of 6 or above (cf. Cherington, 1981). It has been estimated that 0.05-0.5μg of the purified neurotoxin (orally administered) represents one human lethal dose. The clinical symptoms of botulism occur within 12-36 hours after ingestion of contaminated food, beginning in the cranial nerve territory and then descending (Cherington, 1981); respiratory paralysis can follow quickly and is often fatal, even if treated. Recovery, if it occurs, is prolonged, but
almost total; treatment involves providing artificial ventilation, often for as long as weeks or months, and guanidine and 4-aminopyridine have also been used to relieve the symptoms of the intoxication.

Wound botulism is the rarest form of botulism and only type A and type B cases have been reported (Sugiyama, 1980). This form of the intoxication results when Clostridium botulinum infects a wound and produces toxin, which reaches other parts of the body by way of the bloodstream. Infant botulism occurs in babies aged between 3 and 26 weeks and type A cases are slightly more frequent than B cases. Spores are probably the infective form and are most likely acquired by swallowing; the illness is a toxico-infection in which the organism colonises and produces toxin in the intestinal tract (Arnon et al., 1977).

**BOTULINUM NEUROTOXIN.**

1.4.1 Purification and radio-iodination of the neurotoxic moiety of botulinum toxin.

Of the seven forms of botulinum toxin, type A is the most potent and well studied. When it was first isolated as an apparently single protein, it was shown to have a molecular weight of 900,000 (Lamanna et al., 1946). It was later found to be a complex of two major protein components, one of which displayed haemagglutinin activity and the other which was highly neurotoxic (DasGupta et al., 1966). A necessary step towards realising the potential of botulinum toxin as a probe for studying the mechanism of neurotransmitter release was the isolation of the neurotoxic moiety in large quantities. DasGupta and Boroff (1968) separated the neurotoxic protein from crystalline toxin-haemagglutinin complex by DEAE-Cellulose chromatography and showed it to have a molecular weight of 150,000. The haemagglutinin was found to exist in several states of aggregation with molecular weights of 290000, 500000 and 900000. Moberg and Sugiyama (1978) purified the neurotoxin by
affinity chromatography using p-aminophenyl-β-D-thiogalactopyranoside, an inhibitor of haemagglutinin, coupled to Sepharose beads. Homogeneous neurotoxin was obtained by use of a subsequent chromatographic step on DEAE Sephacel, which removed the tightly associated contaminant shown to have a different molecular weight (~130,000), subunit pattern, immuno-reactivity and very low toxicity compared to the neurotoxin (Tse et al., 1982). Type B botulinum neurotoxin (BoNT) has also been purified to homogeneity by this method (Kozaki and Sakaguchi, 1975; DasGupta and Sugiyama, 1976; Williams, 1984). Early studies on the binding and action of the toxin involved crude or partially purified preparations of its high molecular weight complex with haemagglutinin. As shown by Dolly et al., (1981), the complex is unsuitable for biochemical and structural localisation studies due to the non-specific interaction of the haemagglutinin moiety with neuronal membranes. Thus, purification of the neurotoxin represented a major advance in the study of these powerful agents.

The value of the neurotoxin as a probe for investigating neurotransmission has also been greatly enhanced by the recent production of biologically active [125I]-iodinated types A (Williams et al., 1983) and B (Williams 1984) BoNT of high specific radioactivity (700-1750Ci/mmol), with retention of neurotoxicity and ability to bind specifically to neuronal membranes in the central nervous system. Hence, the goals in botulinum toxin research to localise and isolate the tissue acceptor and identify the cellular process that is poisoned may now be achieved.

1.4.2 Structure and function of the neurotoxin molecule.

All the botulinum neurotoxins are synthesised intracellularly. It is thought that toxin production may be mediated by bacteriophages or plasmids (Inoue and Iida, 1970; 1971), as has been observed with
diphtheria toxin (Pappenheimer, 1977) and suggested for tetanus toxin (Mellanby and Green, 1981). They are produced as single chain inactive precursors which may be acted on by endogenous proteolytic enzymes to produce fully active dichain structures by a process called 'nicking' (Sugiyama, 1981). Nicking can also be carried out in vitro by mild trypsinisation (Krysinski and Sugiyama, 1981). Type C has never been isolated in an unnicked form. Some strains of type E, in contrast, only produce the single chain molecule; these type E cultures apparently lack the enzyme that converts the single chain to the dichain form (DasGupta and Sugiyama, 1977) but this can be achieved by proteolytic cleavage with trypsin as discussed above. The case of type B is interesting as late cultures (4-7 days) consist of a mixture of nicked and unnicked molecules (Kozaki and Sakaguchi, 1975; DasGupta and Sugiyama, 1976); the presence of the dichain form suggests that the nicking enzyme is available, but the reason for the incomplete conversion of the single chain molecules to dichains is unknown.

The generalised structure of the botulinum neurotoxin molecule is shown in Fig. 1.4. The two polypeptide chains, known as the heavy (H) ($M_r \approx 100,000$) and light (L) ($M_r \approx 50,000$) subunits, are linked by a disulphide bond which, when reduced, allows the chains to separate. Isolation of the subunits can be achieved by overnight treatment of BoNT with dithiothreitol and urea (Kozaki et al., 1981; Williams et al., 1983; Williams, 1984). However, as shown by Sugiyama et al., (1973), the integrity of the disulphide bond is essential for expression of toxicity. Moreover, there is evidence to suggest that the larger subunit is responsible for toxin binding to neuronal membranes in the central nervous system (Kozaki, 1979; Williams et al., 1983).

To date, the relationship between nicking and pharmacological activity of the toxin remains unclear. It is well known that the toxin is synthesised as a single chain inactive precursor and that trypsin-
Fig. 1.4 Generalised structure of botulinum toxin. (adapted from DasGupta, 1981)

NICKING

\[ \sim 150,000 \rightarrow \sim 50,000 \]

\[ \sim 150,000 \rightarrow \sim 100,000 \]

M.W.
like enzymes cause nicking and activation. However, it is unclear whether the two phenomena, i.e. nicking and activation, are related. Most workers believe that nicking is necessary, although perhaps not sufficient, for activation, and that another molecular change in addition to nicking must take place for full activation (DasGupta, 1981). DasGupta suggests that two proteolytic enzymes are responsible for producing two cleavages in the single chain precursor; one for nicking and one for activation (Fig. 1.5).

1.4.3 **Target sites for botulinum neurotoxin.**

The relevance of botulinum toxin to the elucidation of components involved in neurotransmitter release lies in its biological effect: the irreversible inhibition of both spontaneous and evoked release of acetylcholine from cholinergic nerve terminals in the peripheral nervous system (Burgen et al., 1949). In spite of the fact that a number of tissues are vulnerable to the paralysing effects of the toxin, its mechanism of action has been studied almost exclusively at the neuromuscular junction (Burgen et al., 1949; Cull-Candy et al., 1976; Simpson, 1980; Thesleff, 1981). When botulinum toxin is administered in vivo, its potent effects on the skeletal neuromuscular junction, which lead to death of the experimental animal as a result of respiratory paralysis, obscure its known actions on cholinergic terminals of the autonomic nervous system (Ambache, 1951a; Habermann et al., 1980; MacKenzie et al., 1982). Some studies have been carried out on the action of the toxin in non-mammalian tissue innervated by cholinergic neurons (e.g. frog neuromuscular junction). In addition, its effects on adrenergic (Rand and Whaler, 1965; Holman and Spitzer, 1973) and nonadrenergic-noncholinergic (excitatory and inhibitory) nerve terminals (MacKenzie et al., 1982) have been investigated, but in some cases, the results obtained by different investigators are contradictory.
The single chain molecule (Mₚ ~150,000) (left), which has low toxicity, is converted to its fully active dichain form (right) by nicking (position 1) and activation (position 2). During trypsinization, the two events take place, but in growth cultures activation may occur by sequential nicking and activation, or vice versa (upper and lower routes respectively). An endogenous trypsin-like enzyme is thought to produce partial activation without nicking; on nicking, the molecule becomes fully functional (adapted from DasGupta, 1981).
and it remains to be ascertained whether specific acceptors for the toxin exist at these synapses. Finally, attempts have been made to ascertain whether the toxin exerts its pharmacological action in the central nervous system. To date, no central effects have been reported for the neurotoxin administered to animals in vivo, either peripherally (which can be explained by inability of the large toxin molecule to cross the blood/brain barrier) or centrally (Williams in this laboratory, unpublished results). Effects have been reported for the crude toxin-haemagglutinin complex (Polley et al., 1965; Wiegand and Wellhoner, 1977) but these are thought to be induced by non-specific interactions of the haemagglutinin moiety with target tissues.

Acceptors for botulinum neurotoxin have been detected morphologically, using light-microscope autoradiography, at the mammalian neuromuscular junction (Hirokawa and Kitamura, 1975), but their saturability and cellular location were not examined in this study. Prior to the experiments reported in this thesis, attempts at ultrastructural localisation of these acceptors at the neuromuscular junction had proved unsuccessful (Zacks et al., 1962; Habermann, 1974), possibly due to the problems involved in using the crude toxin-haemagglutinin complex and the unavailability of biologically active radiolabelled neurotoxin preparations of high specific radioactivity (cf. Williams et al., 1983). Ultrastructural localisation of acceptors for botulinum toxin type A on the membrane of rat cerebrocortical synaptosomes has been carried out using a double-sandwich immunocytochemical technique (Hirokawa and Kitamura, 1979) but, again, no evidence of the saturability of toxin binding was given in this report. In addition, acceptor sites for $^{125}$I-BoNT types A, B and E have been detected in the central nervous system using biochemical techniques (Habermann and Heller, 1975; Kitamura, 1976; Williams et al., 1983; Williams, 1984). Analysis of equilibrium binding and kinetic data showed heterogenous populations of
saturable acceptors for both types A (Williams et al., 1983) and B (Williams, 1984) $^{125}$I-labeled proteins on rat synaptosomal membranes ($K_D \sim 0.5$ and 20nM, $B_{max} \sim 50$fmol/mg and 3pmol/mg respectively).

1.4.4 Pharmacological action of botulinum neurotoxin.

Electrophysiological studies have allowed the formulation of a general model to explain the actions of botulinum toxin at the nerve terminal (Simpson, 1980). This model involves three discrete steps. There is an initial 'binding' step that is not affected by nerve stimulation, is rapid, essentially irreversible, non-toxic, and of a nature that leaves the toxin accessible to inactivation by antitoxin.

The identity of the cell surface acceptor is unknown, although there is evidence to suggest that a sialic acid-containing protein component is involved in the binding of $^{125}$I-BoNT types A and B to rat cerebrocortical synaptosomes (Williams et al., 1983; Williams, 1984). The second step is one in which the toxin becomes inaccessible to antitoxin and, like the binding step, is not directly responsible for toxicity. It is referred to as the 'translocation' step and is thought to involve internalisation of the toxin, or a fragment thereof, although direct evidence for this is lacking as the conformation of the toxin may change on binding to the membrane resulting in inability to bind the antibody.

The mechanism of this postulated membrane penetration has not been determined, but an active process such as acceptor-mediated endocytosis (see Section 1.5) and/or the formation of protein channels or carriers is likely. The final step results in blockade of neurotransmission; it is much slower than the binding or translocation steps, highly temperature-dependent and is accelerated by nerve stimulation (Hughes and Whaler, 1962). This 'lytic' phase is thought to occur intracellularly and results in the inhibition of neurogenic release of ACh from the nerve terminal (Burgen et al., 1949; Harris and Miledi,
1971; Thesleff, 1981), as described previously. The toxin does not inhibit propagation of the action potential along the nerve trunk nor does it block its flow into the terminal arborizations of the nerve (Harris and Miledi, 1971), and paralysed muscles still respond normally to agonists (Burgen et al., 1949). It blocks the postsynaptic responses that are normally evoked by presynaptic nerve stimulation, or more precisely, it causes reduction in the amplitude of impulse-induced end-plate potentials (epps) with high failure rate of transmitter release (Harris and Miledi, 1971; Cull-Candy et al., 1976; Tse et al., 1982). In addition, botulinum toxin partially blocks spontaneous release; it diminishes the frequency of miniature end-plate potentials (mepps) (initially to about 1% of that seen in controls) (Dolly et al., 1981; Thesleff, 1981) and causes the mean amplitude to decrease progressively for two days after poisoning. In normal preparations, spontaneous mepps have a Gaussian distribution, whereas in poisoned tissues, the amplitude distribution is skewed to the left (lower amplitudes) at all times (Cull-Candy et al., 1976). As paralysis persists, the frequency of mepps increases slightly and a population of mepps with larger than normal amplitude appears (Thesleff, 1981). Sub- and giant mepps are also seen at normal synapses, but the proportion is small relative to the predominant intermediate-sized mepps. The shift in mean mepp amplitude seen in poisoned muscles is accompanied by an increase in mean rise time and a fall in mean rate of rise of mepps (Thesleff, 1981). Several mechanisms have been proposed for the decreased frequency and amplitude of spontaneous mepps. Boroff et al. (1974) suggested that the toxin altered vesicle membranes in such a way as to impair loading or storage of ACh. However, Wonnacott and Marchbanks (1976) have shown that the vesicular content of transmitter is unaffected by botulinum toxin. Moreover, uptake of choline and synthesis of ACh are normal at poisoned nerve terminals. In addition, the large number of vesicles
(2-5 x 10⁵/nerve terminal) and the low number of toxin molecules required for paralysis (e.g. 10⁸ molecules/mouse) preclude a direct action of the toxin on individual vesicles (cf. Simpson, 1981). An alternative explanation was put forward by Kriebel et al., (1976). This involves the proposal that mepps are the result of the synchronous release of several quanta of ACh, and not to the release of only one as suggested by del Castillo and Katz (1957). According to this proposal, one quantum would evoke a subminiature end-plate potential (submepp) and mepps would occur when several of these are released simultaneously. Kriebel et al., (1976) thus suggest that the shift in mepp amplitude observed in botulinized muscles is the result of desynchronization of release of submepps. Finally, Thesleff (1981) has put forth an interesting explanation involving different populations of vesicles. He proposes that there are two kinds of vesicular, quantal release: the first occurs at the 'active zones', directly opposite the highest postsynaptic density of ACh receptors, and evokes mepps and epps of rapid and uniform rise time. The second type, which is independent of intracellular Ca²⁺ concentration and of transmembrane Ca²⁺ flux and, therefore, uninfluenced by nerve stimulation, occurs from areas dispersed around the terminal (some closer and some more distant from the postsynaptic folds). Quantal release from these areas is responsible for mepps with variable, greatly prolonged rise times. The amplitude of these mepps will depend on whether one or several vesicles are released from these sites at a given time. Thesleff proposed that release from these sites is responsible for the small portion of sub- and giant mepps seen at normal end-plates and that this release is not affected by BoNT. In this scheme, the toxin only affects release of quanta from the active zones.

As mentioned earlier, the toxin-induced inhibition of release is not absolute as occasional quanta are released by nerve stimulation.
Moreover, it has been shown that raising the extracellular Ca\(^{++}\) concentration in conjunction with use of CA\(^{++}\) ionophores (Cull-Candy et al., 1976; Kao et al., 1976), or use of drugs which raise intracellular levels of Ca\(^{++}\) (e.g. 4-aminopyridine; Lundh et al., 1976), result in mepps of normal frequency and amplitude. This suggests that the release mechanism has not been destroyed. Since there is no evidence to suggest that BoNT blocks Ca\(^{++}\) channels or prevents Ca\(^{++}\) flux into the nerve terminal (Gundersen et al., 1982), it appears that BoNT acts by interfering with the sensitivity of a step in the release process to Ca\(^{++}\) (Cull-Candy et al., 1976; Thesleff, 1981). The mechanism by which the toxin exerts this interference is unknown (Simpson, 1981).

1.5 ENDOCYTOSIS.

A number of toxins, viruses and glycoprotein hormones which act intracellularly are thought to be taken up into target cells by the process of acceptor-mediated endocytosis (Neville and Chang, 1978). Since BoNT also appears to act intracellularly (Simpson, 1980), a discussion of this process of uptake is relevant.

The term endocytosis is used to describe how cells internalise molecules: a patch of the plasma membrane surrounds the material to be taken in; it then invaginates and pinches off to form a vesicle inside the cell. There are three kinds of endocytosis: phagocytosis, pinocytosis and acceptor-mediated endocytosis (see Fig. 1.6). In phagocytosis, the binding, or close proximity, of a very large particle or molecular complex to the surface of the cell triggers an expansion of the membrane around the material, which is incorporated into the cell in a bag-like vesicle that can be several microns in diameter (Dautrey-Varsat and Lodish, 1984; Steinman et al., 1983). This is the process by which protozoans ingest food particles and macrophages take up
In phagocytosis (left), the cell's plasma membrane expands to wrap around a large particle such as a bacterium and engulf it within the cell. In pinocytosis (centre), a droplet of the liquid extracellular medium is surrounded by a patch of the membrane, which folds inwards and 'buds off' to form a membrane-bounded vesicle enclosing the droplet and any small molecules dissolved in it. Acceptor-mediated endocytosis (right) is a mechanism for the selective uptake of large molecules or particles. A ligand binds to its specific acceptor on the plasma membrane, triggering the internalisation of the acceptor-ligand complex in an invagination of the plasma membrane. The vesicle thus formed buds off inward, carrying the ligand into the cell (adapted from Dautrey-Varsat and Lodish, 1984).
intruders such as bacteria. The internalised vesicles are rapidly attacked by lysosomes and degradation of the ingested material ensues. The process of pinocytosis, on the other hand, results in non-specific uptake of extracellular fluid (Fig.1.6). A minute droplet of fluid is surrounded by a patch of the plasma membrane and is internalised by a vesicle only about 0.1μm in diameter, bringing with it the ions and small molecules that happen to be in the droplet. These small vesicles are short-lived as they fuse with lysosomes within a few minutes of entry (Dautrey-Varsat and Lodish, 1984; Pastan and Willingham, 1981). Both phagocytosis and pinocytosis are nonconcentrative forms of endocytosis in which trapped fluid contents or materials adsorbed to the cell surface are internalised, transferred to lysosomes, and rapidly degraded.

The process of acceptor mediated endocytosis, in contrast, is very specific (Fig.1.7) (Goldstein et al., 1979; Pastan and Willingham, 1981a; Brown et al., 1983). By this process, cells are able to carefully regulate their internal environment; they can control which molecules are allowed to enter and which are not. The acceptors have a binding site specific for a particular ligand and they essentially pluck one ingredient from the extracellular fluid, even if it is present at extremely low concentrations amidst a vast excess of unrelated molecules, and concentrate it on the membrane. A wide variety of ligands are brought into cells by acceptor-mediated endocytosis and, once inside, they are disposed of differently. Some internalised ligands enter endosomes and lysosomes (e.g. low density lipoprotein and asialoglycoproteins), whereas others are transported in extra-lysosomal compartments long distances from the site of uptake without being destroyed (e.g. nerve growth factor). Some are even carried through the cell into another compartment (e.g. prolactin, antibodies, tetanus toxin) and others enter the cytosol of the target cell (diphtheria toxin, abrin and ricin) (for review, see Neville and Chang, 1978).
Fig. 1.7 Acceptor-mediated endocytosis.

a. The ligand binds to acceptors on the cell surface which then collect in coated pits. The pits invaginate and are internalised as coated vesicles whose fusion gives rise to endosomes and then to a CURL (compartment of uncoupling of receptors and ligands). In the acidic CURL environment, ligand is dissociated from acceptors. The ligand accumulates in the vesicular lumen of the CURL and the acceptors are concentrated in the membrane of an attached tubular structure, which then becomes separated from the CURL. The vesicular part moves deeper into the cell and fuses with a lysosome, to which it delivers the ligand for degradation. The membraneous tubular structure is thought to recycle acceptors to the plasma membrane (adapted from Dautrey-Varsat and Lodish, 1984).

Pathway used by opportunistic ligands such as toxins and viruses.

b. The ligands bind to cell surface acceptors which cluster in coated pits. The pits pinch off to form coated vesicles which then lose their coats and fuse to form endosomes. Opportunistic ligands take advantage of the low pH in these compartments to gain access to the cytoplasm. Their acceptors may be recycled to the cell surface in the tubular portions of the CURL.
a

- ligand
- clathrin
- coated pit
- coated vesicle
- endosome
- tubular portion
- CURL
- lysosome

b

- opportunistic ligands such as toxins and viruses
- acceptor
- coated vesicle
- endosome

plasma membrane
Animal cells use the process of acceptor-mediated endocytosis to take up nutritional and regulatory factors from the extracellular fluid (Goldstein et al., 1979). The internalisation process is effectively coupled to binding and has a half-time of less than 10 minutes. The process involves initial binding of the ligand to mobile acceptors, which are distributed randomly on the cell surface (Fig. 1.7a), with subsequent clustering of the ligand-acceptor complexes in specialised regions of the plasma membrane known as coated pits (Roth and Porter, 1964). The coat for which the pits are named, a thick proteinaceous layer on the inner side of the plasma membrane under each of these specialised indentations, is mainly clathrin (Pearse, 1976). The aggregation of ligand-acceptor complexes over coated pits is thought to be promoted by one of a group of intracellular enzymes found in many types of cells known as transglutaminases (Davies et al., 1980). Some of these enzymes covalently cross-link proteins by forming $\gamma$-(\varepsilon$\text{-glutaminy}l)lysine cross bridges in the presence of Ca$^{++}$, and this process can be inhibited by a variety of amines (e.g. ammonium chloride and methylamine) (Maxfield et al., 1979). The following mechanism for acceptor-ligand clustering which includes a role for transglutaminase has been proposed by Davies et al., (1980): when acceptors are distributed diffusely over the cell surface, the enzyme in the cell is largely inactive because the levels of intracellular Ca$^{++}$ are too low (0.1-1mM). Following binding of ligand, occupied acceptors may undergo an allosteric change so that they are recognised by the trapping mechanism in the pit. Davies et al. proposed that the formation of small reversible aggregates in coated pits promotes Ca$^{++}$ influx and local activation of transglutaminase. This could result in covalent cross-linking of proteins in the complex; acceptors may be coupled to intrinsic membrane proteins or cytoskeletal components, or coupled to each other. This would make the aggregation process irreversible, and
would result in progressive accumulation of acceptor-ligand complexes in the coated pit. It must be emphasised, however, that this hypothetical scheme may not be applicable to all cases of acceptor-mediated endocytosis (Anderson et al., 1977).

The coated pit region of the membrane, containing aggregates of acceptor-ligand complexes, then invaginates to form a coated vesicle (Fig. 1.7a). This process is continual; coated pits keep folding inwards to form coated vesicles and are constantly regenerated on the cell surface. The clathrin coat envelops the vesicle membrane (Linden and Roth, 1983) in a fibrous network of pentagons and hexagons called triskelions. As the coated vesicles move deeper into the cytoplasm, they shed their clathrin coat (15-60 seconds) (Pearse and Bretscher, 1981) and fuse with one another to form larger, smooth-surfaced vesicles called endosomes. A newly formed endosome is about 250-400nm in diameter. These structures are heterogeneous in their morphology and cellular distribution (Pastan and Willingham, 1981b; Helenius et al., 1983). They often have an electron-lucent interior and a smooth, continuous membrane. Attached to the inner surface is a proteinaceous layer, probably representing ligand-receptor complexes. In some preparations, a small intraluminal vesicle about 60nm in diameter is observed and one edge of the endosome is often thickened and has a frill-like appearance. The endosomes are surrounded by small tubular or round vesicular profiles which can form anastomosing networks (Pastan and Willingham, 1981b; Helenius et al., 1983; Geuze et al., 1983). It has only recently been appreciated that the first vesicle in which the ligand appears after cellular entry is a special organelle and not some kind of lysosome (Willingham and Pastan, 1980). Endosomes do not contain cytochemically detectable hydrolytic enzymes and are, therefore, different from lysosomes which are phase-dense and rich in such enzymes. Electron-microscope studies have shown that ligand resides for
20 minutes in intermediate vesicles prior to delivery to lysosomes (Anderson et al., 1977). It has also been shown (Dunn et al., 1980) that fusion of endosomes and lysosomes does not occur at 4°C, an indication that the two compartments are distinct. The buoyant density of the endosomal vacuoles varies between 1.02 and 1.12g cm\(^{-1}\) and is much lower than that of lysosomes, which suggests that they contain appreciably less protein (Marsh et al., 1983).

One of the most important of the endosome's known characteristics is its low internal pH. Tycko and Maxfield (1982) took advantage of the pH-dependent fluorescence spectrum of fluorescein-conjugated \(\alpha_2\)-macroglubulin to show that this ligand reached an acidic pH (5.0) rapidly (15-20 minutes) following internalisation. There is evidence to suggest that an ATP-driven proton pump exists in endosomes similar to that described for lysosomes (Schneider et al., 1981; Ohkuma et al., 1982). An important function of endosomes is that of sorting acceptors and ligands and directing them to their appropriate destinations in the cell. Whereas internalised solutes and acceptor-bound ligands are typically routed with high efficiency into lysosomes, the membrane acceptor is returned, or recycled, to the cell surface (Fig. 1.7a). Experiments with purified acceptors and ligands have shown that, while acceptors bind their ligands tightly at neutral pH, the ligands rapidly dissociate at pH below 5.5. Thus, when an acceptor-ligand complex enters the acidic endosome, the ligand is separated from the acceptor and accumulates in the vesicular lumen; the acceptor remains bound to the membrane and is transferred to the tubular portions of the endosome from which vesicles pinch off and travel back to the cell surface. Dissociation of acceptor-ligand complexes in the endosome compartment has recently been demonstrated by ultrastructural immunocytochemical techniques (Geuze et al., 1983). These workers have named the vesicle-plus-tubule complexes CURL (or compartment of uncoupling of receptors and ligands).
Although different ligands have different destinations (as mentioned above), most of them are delivered to the lysosomes, which also have an acid pH (4.5 to 5 in contrast to the neutral pH 7 to 7.4 of the cytoplasm). The vesicular portions of CURL eventually fuse with these structures, where the ligands are degraded (as explained previously, the acceptors escape lysosomal processing as the tubular portions of CURL somehow transport them back to the cell surface). Lysosomes are identified on the basis of two criteria: a surrounding membrane, as detected by electron-microscopy, and the occurrence of one or more acid hydrolases, most often acid phosphatase, as revealed by cytochemical staining. They cannot be identified by the usual morphological criteria of size, shape and internal structure, because of the great variability of these properties. In the electron-microscope, membrane-enclosed bodies containing dense deposits, membraneous whorls or other materials with a 'messy' appearance, are most probably lysosomes (de Duve, 1983). These vesicles contain about 40 different digestive enzymes which are responsible for degradation of endogenous and exogenous ligands. The digestion products are either eliminated from the cell or exported into the cytoplasm as raw materials.

There are several interesting variations of the scheme described above (Fig. 1.7b). Invasive agents such as enveloped viruses and bacterial toxins, which introduce their genomes or their toxic subunits into the cytosol, are thought to use the process of acceptor-mediated endocytosis to reach targets within this intracellular compartment. In the viral systems (toga viruses, orthom ixoviruses and rhabdo viruses), the low pH of the endosomes induces a conformational change in the viral spike glycoproteins. This activates a membrane fusion activity which leads to the fusion of the viral membrane with the limiting membrane of the endosome (Marsh et al., 1983). Bacterial toxins, such as diphtheria and tetanus toxin, are believed to bind to cell surface acceptors and
then enter the acidic endosome, where the low pH induces a conformational change that allows an active portion of the toxin molecule to cross the membrane and enter the cytosol (Donovan et al., 1982; Boquet and Duflot, 1982). It has been shown for diphtheria toxin that lowering the pH of the external medium allows the toxin to gain access to the cytosol directly from the cell surface (Sandvig and Olsnes, 1980). The pathway normally taken by opportunistic ligands is depicted in Fig. 1.7b. The presence of the endosome explains how these ligands can enter the cytosol so quickly and escape lysosomal degradation.

Agents which increase the pH in endosomes and lysosomes inhibit acceptor-mediated endocytosis by preventing dissociation of acceptor-ligand complexes and accordingly, acceptor re-use. These agents are known as lysosomotropic agents; they accumulate in acidic compartments of the cell and cause a rise in the endo-lysosomal pH up to values that may be as high as 6.5 (Ohkuma and Poole, 1978; Poole and Ohkuma, 1981) within minutes. As the amount of compound taken up increases, osmotic attraction of water causes swelling of the endocytic vesicles, leading to the extensive vacuolation that is seen after an hour or more of exposure (de Duve, 1983). Lack of detachment of ligand from its acceptor may cause it to be returned to the cell surface with recycling membrane patches, and thereby result in severe inhibition of net endocytic uptake (Schneider and Trouet, 1981). Also, immobilisation of membrane in expanding endocytic vesicles may interfere with the recycling process itself. Examples of such agents are chloroquine, ammonium chloride and methylamine, all of which are weak bases.

Although several different molecules may enter the cell via acceptor-mediated endocytosis (Pastan and Willingham, 1983) and cluster in the same coated pits and endosomes, they can exhibit quite different kinetics. It is not known what determines the final destination of an
internalised ligand, or how the internalised patch of plasma membrane and other membranes retain individuality through continual invagination, fusion and recycling events. More detailed understanding of acceptor-mediated endocytosis is required to answer some of these questions.

1.6 AUTORADIOGRAPHY

In both light- and electron-microscope autoradiography, the pattern of radiation in a specimen is detected in a thin layer of photographic emulsion and visualised, as silver grain deposits, by chemical or physical development. As the specimen will absorb and scatter its own radiation, the final image reflects the distribution of radioactivity within the specimen as influenced by its structure. The techniques can be used to localise and quantitate radioactive material at cellular and subcellular levels. In brief, they involve preparation and mounting of a specimen section on a support, coating it with emulsion, developing it after appropriate exposure time, and analyzing the resulting autoradiograms (Rogers, 1979).

Autoradiography is an old technique. It was known before radioactivity was discovered and contributed towards its recognition. In 1867, Nièpce de St Victor published the observation that blackening was produced on emulsions of silver chloride and iodide by uranium nitrate and tartrate. Becquerel (1896) observed that this blackening could take place in the dark, without prior exposure to sunlight. Through this work, and the work of the Curies in 1898, radioactivity was first demonstrated. The development of autoradiography progressed little until the 1940's when advances in nuclear physics resulted in the increasing availability of artificially produced radioisotopes of elements such as hydrogen, sulphur, carbon and phosphorus. In 1940, Hamilton et al. demonstrated the uptake of radioactive iodine by the thyroid gland and Leblond (1943) then showed its distribution within the
gland in autoradiograms. Electron-microscope autoradiography was introduced by Liquier-Milward (1956); since then improved techniques have been developed, with new nuclear emulsions being produced, so the process is now routinely used in many laboratories.

For a detailed description of the autoradiographic technique, see Rogers (1979). Briefly, the emulsion used in autoradiography consists of a large number of silver halide crystals, suspended in a solid phase which is usually gelatin. Each crystal is a separate detector, insulated from the rest of the emulsion by its capsule of gelatin. It can respond to the passage through it of a charged particle, with the formation of a latent image which may persist throughout the exposure period, and is made permanent by development. The crystals of silver halide (chiefly bromide) in nuclear emulsions range from 0.07 to 0.4 μm in diameter depending on the emulsion, and are reasonably uniform in size in any one type. Silver bromide crystallises in a cubic pattern, with ions of silver and bromide regularly spaced; each silver ion is surrounded by six bromide ions, and vice versa. Each crystal is likely to contain a number of faults in the regular lattice (which are essential for sensitivity of the emulsion to radiation, and are introduced during manufacture by insertion into the crystal of atoms of other elements such as sulphur). When a charged particle enters the crystal, it has a high probability of giving up its energy to one electron within the lattice, which then enters a conductivity band and travels through the crystal. Its journey usually ends at a fault in the lattice where it is thought to be captured by a silver ion which then becomes an atom of metallic silver. For each atom of metallic silver which is thus withdrawn from the crystal lattice, an ion of bromide is converted to an atom of bromine. Bromine is liberated from the surface of the crystal and migrates to acceptor sites in the gelatin. The silver constitutes the latent image; silver atoms deposited at
sensitivity specks may rejoin the crystal lattice in a process known as latent image fading. This fading is encouraged by the presence of oxidising agents and moisture and is controlled by thorough drying of the emulsion layer prior to exposure. Latent images may also be produced by pressure, heat, and many chemicals, particularly reducing agents, so care must be taken in the interpretation of autoradiograms.

The various particles produced during radioactive decay have different effects in nuclear emulsions. Charged α and β particles form the main radiations of interest to autoradiographers. These particles can exert an effect, even at a distance, on the electrons in orbit about the atomic nuclei of silver and bromine which make up the crystal. Uncharged particles such as neutrons and electro-magnetic radiations such as X-rays and γ-rays only lose energy by direct collision with electrons or nuclei. Since such collisions are unlikely, these particles may travel considerable distances through the emulsion without leaving a latent image to indicate their passage. α particles consist of two protons and two neutrons, and therefore carry two positive charges. They cause tremendous havoc to the electron shells through which they pass and produce very dense, straight tracks, usually relatively short (15-50μm long) as they dissipate energy very rapidly. β particles are essentially electrons of nuclear origin; they have the same mass as electrons and the same single negative charge. Unlike α particles, β particles emitted by one particular isotope do not all have the same initial energy; they show a range of energies from a maximum value down to zero. The tracks of β particles in the nuclear emulsion are very irregular and tortuous, showing many changes of direction, and are composed of separate grains which are randomly spaced. The rate of energy loss is lower for a high energy β particle than for one of low energy, which is of considerable importance in relation to emulsion sensitivity. As they pass through the cloud of orbital electrons around...
an atom, low energy particles will collide with the latter more frequently, giving rise to more closely spaced silver grains along their tracks in the emulsion, than high energy ones. Similarly, collisions will be more frequent at the end of the track of a high energy particle than at the beginning.

Some isotopes, such as \(^{125}\text{I}\), undergo rearrangements of the neutrons and protons in their nuclei without the ejection of a charged particle, losing energy as a photon. This photon may lose its energy to one of the orbital electrons in the inner K or L shells. The electron will leave the atom behaving like a β particle but, as it is not of nuclear origin, it is called an electron of internal conversion. The space left in the inner electron shell is filled by an electron from one of the outer shells and this transition is accompanied by the emission of low energy photons which are called X-rays. These X-rays may, in turn, eject an outer electron (called an Auger electron) from orbit. The decay schemes of such isotopes may, therefore, be very complex, with production of a number of electrons of specified energies and probabilities of occurrence. These radionuclides (e.g. \(^{125}\text{I}\)) which decay by internal conversion are useful for autoradiography as the electrons emitted tend to have very low initial energies, in the range of those emitted by tritium (lower values). They, therefore, give good resolution in autoradiographs.

Latent images can be visualised and made permanent by development. In chemical development, the emulsion is placed in a solution which reduces silver bromide to metallic silver and hydrogen bromide. Most developing agents have the appropriate reducing potential at alkaline pH. Development is a self-catalytic process, proceeding faster in crystals where reduction has started: thus, where aggregation of silver atoms has occurred (i.e. at a sensitivity speck) there is higher probability of further silver being added. Development is thus a
process of amplification, increasing the size of a deposit of metallic silver in a crystal until it reaches a threshold at which it can be recognised; at this threshold, the crystals become silver grains which look like a coiled filament, and can grow to occupy a volume up to three times that of the original crystal. The longer development proceeds, the more crystals are fully reduced, until the entire emulsion may be fully developed. Development can, therefore, be stopped at different stages; ideally, a situation in which only crystals with latent images are developed and not others must be achieved. This critical point must be determined empirically. The process of chemical development described above, therefore, uses the silver bromide of each crystal as the source for the silver of a developed grain. In physical development, the silver is deposited from solution (e.g. Phenidone) on the silver deposits at sensitivity specks. This produces a concentric growth of the deposit, giving a round grain the size of which can be controlled.

After development, fixation dissolves away the silver bromide crystals that remain in the emulsion. This is normally carried out in a solution of sodium thiosulphate. The developed autoradiograms can then be viewed in a light or electron microscope; as the silver grains will be located on or near the source of radioactivity, the pattern of labelling will reflect visually the distribution of radiation within the specimen. The radiation can be attributed to particular cells and subcellular structures therein.

Quantitative autoradiographic methods at the electron microscope level have improved greatly in recent years and it is possible not only to compare the levels of radioactivity in microscopic sources, but also to measure them in absolute terms (Porter and Barnard, 1975; Fertuck and Salpeter, 1976). Important calibration tests for quantitative autoradiography include determination of resolution and efficiency of the autoradiographic technique used (discussed in detail in Chapter 4).
As the track of the β particle may be long and tortuous, silver grains may be formed at a distance from the radioactive source. The grain density will be highest over the source, falling off symmetrically as the distance increases around it. Resolution is usually defined in terms of the distribution of silver grains around a linear source of radioactivity (Salpeter et al., 1969; 1977). The half distance (HD) is the distance from the source to lines on either side of it which enclose half the silver grains produced by the source. The spread of silver grains depends on geometric factors (such as section and emulsion thickness) and on photographic factors (i.e. diameter of both the silver halide crystal of the emulsion and the developed grain). The shape of the distribution is independent of these factors and dependent on the shape of the source. Thus, particular grain distributions can indicate the geometry of the radioactive source (point, line, hollow circle, etc) (Salpeter, et al., 1969).

The efficiency of the autoradiographic method used can be defined as the number of silver grains produced for every 100 desintegrations in the specimen. Factors which affect efficiency include section thickness, separation of the specimen from the emulsion, density of the specimen, thickness of the emulsion layer, crystal size, development conditions, probability of emulsion saturation, and latent image fading. Ideally, resolution and efficiency should be determined for the autoradiographic method used; values obtained from the literature can only be approximations. As efficiency and resolution have conflicting demands (e.g. for best resolution, the section should be extremely thin and the monolayer of emulsion of the smallest crystal diameter available; however, for best efficiency thicker sections and larger crystals are desirable as this will provide more radioactivity and a higher probability of its detection in the emulsion layer), the technique for any particular experiment is nearly always a compromise between best resolution and best efficiency.
1.7 **THE PRESENT STUDY.**

Botulinum toxin will undoubtedly prove to be a very powerful tool in neurobiology for the study of components of cholinergic nerve endings and for the elucidation of the mechanisms involved in neurotransmitter release in chemically-transmitting nerves. It may also prove invaluable in studies of acceptor-mediated protein transport. In order to realise the full potential of this powerful neurotoxin, a study of its mechanism of action is essential.

The short term goals of this study were to localise (at the ultrastructural level) and quantitate acceptor sites for $^{125}$I-BoNT types A and B at the neuromuscular junction (Chapters 3 and 4), to study the process by which the toxin is internalised (Chapter 5), and to investigate its interaction with other synapses in the peripheral and central nervous systems of different species (Chapter 6). In Chapter 7, the results are discussed in relation to findings obtained from electrophysiological studies, and a possible model for access of the toxin to its target site is discussed.
CHAPTER II

MATERIALS AND METHODS
A. THE NEUROMUSCULAR JUNCTION.

I. In vitro studies

2.1. DISSECTIONS.

Diaphragms of 5-6 week old male or female TO mice were used in most experiments involving the neuromuscular junction (NMJ). Dissection was carried out as follows:

1. Animals were sacrificed by cervical dislocation; chest and abdominal walls were exposed by removing the skin.
2. The abdominal cavity was opened, the liver removed or pushed away from the diaphragm and the oesophagus and aorta were cut.
3. A small incision was made in the diaphragm just below the lower end of the sternum and the muscle was carefully detached from the rib cage and spine using small dissection scissors.
4. The tissue was quickly transferred to a large (90mm) petri dish containing Krebs Ringer solution (0.75mM CaCl₂, 10mM glucose, 124mM NaCl, 5mM KCl, 1.3mM MgSO₄, 1.2mM KH₂PO₄, 20mM Na₂HPO₄) previously gassed for at least 30 minutes with 95% O₂/5% CO₂ and adjusted to pH 7.4 at 22°C.

2.2. INCUBATIONS.

Incubations were performed in small (25mm) petri dishes containing a thin layer (~3mm thick) of dental wax at the base. The diaphragm was usually cut in two along the midline; each hemidiaphragm was then pinned out (to avoid contraction of the muscle fibres) in a separate petri dish. Sometimes the hemidiaphragms were also cut in two, and corresponding tissue sections were used as tests and controls. To minimise the amount of radiolabelled toxin required, small incubation volumes (0.5 - 1ml) were routinely used in localisation experiments.
However, the same results were obtained using larger volumes of continuously aerated incubation medium, as shown in Section 4.3.2. Specific conditions are described in detail in the Methods section and figure legends of each Results chapter.

2.3 FIXATION OF TISSUE AND LOCATION OF ENDPLATE REGIONS.

Fixation was carried out routinely for 60 minutes at 22°C in 2% glutaraldehyde (diluted from a 25% aqueous stock solution with Krebs Ringer).

Glutaraldehyde is a five-carbon dialdehyde (molecular weight 100.14) which is the most widely used primary fixative for electron microscopy (Glauert, 1975). It is an excellent fixative for proteins even though it causes significant irreversible changes in their molecular structure (Lenard and Singer, 1968). These changes, however, are unlikely to be accompanied by morphological effects detectable in electron micrographs of thin sections. The nature of the reaction of glutaraldehyde with proteins remains uncertain, although it appears to involve $\epsilon$-amino groups of lysine side chains (Hayat, 1981). When used alone, glutaraldehyde is not an adequate fixative for general purposes, since certain cell components (e.g. lipids) are not fixed and may be extracted during dehydration. Double fixation with glutaraldehyde and osmium tetroxide ($\text{OsO}_4$) is therefore required for optimal preservation of the tissue (see below).

Following fixation, the tissue was washed in Krebs Ringer (two changes, 5 minutes each).

To locate the end-plate region, histological staining to detect acetylcholinesterase was carried out using the method of Tsuji (1974), with slight modifications. The tissue was immersed in 0.1M acetate buffer, pH 5.0, for a few minutes. It was preincubated for 45-60 minutes at 22°C in 'preincubation medium' containing acetate buffer
(9ml) and 1ml freshly prepared glycine solution (consisting of 0.5M glycine and 0.1M CuSO₄·₅H₂O). This was followed by incubation for 45 minutes at 22°C in a solution of acetylthiocholine iodide (30mg) in 'preincubation medium' (10ml). The tissue was washed extensively in acetate buffer, stained with 0.1% ammonium sulphide for approximately 15 minutes (or until end-plates were visible), and washed again. The end-plates appeared as small black dots in a band coinciding with the branches of the phrenic nerve, which are visible without staining (see diagram below). In this staining method, the thiocholine liberated through enzyme hydrolysis (acetylthiocholine iodide is used as substrate for the enzyme acetylcholinesterase) is trapped in a copperthiocholine complex. The copper is then displaced by ammonium sulphide and rendered visible, at the site of the reaction, as a black precipitate of copper sulphide.

Mouse hemidiaphragm

endplate regions dissected for autoradiography

phrenic nerve

The end-plate regions were cut out in strips no more than 1mm wide; when the tissue was unstained, areas of the diaphragm containing the phrenic nerve were located visually and cut out. The strips were cut transversely into sections ~0.5mm wide. To facilitate location of nerve terminals, the sections were made as small as possible; they were
cut into rectangular shapes to make orientation possible after post-fixation:

The small tissue sections were post-fixed in 1% aqueous OsO$_4$ for 1hr at 22°C and washed thoroughly in Krebs Ringer solution for 10-15 minutes with several changes (at this stage they could be left overnight at 4°C).

The post-fixation was necessary as OsO$_4$ reacts with components of tissues, particularly lipids, that are not fixed by aldehydes. In addition, it acts as a stain, making the structures in the tissue electron dense (Millonig and Marinozzi, 1968). However, like glutaraldehyde, OsO$_4$ is not suitable as a general fixative when used alone. Its main disadvantage is that it penetrates and reacts with tissues so slowly that considerable changes in structure can occur before fixation is complete. Specimens for fixation must therefore be very small (0.5 - 1.0mm thick). Despite these drawbacks, OsO$_4$ is a very useful second fixative and is used routinely following aldehyde fixation.

The fixative is supplied as yellow crystals (1.0g) in glass ampoules. To prepare stock solutions of 2% OsO$_4$ (aqueous), the ampoule was broken and dropped into a brown glass bottle. Distilled water (50ml) was added and the mixture stirred until the OsO$_4$ had dissolved. The solution is stable for several months, but must be stored away from direct sunlight.

**Warning:** OsO$_4$ volatilises readily at room temperature. Vapours are harmful to eyes, nose and throat. The fixative must only be handled in a fume cupboard with good ventilation.
2.4 DEHYDRATION, INFILTRATION AND EMBEDDING.

Dehydration of osmicated tissue sections was carried out by sequential treatment with different concentrations of absolute alcohol at room temperature: 25%, 50%, 75% and 100%, for 5 - 10 minutes each with occasional agitation. After incubation in "dry" ethanol (with added dehydrating agent, i.e. NaSO₄) for approximately 20 minutes, the sections were prepared for embedding in Spurr's epoxy resin (Spurr, 1969). This resin is based on ERL-4206 (vinyl cyclohexene dioxide) and the "soft" mixture was used. Its composition is: epoxy resin ERL-4206 (10g), D.E.R. 736 flexibilizer (diglycidyl ether of polypropylene glycol) (8g), anhydride hardener NSA (nonenyl succinic anhydride) (26g), and S-1 accelerator (dimethylaminoethanol or DMAE) (0.4g). Each component of the medium was added in turn and the final mixture agitated gently for several minutes. It was stored at -20°C in a bottle with a well-fitted cap. This resin is not miscible with water and must be protected from excessive exposure to water vapour in the air.

Infiltration was carried out through a graded series of Spurr's resin in ethanol (25%, 50%, 75%, 100%), for at least 1 hr each, with an additional change in the 100%. Continuous gentle agitation of the mixture was provided by placing the vials on a rotating motor-driven wheel.

For embedding, the specimens were transferred, using a toothpick, to oven-dry silicone rubber embedding moulds and orientated so as to allow transverse sectioning through the muscle fibres. The moulds were filled with fresh embedding medium and polymerization was carried out in an oven at 70°C for 10 hrs.
2.5 LIGHT-MICROSCOPE AUTORADIOGRAPHY.

2.5.1 Sectioning

Blocks were trimmed with a razor blade and 1µm sections of the embedded muscle tissue were cut on an LKB microtome using a glass knife (made using an LKB knife maker as described below). Sections were viewed in a Leitz Ortholux light microscope to locate the nerve. Innervated areas of the block were examined for the presence of nerve terminals by cutting pale gold/silver sections for viewing in a Hitachi-600 electron microscope. Sections were collected on 200-mesh copper grids and stained with 12% uranyl acetate in methanol for 3 - 5 minutes. In sections from blocks where the tissue had been stained previously for acetylcholinesterase, nerve ending-containing areas could be detected in the light microscope.

Once the appropriate areas had been located in several different blocks, the tissue was prepared for light-microscope autoradiography:

1. Dust was cleaned from ordinary 3" x 1" glass microscope slides with vellum tissue.
2. 1µm thick plastic sections were cut and transferred to slides using a platinum (0.2mm wire, 3 - 4mm diameter) loop. The sections were arranged as shown in Fig. 2.1 and allowed to dry and become wrinkle-free on a hot plate at ~50°C.

Preparation of knives: glass knives for ultramicrotomy were made from sheet glass supplied in strips of 25mm width (LKB 7890-04, 400 x 25mm, thickness 6 - 7mm). The strips were cleaned in detergent (e.g. Teepol), scored transversely in the knifemaker (LKB 7800-B) and broken off into squares. The glass squares were then scored diagonally and broken into two pieces, each of which had a sharp and a blunt edge.
Tissue sections (1μm thick) were cut using an LKB ultramicrotome and transferred to glass microscope slides using a platinum wire loop. Test sections from a minimum of two different blocks were arranged in groups along one side of the slide, while groups of control sections were placed on the other. This ensured that test and control tissue was exposed under the same conditions.
2.5.2 Dipping.

Tissue sections were covered with emulsion for light-microscope autoradiography as follows (all steps were carried out in the dark):

1. Ilford L4 emulsion (10g) and distilled de-ionized H₂O (15ml) were mixed in a film container and warmed to 43°C in a water bath (≈15 minutes).

2. The mixture was stirred carefully with a clean glass slide and left for a few minutes until all the air bubbles had disappeared. A sample slide was dipped to ensure uniformity of the emulsion layer.

3. Test slides were dipped vertically into the molten emulsion (at 43°C) and removed after a few seconds. They were drained briefly on paper before wiping the backs with tissue paper.

4. Dipped slides were put on a cold tile for 20 minutes (to allow the emulsion to set) before being transferred to an uncovered slide box in a desiccator to dry overnight.

5. Slides were stored at 4°C in plastic slide boxes containing a small packet of silica gel to maintain dryness. To ensure darkness, boxes were sealed with tape and wrapped in black plastic bags.

2.5.3 Developing.

The emulsion was developed in Kodak D-19 developer (4 minutes at 22°C), with no agitation to minimise background levels. Slides were washed in distilled deionized H₂O for 30 seconds and the emulsion was fixed in 25% sodium thiosulphate for 3 minutes. This was followed by extensive washing in 5 changes distilled deionized H₂O (2 - 3 minutes each). After drying in a warm oven, tissue sections were mounted permanently with 'DEPEX' mounting medium and a coverslip.
2.6 ELECTRON-MICROSCOPE AUTORADIOGRAPHY.

2.6.1 Sectioning.

The procedure followed for electron-microscope autoradiography was essentially the flat substrate method of Salpeter and Bachmann (1972). After locating the end plate region in several test and control blocks (as for light microscopy; see Section 2.3), pale gold/silver sections were cut on an LKB microtome using glass knives. Ultrathin sections were spread with chloroform vapour and collected in groups of 3 - 5 with a clean platinum wire (0.1mm) loop (2mm in diameter); if the thickness of the sections was important, as in quantitative experiments, only pale gold sections were collected. Droplets containing the sections were transferred to glass microscope slides (3" x 1"), previously cleaned with vellin tissue and coated with 0.5% formvar (w/v) in chlorofrom (the dried formvar film gave a silver to gold interference colour when stripped off on water). Sections, arranged as shown in Fig 2.2, were dried on a hot plate at \(\sim 60^\circ C\).

2.6.2 Dipping.

All steps were carried out in the darkroom. Distilled deionized \(H_2O\) (24ml) was added to Ilford L4 emulsion (10g) in a film container which was then placed in a water bath at 43°C for \(\sim 15\) minutes. When the emulsion had melted, the mixture was stirred carefully with a glass slide until homogeneous. The container was transferred to a semi-automatic coating device and left for 1hr to allow the emulsion to cool to 32°C and become free of air bubbles. A blank slide was dipped in the emulsion and withdrawn at a randomly selected speed. It was taken out of the darkroom to check for a purple interference colour (indicating a monolayer of emulsion crystals, \(\sim 170\)nm). The speed of withdrawal was adjusted according to the thickness of the emulsion layer on the blank.
Ultrathin sections (pale gold to silver) were cut using an LKB ultramicrotome and transferred to formvar-coated microscope slides using a small platinum wire loop. Test tissue from different blocks was arranged in at least 8 groups of 3-5 sections each along one side of the slide. Control sections were arranged along the other. In this way test and control sections were exposed under identical conditions.
slide - if too thick, the speed was reduced; if too thin, it was increased. Once adjusted to the required speed, test slides were dipped and placed on a cold glazed tile protected from dust to allow the emulsion to set. If a large number of slides was being dipped, the thickness of the emulsion layer was occasionally checked. After 1 hr, emulsion-coated slides were put into a slide box with a small packet of silica gel and left uncovered in a desiccator overnight. The following morning, the box was covered, sealed with tape, wrapped in black plastic and stored at 4°C.

2.6.3 Exposure.

Exposure was carried out at 4°C for varying periods of time. Most slides were exposed for 3 weeks prior to development.

2.6.4 Developing.

Slides were warmed to room temperature and developed, one at a time, in Kodak D-19 developer at full strength for 2 minutes at 22°C, without agitation. This was followed by a brief wash (30 seconds) in distilled deionized H₂O prior to fixation in 25% sodium thiosulphate for 4 minutes. Slides were washed extensively in distilled deionized H₂O for 30 minutes (6 changes).

2.6.5 Stripping.

After a brief final rinse in distilled deionized H₂O, slides were drained vertically on paper and the backs were dried with tissue. Without allowing the formvar to dry completely, the edges of the formvar film were scraped with sharp forceps and the slide gently immersed in a large petri dish of distilled H₂O at an angle of about 30°. The formvar slowly separated from the slide, floating onto the surface of the water. Grids (copper, 200-mesh) were dropped, matt side up, on to
the sections and the whole formvar film carrying sections, grids and
developed emulsion was collected using a piece of parafilm (see Fig.
2.3). The assembly was allowed to dry in a dust-free atmosphere and
 grids were subsequently cut free from the formvar film.

2.6.6 Staining.

Sections were stained by immersing the grids in droplets of 12%
uranyl acetate in methanol (w/v) for 20 - 30 minutes, followed by
extensive washing in methanol and distilled water. Uranyl ions combine
in large amounts with nucleic acids and phosphate groups. They also
complex at cell surfaces with carboxyl and phosphoryl groups, but not
with sulphhydril groups (Hayat, 1972). These ions are very effective in
scattering electrons due to their large size.

2.6.7 Viewing in the electron microscope.

After the grids had dried, they were viewed in an Hitachi-600
electron microscope operated at 70KV. The thickness of the sections was
determined by the method of Small (1968) and Reedy (1968) using certain
folds in the tissue sections. These folds are routinely produced as the
section dries down on the formvar-coated slide. After staining, the
width of the penetrated stain shows the inner surfaces of a fold to be
virtually in contact (about 1 - 2mm apart), and also perpendicular to
the plane of the section (see Fig. 2.4). The folds have sharply defined
external edges, indicating that they do not bulge at the top. The total
width of the fold is therefore double the section thickness.
Electron microscope autoradiograms were prepared by the flat substrate method (Salpeter et al., 1969). The ribbons of sections were transferred to a formvar-coated slide in a droplet of water (a) and the slide was dried on a hot plate at approximately 60°C (b). This was followed by dipping into molten emulsion (Ilford L4) (c), and draining onto tissue paper (d). A diagram of the various layers on the slide during exposure is shown in (e). After exposure the emulsion was developed and fixed (f). The formvar film, carrying the sections and the emulsion layer was stripped off the slide onto the surface of distilled water (g). Grids were placed over the sections and the film with sections and grids on it was picked up from the water surface with parafilm and dried prior to staining the tissue with uranyl acetate and viewing in the electron microscope.
Staining of the section using 12% uranyl acetate shows that the inner edges of the fold are virtually in contact with one another (arrow) and perpendicular to the plane of the section. The outer edges of the fold are sharply defined indicating that the top does not bulge. The thickness of the section is therefore the distance from the outer edge of the fold to the line of contact between the inner edges (arrows). Magnification of the micrograph is 77600.
II In vivo studies

2.7 TISSUE PREPARATION

Animals were injected intraperitoneally with radiolabelled botulinum neurotoxin (BoNT) and, after death, the tissue required was dissected. Details of experimental procedure are given in the Methods section of the Results chapters.

B. THE CENTRAL NERVOUS SYSTEM.

2.8 CRYOSAT - SECTIONING OF BRAIN TISSUE.

2.8.1 Perfusion of the animal.

Sprague Dawley rats (200 - 250g) were anaesthetized with ether and 1ml Sagatal (60mg/ml pentobarbitone) injected intraperitoneally, before intracardiac perfusion with phosphate buffered saline (PBS) (50ml, containing 10mM Na$_2$PO$_4$, 10mM Na$_2$HPO$_4$, and 0.9% NaCl, pH 7.4) to remove blood. This was followed by 0.1% (w/v) paraformaldehyde/PBS pH 7.4 (250ml), over a period of 20 -25 minutes. Stock paraformaldehyde solutions were prepared by heating a mixture of paraformaldehyde powder (5g) and distilled H$_2$O (50ml) in a fume cupboard (with stirring) until steaming, followed by dropwise addition of concentrated NaOH until the mixture cleared. The solution was then allowed to cool and stored at 4°C for up to 1 week.

2.8.2 Dissection, mounting and freezing.

After perfusion to fix the tissue, brain areas were dissected. The cerebellum was detached from the forebrain, cut in two along the midline and each half mounted on a small, flat piece of cork using Tissue-Tek II (cut surface down). The forebrain was cut transversely in half and each piece was mounted on a piece of cork, cut side down. The tissue was frozen quickly by immersion in isopentane at -40°C.
2.8.3 Cryostat sectioning, mounting and hardening.

Blocks of frozen material were placed in a Bright cryostat microtome (model OTF/AS/M/V) for approximately 30 minutes to adjust the temperature of the tissue. Serial sections, 10μm thick, were cut and thaw-mounted in pairs on glass slides (3" x 1") previously subbed with gelatin. Slides were subbed as follows:

1. Distilled H₂O (300ml) was heated until steaming.
2. Chrome alum (0.15g) was added and stirred until dissolved.
3. Gelatin (1.5g) was added and the mixture boiled for 10 minutes with stirring.
4. The gelatin mixture was cooled and filtered into a square glass container.
5. Dust-free slides were dipped into the gelatin (in groups of 25 in a slide holder) for 30 seconds.
6. Excess gelatin was drained onto tissue paper and slides were left to dry overnight in a warm oven (30°C).

Mounted cryostat sections were placed in slide boxes containing silica gel to maintain dryness. Boxes were sealed with tape and stored at -20°C for at least 48 hours to allow the sections to harden.

2.8.4 Preparation of slides for use of ³H-ultrofilm, exposure and development.

After sections had been incubated with radiolabelled toxin and washed thoroughly as described for each experiment performed, they were immersed in 3% paraformaldehyde (for 15 minutes) to inactivate free toxin not removed in the washes. They were rinsed in a large volume of distilled water, drained on tissue paper, and dried with a hair-dryer at 30°C. The slides were placed in slide boxes which were left uncovered in a desiccator containing silica gel. The following day, they were arranged in an autoradiogram cassette with double-sided
Sellotape (in some cases, they had to be cut down so they would all fit in one cassette and thus be exposed under the same conditions).

2.8.4.1 Exposure.

A sheet of LKB $^3$H-ultrofilm was fixed, emulsion side up, to the cassette lid with very small pieces of double-sided Sellotape. (Gloves were worn when handling the film to avoid fingerprints and other artefacts.) The cassette was then closed and wrapped in a black plastic bag with a packet of silica gel to maintain dryness. Exposure was carried out at 4°C.

2.8.4.2 Development.

All steps were performed at 18 - 20°C. The film was developed in Kodak D-19 developer for 5 minutes, washed for 5 minutes in running tap water to remove the developer, and fixed in 25% Sodium thiosulphate for 6 minutes. It was washed extensively for 20-25 minutes and air-dried.

2.8.4.3 Histological staining of brain sections.

After the exposure period, tissue sections were stained with 0.2% safranin (aqueous) for 2 minutes at 22°C, washed in distilled water and differentiated in absolute ethanol. The safranin stained the nuclei red.

2.9 ELECTRON-MICROSCOPE AUTORADIOGRAPHY USING UNFIXED BRAIN TISSUE.

2.9.1 Dissection of the cerebellum and preparation of cerebellar slices.

The preparation of surface slices from cerebellar vermis was carried out essentially by the method of Garthwaite (personal communication). Gillette Techmatic cutting ribbon (9.5cm), trimmed to width of about 1mm with sharp scissors, and secured under tension in a holder as shown in Fig. 2.5, was used to cut the slices. The thickness
Fig. 2.5 Cutter used for preparation of cerebellar slices.
(designed by J. Garthwaite, Institute of Neurology).

FRAME: This is made from steel wire (3mm in diam.) bent to the shape illustrated above (actual size).

BLADE: Gillette Techmatic cutting ribbon trimmed to a width of about 1mm.
of the sections was determined using a guide (Fig. 2.6) made from a piece of glass 76mm x 38mm and 2 No.2 coverslips (about 200µm thick, 22mm x 64mm) (Chance Propper Ltd.). The cleaned coverslips were cut longitudinally with the aid of a ruler and diamond, and a resulting half-coverslip was glued with Loctite glass bond (which remains fluid until exposed to UV light) to each side of the clean rectangle of glass. The assembly was warmed (60 - 70°C) to allow the glue to spread thinly and then exposed to UV light to harden it. The process was repeated with the two remaining half-coverslips. Any spaces were filled in with additional glue and the excess was removed with a razor blade. The depth of the resulting central recess was measured accurately with a micrometer and came to be 400µm. Cutting was carried out on a "cutting table" consisting of a cylindrical brass base (6.5cm high x 5cm in diameter) to which a circular polished perspex top (1.2cm high x 5cm in diameter) was attached.

An adult Sprague-Dawley rat (200 - 250g) was killed by stunning; the cerebellum was excised and placed, dorsal surface upwards on a piece of 5.5cm hardened filter paper (Whatman No.54) moistened with fresh Krebs solution (containing 118mM NaCl, 4.7mM KCl, 2.5mM CaCl₂, 25mM NaHCO₃, 1.18mM KH₂PO₄, 1.19mM MgSO₄, 7H₂O, 11mM glucose, gassed with 95% O₂/5% CO₂ and adjusted to pH 7.4 at room temperature) and placed on the cutting table. The cutter, with moistened blade, was picked up with the right hand and placed below the guide (held in the left hand with thumb uppermost) and a slight upward pressure was maintained against it (Fig. 2.7). The guide was then lowered onto the surface of the vermis and the tissue only very slightly compressed. Using a side-to-side motion (2 - 5mm) and a slight forward pressure of the cutting ribbon, a slice was cut and rapidly transferred to a petri dish containing fresh Krebs solution at room temperature. Only one slice was taken from each cerebellum and was obtained about 70 seconds
The guide was made from a piece of glass 76mm x 38mm and 2 No.2 coverslips, cut in two longitudinally. Two half-coverslips were glued, one above the other, to each side of the piece of glass with Loctite glass bond. The depth of the resulting central recess measured 400µm.
The cutter, with moistened blade, was held in the right hand and placed under the guide, which was held with the left (thumb uppermost). The guide was then lowered onto the surface of the cerebellum, and the tissue was compressed slightly. Using a side to side motion and a slight forward pressure of the ribbon, a slice was cut and transferred to a petri dish containing Krebs Ringer solution at 22°C.
after killing the animal. Speed in cutting slices was essential because of the known detrimental effects of hypoxia on brain tissue. The slices were carefully transferred to incubation vessels with a spatula.

2.9.2 Incubations.

10ml conical flasks, sealed with rubber stoppers through which two syringe needles were inserted, were used for incubations. The incubation volume used was 3ml. The surface of the incubation medium was gassed continuously with 95% \( O_2 \)/5% \( CO_2 \) through one of the needles; 95% \( O_2 \)/5% \( CO_2 \) was bubbled through water in a 500ml Drechsel gas washing bottle with a sintered Drechsel bottle head and distributed to the incubation flasks through small gassing tubes. The other needle served as a gas outlet. The flasks were placed in a shaking water bath at 37°C for the incubation period. Washes were carried out at 37°C with pre-gassed Krebs solution and the slices were transferred to fresh medium using a spatula. Details of conditions used in experiments are given in Chapter 6, Materials and Methods section.

2.9.3 Fixation and osmication.

The tissue was fixed in 2.5% glutaraldehyde/4% paraformaldehyde/0.1M phosphate buffer (pH 7.4) for 1 hour at room temperature. After rinsing in phosphate buffer, it was osmicated for 1 hour in 1% \( OsO_4 \)/0.1M cacodylate buffer and subsequently washed extensively (3 times, 5 minutes each) in the same buffer.

2.9.4 Electron-microscope autoradiography.

The osmicated cerebellar slices were dehydrated in ethanol and embedded in Spurr's resin as described in Section 2.4. Before putting them into moulds, they were cut into smaller pieces and orientated with the dorsal surface uppermost, so as to ensure that both the molecular
and the granular layers as well as some myelin could be seen in ultrathin sections cut for electron-microscope autoradiography. These pieces were prepared and processed as described for the neuromuscular junction (Section 2.6).

C. SYNAPTOSOMES AND CELL LINES.

2.10 SYNAPTOSOMES.

2.10.1 Preparation.

Rat cerebrocortical synaptosomes were prepared by the method of Cotman and Matthews (1971). Adult female Sprague-Dawley rats (200 - 250g) were killed by stunning and the whole cerebral cortex was rapidly excised and placed in ice-cold 2mM HEPES, pH 7.5, containing 0.32M sucrose. Using a glass-perspex homogenizer, a 10% (w/v) homogenate was prepared and centrifuged in a Beckman L565 Ultracentrifuge using a Beckman type 30 rotor (10000 xg for 10 minutes). The pellet (P1) obtained was discarded and the supernatant (S1) was centrifuged (type 30 rotor, 20 minutes at 20000 xg) to yield the P2 and S2 fractions. The crude mitochondrial pellet (P2) was resuspended by homogenisation in 2mM HEPES, pH 7.5/0.32M sucrose, and 15ml of this suspension was carefully layered onto a discontinuous density gradient consisting of 8% (w/v) Ficoll (20ml); all Ficoll solutions contained 2mM HEPES buffer, pH 7.5. The Ficoll gradients were centrifuged (76000 xg for 1 hour using a Beckman SW25.1 rotor) to yield three fractions: P2A, P2B, and P2C. The synaptosomal fraction (P2B) was collected at the interface between the 8% and 13% Ficoll solutions, in approximately 10-11%. This was diluted with 1.5 volumes of 0.32M sucrose. The purified synaptosome suspension was sedimented at 54000 xg for 20 minutes (type 30 rotor), and the pellet was resuspended in the required volume of Krebs phosphate medium for subsequent experiments.
2.10.2 Processing for electron-microscope autoradiography.

After the final wash following incubation with toxin, the labelled synaptosomes were centrifuged at 9000 xg for 5 minutes in an Eppendorf centrifuge to form a well-packed pellet. The supernatant was then removed and replaced with 0.5ml 2% glutaraldehyde (diluted from a 25% stock with Krebs Ringer) for 1 hour at room temperature. The pellet was carefully dislodged from the bottom of the tube using a toothpick and washed twice for 5 minutes in Krebs Ringer prior to postfixation for 1 hour in 1% OsO₄. After extensive washing in Krebs Ringer solution, the pellet was dehydrated and embedded following the procedure used for the neuromuscular junction (Section 2.4). Sections were process for electron-microscope autoradiography as described in Section 2.6.

2.11 CELL LINES.

2.11.1 Cell lines tested for ability to bind ¹²⁵I-BoNT type A.

Localisation of acceptor sites for ¹²⁵I-BoNT type A was attempted on several neuroblastoma cell lines (obtained from Dr. J. Kemshead, Imperial Cancer Research Fund): these included CHP100, MR32, NB212, Lan 1 and TR14 (believed to be cholinergic; T. Rupniak, personal communication). The leukaemic cell line HL60 was used as control. The binding of ¹²⁵I-BoNT type A to PC12 cells (Pheochromocytoma cell line, derived from the adrenal medulla) was also studied (cells were kindly provided by Mr. Anthony Ashton of this laboratory). Details of incubation conditions are given in Chapter 6.

2.11.2 Preparation of cells for electron-microscope autoradiography.

After a final wash in growth medium (+ foetal calf serum, FCS) or Krebs Ringer solution (+ bovine serum albumin, BSA), the cells were
pelleted by centrifugation and fixed, dehydrated and embedded following the same procedure as that described for synaptosomes (Section 2.10.2).

D. \( ^{125}\text{I-RAIODLABELLING OF PROTEINS.} \)

2.12.1 \( ^{125}\text{I-Iodination of BoNT type A for autoradiographic studies.} \)

\textit{Clostridium botulinum} type A neurotoxin, purified to homogeneity by affinity chromatography on p-aminophenyl-\(\beta\)-D-thiogalactopyranoside CH-Separose-4B followed by DEAE-Sephacel ion-exchange chromatography (Tse et al., 1982), was routinely labelled with \( ^{125}\text{I} \) using a modification of the Chloramine-T method (Greenwood et al., 1963) as described by Williams et al., (1983). BoNT (100 - 200\( \mu \)g) in a maximum volume of 200\( \mu l \) was added to 5m Cl (50\( \mu l \)) carrier-free Na\( ^{125}\text{I} \) (Amersham International, Amersham) in a plastic microfuge tube, and the reaction was initiated by the addition of a freshly prepared solution of Chloramine-T (10\( \mu l \); final concentration 0.22mM) in 100mM sodium phosphate buffer, pH 7.4, containing 150mM NaCl. After 30 seconds (with gentle agitation), the reaction was quenched by the addition of an excess of L-tyrosine (25\( \mu l \); 2mg/ml in 100mM sodium phosphate buffer containing 150mM NaCl, pH 7.4). \( ^{125}\text{I-BoNT} \) was separated from L-tyrosine and free iodine by gel-filtration on a Sephadex G-25 (superfine) column (5 x 1.5cm), pre-equilibrated with 100mM sodium phosphate buffer, pH 7.4, containing 150mM NaCl. To ensure stability of the neurotoxin in dilute solution, 2.5\% (w/v) gelatin was added to a final concentration of 0.25\% (w/v), after removal of aliquots for determination of protein (2 x 200\( \mu l \)) and radioactivity (2 x 10\( \mu l \)). The amount of free radiolabel present in a labelled-toxin sample (5- 10\( \mu l \)) was determined by precipitation with trichloroacetic acid (10\% v/v), using bovine serum albumin (100\( \mu g \)) as a carrier protein. The specific activity was accurately determined from the radioactive and protein
contents [obtained by $\gamma$-counting in a Wallac $\gamma$-counter and colorimetrically by the method of Lowry et al., (1951), respectively]. High specific radioactivity (450-1750 Ci/mmol) was obtained routinely and the preparations retained appreciable levels of biological activity (approximately 60 - 85% of the original, equivalent to $2 \times 10^8$ mouse lethal doses/mg protein) as shown by serial, intraperitoneal injections into mice. The majority (90% - 97%) of the radioactivity in the preparations was acid-precipitable (indicating low levels of free $^{125}$I-iodine) and both of the toxin's subunits were found to be labelled (Williams et al., 1983). The high levels of specific radioactivity and neurotoxicity of these preparations rendered them suitable for autoradiographic studies. In most cases, the $^{125}$I-BoNT type A used was kindly provided by Dr. Richard Williams of this laboratory; however, a few experiments were carried out using my own preparation of radiolabelled toxin (Chapters 3 and 6). Before performing localisation experiments, the $^{125}$I-labelled toxin was shown to be active by its ability to bind saturably to rat cerebrocortical synaptosomes.

2.12.2 $^{125}$I-Iodination of botulinum neurotoxin type B for autoradiographic studies.

Type B BoNT was $^{125}$I-radiolabelled following the procedure used for type A neurotoxin (Section 2.12.1). $^{125}$I-BoNT type B of high specific radioactivity (600 - 1000 Ci/mmol) was obtained routinely with a neurotoxicity approximately 60% of that displayed by native type B neurotoxin. (Labelled preparations were kindly proved by Dr. R. Williams.)
2.12.3 $^{125}$I-Iodination of bovine serum albumin for efficiency determinations in autoradiography.

Bovine serum albumin (BSA) was radiolabelled with $^{125}$I using essentially the Chloramine-T method of Greenwood et al. (1963). BSA (250μg) in a volume of 50μl phosphate buffered saline (PBS) pH 7.4 was added to 1mCi (10μl) carrier-free Na$^{125}$I (Amersham International, Amersham) in a plastic microfuge tube. The reaction was started by the addition of a freshly prepared solution of Chloramine-T (25μl; final concentration, 267μg/ml) in PBS. After 2 minutes incubation with agitation, the reaction was quenched by the addition of a fresh solution of sodium metabisulphite (25μl; final concentration 227μg/ml) in PBS. PBS (100μl) and sodium iodide (25μl; final concentration, 192mM) were also added. $^{125}$I-labelled BSA ($^{125}$I-BSA) was separated from free iodine by gel filtration on a Sephadex G-25 superfine column (5 x 1.5cm), pre-equilibrated with PBS, pH 7.4. Aliquots from the fractions collected were counted in a Wallac $\gamma$-counter (50% efficiency) (Fig. 2.8), and those containing the $^{125}$-labelled protein peak were pooled. The specific radioactivity of the labelled BSA was determined accurately from the radioactive and protein contents. A specific activity of 400 Ci/mmol was obtained for $^{125}$I-BSA; the majority of the radioactivity (99%) in the pooled fractions was precipitated with trichloroacetic acid (12%; w/v) indicating low content of free $^{125}$I-iodine.
Fig. 2.8 Separation of $^{125}\text{I}$-BSA from free $^{125}\text{I}$-iodine by gel filtration.

$^{125}\text{I}$-BSA was separated from free $^{125}\text{I}$ by gel filtration on a column of Sephadex G-25 superfine (5 x 15cm) equilibrated with phosphate buffer saline, pH 7.4. Eluted fractions were collected and 10μl aliquots were counted in a Wallac γ-counter. Arrow (↓) points to the radiolabelled protein peak; fractions 5-10 were pooled and the specific radioactivity of the $^{125}\text{I}$-BSA was determined from the radioactive and protein contents.
CHAPTER III

LOCALISATION OF ACCEPTOR SITES FOR BOTULINUM NEUROTOXIN TYPES A AND B
AT THE MURINE NEUROMUSCULAR JUNCTION.
3.1 INTRODUCTION.

Pharmacological and biochemical studies have pinpointed the cholinergic nerve terminal as the target for several of the botulinum toxin types. Toxin administered to mammals in vivo exerts its effects at four different sites: the neuromuscular junction (Dickson and Shevky, 1923a,b; Edmunds and Long, 1923; Burgen et al., 1949), autonomic ganglia (e.g. Ambache, 1952), postganglionic parasympathetic nerve endings (e.g. Bigalke and Habermann, 1980) and postganglionic sympathetic nerve endings that release acetylcholine (Ambache, 1951a,b). However, most studies dealing with the mechanism of action of the toxin have focused on the neuromuscular junction. Dickson and Shevky (1923a,b) and Edmunds and Long (1923) showed that botulinum toxin does not impair muscle activity (muscles still responded to direct stimulation and to appropriate agonists) and does not block impulse propagation in the nerve trunk or its flow into the terminal arborizations of the nerve. These collective findings suggested that the toxin acts at a point in the transmission between nerve and muscle. Using isolated neuromuscular preparations, Burgen et al., (1949) showed that botulinum toxin diminishes neurogenic release of acetylcholine. Subsequent studies showed that blockade does not result from inhibition of synthesis, storage or metabolism of acetylcholine (at least in the CNS) (Wonnacott and Marchbanks, 1976; Gundersen and Howard, 1978; Gundersen, 1980); the toxin appears to exert its paralytic action by antagonising events triggered by calcium which culminate in neurotransmitter release (Cull Candy et al., 1976; Lundh et al., 1976; Lundh et al., 1977). Early investigations showed that target tissues bind the toxin long before toxicity is expressed (Burgen et al., 1949), indicating that binding is probably not directly responsible for paralysis. Onset of neuromuscular blockade has been found to be related to rate of nerve stimulation (Hughes and Whaler, 1962): nerves stimulated rapidly become paralysed
more quickly than quiescent or slowly stimulated nerves. Collectively, these findings suggest that the nerve terminal, or a site nearby, is the target for the toxin's actions.

Attempts to localise the peripheral site of action of the toxin by histological and autoradiographic techniques at the ultrastructural level have been unsuccessful. Zacks et al., (1962), using ferritin-labelled type B botulinum toxin-haemagglutinin complex and electron-microscopy, showed binding to the amorphous surface material in the primary and secondary clefts at the murine neuromuscular junction. However, with the technique of electron-microscope autoradiography, Habermann (1974) was unable to reproduce these results. Studies at the light microscope level have been more informative. Using fluorescein-labelled type A toxin or antitoxin antibodies, Zacks et al. (1968) showed binding at axon terminals and/or in the subneural apparatus in skeletal muscle but not in other tissues such as kidney, heart, liver, and brain. Hirokawa and Kitamura (1975) demonstrated specific binding at the neuromuscular junction using high concentrations of $^{125}$I-labelled toxin type A; however, saturability of binding was not tested in this study. Moreover, the limits in level of resolution of the technique did not permit localisation of the toxin at a pre- or post-synaptic site. Toxin-haemagglutinin complex was used in the localisation studies of Zacks et al., making their results somewhat suspect because of the observed ability of tritiated complex to bind avidly to carbohydrate-containing moieties on a variety of membranes (Dolly et al., 1981).

Using data obtained from a series of pharmacological experiments, Simpson (1973, 1974, 1978, 1980) proposed a model for the mechanism of action of botulinum toxin at the neuromuscular junction. He suggested that intoxication is the result of a complex multi-phasic process involving a binding step, a translocation step and an intraterminal
paralytic step. In this scheme, binding to cell surface acceptors is essential to the development of paralysis, but in itself does not produce toxic effects. Likewise, the translocation step, during which the bound toxin (or a fragment thereof) is taken up into the nerve terminal, is essential but produces no detrimental effects. Finally, the internalised toxin interferes with an intracellular target, the outcome of which is blockade of neurotransmitter release. However, this model was based on results obtained by indirect methods and no morphological data were available to support it.

Other investigators have suggested that the toxin acts directly by physically blocking a process involved in neurotransmitter release. Tentative models include the 'lock and key' mechanism proposed by Hanig and Lamanna (1979) in which toxin blocks a gate involved in regulating transmitter efflux, and interference with a calcium channel suggested by Hirokawa and Heuser (1981). There is no firm evidence to support either of these proposals; direct demonstration of the interaction of botulinum toxin with target cells, as well as quantitation of binding sites, is necessary to establish the validity of any of the suggested models.

Competition studies in the central nervous systems have shown that, in most cases, the antigenically different botulinum neurotoxin (BoNT) types bind specifically to distinct sites at isolated nerve terminals. There is overlap in the binding of types A and B and of types A and E but types B and E do not appear to compete for binding sites (Kozaki, 1979; Williams, 1984). Similar studies have not been carried out at the neuromuscular junction.

A major obstacle to localisation studies has been the unavailability of a biologically active, radiolabelled toxin derivative of high specific radioactivity which remained stable upon storage. The successful preparation of $^{125}$I-BoNT types A and B in this laboratory
(Williams et al., 1983; Williams, 1984) was an important step towards the elucidation of the toxin's site of action.

In this Chapter, the interaction of $^{125}$I-BoNT types A and B with the neuromuscular junction is investigated by light- and electron-microscope autoradiography, both in vitro and in vivo. Competition studies are carried out to establish whether the toxins bind to the same site(s) at the neuromuscular junction.

3.2 MATERIALS AND METHODS

Localisation of $^{125}$I-BoNT types A and B in a murine nerve-muscle preparation

3.2.1 Studies at the light microscope level.

For in vitro localisation studies, nerve-muscle preparations were treated with $^{125}$I-BoNT type A (0.2nM or 15nM) in 0.5ml Krebs Ringer, pH 7.4, for 90 or 180 minutes at room temperature. The tissue was then washed (for 20-30 minutes at 4°C or 120 minutes at 22°C) and fixed in 2% glutaraldehyde. Half of the fixed tissue was stained to detect acetylcholinesterase at the neuromuscular junction by the acetylthiocholine technique (Tsujl, 1974) (see Section 2.3). Control samples were incubated with radiolabelled toxin (as for the test tissue) with the addition of a 100-fold molar excess of native neurotoxin.

For in vivo localisation of the toxin in diaphragm tissue, $^{125}$I-BoNT type A [100μl of a 75nM solution; 1.05μg or $\sim 8.8 \times 10^4$ MLD$_{50}$ in Krebs Ringer/1% BSA (1 MLD$_{50}$ corresponds to the amount of toxin sample, injected intraperitoneally, required to kill 50% of a representative mouse population after 4 days; Williams, 1984)] was injected intraperitoneally into a mouse. Death occurred as a result of respiratory failure after 80 minutes. The diaphragm was dissected and treated as above (in vitro studies). A control animal was injected with
100μl Krebs Ringer/1% BSA containing 1.05μg $^{125}$I-BoNT type A and a 50-fold excess of native neurotoxin type A (total: $\leq 4.5 \times 10^6$ MLD$_{50}$).

All tissue was processed for light microscope autoradiography as described in Chapter 2 and slides were exposed for 2 weeks at 4°C.

3.2.2 Studies at the ultrastructural level.

3.2.2.1 Interaction of $^{125}$I-BoNT types A and B with the neuromuscular junction.

For in vitro and in vivo localisation studies, nerve-muscle preparations (mouse diaphragm) were treated with $^{125}$I-BoNT type A (or B) as described in Section 3.2.1 and processed for electron microscope autoradiography as described in Chapter 2 (with the omission of acetylcholinesterase staining). Slides were exposed for 3 weeks to 6 months at 4°C.

In order to localise the acceptor sites for $^{125}$I-BoNT type A (or B) at the murine motor nerve terminal, diaphragm tissue was preincubated with the inhibitors of mitochondrial oxidative phosphorylation azide (15mM) or dinitrophenol (0.5mM) for 20-30 minutes at room temperature, prior to incubation with $^{125}$I-BoNT type A (or B).

The presence of acceptor sites for BoNT type A on the plasma membrane of areas of the motor nerve which are myelinated could not be determined in adult mice, as the protective myelin sheath does not allow access of the toxin to the axonal membrane. Because motor nerves in the diaphragm of newborn rats are incompletely myelinated at this stage in development, this tissue was used as a model in which to determine the extent of binding of $^{125}$I-BoNT type A along the nerve axon. Newborn rats were killed by stunning and the diaphragm was dissected and detached from the rib cage before treatment with radiolabelled toxin (30nM).
3.2.2.2 Effect of nerve stimulation on the distribution of toxin molecules at the murine motor nerve terminal.

Diaphragms were dissected from 2 mice with the phrenic nerve intact as follows:

1. The chest wall was exposed by removing the skin.

2. Cuts were made alongside the sternum (1) and laterally at the lower end of the thorax (2) (after the first cut, the inside of the thorax was inspected to ensure that the nerve had not adhered to the chest wall). The right side of the thorax was also cut (3) and then the rest of the sternum, as shown below (4);

![Diagram of dissection procedure](image)

Fig. 3.1. Procedure for dissection of a murine phrenic nerve-hemidiaphragm preparation.

3. The upper part of the sternum was removed to expose the phrenic nerve running from the diaphragm towards the thymus gland.

4. The lung was removed and the nerve followed as high as possible, carefully teasing it out. The end was tied with a piece of cotton and cut.

5. The diaphragm was then removed by cutting through the backbone and placed in Krebs' Ringer/0.5% BSA. It was cut in two along the midline.
Each hemidiaphragm was attached to an electrode by means of two small pins through the rib cage. The phrenic nerve was placed in the appropriate holder provided (see Fig. 3.2). A length of cotton was attached to the hemidiaphragm by means of a small hook through the connective tissue at the apex. Each nerve-muscle preparation was immersed in 8ml Krebs Ringer/0.5% BSA, gassed gently with 95% O2/5% CO2 at room temperature; the attached cotton was tied to a transducer for recording of the trace in one case (test sample) and to a suitable object (e.g. clamp or a retort stand) in an equivalent position in the other (control sample). 125I-BoNT type A (600 Ci/mmol) was then added to each bath to a final concentration of 10nM and one hemidiaphragm was stimulated electrically at 0.25Hz until complete blockade of neurotransmission was observed (150 minutes). The hemidiaphragms were washed twice in the organ baths for 3 minutes with Krebs Ringer/0.5% BSA at 4°C, and then removed, detached from the rib cage and pinned out on wax in separate 25mm petri dishes. Thorough washing was continued for a further 20 minutes and followed by fixation and processing for electron-microscope autoradiography.

After 4 months exposure at 4°C, the developed and stained tissue sections were viewed in the electron microscope. The extent of labelling in test and control sections was quantitated as follows: about 20-30 end-plates from each preparation were photographed at 6000x magnification and autoradiograms were printed at a final magnification of 18,000x. The nerve terminal plasma membrane was traced onto acetate sheets and its total length was determined by digitisation using a Hewlett-Packard digitiser. The silver grains associated with the nerve terminals were then counted. The section thickness was determined by the
Fig. 3.2 Set-up for electrical stimulation of a nerve-muscle preparation. (see text for details)
method of Small (1968) (see Section 2.6.7) and only those of similar thickness were photographed. Grain density for each preparation was expressed as number of developed silver grains/μm of membrane (on the assumption that all internalised grains came from the plasma membrane). A comparison was also made between the number of grains internalised in stimulated and unstimulated nerve terminals.

3.2.2.3 Interaction of BoNT types A and B at the motor nerve terminal.

To study the effect of BoNT type B on the binding of $^{125}$I-BoNT type A, a small piece of mouse diaphragm tissue was preincubated in 0.5ml Krebs Ringer/~1.5μM native type B BoNT for 1hr at 22°C. $^{125}$I-BoNT type A was then added to a final concentration of 15nM and incubation was continued for a further 90 minutes at 22°C prior to extensive washing. A control sample of tissue was preincubated in 0.5ml Krebs Ringer for 1 hr at 22°C and then treated with $^{125}$I-BoNT type A as above.

After 3 weeks exposure, sections were developed, stained and viewed in a Hitachi-600 electron microscope. About 20-30 end-plates from each sample were photographed at 10,000x magnification; prints were made at a final magnification of 19,000x and the total presynaptic membrane lengths determined by digitisation. The number of grains per unit length of membrane was determined for test and control preparations.

The effect of BoNT type A on the binding of $^{125}$I-BoNT type B was investigated using the same procedure as that described above except that the preincubation medium for the test sample contained BoNT type A at a concentration of 1μM (instead of type B). The incubation medium for both test and control samples
contained 10nM $^{125}$I-BoNT type B. Prints were made at a final magnification of 30,000x.

3.3 RESULTS

Part A Localisation of $^{125}$I-BoNT type A in a murine nerve-muscle preparation.

Light-microscope autoradiography

3.3.1 In vitro and in vivo localisation of $^{125}$I-BoNT type A.

Samples of tissue, previously incubated with $^{125}$I-BoNT type A in vitro, were examined after 2 weeks exposure. Silver grains were seen in discrete clusters on several muscle fibres in the unstained sections, with both concentrations used (15nM and 0.2nM) (Fig. 3.3a,c). These clusters appeared at the edge of the fibres (in transverse section) and were comparable in contour and size to the neuromuscular junction (as observed by acetylcholinesterase staining). No silver grains were detected on nerve bundles or blood vessels. In test sections where autoradiography had been combined with histochemical localisation of acetylcholinesterase, the clusters of silver grains corresponded with the sites reacting with acetylthiocholine (Fig. 3.4a). Owing to limitations of resolution in the technique used, it was not possible to establish whether the observed binding was pre- or postsynaptic, or both. Control sections, both stained and unstained, were totally devoid of silver grain deposits (Figs. 3.3b, 3.4b) indicating that the binding was saturable as well as specific for the synaptic areas. Silver grain deposits were also clearly visible when the radiolabelled toxin was applied at a concentration of 0.2nM and the tissue was subsequently washed for 2 hours at room temperature (Fig. 3.3c). The extent of
Fig. 3.3  Localisation in vitro of $^{125}$I-BoNT type A target sites in mouse diaphragm muscle by light-microscope autoradiography.

Test diaphragm tissue (a) was incubated with $^{125}$I-BoNT type A (15nM) in 0.5ml Krebs Ringer, pH 7.4, for 90 minutes at 22°C. A control sample (b) was treated similarly, except that a 100-fold excess of unlabelled neurotoxin was included in the incubation medium. After washing for 30 minutes at 4°C, the specimens were fixed, embedded and processed for light microscope autoradiography. Silver grains were seen in discrete clusters in the test specimens after 2 weeks exposure; no grains were detectable in the controls.

Another sample (c) was treated with $^{125}$I-BoNT type A at a more physiological concentration (0.2nM) for 3 hours at 22°C followed by extensive washing for 2 hours at 22°C. It was then processed as described above. Silver grains were still detectable in discrete accumulations at the edges of muscle fibres in transverse section.
Test (a) and control (b) specimens were treated with $^{125}\text{I-BoNT}$ type A as described in Fig. 3.3 (a,b) and then stained for acetylcholinesterase by the acetylthiocholine technique (Tsuji, 1974) (Section 2.3). Silver grain clusters in test specimens coincided with areas stained for the enzyme, indicating that the toxin binds specifically to the neuromuscular junction. The binding is saturable as no grains were detected at stained areas in control samples.
labelling was qualitatively indistinguishable from that seen when the tissue was washed for only 20-30 minutes at 4°C (data not shown) suggesting that the interaction of the toxin with the neuromuscular junction was essentially irreversible.

In tissue treated with $^{125}$I-BoNT type A in vivo, silver grain deposits were also observed at the edge of several muscle fibres (Fig. 3.5a). In stained sections (Fig. 3.6a), these accumulations coincided with acetylcholinesterase staining and were, therefore, localised at the neuromuscular junction as observed in the in vitro localisation studies. Control preparations were completely lacking in silver grain clusters (Figs. 3.5b, 3.6b) indicating the saturability of the binding sites involved. No other structures in these sections showed specific labelling (e.g. nerve bundles, blood vessels).

Localisation studies at the light microscope level showed, therefore, that $^{125}$I-BoNT type A, applied to a mouse diaphragm in vitro or in vivo interacts saturably and specifically with the neuromuscular junction areas of the tissue.

Electron microscope autoradiography

3.3.2 In vitro and in vivo localisation of $^{125}$I-BoNT type A at the ultrastructural level.

Test sections, incubated with radiolabelled toxin in vitro and exposed for 3 weeks, showed deposition of silver grains at the motor nerve terminal, both on the presynaptic plasma membrane and within the nerve terminal cytoplasm (Fig 3.7a). The majority of the developed grains, i.e. 62% of the total, were on the membrane (Table 3.1). The internalised radioactivity, which represented 38% of the total associated with the nerve terminal, did not appear confined to any particular intraterminal compartment (although grains were sometimes
Fig. 3.5 Localisation in vivo of the target sites for $^{125}$I-BoNT type A in mouse diaphragm muscle by light-microscope autoradiography.

$^{125}$I-BoNT type A (1.05µg or $8.8 \times 10^4$ MLD$_{50}$) was injected intraperitoneally into a mouse. Death occurred as a result of respiratory failure after 80 minutes. The paralysed diaphragm was excised, washed extensively and processed for light-microscope autoradiography. A control animal was injected with 1.05µg $^{125}$I-BoNT type A and a 50-fold excess of native type A neurotoxin (total: $\sim 4.5 \times 10^6$MLD$_{50}$). Silver grains clusters were seen at the edge of several muscle fibres (arrows) in test specimens (a) as previously observed in preparations exposed to toxin in vitro; however, no grains were seen in the controls (b).
Fig. 3.6 Localisation of $^{125}$I-BoNT type A target sites in vivo in tissue stained to detect acetylcholinesterase at the neuromuscular junction.

Test and control tissue samples, treated as described in Fig. 3.5 (a, b), were stained to detect acetylcholinesterase as described in Chapter 2 (Section 2.3). In test specimens (a), silver grains were seen accumulated in areas which were also stained histologically, indicating that the target site for $^{125}$I-BoNT type A is the neuromuscular junction in vivo as well as in vitro (Fig. 3.4a). Binding of toxin to its target sites is saturable as no grains were detectable in any areas of control sections (b).
Fig. 3.7 Interaction of $^{125}$I-BοNT type A with the murine motor nerve terminal.

a. Pale gold sections from a nerve-muscle preparation treated with $^{125}$I-BοNT type A (15nM) in 0.5ml Krebs Ringer, pH 7.4, for 90 minutes at 22°C, washed extensively for 30 minutes at 4°C and processed for electron-microscope autoradiography. Slides were exposed for 3 weeks at 4°C and developed in D-19.

b. Control preparation treated as described in (a) in the presence of a 100-fold excess of native BοNT.
Table 3.1 Distribution of silver grains at the motor nerve terminal under different conditions.

<table>
<thead>
<tr>
<th>Labelled Structures</th>
<th>Treatment</th>
<th>Distribution of silver grains with respect to the plasma membrane of the motor nerve (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>on (or close to it)</td>
</tr>
<tr>
<td>Synaptic boutons</td>
<td>( ^{125}\text{I-BoNT type A} ) (15nM) (in vitro)</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>( ^{125}\text{I-BoNT type A} ) (in vivo)</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>( ^{125}\text{I-BoNT type A} ) (11nM) + Na Azide (15mM)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>( ^{125}\text{I-BoNT type A} ) (10nM) + nerve stimulation</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>( ^{125}\text{I-BoNT type B} ) (10nM) (in vitro)</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>( ^{125}\text{I-BoNT type B} ) (20nM) + Na Azide (15mM)</td>
<td>100</td>
</tr>
<tr>
<td>Myelinated motor nerve axons</td>
<td>( ^{125}\text{I-BoNT type A} ) (in vivo)</td>
<td>58</td>
</tr>
</tbody>
</table>

Large numbers (20-50) of nerve endings or axons from preparations treated with radiolabelled toxin types A or B under different conditions were photographed. The silver grains on the nerve membrane and inside the synaptic bouton or axon were counted independently and expressed as % of the total. A grain was considered to be 'on the membrane' if it was located within 60-70nm of the plasma membrane (see Section 4.3.5 on resolution in electron-microscope autoradiography with \( ^{125}\text{I} \)).
associated with the membrane of endosome- or lysosome-like structures, see Fig. 3.8). The exact location of the external acceptor sites could not be established from these autoradiograms as they were masked by internalised radioactivity. However, labelling was not restricted to the synaptic bouton as grains were also deposited on or near the plasma membrane of all unmyelinated axons (i.e. areas of the motor nerve distal to the final node of Ranvier that lead to the synaptic bouton) (Fig 3.9). No grains were seen on muscle fibres, Schwann cells, blood vessels, myelin, or connective tissue (Fig. 3.7a). Control sections were virtually devoid of grains (Fig. 3.7b); native BoNT type A reduced the extent of labelling to less than 4% of that seen at test nerve terminals. Collectively, these results showed that the interaction of 125I-labelled type A toxin with its target tissue (i.e. all unmyelinated areas of the motor nerve terminal) was both specific and saturable.

The source of the silver grains associated with unmyelinated axons could not be established from these autoradiograms. The grains could represent toxin molecules bound to an external site on the axonal plasma membrane. Alternatively, toxin previously bound to sites on areas of the terminal membrane opposite the postsynaptic folds could have been taken up and carried to an internal site on the axolemma by retrograde intra-axonal transport (see below). Internal and external sites on the plasma membrane could not be distinguished due to limits in resolution of the autoradiographic technique.

The labelling observed in sections from preparations treated with toxin in vivo was comparable to that seen in the in vitro samples. Grains were deposited on the nerve terminal plasma membrane and within the cytoplasm (Fig. 3.10a); unmyelinated axonal membrane was labelled and, interestingly, silver grains also appeared within myelinated axons suggesting that retrograde intra-axonal transport of toxin had occurred
Fig. 3.8 Binding of $^{125}$I-BoNT type A to the membrane of intracellular vesicular structures.

Nerve endings from a hemidiaphragm preparation treated with $^{125}$I-BoNT type A (15nM) for 90 minutes at 22°C, washed extensively and processed for electron-microscope autoradiography. Silver grains were seen associated with the presynaptic membrane and also with the membrane of intracellular vesicles (arrows).

Fig. 3.9 Association of $^{125}$I-BoNT type A with the plasma membrane of unmyelinated axons.

Transverse section through an unmyelinated axon (a) which is surrounded loosely by Schwann cell processes (s). The tissue was treated with $^{125}$I-BoNT type A as described in Fig. 3.7a. Silver grains are seen associated with the plasma membrane and also with intra-axonal vesicles (arrow).
Fig. 3.10 Interaction of $^{125}$I-BoNT type A with motor nerves after in vivo administration of radionlabelled toxin to mice.

a. Motor nerve terminals labelled with $^{125}$I-BoNT type A after intraperitoneal injection of $\mu$g of radio-iodinated toxin (in 100μl Krebs Ringer/1% BSA) into a mouse. The animal died after 80 minutes; the diaphragm was excised, washed extensively with Krebs Ringer at 4°C (6 changes, 5 minutes each) and processed for electron-microscope autoradiography. Slides were developed after 3 weeks exposure.

b. Unmyelinated axon from a mouse hemidiaphragm treated as described in (a). The labelled axonal membrane is surrounded loosely by Schwann cell processes (s).

c. Transverse section through myelinated axons treated as described in (a). (1) Silver grains are associated with the axoplasm suggesting that the toxin is carried up the axon by retrograde axonal transport. (2) Silver grains are seen close to the axonal membrane (arrow) suggesting that the toxin is carried by the fast component of axonal transport in tubular (or vesicular) structures which lie directly below the plasma membrane.
(Figs. 3.10b,c). The ratio of internalised to membrane-bound grains at the terminal was 37:63; the proportion of internalised grains was thus comparable to that observed at nerve terminals exposed to radiolabelled toxin in vitro (where the ratio was 38:62). Silver grains seen within myelinated axons were associated with the axonal membrane (58% of the grains seen were on or close to axolemma, see Fig. 3.10c2) and also with the axoplasm (42%, see Fig. 3.10c1). No labelling of muscle fibres, Schwann cells, blood vessels, collagen or myelin was detected in any of the sections. The specificity of the interaction of $^{125}$I-BoNT type A with neuronal tissue was re-emphasised in this experiment; also, the similarity of the labelling in preparations exposed to toxin in vivo and in vitro suggests that the in vitro model is valid for localisation studies.

3.3.3 Localisation of the acceptor sites for $^{125}$I-BoNT type A at the motor nerve terminal.

Initial experiments, performed in vitro and in vivo, showed that $^{125}$I-BoNT type A interacts with the motor nerve terminal specifically and saturably. Grains were seen on the plasma membrane and inside the synaptic bouton; however, the exact location of the saturable acceptor sites was not apparent as they were masked by internalised grains. In the presence of 15mM sodium azide, an inhibitor of mitochondrial oxidative phosphorylation, the internalisation of radioactivity was prevented totally (Fig.3.11a) (90% of the grains counted were found on the membrane itself, 10% were within 150nM of the latter and could be attributed to expected scatter, see Table 3.1 and Chapter 4). The total number of grains was slightly diminished compared to samples untreated with azide.

Since sodium azide inhibits the production of energy within the cell, the internalisation of $^{125}$I-labelled toxin molecules is probably
Fig. 3.11 Localisation of acceptor sites for $^{125}$I-BoNT type A at the murine motor nerve terminal.

Nerve muscle preparations were treated with sodium azide (15mM) for 30 minutes at 22°C. The tissue was then incubated with $^{125}$I-BoNT type A (11nM) in the presence of the metabolic inhibitor for 90 minutes (22°C) and washed extensively with Krebs Ringer/15mM azide (6 changes, 5 minutes each) prior to processing for electron-microscope autoradiography. Slides were developed after 3 weeks exposure at 4°C in D-19.

a. Motor nerve terminals labelled with $^{125}$I-BoNT type A. Silver grains show the location of acceptor sites for the toxin.

b. Motor nerve terminals treated with labelled BoNT type A in the presence of excess (100-fold) native neurotoxin. Absence of silver grains suggests saturability of the acceptor sites.
energy-requiring process. By preventing uptake, the precise location of the external binding sites became apparent; radioactivity was restricted to the presynaptic membrane but extended uniformly to all the unmyelinated terminal arborizations of the nerve (Figs. 3.11a, 3.12). No grains were detectable on the protective myelin sheath, but the possibility that binding sites are also present on the plasma membrane of the myelinated axons (to which the toxin has no access) could not be excluded. The nerve terminal membrane in control sections was virtually devoid of grains; this re-emphasised the saturability of the acceptor sites for $^{125}$I-BoNT type A (Fig. 3.11b). Dinitrophenol ($5 \times 10^{-4}$M), another inhibitor of energy production, also prevented uptake of radioactivity into the nerve terminal. Grains were detectable on all unmyelinated areas of the nerve terminal plasma membrane in test sections (Fig. 3.13), but not on those of controls (data not shown). The binding was thus saturable and the internalisation energy-dependent.

3.3.4 In vitro localisation of $^{125}$I-BoNT type A in a nerve-muscle preparation from newborn rats.

Newborn rats are highly susceptible to intoxication by $^{125}$I-BoNT type A, dying within a few hours of intraperitoneal injection of low ($< 1$MLD$_{50}$ for adults) doses of the toxin. Accordingly, binding of $^{125}$I-BoNT type A was detected at the developing motor nerve terminal in the diaphragm of newborn rats (Fig. 3.14a). As motor neurons innervating the diaphragm are still unmyelinated at this stage of development (Zacks, 1973) and the Schwann cells that loosely surround the axons do not appear to prevent access of the toxin to the axonal plasma membrane, the possible distribution of acceptor sites could be evaluated along the entire length of the nerve in this preparation. It was found that some (although few) grains were present on the axonal areas nearest the developing terminal matrices, but no labelling was
Fig. 3.12 Saturable acceptors for $^{125}$I-BoNT type A on the membrane of unmyelinated axons.

Nerve-muscle preparations were treated as described in Fig. 3.11.

Autoradiogram of a longitudinal section through an unmyelinated axon and a synaptic bouton. Silver grains are seen on the axonal plasma membrane (arrow) suggesting that acceptor sites for $^{125}$I-BoNT type A are present in these areas of the nerve terminal as well as on the plasma membrane of the synaptic bouton. Note the Schwann cell processes (s) loosely surrounding the axon.
Fig. 3.13 Binding of $^{125}\text{I-BoNT}$ type A to the murine motor nerve terminal in the presence of dinitrophenol.

Mouse diaphragm tissue was treated with the metabolic inhibitor dinitrophenol (0.5mM) for approximately 20 minutes. It was then incubated with 0.5ml Krebs Ringer solution/11nM $^{125}\text{I-BoNT}$ type A/0.5mM dinitrophenol for 90 minutes at 22°C. Following extensive washing in Krebs Ringer/0.5mM dinitrophenol for 30 minutes (5 changes; 6 minutes each), the tissue was fixed and processed for electron-microscope autoradiography. Slides were exposed for 3 weeks at 4°C. The autoradiogram shows a nerve terminal labelled with $^{125}\text{I-BoNT}$ type A in the presence of dinitrophenol. Silver grains represent membrane acceptors for the toxin.
Fig. 3.14 Interaction of $^{125}$I-BoNT type A with developing motor nerve terminals in newborn rats.

The diaphragm of a newborn rat was dissected, detached from the rib cage, and pinned out on wax. The tissue was incubated with $^{125}$I-BoNT type A (30nM) in 0.5ml Krebs Ringer, pH 7.4 for 90 minutes at 22°C, prior to extensive washing for 30 minutes with Krebs Ringer at 4°C. It was then fixed and processed for electron-microscope autoradiography. Slides were exposed for 4 weeks at 4°C.

a. Developing motor nerve terminals labelled with $^{125}$I-BoNT type A. Note the postsynaptic folds in the muscle cell (f) and the numerous small axon terminals (nt) embedded in an overlying Schwann cell (s).

b. Larger nerve terminal (nt) from a newborn rat diaphragm labelled with $^{125}$I-BoNT type A. Note the internalised silver grain (arrow) possibly associated with a vesicular structure (v), the overlying Schwann cell (s) and the postsynaptic folds in the muscle cell (f).

c. Control nerve terminal (nt) treated with radiolabelled type A BoNT in the presence of excess (3µM) native neurotoxin. The absence of silver grains indicates saturability of the acceptor sites.

d. Longitudinal section through unmyelinated axons (a) loosely wrapped by Schwann cells (s) in a newborn rat diaphragm preparation treated as described above. Although the nerve terminal areas (nt) are labelled with $^{125}$I-BoNT type A, no silver grains are detectable on the unmyelinated axonal membrane.

e. Transverse section through axon bundles (a) surrounded by Schwann cells (s) which are destined to become myelinated in the adult. Absence of silver grains on the axonal membrane suggests that the acceptor sites for $^{125}$I-BoNT type A are found only on the nerve terminal membrane.
detectable in the large bundles of axons more distant from terminal regions (Fig. 3.14d,e). These observations suggest that the binding sites are located only in those areas of the axonal plasma membrane which are not destined to become myelinated, i.e. the terminal areas, although the possibility that the binding component appears at a later stage of development cannot be excluded. The labelling of newborn rat motor nerve terminals was saturable, as controls were completely devoid of silver grains (Fig. 3.14,c). Most of the terminals were too small to allow detection of internalisation but, in those that were slightly larger, uptake was apparent (Fig 3.14b, arrow).

3.3.5 Localisation of $^{125}$I-BoNT type A in a preparation stimulated electrically until transmission was blocked.

In this experiment, both test and control preparations were set up in exactly the same way - each hemidiaphragm was attached to an electrode and immersed in a bath of Krebs Ringer/0.5% BSA containing $^{125}$I-BoNT type A (10nM) and gassed continuously with 95% O$_2$/5% CO$_2$ at 22°C, although only one of these was electrically stimulated. The twitch response in this preparation was abolished after 150 minutes (Fig. 3.15a). After four months exposure of ultrathin sections cut from test and control blocks, silver grains were seen at nerve endings and on unmyelinated axons in both preparations (Fig. 3.15b,c). Grains at the nerve terminal appeared on the plasma membrane and in the cytoplasm indicating that uptake of toxin had occurred but, interestingly, the ratio of membrane-bound to internalised grains was different in unstimulated and stimulated preparations. In the former, the silver grains on 22 nerve endings were counted; the total came to 51 grains, 61% of which were on the membrane and 39% within the terminal. This accords very closely with the ratio obtained in preliminary in vitro experiments (62%:38%). In the test preparation, however, of 102 grains
Fig. 3.15 Localisation of $^{125}\text{I-BoNT}$ in a preparation stimulated electrically until transmission was blocked.

A nerve-muscle preparation (mouse hemidiaphragm) was attached to an electrode and immersed in 8ml Krebs Ringer/0.5% BSA gassed gently with 95% O$_2$/5% CO$_2$ at 22°C. It was stimulated electrically at 0.25 Hz for 15 minutes prior to the addition of $^{125}\text{I-BoNT}$ type A to a final concentration of 10nM. When complete blockade of neurotransmission was observed, the hemidiaphragm was washed thoroughly, fixed and processed for electron-microscope autoradiography. Slides were exposed for 4 months at 4°C.

a. Trace of the muscle response to nerve stimulation in the presence of $^{125}\text{I-BoNT}$ type A. Twitch was abolished after 150 minutes.

b. Autoradiogram showing the effect of stimulation on the distribution of $^{125}\text{I-BoNT}$ type A molecules at the motor nerve terminal. Silver grains are seen on the nerve terminal membrane and in the cytoplasm but the proportion of internalised radioactivity is greater than in controls (by 50%, see Table 3.1).

c. Transverse section through an unmyelinated axon from a twitch preparation labelled with $^{125}\text{I-BoNT}$ type A.
counted (19 nerve terminals), 41% were membrane-bound and 59% had been translocated across it (Table 3.1). The total number of grains per μm of membrane was the same in each case (0.31 for the test and 0.31 for the control). The results obtained in this experiment showed that binding of the toxin to cell surface acceptors was unaffected by nerve stimulation; the translocation step, however, appeared to be in some way enhanced by it.

Part B  Localisation of $^{125}$I-BoNT type B in a murine nerve-muscle preparation.

3.3.6 In vitro localisation of $^{125}$I-BoNT type B under physiological conditions and in the presence of metabolic inhibitors.

The similar overall pharmacological actions exerted by BoNT Type A and B at the neuromuscular junction prompted an investigation of the interactions of the latter with the murine motor nerve terminal. After 11 days exposure, nerve terminals incubated with 10nM (and 30nM, data not shown) $^{125}$I-BoNT type B were labelled with silver grains in a manner similar to those incubated with $^{125}$I-BoNT type A (Fig. 3.16a). Grains were deposited both on the plasma membrane and within the synaptic bouton itself, suggesting that uptake of the toxin, or a fragment thereof, had taken place. However, the extent of internalisation was considerably reduced compared to that seen with $^{125}$I-BoNT type A; whereas the ratio of membrane-bound to internalised grains for type A was approximately 62% to 38%, that for type B was 75% to 25% (25 end plates counted) (Table 3.1). As with A, in many cases a number of the intra-terminal grains were associated with endosome-lysosome-like structures (Fig. 3.16a). The binding was saturable as no labelling was detectable at control terminals treated with excess native type B neurotoxin (Fig. 3.16b). Saturable binding to axonal plasma membrane was
Fig. 3.16 Interaction of $^{125}$I-BoNT type B with the murine motor nerve terminal.

A small sample of tissue was treated with $^{125}$I-BoNT type B (10nM) in 0.5ml Krebs Ringer, pH 7.4, for 90 minutes at 22°C. It was then washed extensively with the same buffer for 30 minutes fixed and processed for electron-microscope autoradiography. Slides were exposed for 11 days at 4°C.

a. Five synaptic boutons labelled with $^{125}$I-BoNT type B. Note silver grains on the presynaptic plasma membrane and within the terminal cytoplasm where they are associated with vesicular structures (arrows).

b. Control nerve terminals treated with $^{125}$I-BoNT type B in the presence of excess (100-fold) native toxin. Control samples showed ~1% of the labelling seen in test sections indicating that the interaction of $^{125}$I-BoNT type B with the motor nerve terminal is saturable.
also observed; this extended as far as the terminal node of Ranvier (Figs. 3.17a,b). Muscle fibres, Schwann cells, myelin, blood vessels and connective tissue were all unlabelled. Some retrograde axonal transport was detectable (Fig. 3.18) as seen previously with type A BoNT.

Acceptor sites for $^{125}$I-BoNT type B were detectable at the motor nerve terminal in the presence of 15mM azide (Fig. 3.19a). They were uniformly distributed on the presynaptic plasma membrane, including the unmyelinated axonal branches leading to the synaptic bouton (Fig. 3.20) as observed previously with type A BoNT. 90% of the grains counted (299 in total) were on the plasma membrane, the other 10% were very close to it. The binding was saturable (as no grains were detectable in control preparations) as well as specific for nerve terminal areas (Fig. 3.19b). Since sodium azide is an inhibitor of mitochondrial oxidative phosphorylation, uptake of toxin into the cytosol must be an energy-dependent process.

Part C Interaction of types A and B Botulinum neurotoxins.

3.3.7 Effect of BoNT type B on the binding of $^{125}$I-BoNT type A at the murine motor nerve terminal.

The total number of silver grains deposited at 20 nerve endings in test (type B + $^{125}$I-BoNT type A) and control ($^{125}$I-BoNT type A alone) preparations was determined and expressed per total length terminal plasma membrane (The latter was measured in µm by digitisation). This was carried out on the assumption that any internalised grains had come from the surrounding plasma membrane. Pre-incubation with a 100-fold excess of type B neurotoxin and incubation in the presence of the latter did not prevent binding and internalisation of $^{125}$I-BoNT type A at the motor nerve terminal (Fig. 3.21a). Quantitative analysis showed that, in sections incubated with radio-
Mouse diaphragm tissue was treated with $^{125}$I-BoNT type B as described in Fig. 3.16.

a. Unmyelinated axon labelled with $^{125}$I-BoNT type B right up to the final node of Ranvier. Note that, although this preparation was treated in the absence of metabolic inhibitors, all silver grains are associated with the neuronal plasma membrane. The proportion of internalised grains seen at nerve endings exposed to $^{125}$I-BoNT type B was considerably lower than that observed at endings exposed to $^{125}$I-BoNT type A (25% and 40% of the total, respectively).

b. The acceptor sites for $^{125}$I-BoNT type B on axonal membrane are saturable as excess native neurotoxin prevented the binding of labelled derivative completely.
Fig. 3.18 Retrograde intra-axonal transport of $^{125}$I-BoNT type B.

Final node of Ranvier (arrows) in a preparation treated with $^{125}$I-BoNT type B as described in Fig. 3.17. Note the labelling of axoplasm (a) suggesting occurrence of retrograde axonal transport of radio-iodinated toxin molecules, or fragments thereof.

x13500
Fig. 3.19 Acceptor sites for $^{125}$I-BoNT type B at the murine motor nerve terminal.

A small sample of diaphragm tissue was preincubated in Krebs Ringer/15mM sodium azide for 30 minutes at 22°C. It was then incubated in 0.5ml Krebs Ringer/20nM $^{125}$I-BoNT type B/15mM Na azide for 90 minutes at 22°C followed by extensive washing. The tissue was then fixed, processed for electron-microscope autogradiography and the slides were exposed for 11 days at 4°C.

a. Autoradiogram of a motor nerve terminal showing the acceptor sites for $^{125}$I-BoNT type B. Like those for $^{125}$I-BoNT type A, they are located on all areas of the synaptic bouton plasma membrane.

b. Control nerve terminal treated with $^{125}$I-BoNT in the presence of excess (100-fold) unlabelled toxin. The absence of silver grains shows the saturability of the acceptor sites.
Fig. 3.20 Acceptor sites for $^{125}$I-BoNT type B on the plasma membrane of unmyelinated axons.

A mouse diaphragm was treated as described in Fig. 3.19. Acceptor sites for $^{125}$I-BoNT type B are detectable on the axolemma (arrow) as previously observed with $^{125}$I-BoNT type A. Note the uniform distribution of silver grains on all areas of the motor nerve terminal membrane.
Fig. 3.21 Interaction of $^{125}$I-BoNT type A with motor nerve terminals pretreated with native BoNT type B.

a. Four synaptic boutons labelled with $^{125}$I-BoNT type A in the presence of type B BoNT. The tissue was preincubated with native type B BoNT (1.5μM) for 1 hour at 22°C prior to the addition of radiolabelled type A BoNT to a final concentration of 15nM. Incubation was continued for a further 90 minutes, followed by washing and processing for electron-microscope autoradiography. Slides were exposed for 3 weeks at 4°C.

b. Control nerve terminals labelled with $^{125}$I-BoNT type A alone. The tissue was preincubated in Krebs Ringer for 1 hour at 22°C and then treated with $^{125}$I-BoNT type A as above.
labelled type A alone (Fig. 3.21b), 0.67 silver grains occurred per μm of membrane (134 grains/201μm) (Table 3.2). In sections preincubated with B native neurotoxin, 0.71 grains were detected per μm membrane (or 175 grains/248μm). Expressed relative to the binding in control sections (100%), the amount of binding in test preparations was 106%. Thus, there was no effect of type B BoNT on the binding of $^{125}$I-BoNT type A (the slight differences can be attributed to experimental error). It appears, from these results, that types A and B botulinum neurotoxins have different acceptor sites at the murine motor nerve terminal.

3.3.8 Effect of BoNT type A on the binding of $^{125}$I-BoNT type B at the murine motor nerve terminal.

Type A BoNT did not inhibit the binding of $^{125}$I-BoNT type B totally, as silver grains were still seen at terminals pre-incubated with type A toxin (Fig. 3.22a). A quantitative analysis of the results obtained showed that 1.10 grains occurred per μm of plasma membrane (333 grains/304μm) in sections incubated with $^{125}$I-BoNT type B alone (Table 3.2, Fig. 3.22b). Terminals pre-incubated with type A native neurotoxin showed 0.84 grains per micron of membrane (272 grains/325μm). Specimens exposed to type A BoNT, therefore, showed only 75% of the binding of radiolabelled type B BoNT seen at control nerve endings. In contrast to the observations made when terminals were pre-incubated with type B BoNT followed by incubation with $^{125}$I- BoNT type A, the results shown here suggest that type A may interact with some acceptor sites for type B and thus prevent access of the latter to these sites. Type A need not necessarily bind to the same site as type B to prevent its binding; interaction with its own site could produce steric interference which may occlude some of the binding sites (24%) for type B on the nerve terminal membrane.
Table 3.2 Interactions of types A and B Botulinum neurotoxins.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Number of grains/μm plasma membrane</th>
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</thead>
<tbody>
<tr>
<td>$^{125}$I-BoNT type A (15nM)</td>
<td>0.67 (100%)</td>
</tr>
<tr>
<td>Type B BoNT (1.5μM) + $^{125}$I-BoNT type A (15nM)</td>
<td>0.71 (106%)</td>
</tr>
<tr>
<td>$^{125}$I-BoNT type B (10nM)</td>
<td>1.10 (100%)</td>
</tr>
<tr>
<td>Type A BoNT (1μM) + $^{125}$I-BoNT type B (10nM)</td>
<td>0.84 (76%)</td>
</tr>
</tbody>
</table>

Tissue samples were treated with BoNT types A and B as detailed in Section 3.2.2.3 of Materials and Methods. After 3 weeks exposure, the emulsion was developed in Kodak D-19 developer and tissue sections were stained with 12% uranyl acetate. They were viewed in the electron microscope and about 20-30 end-plates from each preparation were photographed. The total length of presynaptic membrane seen in the prints was measured in μm by digitisation; the number of grains associated with the nerve terminals was determined and grain density was expressed as no. of grains/μm plasma membrane (for each different preparation). This allowed the labelling observed under different conditions to be compared in a quantitative manner.
Fig 3.22 Interaction of $^{125}$I-BoNT type B with motor nerve terminals pretreated with native type A BoNT.

a. Four synaptic boutons treated with $^{125}$I-BoNT type B in the presence of native BoNT type A. Tissue samples were pretreated with BoNT type A (1μM) for 1 hour at 22°C. $^{125}$I-BoNT type B was then added to a final concentration of 10nM and incubation was continued for a further 90 minutes. The tissue was then washed and processed for electron-microscope autoradiography. Slides were exposed for 3 weeks at 4°C. Note that there were fewer grains than in control sections (see below; Table 3.2).

b. Nerve terminals treated with $^{125}$I-BoNT type B alone.
3.4 DISCUSSION

3.4.1 Interaction of $^{125}$I-BoNT type A with the murine neuromuscular junction: a light-microscope autoradiographic study.

Light-microscope autoradiographic studies showed that $^{125}$I-BoNT type A binds specifically to structural components of the neuromuscular junction in the mouse diaphragm, thus confirming results obtained previously (Hirokawa and Kitamura, 1975). Furthermore, these investigations showed that the binding is detectable at relatively low (and thus more physiological) toxin concentrations ($1.25 \times 10^3 \text{MLD}_{50}/\text{ml}$ or 0.2nM) compared with the $1.3 \times 10^6 \text{MLD}_{50}/\text{ml}$ used in the earlier study. In addition, intraperitoneal administration of the radiolabelled toxin in vivo gave a similar pattern of labelling at synaptic areas, emphasising the validity of the in vitro model for studies of the interaction of BoNT with target tissues. The acceptor sites were found to be saturable as control specimens, incubated with excess native neurotoxin, showed no localised silver grain accumulations. Finally, the interaction of BoNT with the neuromuscular junction was essentially irreversible: the extent of labelling was qualitatively indistinguishable after washing for 2 hours at room temperature or for 20-30 minutes at 4°C. This irreversibility of binding can be explained, in part, by subsequent uptake of toxin molecules into the nerve terminal (Simpson, 1980; see below) or sequestration within the plasma membrane as suggested by Yavin et al., (1983) for tetanus toxin (see below).

3.4.2 $^{125}$I-BoNT at the motor nerve terminal: an ultrastructural study of binding and internalisation.

The level of resolution of the light-microscope autoradiographic technique did not permit precise localisation of the saturable acceptor
sites for BoNT in the synaptic region. Ultrastructural studies were necessary to distinguish between pre- and postsynaptic sites and membrane-bound and/or intracellular targets.

Histological localisation of botulinum toxin at the ultrastructural level was attempted by Zacks et al., (1962) using ferritin-labelled type B toxin. However, these workers used the toxin-haemagglutinin complex in their study. The large numbers of ferritin particles they observed in the amorphous surface material of primary and secondary clefts of the postsynaptic apparatus, therefore, may be attributed to interactions of the latter with the haemagglutinin moiety of the type B toxin complex used. In the present study, direct evidence of the interaction of $^{125}$I-BoNT types A and B with the motor nerve terminal is presented for the first time. After labelling of nerve-muscle preparations with $^{125}$I-BoNT type A or type B in vitro, in conditions known to block neurotransmission (at least in the case of type A as shown in Section 3.3.5), electron-microscope autoradiograms showed distinct deposition of silver grains at motor nerve endings. The labelling was saturable as control samples treated with excess native neurotoxin contained few, if any, silver grains. The specificity of the interaction of these BoNT types with neuronal tissue was emphasised by the absence of grains on muscle cells, Schwann cells, blood vessels, connective tissue and myelin. The labelling was not restricted to the plasma membrane of the synaptic bouton as silver grains were also present within the terminal cytoplasm. As shown previously (Simpson, 1980), the kinetics of the toxin's neuroparalytic action are consistent with the involvement of a target site within the nerve terminal; the findings presented here provide the first direct demonstration of the internalisation of BoNT and thus support the results of earlier pharmacological studies. (However, it is not possible to ascertain from these results whether internalised silver grains represent the intact
toxin molecule or a fragment of it, see Chapter 5).

In the case of $^{125}$I-BoNT type A, approximately 60% of the grains associated with the nerve terminal were found on the plasma membrane, the remaining 40% being inside. The proportion of internalised grains at nerve endings labelled with $^{125}$I-type B BoNT was consistently 25% of the total (75% were membrane-bound). A possible reason for this pattern of labelling is that the majority of the molecules in the type B toxin preparation used were un-nicked (DasGupta, 1981; Williams, 1984) and probably in an inactive form. The neurotoxin is synthesised as a single chain and then undergoes two post-translational modifications: a cleavage known as 'nicking' which converts the single chain molecule into the dichain form, and another which produces activation (a molecular change still unidentified) (see Introduction). These modifications are probably essential for the uptake of toxin into the nerve terminal. In the case of type A BoNT, all molecules in the preparation used were both nicked and activated. It is interesting that type B is considerably less toxic (1.1. x $10^8$ MLD$_{50}$/mg) than type A botulinum toxin ($2 \times 10^8$ MLD$_{50}$/mg) in mice (Williams, 1984). If, as Simpson has suggested (1980), internalisation of toxin is required for expression of toxicity, the demonstrable reduction in the proportion of translocated radioactivity (~40% less uptake relative to that seen with type A) could account for lower toxicity of BoNT type B because nicking and activation may be required for entry of toxin into the cytosol. Moreover, in this scheme, unnicked toxin molecules taken up in endocytic vesicles could remain bound to their acceptors and be returned to the cell surface during acceptor-recycling. This would result in a net increase in the proportion of membrane-bound silver grains (see Chapter 5) as observed at nerve endings labelled with $^{125}$I-BoNT type B.

Another relevant observation involves the distribution of radio-label within the toxin molecule; the heavy and light subunits of
I-BoNT type A contain 60% and 40% of the radiolabel respectively (Williams et al., 1983) whereas, in the case of type B, the heavy subunit contains nearly all the radioactivity (Williams, 1984). The distribution of silver grains might therefore reflect a mechanism in which the intact toxin binds the neuronal membrane and the light subunit, thought to be responsible for toxicity, subsequently enters the cytosol by direct penetration of the cell membrane to exert its paralytic action.

In autoradiograms of tissue treated with \(^{125}\text{I}-\text{BoNT}\) under physiological conditions, acceptor sites for type A and B were masked by internalised radioactivity. However, toxin uptake could be prevented totally by inhibitors of oxidative phosphorylation (and thus of energy production in the cell), such as azide and dinitrophenol, indicating that internalisation is an energy-requiring process. In samples treated with metabolic inhibitors, membrane-bound silver grains were uniformly distributed on all unmyelinated areas of the nerve terminal; acceptor sites are therefore not located solely in the synaptic region but can also be found on axonal membrane. In contrast to previous suggestions (Hanig and Lamanna, 1979), it appears that the binding sites are not involved directly in the process of neurotransmitter release as their location is not restricted to areas of the membrane opposing the postsynaptic folds (active zones), where release is thought to occur (it must be emphasised that this pattern of binding was also seen at low toxin concentrations, e.g. 1.5nM; see Chapter 4). Since the protective myelin sheath did not allow access of the toxin to the axonal plasma membrane it encloses, the presence of toxin acceptors in areas beyond the terminal node of Ranvier was investigated in newborn rats. Myelination is incomplete at this stage in development (Kelly and Zacks, 1969), so diaphragms of newborn rats were useful as a model for such studies. Newborn rats are highly susceptible to intoxication by BoNT
type A (data not shown) and accordingly, a saturable acceptor was found at the developing nerve terminal. The large bundles of nerves destined to become myelinated however, were not labelled by the toxin. It appears, therefore, that the binding site(s) is restricted to unmyelinated terminal arborizations of the motor nerve. Thus, these autoradiographic studies are the first to show saturable sites for a presynaptic toxin selectively located on the motor nerve terminal membrane.

3.4.3 Possible mechanism of toxin uptake.

In samples treated under physiological conditions, excess native neurotoxin inhibited binding of radiolabelled BoNT completely and no grains were detected within the nerve terminal cytoplasm. Thus bulk endocytosis, which is unsaturable (Steinman et al., 1983), can be ruled out as the main mechanism of entry of toxin into the cytoplasm. It would seem, therefore, that the binding step is essential for translocation.

The process by which uptake occurs cannot be determined with certainty from these autoradiograms; however, the fact that silver grains were often associated with the membrane of endosome or lysosome-like structures suggests that acceptor-mediated endocytosis (Goldstein, 1979) is the mechanism involved. There is no evidence that BoNT possesses enzymic activity (Simpson, 1981) and the molecule is too large to penetrate any of the ion channels known to be present in neuronal membranes. Thus, the possibility of an active process inherent in the membrane such as endocytosis, which is almost universal among cells (Steinman et al., 1983) including those in the nervous system (Heuser and Reese, 1973), is attractive. The intracellular structures involved in acceptor-mediated endocytosis (i.e. coated pits, coated vesicles, endosomes and lysosomes; see General Introduction) are present in nerve endings. A number of characteristics of this process are relevant to
the uptake of BoNT. As reported by Ceccarelli et al., (1973) the rate of endocytosis is proportional to exocytosis. Thus, nerves that are stimulated rapidly will undergo vigorous exocytosis, with concomitant accelerated endocytosis, relative to nerves at rest. The rapid rate of endocytosis would result in uptake of a greater proportion of toxin molecules bound to the presynaptic membrane. There is evidence to suggest that intoxication by BoNT occurs more rapidly in stimulated than in quiescent nerves (Hughes and Whaler, 1962). Moreover, results presented in this Chapter show that the proportion of internalised grains in preparations that were electrically stimulated during the incubation period (with $^{125}$I-BoNT type A) was 50% greater than that of nerves incubated at rest. Increased uptake could account for greater toxicity; this finding re-emphasises the possibility of an intracellular target for the toxin. The findings reported in this Chapter are so far consistent with Simpson's (1980) three-step mechanism of intoxication. The toxin binds to cell surface acceptors which do not appear to be directly involved with transmitter release, but which are responsible for toxin translocation into the cell. It then appears to be actively internalised in vesicles (by acceptor-mediated endocytosis) from which it probably enters the cytosol to exert its 'lytic' action.

It must be emphasised that there is no direct evidence at this stage to confirm that the bound or endocytosed toxin is the same form of BoNT that causes blockade of neuromuscular transmission (but see Chapter 5). However, the fact that the morphological data shown here is so consistent with Simpson's model of toxin action suggests that, at least some of it, is biologically significant.
3.4.4 Retrograde intra-axonal transport of $^{125}$I-BoNT type A in vivo.

Autoradiograms of tissue treated with $^{125}$I-BoNT type A in vivo also showed deposition of silver grains at the nerve terminal, with 60% of the radioactivity occurring on the plasma membrane and 40% in the nerve terminal cytoplasm. Unmyelinated axons were also labelled. The presence of silver grains within myelinated axons suggests that the toxin, or a fragment thereof, undergoes retrograde intra-axonal transport as has been shown for tetanus toxin (Price et al., 1975) and nerve growth factor (Hendry, 1977) (type B BoNT appears to be transported also, as shown by in vitro studies.) The pathway for retrograde axonal transport of exogenous proteins usually begins with uptake by endocytosis at the nerve ending or unmyelinated portions of the pre-terminal axon (Grafstein and Forman, 1980). Thus, some of the transported toxin may never have entered the synaptic bouton. The protein is carried in the axon inside various vesicular or tubular organelles to the cell body where it may be transferred to lysosomes (Holtzman, 1977), to some other compartment in the cell, or to presynaptic terminals that synapse on that cell body (e.g. tetanus toxin; Mellanby and Green, 1981). A significant number of the silver grains associated with myelinated axons were located very close to the plasma membrane; this could be attributed to the existence of an internal site or to transport in vesicles along the periphery of the axon. The presence of such structures in axons of motor nerves is evident in Fig. 3.9. It is believed that the fast component of retrograde axonal transport (~300mm/day) takes place in vesicles and in the agranular endoplasmic reticulum of the axon (Lasek, 1980) and these structures have been shown to occur just inside the axonal plasma membrane. It is likely, therefore, that the radioactivity observed in myelinated axons was carried by this fast component of axonal transport. Neural ascent of BoNT type A was reported previously by Habermann (1974) and Wiegand and Wellhoner (1977). Habermann
injected $^{125}$I-derivative botulinum toxin type A into the left gastrocnemius or the left foreleg of rats and after 24 hours, examined the spinal cord for the presence of radioactivity. Injection into the hind limb caused radioactivity to appear in the lumbar cord, whereas injection into the forelimb led to radio-activity in the cervical cord. However, the labelled material that was transported centrally was not shown to bind to the neuromuscular junction, a fact difficult to reconcile with the known action of botulinum toxin at cholinergic nerve terminals. Also, the material transported was not isolated to determine whether it was intact toxin or one of its subunits, and the levels of radioactivity were so low that these studies are regarded as somewhat suspect. Wiegand and Wellholner (1974) traced the labelled toxin to the spinal cord in cats with local botulism. Efforts were made to correlate this neural ascent of toxin with blockade of neurotransmission between α-motoneuron collaterals and Renshaw cells. As this junction is cholinergic, it was expected to be susceptible to the toxin. Interestingly, it was found that Renshaw responses were not affected by toxin injected into muscle and allowed to ascend the nerve or by toxin injected into the ventral roots. Only direct injection into the spinal cord produced altered responses; these results suggest that a modified (inactive) form of the toxin is involved in retrograde axonal transport and that it cannot cross the synaptic cleft to interact with nerve terminals once it has reached the motoneuron cell body. Clearly, botulinum toxin must be transported in a different manner to tetanus toxin which is known to reach the central nervous system in an active form; in fact central nerve terminals are known to be the main target of this neurotoxin. It has been suggested that tetanus toxin may make use of a pre-existing physiological mechanism that normally transfers endogenous macromolecules through chains of neurons and may be responsible for some
Transport of BoNT to the cell body may occur for a different reason: whereas the neuronal cell body is rich in both primary and secondary lysosomes, these structures are less common in the axon and nerve endings (Holtzmann, 1971). Retrograde axonal transport may, therefore, be responsible for carrying botulinum toxin or a toxin fragment to the cell body for degradation.

3.4.5 Interaction of BoNT types A and B at the murine motor nerve terminal.

The interactions of antigenically different BoNT types have been studied in the central nervous system (Kozaki, 1979; Williams et al., 1983) but not at the neuromuscular junction. Using rat cerebrocortical synaptosomes, Kozaki studied the interaction of types A, B and E derivatives of botulinum toxin and found that they do not bind to the same site in all cases. He found that types A and E inhibited each other's binding completely and thus share a similar binding site(s). Type A partially inhibited the binding of type B, but the latter was not affected by type E. Finally, type B did not compete with either type A or type E toxins for binding sites. The interaction between types A and B BoNT was also reported by Williams (1984), who observed a number of differences as well as similarities in the nature of the binding sites involved. Acceptor sites for type A BoNT (both high and low affinity) are trypsin- and neuraminidase-sensitive and heat-labile. Those for type B show various characteristics: there is a trypsin-sensitive, heat-resistant site and a trypsin-resistant, heat-labile site, both of which are neuraminidase-sensitive. It is not surprising, therefore, that the toxins do not compete significantly for the same sites at the neuromuscular junction; type B has no effect on the binding of type A; type A, however, inhibits type B binding by 24%. The results obtained are exactly the same as those reported for the interaction of types A
and B in the central nervous system (Kozaki, 1979; Williams, 1984). These findings suggest that binding of type A BoNT interferes, perhaps sterically, with the binding of type B to some of its sites; the interaction must be complex as inhibition is not mutual. Further studies are necessary to clarify the nature of this interaction. It appears that although type A BoNT may have a single set of sites at peripheral nerve terminals (this is not the case in the central nervous system; Williams et al., 1983), there probably are at least two populations of sites for type B, one of which is blocked by type A.

In the next Chapter, quantitative studies on the binding and internalisation of $^{125}$I-BoNT types A and B at the murine nerve terminal are described. Similarities and differences between toxin types are emphasised and discussed.
CHAPTER IV

CHARACTERISTICS OF BINDING AND INTERNALISATION OF
$^{125I}$-BOTULINUM NEUROTOXIN TYPE A AND QUANTITATION OF ACCEPTOR SITES
FOR $^{125I}$-BOTULINUM NEUROTOXINS TYPES A AND B

AT THE MURINE NEUROMUSCULAR JUNCTION
4.1 **INTRODUCTION**

Owing to the difficulties involved in performing kinetic studies on the acceptor(s) for BoNT types at the neuromuscular junction, cerebrocortical synaptosome suspensions have been used as a model in a number of such investigations (Habermann and Heller, 1975; Kitamura, 1976; Kozaki, 1979; Kozaki and Sakaguchi, 1982; Williams et al., 1983). The recent successful preparation of biologically active, radiolabelled derivatives of BoNT types A (Williams et al., 1983) and B (Williams, 1984) has enabled notable advances in the characterisation of the binding component(s) in the central nervous system. Saturable binding sites for \(^{125}\)I-BoNT types A and B have been detected on brain synaptosomes as demonstrated by equilibrium and kinetic methods. A fraction of the binding sites for each of these toxin types exhibited high affinity for their respective ligand (\(K_D\) 0.6nM, \(B_{max}\) 60 fmol/mg protein for type A BoNT; \(K_D\) 0.5nM, \(B_{max}\) 30 fmol/mg protein for type B), the remainder being of lower affinity (\(K_D\) 20-25nM, \(B_{max}\) 2 pmol/mg protein for type A; \(K_D\) 20nM, \(B_{max}\) 3 pmol/mg protein for type B). The binding of type A BoNT appeared selective as it was unaffected by bound type B neurotoxin (Williams et al., 1983; Chapter 3); however, type A had a slightly inhibitory affect on the binding of \(^{125}\)I-BoNT type B (~30%) (Williams, 1984; Chapter 3). The radio-labelled toxin derivatives used in these investigations were also used in experiments at the neuromuscular junction described herein.

Only indirect methods have been used to characterise the interactions between botulinum toxin type A and the cholinergic neuromuscular junction (Simpson, 1980). Using pharmacological techniques, kinetic studies were performed using a fixed toxin concentration (0.1nM) and the half-times and rate constraints of the binding, translocation and lytic steps were determined. A simple kinetic model was presented to account for the interaction of botulinum toxin type A with the cholinergic nerve terminal:
According to this model, the toxin (BT) binds ($K_B$) to an acceptor ($R_1$) on the surface of the nerve ending and is subsequently translocated across the membrane ($K_T$). The translocated intact toxin, or a fragment thereof, then binds to a new site ($S$) within the cytosol and finally interacts with an intracellular substance ($R_2$) causing blockade of neurotransmitter release. In the absence of nerve stimulation, the half-time of the binding step was $\approx 12$ min (at $37^\circ$C) and had a rate constant of $\approx 0.058$ min$^{-1}$. This binding had a low temperature dependence ($Q_{10} \approx 1.6$). The translocation step had a halftime of 4.9 min and a rate constant of $\approx 0.141$ min$^{-1}$, and the lytic step a halftime of $\approx 55$ min with a rate constant of $\approx 0.013$ min$^{-1}$. The lytic step had a high temperature dependence ($Q_{10} \approx 4.2$). Using similar techniques, Simpson (1980) also observed that the time to paralysis is dependent on toxin concentration.

To date, very little is known of the nature of the acceptor sites for BoNT types A and B at the neuromuscular junction. No absolute quantitation of these sites has been carried out, as such studies were not possible before the availability of biologically active, radio-labelled probes. Knowledge of the number of sites involved could prove helpful in the identification of the membrane component(s) with which the toxin types interact.

In this Chapter, radiolabelled toxins types A and B were used in ultrastructural autoradiographic studies to investigate the characteristics of toxin binding and internalisation at the neuromuscular junction. Taking advantage of the fact that metabolic inhibitors such as sodium azide prevent the translocation step, two phases in the intoxication process (Simpson, 1980) were investigated separately. The optimum conditions for saturating all the acceptor
sites for each toxin type were determined, and this information was used to quantitate the sites by the method of Fertuck and Salpeter (1976). In order to carry out quantitative studies, calibration of the autoradiographic technique was essential; percent efficiency and resolution of the emulsion-developer combination used were, therefore, carefully determined.

4.2 MATERIALS AND METHODS

4.2.1 General methodology for studying BoNT binding and internalisation.

For studies on concentration dependence of binding and internalisation of $^{125}$I-BoNT types A and B, all incubations were carried out in 0.5ml Krebs Ringer, pH 7.4 at 22°C for 90 minutes. Small pieces of mouse diaphragm tissue were incubated with increasing concentrations of radiolabelled toxin in the presence and absence of the metabolic inhibitor Na azide (15mM).

The time course of toxin binding and internalisation was investigated by incubating tissue preparations with 0.5ml Krebs Ringer/15nM $^{125}$I-BoNT type A for different periods of time at 22°C in the presence and absence of Na azide (15mM). To ensure that the internalisation process was not being affected by lack of oxygen or depletion of nutrients from the incubation medium, a small piece of tissue was incubated with radiolabelled toxin in 3ml Krebs Ringer (pre-gassed and adjusted to pH 7.4) continuously aerated with a fine stream of 95%O$_2$/5%CO$_2$ for 180 minutes. Two other preparations were incubated in 8 and 10ml Krebs Ringer/$^{125}$I-BoNT type A for 150 and 240 minutes, respectively.

For temperature-dependence studies of the different steps in the interaction of BoNT with motor nerve terminals, samples of tissue were
incubated in 0.5ml Krebs Ringer/11mM $^{125}$I-BoNT type A at 4°C and 22°C for 2 hours.

4.2.2 Development and analysis of results.

Following the standard processing for autoradiography, the sections were exposed for 4 weeks at 4°C and the emulsion was then developed as described in Chapter 2 (Section 2.6.4). Sections were stained and viewed in a Hitachi-600 electron microscope. Photographs were taken of at least 20 different nerve endings from each preparation (50-330 silver grains); prints were made at a final magnification of 19,000x. The pre-synaptic plasma membrane was traced onto acetate sheets and its length determined (in μm) by digitisation using a Hewlett-Packard digitiser. The extent of binding was expressed as number of silver grains per μm of plasma membrane; in the case of nerve endings incubated in the absence of 15mM azide, the binding was expressed as total number of grains (i.e. membrane-bound and internalised) per μm of membrane, on the assumption that all internalised grains came originally from the surrounding plasma membrane. The extent of uptake was determined by counting the grains on or close to the plasma membrane (to a distance of ~70nm) and those within the nerve terminal separately, and expressing each as a percentage of the total.

4.2.3 Determination of percent efficiency in electron-microscope autoradiography using $^{125}$I.

A small volume (10μl) of $^{125}$I-bovine serum albumin ($^{125}$I-BSA), prepared as described in Section 2.12.3, was added to 100μl of a 1mg/ml solution of unlabelled BSA on ice. Trichloroacetic acid (TCA) was then added to a final concentration of 12% and the mixture was left on ice for 30 minutes. It was then centrifuged for 5 minutes at
9000 x g and the pellet was washed in ice cold 12% TCA. After the final wash, the pellet was fixed in 2% glutaraldehyde for 1 hour, post-fixed in 1% OsO₄ for 1 hour, dehydrated through a series of concentrations of ethanol and finally embedded in Spurr's resin. The pellets were left in 100% resin for two days (with several changes) in order to ensure complete infiltration.

After polymerization, sections (1µm thick) were cut with an ultramicrotome and transferred to formvar-coated slides with a wire loop (10 1µm sections/slide). Each section was then viewed in a Leitz Ortholux light microscope and photographed using Ilford FP4 film. Prints were made at a final magnification of 219x. The outline of the embedded 125I-BSA was traced onto acetate sheets and the area was determined by digitisation using a Hewlett-Packard digitiser. As the thickness of the sections was known, the total volume of radioactive material on each slide could be calculated.

Ultrathin (pale gold) sections were cut and transferred to formvar-coated slides. At least 8 sections were placed on each of 6 slides. On the day the slides were dipped in Ilford L4 emulsion for electron-microscope autoradiography, the formvar films supporting the 10 1µm-thick sections were floated onto the surface of water, picked up with parafilm and their radioactive content was determined using a Wallac γ-counter at 50% efficiency (sections from 3 different slides were counted). From the total volume of radioactive material counted and the number of counts per minute obtained, the number of disintegrations per unit volume of 125I-BSA at the start of the exposure period could be calculated.

The slides prepared for electron-microscope autoradiography were exposed for varying lengths of time; 4, 8, 12, 21, 35 and 42 days. After development in Kodak D-19 developer, the sections were viewed in the electron microscope and photographs were taken of 10 different
labelled areas randomly selected from each slide at a magnification of 10,000x. Prints were made at a final magnification of 25,000x and the silver grains in each area were counted. The volume of radioactive material could be calculated from the size of the photographed area and the thickness of the section (determined by the method of Small, 1968).

The percent efficiency of the autoradiographic technique used could then be determined from the total number of disintegrations occurring per unit volume of labelled material during the time of exposure, given by equation (1) (Fertuck and Salpeter, 1974):

\[ D = D_0 \times 124,800 \left(1 - e^{-0.01155(t)}\right) \]  

\[ D = \text{total disintegrations/unit volume during time } t \]
\[ D_0 = \text{disintegrations/unit volume at the start of exposure} \]
\[ t = \text{exposure time in days} \]

and the number of grains produced as a result of these decays:

\[
\% \text{ efficiency} = \frac{\text{No of grains}}{\text{No of decays}} \times 100\%
\]

4.2.4 Resolution in electron-microscope autoradiography using $^{125}$I.

The distance from a radioactive line source within which half the developed grains are located is called the half distance, HD. When properly obtained, the HD value can be a direct measure of resolution. In the presence of azide, $^{125}$I-BoNT types A and B remain on the membrane which, therefore, acts as a "radioactive line source"; the grain distribution around it can be analysed and the HD value can thus be determined. This was carried out using the method of Salpeter et al., (1969). The developed autoradiograms were photographed; enough presynaptic membrane was included to yield approximately 500 grains. The distance from the midpoint of every developed grain was measured to the nearest point of the presynaptic membrane. To determine the midpoint of a grain, a plastic mask which had a series of concentric
circles marked around a common perforated centre was placed over the grain, and the latter was fitted symmetrically into the smallest circle which fully circumscribed it. The centre of the circle was then marked on the autoradiographic print with a fine ball-point pen. The measurements were done on prints at a magnification of 28,320x and grains were counted to a cut-off distance of 0.5µm on either side of the 'line'.

4.2.5 Quantitation of 125I-BoNT types A and B binding sites at the murine motor nerve terminal.

As shown previously (Chapter 3, Section 3.3.3), de-energisation of a nerve-muscle preparation, produced by inhibitors of oxidative phosphorylation such as sodium azide and dinitrophenol, prevents uptake of 125I-BoNT types A and B into the nerve terminal. As the toxin molecules remain bound to their acceptor sites, quantitation of the latter can be carried out using the procedure described by Fertuck and Salpeter (1976) for the quantitation of junctional and extrajunctional acetylcholine receptors using 125I-α-bungarotoxin.

For quantitation of 125I-BoNT type A acceptor sites, a very small piece of mouse diaphragm tissue containing part of the end-plate region was preincubated in Krebs Ringer solution/15mM sodium azide for 30 minutes. It was then incubated with 150nM 125I-BoNT type A (1377 Ci/mmol) in 250µl Krebs Ringer/15mM azide for 90 minutes at room temperature. This toxin concentration is known to saturate all the binding sites in labelling conditions that minimise structural damage to the tissue (see Section 4.3.1). Following incubation, the tissue was washed extensively (7 times, 5 minutes each), fixed in 2% glutaraldehyde for 1 hr and processed for electron-microscope autoradiography as described in Chapter 2. In order to ensure that the binding observed was saturable, a control sample was treated with 100-fold excess of native BoNT type A.
Sections were cut from two different test blocks containing a large number of nerve endings and one control block (also rich in nerve terminals). At least 9 groups of 3 or more test sections were transferred to one side of 6 formvar-coated slides, and at least three groups of control sections were dried down on the other. The slides were exposed at 4°C, and one of these was developed in Kodak D-19 developer every week for 6 weeks in order to investigate the time course of grain production. Each week, stained sections were viewed in the electron microscope and photographs of 22 to 34 different nerve endings were taken at a magnification of 10,000x. Prints were made at a final magnification of 28,320x, the presynaptic membrane was traced onto acetate sheets and its length determined by digitisation using a Hewlett-Packard digitiser. The thickness of each section photographed was determined by the method of Small (1968) as described previously (Chapter 2, Section 2.6.7).

The grain density (grains per square micrometer of membrane surface area) was determined separately for each tissue section viewed. This was carried out by counting all the developed silver grains on every nerve ending photographed from a particular section, and dividing this number by the length of the membrane (measured by digitisation) and the section thickness determined as described above. The average grain density was calculated for each exposure time (7, 14, 21, 28, 35 and 42 days) by determining the grain density of several sections on each slide.

The density of $^{125}$I-BoNT type A binding sites was then calculated for each exposure time using the following equation (Fertuck and Salpeter, 1976):
sites/µm² membrane = \( \frac{G \times d}{124,800 \left(1 - e^{-0.01155t}\right)} \times \frac{A}{S_0 C} \)

where \( G \) = grain density (grains per square micrometer of membrane surface area), \( d \) = decays needed for one developed grain (calculated from percent efficiency values - Section 4.2.3), \( t \) = exposure time in days, \( S_0 \) = specific activity of the \(^{125}\text{I-BoNT Type A} at the beginning of the exposure period (Ci/mmol), \( C = \) disintegrations per minute or \( 2.22 \times 10^{12} \), \( A = \) Avogadro's number in the same units as used for \( S_0 \) (6.023 \( \times \) \( 10^{20} \) molecules/mmol).

A similar procedure to that described above was used to quantitate the number of acceptor sites labelled using 15 and 35nM \(^{125}\text{I-BoNT type A} (saturating concentrations). This was carried out in order to determine whether sites of lower affinity were being detected at higher toxin concentrations (i.e. 150nM), well above those known to saturate all the acceptor sites for type A at the murine motor nerve terminal (15-20nM).

Quantitation of acceptor sites for \(^{125}\text{I-BoNT type B} was also carried out, using a toxin concentration of 100nM. Sections were cut from two different blocks and the results were analysed after 11 days exposure.
4.3 RESULTS

Part A: Characterisation of the binding and internalisation of $^{125}$I-BoNT type A at the murine neuromuscular junction and concentration dependence of binding of $^{125}$I-BoNT type B.

4.3.1 Concentration dependence of $^{125}$I-BoNT type A binding and internalisation.

Binding: As shown in Chapter 3 (Sections 3.3.2, 3.3.3), $^{125}$I-BoNT type A binds saturably to the plasma membrane of murine motor nerve terminals both in the presence and absence of 15mM sodium azide. This was established by including excess native toxin in the incubation medium. In this section, a quantitative study of the saturability of binding was carried out by incubating nerve-muscle preparations with increasing concentrations of $^{125}$I-BoNT type A (1.5nM, 15nM and 35nM).

Whereas the extent of binding at a toxin concentration of 1.5nM was only 36% and 42% of that seen at 15nM for the untreated and azide-treated samples respectively (Table 4.1, Fig. 4.1), when the concentration was increased from 15nM to 35nM, the number of silver grains associated with nerve terminals increased by only 2% in both cases (in untreated samples, total binding was expressed as membrane-bound grains + internalised grains per μm plasma membrane). Saturation of acceptor sites in this system, therefore, appeared to occur when $^{125}$I-BoNT type A was applied at a concentration of approximately 15-20nM, for 90 minutes at room temperature. The data obtained (Fig. 4.1) suggested that the affinity of the toxin for its acceptor sites at the murine neuromuscular junction is comparable with that for the high affinity sites on rat cerebrocortical synaptosomes ($K_D$ 0.6nM; Williams et al., 1983). The low affinity acceptor sites have a $K_D$ of 20-25nM which is too high to account for the affinity of binding at the motor
Table 4.1 Concentration dependence of binding of $^{125}\text{I-BoNT}$ type A.

The concentration dependence of toxin binding to the motor nerve terminal was studied in the presence and absence of Na azide (15mM). Small sections of mouse diaphragm tissue were incubated with increasing concentrations of $^{125}\text{I-BoNT}$ type A (1.5nM, 15nM and 35nM) in 0.5ml Krebs Ringer (or Ringer/15mM Na azide) for 90 minutes at 22°C. Washes were carried out in Krebs Ringer or (Krebs Ringer/15nM Na azide) for 30 minutes at 4°C (6 changes, 5 minutes each). The tissue was then fixed, embedded and processed for electron-microscope autoradiography. After 4 weeks exposure, the emulsion was developed, sections were stained with 12% uranyl acetate and 20-30 end-plates were photographed from each preparation. The grain density (no. of grains/μm plasma membrane) was then determined for each preparation as described in Section 4.2.2. Sources of error in these determinations included measurement of section thickness and sampling error in autoradiographic grain counting (as the number of grains in autoradiograms is Poisson distributed). The overall autoradiographic error was determined using the following equation (Matthews-Bellinger and Salpeter, 1978):

$$\sqrt{\frac{100^2}{n}} + (\frac{s}{n})^2$$

where $n$ = the number of grains counted, 100/ $\sqrt{n}$ is the percentage sampling error in grain counting, and 5% is the error in section thickness determination.

Concentration dependence of the internalisation step was investigated in preparations treated with increasing concentrations of toxin under physiological conditions. To determine the distribution of radioactivity with respect to the plasma membrane, grains were counted at end-plates from sections of similar thickness: silver grains on or close to the plasma membrane (to a cut-off distance of 70nm) and in the cytoplasm were counted separately and expressed as % of the total.

* % relative to extent of binding in the absence of Na azide.
Table 4.1 Concentration dependence of binding and internalisation of 125I-BoNT type A.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>BINDING (number of grains/μm plasma membrane)</th>
<th>INTERNALISATION (distribution of grains with respect to the plasma membrane) % of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Azide</td>
<td>+Azide (15nM)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.22±0.03</td>
<td>0.23±0.03 (105%)*</td>
</tr>
<tr>
<td>15.0</td>
<td>0.61±0.05</td>
<td>0.55±0.06 (90%)*</td>
</tr>
<tr>
<td>35.0</td>
<td>0.62±0.06</td>
<td>0.56±0.06 (90%)*</td>
</tr>
</tbody>
</table>
Fig. 4.1 Concentration dependence of binding of $^{125}$I-BoNT Type A in the presence and absence of Na azide.

Graphical expression of the data tabulated in Table 4.1. Note the difference in the extent of labelling seen at nerve terminals treated with higher concentrations of $^{125}$I-BoNT type A (10-35nM) in the presence (●) and absence (○) of Na azide (15nM).
nerve terminal (where the sites are saturated at a toxin concentration of ~18nM). Moreover, almost 50% of the total binding occurred when the toxin was applied at a concentration of 1.5nM. The high concentrations required for saturation can be attributed to hindered diffusion in the tissue. The extent of binding seen with 1.5nM $^{125}$I-labelled toxin was the same in azide-treated and untreated samples (Table 4.1). However, at 15nM and 35nM toxin concentrations, azide-treated terminals showed 90% of the binding seen in untreated samples, possibly due to dissociation of the $^{125}$I-BoNT molecules during washes. In untreated samples, energy-dependent internalisation would have occurred, so less toxin would be likely to dissociate (Simpson, 1980). Also, as discussed earlier, the possibility that the toxin was sequestered within the plasma membrane by an energy-requiring process (Yavin et al., 1983) prior to being internalised (which would render the binding virtually irreversible), and/or that some acceptor-recycling took place during the incubation period, cannot be excluded. The latter would allow more toxin to associate with the nerve terminal.

Internalisation: The extent of energy-dependent internalisation of $^{125}$I-BoNT type A into the nerve terminal (in untreated samples) did not change with increasing toxin concentrations (Table 4.1). The ratio of membrane-bound to internalised grains was always approximately 60:40.

4.3.2 Time course of binding and internalisation of $^{125}$I-BoNT type A.

A significant amount of radioactivity was seen associated with motor nerve terminals after only 20 minutes incubation (with 15nM $^{125}$I-BoNT) in both untreated and azide-treated samples (Table 4.2, Fig. 4.2). Taking into consideration the problem of hindered diffusion into the tissue preparation used, the association rate can be said to be rapid. When the incubation time was increased from 20
Table 4.2 Time course of binding and internalisation of $^{125}$I-BoNT type A.

This was studied in the presence of metabolic inhibitors and under physiological conditions. Samples of tissue were incubated with 0.5ml Krebs Ringer containing radiolabelled toxin (15nM) for 20, 90 and 150 minutes at 22°C. After extensive washing with Krebs Ringer or Krebs Ringer/15mM Na azide at 4°C, the tissue was fixed and prepared for electron-microscope autoradiography. Results were analysed after 4 weeks exposure as described in Section 4.2.2.

* Experiment carried out duplicate: the grain densities obtained after quantitative analysis of the data from each of the preparations were 0.55 and 0.54 grains/μm plasma membrane. This result emphasises the reproducibility of the experimental method used. Overall-all autoradiographic error in the results obtained was determined as described in Table 4.1.

The time course of the internalisation process was studied under the conditions described above, in the absence of Na azide. In addition, in order to ensure that the uptake mechanism had not been affected by the incubation conditions used (small incubation volume, no oxygenation), a small sample was treated with toxin (10nM) in 3ml Krebs Ringer (pre-gassed and adjusted to pH 7.4) continuously aerated with a fine stream of 95% O$_2$/5% CO$_2$ for 180 minutes at 22°C. Saturability of binding was tested by treating a sample with excess native BoNT. A control for this experiment consisted of a tissue section incubated with 0.5ml 10nM $^{125}$I-BoNT type A in pre-gassed Krebs Ringer solution, pH 7.4, for 180 minutes at room temperature without continuous bubbling (as in previous experiments).

Another diaphragm section was incubated in 10ml Krebs Ringer/5nM $^{125}$I-BoNT type A for 240 minutes at room temperature with no oxygenation. Finally, a sample was treated with 10nM $^{125}$I-BoNT type A
in 8mL Krebs Ringer for 150 minutes with constant gassing with 95% 
O₂/5% CO₂ as described previously.

Results were analysed after 4 weeks exposure as described in 
Section 4.2.2.

** no oxygenation
Table 4.2 Time course of binding and internalisation of $^{125}$I-BoNT type A.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>-Azide</th>
<th>+Azide</th>
<th>% binding relative to untreated sample</th>
<th>0.5ml incubation medium, no oxygenation</th>
<th>3ml (or more) incubation medium, with oxygenation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% on membrane</td>
<td>% within</td>
</tr>
<tr>
<td>20</td>
<td>0.50±0.05</td>
<td>0.46±0.08</td>
<td>92</td>
<td>79 (±18, n=16)</td>
<td>21 (±18, n=16)</td>
</tr>
<tr>
<td>90</td>
<td>0.61±0.06</td>
<td>0.55±0.06*</td>
<td>90</td>
<td>62 (±21, n=11)</td>
<td>38 (±21, n=11)</td>
</tr>
<tr>
<td>150</td>
<td>0.66±0.06</td>
<td>0.53±0.09</td>
<td>80</td>
<td>57 (±17, n=15)</td>
<td>43 (±17, n=15)</td>
</tr>
<tr>
<td>180</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>240</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indicates significant difference from -Azide condition. ** Indicates highly significant difference from -Azide condition.
Fig. 4.2 Time course of binding of $^{125}$I-BoNT type A in the presence and absence of Na azide.

The data presented in Table 4.2 shown graphically. Note the difference in the extent of binding seen in azide-treated (●) and untreated (○) preparations. Saturation occurred after 40 to 50 minutes incubation with 15nM $^{125}$I-BoNT type A.
minutes to 90 minutes, the extent of binding increased by a factor of 1.2 in both samples. The difference in the amount of binding seen in azide-treated samples relative to untreated samples (8% less at 20 minutes and 10% less at 90 minutes) can be attributed to a combination of loss of radioactivity during washes in the treated sample and to the possible occurrence of a small amount of acceptor-recycling in the untreated sample. Extending the incubation time to 150 minutes did not significantly affect the extent of binding in azide-treated samples [in fact, there was a small decrease (4%) in the amount of toxin bound, attributable to onset of necrosis in the membrane by this time]. It appears that, in treated samples, saturation occurred after approximately 40-50 minutes incubation at the $^{125}$I-BoNT concentration used (15nM); at 20 minutes, about 84% of the maximum binding had already taken place. In untreated samples, however, there was a slight (8%) increase in binding between 90 and 150 minutes incubation (Fig. 4.2); this can probably be attributed to increased levels of acceptor-recycling at this time.

The time course of $^{125}$I-BoNT uptake into the motor nerve terminal was studied by incubating small pieces of mouse diaphragm with 15nM labelled toxin at room temperature for various periods of time. After 20 minutes, 21% of the total radioactivity associated with the terminal was found within the synaptic bouton itself (Table 4.2); the remaining 79% was located on the terminal plasma membrane. The extent of internalisation increased to 38% after 90 minutes and 43% after 150 minutes incubation; if the margin of error in these percentages is taken into consideration, it can be said that the amount of uptake did not change significantly between 90 and 150 minutes. In order to observe the effect on internalisation of a much longer incubation time, a mouse hemidiaphragm was exposed to $^{125}$I-BoNT in a 10ml volume of Krebs Ringer for 4 hours at room temperature. In this
sample, only 34% of the grains seen were located within the nerve terminal; this small decrease in the proportion of grains internalised could be the result of experimental error, but might also be attributed to tissue fatigue after a long incubation period.

The extent of toxin uptake seems, therefore, to remain essentially unchanged after approximately 90 minutes incubation. In order to ensure that this was not an artefact caused by cell death as a result of the incubation conditions used (small volume and no oxygenation during the incubation), a sample of tissue was treated with toxin for 3 hours using a 3ml incubation volume continuously aerated with a fine stream of 95% O₂/5% CO₂. Another sample was incubated in 8ml for 2 1/2 hours, with continuous oxygenation as above. The proportion of internalised grains was 44% and 39% respectively, indicating that the incubation conditions used were not responsible for the results obtained.

In all cases, binding was shown to be saturable as less than 4% of the grains seen in test sections were present in those incubated with a large excess of native neurotoxin.

4.3.3 Effect of temperature on the binding and internalisation of 125I-BoNT at the motor nerve terminal.

Mouse diaphragm tissue was exposed to 125I-BoNT at 4°C for 2 hours. Under these conditions, the toxin still interacted with the nerve terminal membrane as silver grains could be detected in the resultant autoradiograms (Fig. 4.3). However, the extent of binding was only 30% (determined by quantitative analysis of the results as described in Section 4.2.2) of that seen in controls incubated at room temperature, probably due to reduction in the rate of diffusion at this low temperature. The internalisation of toxin molecules was inhibited
Fig. 4.3 Effect of temperature on the binding and internalisation of $^{125}$I-BoNT type A at the motor nerve terminal.

A small sample of the tissue was preincubated with pre-gassed Krebs Ringer, pH 7.4, at 4°C on ice and then incubated with 0.5ml Krebs Ringer/11nM $^{125}$I-BoNT type A (1,600 Ci/mmol) at 4°C for 2 hours. The tissue was then washed, fixed in 2% glutaraldehyde for 75 minutes (at 4°C) and processed for electron-microscope autoradiography as described in Chapter 2 (Section 2.6). Control preparations were treated similarly but at room temperature. After three weeks exposure, the slides were developed and the results analysed as described in Section 4.2.2.

a. Nerve terminal treated with $^{125}$I-BoNT type A at 4°C. Note the reduced number of silver grains associated with the synaptic bouton (30% relative to controls) and the absence of internalised radioactivity.

b. Control preparations treated with $^{125}$I-BoNT at room temperature showing membrane-bound and internalised grains in a ratio of approximately 60:40.
completely under these incubation conditions (Fig. 4.3); silver grains were found on the plasma membrane (or very close to it) but not within it. The translocation step of intoxication is, therefore, not only energy-dependent (Section 3.3.3) but also temperature-dependent and the binding step is temperature-sensitive as fewer grains are associated with the nerve terminal at lower temperatures.

4.3.4 Concentration dependence of binding of $^{125}$I-BoNT type B in the presence of sodium azide.

It was shown in Chapter 3 (Section 3.3.6) that $^{125}$I-BoNT type B binds saturably to acceptor sites on the motor nerve terminal membrane, in a manner comparable to that seen with $^{125}$I-BoNT type A. In this section, quantitative analysis of this binding was carried out; the concentration at which saturation occurred (during a 90-minute incubation period at room temperature) was determined and compared with that seen with type A.

At a toxin concentration of 10nM, 0.53 developed silver grains occurred per micron of plasma membrane (Table 4.3); this represented 65% of the binding seen when a 20nM concentration was used and 56% of that observed at 100nM. Maximum binding seems to have occurred at a toxin concentration of approximately 40-50nM (Fig. 4.4); thus, at 20nM, 86% of the possible binding sites were already occupied. The affinity of $^{125}$I-BoNT type B for its acceptor site(s) at the motor nerve terminal appeared to be lower than that of $^{125}$I-BoNT type A (Figs. 4.4, 4.1) as a higher toxin concentration (approximately twice as much) was required to saturate the binding sites for $^{125}$I-BoNT type B (40-50nM) than for type A (15-20nM) in this tissue.
Table 4.3 Concentration dependence of binding of $^{125}$I-BoNT type B in the presence of sodium azide.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>BINDING (in the presence of 15mM Na Azide) number of grains/μm of terminal plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>20</td>
<td>0.81 ± 0.07</td>
</tr>
<tr>
<td>100</td>
<td>0.95 ± 0.07</td>
</tr>
</tbody>
</table>

Diaphragm tissue was pre-incubated with Krebs Ringer/15mM Na azide for 30 minutes and then incubated with increasing concentrations of $^{125}$I-BoNT type B (831 Ci/mmol) in 0.5ml Krebs Ringer/15mM Na azide for 90 minutes at 22°C. Sections prepared for electron-microscope autoradiography were developed after 11 days exposure and the results were analysed as described in Section 4.2.2. Error in results obtained was determined as described in Table 4.1.
Fig 4.4. Concentration dependence of binding of $^{125}$I-BoNT type B in the presence of Na azide.

The data presented in Table 4.3 shown graphically. Saturation of acceptor sites for $^{125}$I-BoNT type B at the motor nerve terminal occurs at a toxin concentration of ~40-50nm.
Part B: Density of acceptor sites for $^{125}$I-BoNT types A and B.

4.3.5 Calibration of the autoradiographic technique: Determination of the efficiency and resolution of electron-microscope autoradiography using $^{125}$I.

An important calibration procedure for quantitative electron microscopy is the test for efficiency of the autoradiographic method used. Efficiency is defined as the number of silver grains produced in a layer of nuclear emulsion relative to the number of radioactive disintegrations entering it from the source during exposure (Rogers, 1979). It is affected by the initial energy of the particles emitted by the specimen (which determines the distance travelled and the rate of energy loss of the particles), thickness of the source and the emulsion layer, dimensions and packing of the silver halide crystals, sensitivity of the emulsion, stability of the latent image, temperature and duration of exposure, radiation dose dependence, and conditions of development.

In order to determine the percent efficiency of the autoradiographic method used routinely in the experiments described, ultrathin (pale gold) sections containing a known amount of radioactivity (in the form of $^{125}$I-BSA, fixed in glutaraldehyde, post-fixed in OsO$_4$) were coated with Ilford L4 emulsion as described previously. After exposure at 4°C for varying lengths of time, the emulsion was developed and the sections were picked up on microscope grids (see Chapter 2, Sections 2.6.4, 2.6.5); they were subsequently viewed, unstained, in a Hitachi-600 electron microscope.

Figures 4.5 and 4.6a show the average grain density (developed silver grains/9.43 $\mu$m$^2$ radioactive sample) obtained after varying exposure times (4-42 days). It is evident, from Fig. 4.6a, that during the first fifteen days' exposure there was an almost linear increase in the number of silver grains produced; longer exposure times, however, resulted in a slight levelling of the curve.
Fig. 4.5 Grain production as a function of exposure time.

Ultrathin sections of embedded $^{125}$I-BSA, containing a known amount of radioactivity, were processed for electron-microscope autoradiography and developed in Kodak D-19 after various times of exposure (4-42 days). For each time point, photographs were taken of 10 different labelled areas (selected randomly from several sections of similar thickness) at a magnification of 10,000x. Representative prints are shown for (a) 4 days, (b) 8 days, (c) 12 days, (d) 21 days, (e) 35 days, and (f) 42 days exposure at 4°C. Grains could still be counted after an exposure period of 42 days.
Fig. 4.6 Grain density as a function of exposure time and radiation dose.

Photographs of labelled areas in sections of $^{125}$I-BSA were taken as described in Fig. 4.5. Prints of the entire area of the negative (representing 9.23±m of labelled material) were made at a final magnification of 25,000x.

(a) The average number of silver grains produced by this fixed amount of labelled material during different periods of exposure was determined and expressed graphically as a function of time. Note that during the first two weeks of exposure, the increase in the number of grains produced was linear; the curve only levels off significantly after 30 days exposure time.

(b) The same data shown in (a) replotted to give a diagram of grain density as a function of radiation dose. Radiation dose (for a particular area of emulsion during a specified exposure time) was determined using the following equation (Fertuck and Salpeter, 1974):

$$D = D_0 \times 124,800 \left(1 - e^{-0.01155(t)}\right)$$

where:
- $D =$ total disintegrations/unit volume during time $t$
- $D_0 =$ disintegrations/unit volume at the start of exposure
- $t =$ exposure time in days

Note that, in this case, a straight line is produced indicating that radiation dose is directly related to exposure time. Efficiency of grain production is therefore affected little by this range of radiation dose.
a

# grains/9.23 µm³

Exposure time (days)

b

# grains/µm² emulsion

Radiation dose (decays/µm² emulsion)
In the case of $^{125}\text{I}$, the number of disintegrations occurring in the source is not directly related to exposure time. It is best, therefore, to express the complete efficiency information for a given emulsion-developer combination in a curve plotting efficiency as a function of radiation dose. This is relevant as it was shown by Salpeter and Szabo (1972) that percent efficiency is dependent on radiation dose (expressed as total number of decays occurring in the specimen in contact with a specified area of emulsion over a certain period of time and not only the 50% emitted in the direction of the emulsion), with lower doses giving better efficiency. This dose dependence is not caused primarily by saturation of the photographic emulsion (which occurs at higher radiation doses than those used in these calibration experiments). It is thought that this effect is a function of the density of latent images produced in the emulsion (Housley and Fisher, 1975); it appears that the presence of a rapidly developing latent image may suppress in some way the development of other hit crystals in its immediate vicinity. Salpeter and Szabo (1972) also found that the efficiency of grain production showed significantly greater radiation dose dependence if the developer Microdol-X was used than with D-19 and gold-elon asorbic acid, suggesting that the effect is also related to the process of development.

In Fig. 4.6b, the average number of grains obtained per square micron of emulsion, after a particular exposure time, is expressed as a function of the number of decays occurring within the specimen in contact with the specified area of emulsion. The range of radiation doses to which the emulsion was exposed over the six-week period in which efficiency tests were carried out was comparable to that studied by Salpeter and Szabo (1972): in the present study, the range was 0.47 to 4.01 decays/um$^2$ of emulsion whereas Salpeter and Szabo observed the effects of 0 to 3.2 decays/um$^2$ of emulsion. The doses in this
experiment, therefore, were low enough to exclude problems of saturation of the photographic emulsion. The curve obtained by plotting the data in this way indicated that the efficiency of grain production showed relatively little radiation dose dependence (it is almost linear), as would be expected from the combination of Ilford L4 emulsion/D19 developer used here for autoradiography (previous studies have shown that this combination gives high efficiency values). This problem could, therefore, be ignored as the radiation dose to which the emulsion was exposed in test specimens would be unlikely to exceed that found in calibration sections to any significant extent (as shown empirically, see Fig. 4.10).

The curve in Fig. 4.6b could be useful in that it relates grain density to radiation dose - after grain densities in experimental autoradiograms have been determined, the appropriate decays per square micron (and thus specific radioactivity of the labelled area) can be read directly from this curve for any given number of grains. This could be helpful in the determination of efficiency values if there appears to be high radiation dose dependence in the autoradiographic method used.

The percent efficiency values are depicted in Figs. 4.7a and 4.7b. In the former, percent efficiency (or no. of grains/no. of decays x 100%) is related to exposure time and, in the latter, to radiation dose. The relatively low radiation dose dependence is re-emphasised in these determinations: over the range of doses applied (exposure times), efficiency decreased from approximately 63% (almost maximum, see discussion) at the lowest dose (0.47 decays/um²) to 57% at the highest dose (410 decays/um²), the average being 60% ± 3.2 (n=6). This average value could be taken as the percent efficiency in the autoradiographic method employed; it takes into account the emulsion-developer combination, exposure times (i.e. radiation dose), thickness
Fig. 4.7  Efficiency of the autoradiographic procedure and emulsion-developer combination used as a function of exposure time and radiation dose.

Percent efficiency values were obtained from the total number of disintegrations occurring per unit volume of labelled material during a specified time of exposure (given by equation (1), Section 4.2.3) and the number of grains produced as a result of these decays, as shown below:

\[
\text{% efficiency} = \frac{\text{no. of grains}}{\text{no. of decays}} \times 100
\]

These values are expressed graphically as a function of exposure time. (a) They ranged from 63% or almost the theoretical maximum (~64%, see Discussion) (after 4 days exposure) to 57% (after 42 days exposure). (b) Percent efficiency values are plotted as a function of the radiation dose (determined as described in Fig. 4.6b) to which the emulsion was exposed.
Efficiency (%)

Exposure time (days)

Theoretical maximum

Radiation dose (decays/μm²)

Theoretical maximum
of sections, and heavy metal staining with OsO₄, and is thus an accurate representation of the efficiency of the system.

The background grain density was obtained by randomly photographing areas of the sections that contained no ¹²⁵I-BSA, and was found to be negligible.

Another important factor in quantitative electron microscope autoradiography is resolution, that is, the distribution of developed silver grains around the radioactive source. In experiments involving quantitation of plasma membrane acceptors, the radioactive source, or the plasma membrane, represents a line. As the width of the membrane is only 7-9nm and thus negligible, resolution studies should give more accurate results using this 'biological line' than one prepared artificially which will necessarily have a greater thickness (e.g. 50nm; Salpeter et al., 1969). When using a biological specimen to test resolution, it is necessary to make the assumption that the labelling is uniform throughout the 'line'; as autoradiograms of the binding of ¹²⁵I-BoNT to acceptor sites on the presynaptic plasma membrane showed no clustering of grains or restriction in their location (e.g. Fig. 4.9, Section 3.3.3), this biological line source (or hollow 'circle' of large radius which behaves like a line source, Salpeter et al., 1969) appears to satisfy the above requirement. Ultrathin sections of nerve endings, previously incubated in 15mM azide, exposed to ¹²⁵I-labelled BoNT type A and submitted to autoradiography, were used as simple calibration specimens for testing resolution in the radio-autographic method carried out routinely. Factors such as section and emulsion thickness, silver halide crystal size, heavy-metal staining, energy of the isotope, and developed grain size, all influence resolution by affecting the spread of the silver grains relative to the line source.

A useful expression of resolution is the "half distance" (HD) value, defined by Salpeter et al., (1969) as the distance from a radioactive line within which half of the developed grains are located.
The distance from the radioactive line to the midpoint of over 500 silver grains was measured (the growth of a silver grain is not random; it occurs symmetrically around the silver halide crystal from which it was derived), and the data obtained was used to construct a grain distribution histogram (Fig. 4.8a). Following the procedure for analysis of such results outlined by Salpeter et al., (1969), one-sided histograms were also plotted as the first histogram appeared symmetrical on both sides. Thus grains at each specified distance on either side of the membrane were considered equivalent and added together. A smooth curve was then drawn in by eye. The grain density distribution is bell-shaped, indicating that the majority of grains were found on the membrane or very close to it; at a distance of only 100 nanometers, the number of developed grains was already very low. This normal distribution is comparable to that obtained by Salpeter et al., (1977). Fig. 4.8b shows an integrated grain distribution histogram for the same data as shown in Fig. 4.8a; each column gives the total number of grains within that distance from the source. The half distance (HD) value can be determined readily from this histogram: the total number of grains is 532; half the total (266 grains) can be found within approximately 56nm of the source. This half distance is less than that obtained by Salpeter et al., (1977) for $^{125}$I using Ilford L4 emulsion (89±9nm). Inherent differences between batches of emulsion, as well as other differences in experimental technique, may account for this discrepancy. The thickness of the line source could also be a contributory factor. Grains were counted to a cut-off distance of just over 8 HD or 588nm from the radioactive source on either side; this means that the scatter of grains was measured in a strip, approximately one micron wide, about the membrane.

The fact that the distribution of silver grains about the presynaptic plasma membrane was normal, emphasises that acceptors for
Fig. 4.8 Histogram of experimental grain density distribution around a radioactive line source: determination of the HD value.

a. The distance from a biological radioactive line source to the midpoint of over 500 silver grains was measured and a histogram of grain density distribution about the line was constructed with the data. As this histogram appeared symmetrical on both sides (indicating that the source was in fact a line), a one-sided histogram (shaded bins) was also plotted. A smooth curve was then drawn in by eye.

b. The same results as shown in a. were used to plot an integrated grain distribution histogram. For each bin, all the silver grains counted within that distance from the line source were added. The number of grains changed very little after ~300mm and counting was thus stopped at a cut-off distance of ~600mm. The HD, or distance from the source containing half the silver grains, was found to be ~56nm.
a

# of grains

Distance from membrane (nm)

b

# of grains

Distance from membrane (nm)

HD (56 nm)

6 HD

8 HD

266
\(^{125}\text{I}-\text{BoNT}\) type A are indeed located on this biological 'line source' and presence of sites on the postsynaptic membrane can be excluded.

4.3.6 Density of \(^{125}\text{I}-\text{BoNT}\) type A binding sites.

As shown previously, the translocation of radiolabelled toxin into the nerve terminal cytosol can be prevented by including the metabolic inhibitor sodium azide (15mM) in the incubation medium. Quantitation of the binding sites can then be carried out readily using the method of Fertuck and Salpeter (1976). Nerve-muscle preparations, previously incubated with \(^{125}\text{I}-\text{BoNT}\) type A at a concentration known to saturate all the acceptor sites (150nM), were processed for electron- microscope autoradiography. Groups of sections were developed each week for six weeks and a large number of terminals were photographed (Fig. 4.9). The number of grains per micron of plasma membrane was then determined for each exposure time (Fig. 4.10). In order to assign an efficiency value to the groups of autoradiograms, several assumptions were made: as the radioactive source, in this case, was a line of negligible width (7-9nm) and not an area, the grain distribution could not be expressed as grains per square micron of emulsion (see Fig. 4.6b, section 4.3.5) with any accuracy. From studies carried out previously (see section 4.3.5), it can be said that percent efficiency is affected minimally by radiation dose in the autoradiographic method used. This is substantiated by the results depicted in Fig. 4.10 where the number of grains per micron of nerve terminal membrane is plotted as a function of exposure time. It appears that grain production was essentially unaffected by radiation dose during the first 28 to 35 days of exposure, although by 42 days a slight effect had become apparent. Although there was a small increase in scatter of silver grains about the radioactive source (nerve terminal plasma membrane) by this time (see Fig. 4.9f), grains could still be counted as they overlapped only rarely.
Fig. 4.9 Time course of grain development.

One slide, containing ultrathin sections of nerve terminals previously exposed to 150nM $^{125}$I-BoNT type A (in the presence of Na azide), was developed each week for 6 weeks. Representative labelled terminals are shown to illustrate the time course of silver grain production. a. 7 days, b. 14 days, c. 21 days, d. 28 days, e. 35 days, f. 42 days. ($\times 10,000$).

Note the increased scatter of grains after 42 days exposure (f). (arrows).
Fig. 4.10 Grain density on the nerve terminal membrane as a function of exposure time.

20-34 end-plates (100-300 silver grains) were photographed for each time point; grains were counted and the length of the nerve terminal membrane was determined by digitisation. Grain density (no. grains/μm membrane) was then plotted as a function of exposure time. Note that the production of silver grains increased almost linearly as the exposure time was lengthened, although some deviation is seen between 28 and 42 days. Quantitative results will, therefore, be more accurate and meaningful if values are taken from shorter exposure times (i.e. 14-28 days).
The efficiency values obtained experimentally for the exposure times in this study (1 to 6 weeks) ranged from 63% to 57%, the average being 60% (see Section 4.3.5). This value was assigned to all the autoradiograms analysed for absolute quantitation of acceptor sites.

Using this experimental efficiency value (60%), the grain density (grains/µm² membrane) calculated for each exposure time, and the specific radioactivity of the ¹²⁵I-BoNT type A at the beginning of the exposure period, the number of acceptor sites for the toxin per square micron of plasma membrane was determined for each of the six groups of terminals. The average number obtained was 153 per µm² ± 20% (Table 4.4). As the radiation dose given by the radioactive line (plasma membrane) could not be determined, an average value of 60% was used for the efficiency of all exposure times. It is interesting that the number of sites obtained for the group exposed for 42 days was considerably lower than the rest (141); the percent efficiency value assigned was probably too high, and the effect of radiation dose thus underestimated, in this case. If a value of 57% (from Fig. 4.7a) was used instead of 60%, the number of sites became 148 which is closer to the average value obtained (153). The 60% estimate was probably accurate for 21, 28 and 35 day exposures; a higher efficiency would have been more appropriate for 7 and 14 day exposures (using 62% as the efficiency, the number of sites became 152 and 150 for 7 and 14 day exposures respectively). Using these new values, the average was 152 (± 20%) sites per square micron which agrees quite closely with the number (153 sites/µm² ± 20%) previously obtained.

When quantitation of acceptor sites was carried out using 15nM and 35nM ¹²⁵I-BoNT, the numbers obtained were 150 (± 21%) and 146 (± 21%) sites/µm² respectively (see Table 4.4). This emphasises that these concentrations do, in fact, saturate the binding sites for ¹²⁵I-labelled BoNT at the motor nerve terminal (Fig. 4.1). The average
Table 4.4. Density of acceptors obtained with different concentrations of $^{125}$I-BoNT type A.

Using the grain density values obtained for each time point, the experimental value for efficiency (60%) and the initial specific radioactivity of the toxin, the number of acceptor sites for $^{125}$I-BoNT type A at the murine motor nerve terminal was calculated as follows (Fertuck and Salpeter, 1976):

$$\text{sites/\mu m}^2 = \frac{G \times d \times A}{124,800(1-e^{-0.01155(t)}) \times \text{SoC}}$$

where $G = \text{grains/\mu m}^2$, $d$ = decays needed for 1 developed grain or 100/60; $t$ = exposure time in days, $So = \text{specific activity of the } ^{125}\text{I-BoNT type A at the start of the exposure period}$; $C = \text{disintegrations per minute or } (2.22 \times 10^{12})$; $A = \text{Avogadro's number or } 6.023 \times 10^{20} \text{ molecules per mmol}$, $1.248 \times 10^5 = \text{the e-folding time for exponential decay of } ^{125}\text{I in minutes}$; $0.01155 = \text{the inverse of the e-folding time for } ^{125}\text{I in days}$.

The different sources of error in such a calculation and their relative contribution to the overall autoradiographic error are given below (see Matthews-Bellinger and Salpeter, 1978):

1. Sampling error in autoradiographic grain counting (as the number of grains in autoradiograms is Poisson distributed) is given by $100/\sqrt{n}$, where $n$ is the number of grains counted.
2. Error in section thickness determinations: ±5%.
3. Error in specific activity of the $^{125}$I-BoNT type A used: ±10%.
4. Accuracy in determining efficiency values: ±15%.

The percent accuracy in each final value for sites/\mu m$^2$ is the square root of the sum of the squares of all the above errors.
Table 4.4 Density of acceptors obtained with different concentrations of 125I-BoNT type A.

<table>
<thead>
<tr>
<th>Concentration of 125I-BoNT (nM)</th>
<th>Exposure time (days)</th>
<th>Grain Density (per μm²)</th>
<th>Specific activity of the toxin preparation (Ci/mmol)</th>
<th>sites/μm² of plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>150nM</td>
<td>7</td>
<td>4.16</td>
<td>1241</td>
<td>157±33</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>8.26</td>
<td>1241</td>
<td>161±33</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>11.32</td>
<td>1241</td>
<td>153±31</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>14.52</td>
<td>1241</td>
<td>153±30</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>17.47</td>
<td>1241</td>
<td>153±30</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>18.53</td>
<td>1241</td>
<td>141±28</td>
</tr>
<tr>
<td><strong>AVERAGE:</strong></td>
<td></td>
<td><strong>153±30</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35nM</td>
<td>28</td>
<td>7.51</td>
<td>657</td>
<td>150±31</td>
</tr>
<tr>
<td>15nM</td>
<td>28</td>
<td>7.29</td>
<td>657</td>
<td>146±30</td>
</tr>
<tr>
<td><strong>All concentrations used</strong></td>
<td></td>
<td></td>
<td><strong>TOTAL AVERAGE:</strong> 152±31</td>
<td></td>
</tr>
</tbody>
</table>
number of sites per square micron of terminal membrane for all the experiments was 152 ± 20% (or between 121 and 183 sites/μm²).

4.3.7 Quantitation of $^{125}$I-BoNT type B binding sites.

Absolute quantitation of the saturable acceptor sites for $^{125}$I-BoNT B at the motor nerve terminal was carried out as described for $^{125}$I-labelled type A (Section 4.3.6). A study of the concentration dependence of binding of $^{125}$I-BoNT showed that saturation in the system used (see section 4.3.4) occurs at approximately 40-50nM (under conditions that prevent internalisation of toxin molecules). To quantitate the sites, small pieces of mouse diaphragm were exposed to 100nM $^{125}$I-BoNT type B in the presence of 15nM sodium azide for 90 minutes. Pale gold sections from two different blocks were processed for autoradiography; the emulsion was developed after 11 days exposure and a large number of nerve terminals from each group were photographed (~120 silver grains) (see Fig. 4.11). Table 4.5 shows the number of sites determined from nerve terminals in blocks 1 and 2: using equation 3, the values obtained were 623 ± 21% and 631 ± 21% per square micron of membrane giving an average of 627 ± 21%; the range of values is thus 495-759. The number obtained for $^{125}$I-type B is approximately 4 times greater than that for A (152 and 627 respectively).

4.4 DISCUSSION

As direct kinetic studies on the binding component for BoNT at the neuromuscular junction have not been possible to date, autoradiographic methods were used to determine conditions in which all acceptor sites are occupied in a murine diaphragm preparation. This was necessary for absolute quantitation of the acceptor sites for $^{125}$I-BoNT types A and B. In addition, the separable steps of intoxication by BoNT were
Fig. 4.11 Nerve terminal treated with 100 nM $^{125}$I-BoNT type B in the presence of Na-azide for quantitation of the acceptor sites (exposure-11 days).
Table 4.5 Quantitation of $^{125}$I-BoNT type B acceptor sites at the murine motor nerve terminal.

<table>
<thead>
<tr>
<th>Concentration $^{125}$I-BoNT type B (nM)</th>
<th>Exposure time (days)</th>
<th>Efficiency %</th>
<th>Grain Density (per $\mu m^2$)</th>
<th>Specific activity of the toxin preparation (Ci/mmol)</th>
<th>sites/$\mu m^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 1 100</td>
<td>11</td>
<td>62</td>
<td>13.37</td>
<td>630</td>
<td>$623 \pm 129$</td>
</tr>
<tr>
<td>Block 2 100</td>
<td>11</td>
<td>62</td>
<td>13.52</td>
<td>630</td>
<td>$631 \pm 132$</td>
</tr>
</tbody>
</table>

AVERAGE: $627 \pm 131$

Using the same procedure as described for type A (Table 4.4), the binding sites for $^{125}$I-BoNT type B were quantitated. Sections from two different blocks were processed for electron-microscope autoradiography separately and grain densities on the nerve terminal membranes were determined as described in Table 4.4. A value of 62% was used for efficiency as experiments with type A suggested that this was more appropriate for the exposure time (11 days) used in this case (see Section 4.3.5).
studied independently. Thus, concentration dependence, time course and temperature dependence of radiolabelled toxin binding and internalisation were investigated in the presence and absence of metabolic inhibitors. In spite of the limitations of the technique (i.e. diffusion of toxin into the tissue; error in section thickness, specific radioactivity and protein concentration determinations), interesting conclusions can be drawn from the reproducible data obtained. Moreover, results can be correlated with data obtained by pharmacological methods (Simpson, 1980) and with studies performed in the central nervous system. It must be emphasised, however, that numerical values given apply solely to the nerve-muscle preparation used.

4.4.1 Concentration dependence and time course of toxin binding to the motor nerve terminal.

Saturability of binding: In the presence of metabolic inhibitors, $^{125}$I-BoNT type A saturated its acceptor sites on the presynaptic plasma membrane at a concentration of 15-20nM. Taking into account the problem of diffusion into the tissue, the affinity of the toxin for these sites was comparable to that observed in the central nervous system for the high affinity component ($K_D = 0.6nM$; Williams et al., 1983). It is impossible to ascertain, from the autoradiographic data obtained, whether single or multiple sets of sites are involved. However, it appears that all the sites were saturated (both low and high affinity, should they exist at the neuromuscular junction) at a toxin concentration of 15-20nM as there was no difference in the extent of binding between 20 and 150nM. As shown in Chapter 3, $^{125}$I-BoNT types A and B interact not only with the plasma membrane of the synaptic bouton but also with the membrane of unmyelinated terminal axons. A significant number of grains were observed on axonal membrane even at
low toxin concentrations (1.5nM) indicating that the sites involved must be of relatively high affinity. Binding appeared virtually irreversible as washing at 4°C or 25°C for 30 or 90 minutes did not significantly affect the extent of labelling of motor nerve terminals (7%, data not shown).

A study of the concentration dependence of binding of $^{125}$I-BoNT type B to motor nerve terminals showed that saturation occurred at approximately 40-50nM. The affinity of this toxin for its acceptor sites is, therefore, lower than that of radiolabelled type A BoNT for its binding components. The saturation curve obtained (Fig. 4.4.) did not distinguish between populations of sites (as too few points were determined), so a high affinity component may exist and be masked by a large population of lower affinity sites.

**Acceptor recycling:** In the absence of inhibitors of energy production, the number of silver grains associated with nerve terminals when $^{125}$I-BoNT type A had been applied at a concentration of 15nM or 35nM was 10% greater than that observed when sodium azide was included in the incubation medium. It has been shown (e.g. Marsh et al., 1983) that an important part of the process of acceptor-mediated endocytosis of proteins is the replacement of cell surface acceptors that have been internalised. Acceptors often enter cells with their ligands (Steinman et al., 1983) becoming separated at a later stage. In some cases the acceptor, free of its ligand, is returned to the plasma membrane to participate in a new round of endocytosis (acceptor recycling), whereas in others it is destroyed in lysosomes (Pastan and Willingham, 1981a); in the latter case, replacement of acceptors occurs by de novo synthesis within the cell. Both processes are energy-dependent. At low ligand concentrations (e.g. 1.5nM), acceptor-recycling may be undetectable and the extent of binding in azide-treated and untreated samples would be expected to appear the same. However, at higher ligand concentrations,
more acceptors would be internalised and more returned to the cell surface ready to take up more ligand. This possibly accounts for the fact that at a toxin concentration of 1.5nM, the extent of binding was the same in treated and untreated samples, whereas at higher concentrations a difference of 10% became apparent. Dissociation of toxin during washes cannot account for the differences observed at higher toxin concentrations, as washing should also affect toxin bound at low concentrations (but only if all sites have the same affinity for the toxin). However, the possibility that only high affinity sites were occupied when toxin was applied at 1.5nM whereas at 15 and 35nM low affinity sites were also occupied, cannot be excluded.

Time course of the binding step: In de-energised samples, maximum binding of $^{125}$I-BoNT type A occurred after 40-50 minutes (at 15nM, 22°C), with a half-time of approximately 8 minutes. A slight decrease in the number of silver grains associated with the plasma membrane (in azide-treated samples) was observed after 150 minutes incubation. This can be attributed to onset of necrosis in the tissue due to the presence of metabolic inhibitors for an extended period of time. In the absence of sodium azide, maximum binding also occurred after 40-50 minutes incubation. However, even after 20 minutes, there was 7% less binding in treated relative to untreated samples. This suggests some reversibility of the binding step under these non-physiological conditions and/or the possibility that low levels of acceptor-recycling had already occurred in untreated samples by this time (Dautrey-Varsat and Lodish, 1984).

4.4.2 Concentration dependence and time course of the internalisation step.

The internalisation phase of intoxication (referred to as the 'translocation step' by Simpson, 1980) was studied in the absence of
metabolic inhibitors under different incubation conditions. A time course of uptake showed that toxin molecules had already been taken up into the nerve terminal after 20 minutes incubation. The proportion of internalised silver grains at this time was 21% of the total associated with the nerve terminal. This increased to approximately 40% after 90 minutes and remained essentially unchanged, even following prolonged incubation times (e.g. 240 minutes). The proportion of internalised grains was unaffected by toxin concentration; at 1.5, 15 and 35nM the ratio of internalised to membrane-bound silver grains was always approximately 40:60 after 90 minutes incubation. This cannot be attributed to 'cell death' as a result of the incubation conditions used, as studies involving larger volumes with constant oxygenation gave similar results. The steady-state 60:40 ratio of membrane-bound to internalised grains can be explained in various ways. It is possible that two distinct populations of acceptor sites for $^{125}$I-BoNT type A are present on the motor nerve terminal membrane in a ratio of 60:40, and that only one of these populations (40% of the total) is responsible for mediating toxin internalisation. However, these sites would necessarily have the same, or very similar, affinity for the toxin as the same ratio is observed at all concentrations used (i.e. well below and above saturation). It is also possible that after maximum binding has occurred, an equilibrium is eventually reached where the rate of binding and uptake of toxin molecules matches that of degradation and excretion. In this scheme, toxin molecules would bind to their acceptors on the cell surface and subsequently be internalised. Owing to the acid environment of the endocytic vesicle (see General Introduction, Section on acceptor-mediated endocytosis), the subunits would probably separate allowing the smaller subunit to enter the cytosol to exert its toxic effect. Fusion of the endocytic vesicle with a lysosome would lead to degradation of the larger subunit followed by
excretion of degradation products (Steinman et al., 1983); after carrying out its lytic function, the smaller subunit would undergo the same fate (see Chapter 5 and 7). Establishment of this equilibrium would also require replacement of internalised acceptors, either by acceptor recycling or by de novo synthesis.

Situations similar to that described for the internalisation of BoNT are also apparent in other systems. It is interesting that the same distribution of radioactivity is seen when Vero cell mutants (M-91) are exposed to 125I-diphtheria toxin (Middlebrook and Dorland, 1981). This subline is highly resistant to diphtheria toxin, apparently due to a mutation in the elongation factor-2, rendering it refractory to toxin-catalysed ADP-ribosylation. Other measures of toxin-cell interactions such as acceptor affinity, internalisation rate and degradation rate are essentially unchanged in this mutant line. In these cells, an equilibrium is reached when the rate of binding and internalisation equals degradation and excretion such as that proposed for BoNT at the nerve terminal. In wild-type cells, however, protein synthesis is completely inhibited after 1 to 1 1/2 hours. This leads to a loss of acceptors produced by de novo synthesis and hence a block of continuous uptake. The amount of bound radiolabel thus decreases, as does the internalised fraction since degradation and excretion still take place. Just before 1 hour incubation (and thus before protein synthesis is inhibited) the ratio of membrane-bound to internalised toxin is 60:40 in Vero cells also.

Gorden and Orci (1981) performed studies on the binding and uptake of 125I-insulin (a polypeptide hormone) in freshly isolated rat hepatocytes. Within 2 minutes incubation, internalisation of 125I-insulin was made evident by the presence of silver grains in the cytoplasm, and by 30 minutes, which represented steady-state binding, approximately 40% of the radioactivity was located within the cell, and
the remainder (60%) was associated with the plasma membrane. The authors suggest that this steady-state condition is a result of the coupling of binding of the hormone to its degradation within the cell and to loss of its cell surface acceptor.

It appears that BoNT (Simpson, 1981), diphtheria toxin (Olsnes and Sandvig, 1983) and insulin (Gorden and Orci, 1981) all undergo acceptor-mediated endocytosis in target cells. This suggests that the ratio obtained for membrane-bound to internalised grains is a direct result of similar processing pathways.

A third explanation for the observed steady-state proportion of internalised radioactivity could be an effect of the toxin itself. It is possible that once a certain amount of toxin has been taken up into the unstimulated nerve terminal, it causes inhibition of uptake in a manner analogous to its blockade of exocytosis. The 40:60 ratio is not apparent after short incubation times (i.e. 20 minutes) but can always be seen after 90 minutes when onset of paralysis is definitely apparent. It has been shown that choline uptake is depressed by botulinum toxin type A (Gundersen and Howard, 1978; Dolly et al., 1981; Habermann, 1981); however, it is not clear whether inhibition of release leads to eventual inhibition of uptake or whether the reverse situation (depression of release is caused by depression of uptake) actually takes place.

Finally, the distribution of label within the toxin molecule cannot be ignored (as has been discussed in Chapter 3, Section 3.4). On reduction of $^{125}$I-BoNT type A, two radioactive species are observed; these correspond to the heavy and light subunits which contain 60 and 40% of the label respectively (Williams et al., 1983). The smaller subunit of $^{125}$I-BoNT type B contains very little radioactivity relative to the heavy subunit (R. Williams, personal communication). Whereas nerve terminals exposed to $^{125}$I-BoNT type A showed 40% of the radio-
activity in the form of internalised grains, those exposed to type B showed 25% of the grains within the terminal. In each case, the extent of uptake reflected the proportion of label in the lighter subunit, suggesting that only the latter is transported across the membrane. This mechanism of transport has been suggested for cholera toxin (Van Heyningen, 1977), Shigella cytotoxin (Keusch, 1981) and, under certain conditions, for diphtheria toxin (Sandvig and Olsnes, 1981). In this scheme, the assumption that no excretion of toxin takes place must necessarily be made.

It is clear that the elucidation of the exact mechanism by which BoNT is taken up, and the reasons for the distribution of label observed at nerve terminals, require further investigation; this is discussed in the next chapter.

4.4.3 Effects of temperature on binding and internalisation.

Binding of $^{125}$I-BoNT type A at murine nerve terminals was found to be temperature-sensitive. The diminished extent of labelling seen at 4°C (30% of that at 22°C) was probably due to effects on the rate of diffusion into the synaptic matrix as well as direct effects on the interaction between toxin molecules and their acceptors. Internalisation was greatly affected by temperature; at 4°C, this process was totally inhibited.

4.4.4 Density of acceptor sites for $^{125}$I-BoNT types A and B.

The separation of the binding and internalisation steps of intoxication by $^{125}$I-BoNT types A and B using metabolic inhibitors, and studies on concentration dependence of binding, made possible the absolute quantitation of binding sites for both toxin types at the murine motor nerve terminal. In order to carry out such determinations, calibration of the autoradiographic technique was essential. Percent
efficiency and resolution of the emulsion/developer combination used were determined. The energy of the electrons from $^{125}$I is optimal for electron-microscope autoradiography; resolution and percent efficiency values obtained using this isotope are better than for any other studied (Salpeter et al., 1977). $^{125}$I is not a beta emitter like $^3$H or $^{14}$C which emit particles showing a continuous spectrum of energies, ranging from a maximum value down to zero. It produces extranuclear electrons at specific energy levels; most of these have low initial energies ideal for electron-microscope autoradiography. The isotope undergoes rearrangements of the neutrons and protons in its nucleus without ejection of a charged particle; it loses energy as a low energy photon. The latter may lose its energy to one of the orbital electrons in the inner K or L shells which then leaves the atom behaving exactly like a $\beta$-particle. The gap left in the inner electron shell is filled by one of the outer electrons, a transition which is accompanied by emission of low energy photons (X-rays). The X-rays may cause further ejection of Auger electrons (or electrons of the outer shell). In total, per 100 nuclear electrons, the Auger or internal conversion electrons emitted consist of 128 low energy electrons (~3 - 4 keV) and 36 high energy electrons (~23 - 34 keV). In addition, there are 150 photons (that is, X-rays or $\gamma$-rays of 28 - 35 keV) (Fertuck and Salpeter, 1974). The high energy electrons and photons do not contribute significantly to the production of silver grains; the former constitute only 20\% of the electrons emitted and have low probability of being detected in the thin emulsion layers used, and the latter have an even lower probability of detection as they are of even higher energy (although more plentiful: 150/100 nuclear decays). It is the low energy electrons, which constitute 80\% of those emitted, that are optimal for electron-microscope autoradiography. They are not expected to be absorbed within ultrathin sections, but will lose all their energy
within thin emulsion layers. The probability of these low energy electrons producing deposition of a silver grain is relatively high: stable latent images result with high probability of development. As there are 128 low energy electrons \((3-4\ \text{keV})\) emitted for every 100 nuclear decays, the maximum percent efficiency (grains per total decays) obtainable for these electrons is 64\% (the other half (64) are not emitted towards the emulsion). The experimental percent efficiency obtained in this study was almost the theoretical maximum at low radiation dose (60 - 63\%). However, as the radiation dose increased (35 to 42 days), efficiency was slightly reduced (57\%). The low energy electrons emitted are also expected to give high resolution as they will not travel very far from the radioactive source. This was found to be the case; the half distance value obtained was \(56\text{nm}\). The autoradiographic method used was thus of high efficiency and resolution.

In this Chapter, the absolute number of acceptor sites for two presynaptic toxin types is given for the first time. An interesting point arising from the resolution studies involves the location of toxin binding sites. As the number of grains not directly in contact with the nerve terminal membrane was the same on either side of it and the distribution was therefore normal, it can be assumed that all the toxin bound opposite the muscle folds was actually on the presynaptic membrane. This is consistent with the lack of direct toxin-induced postsynaptic effects. If a proportion of toxin molecules were bound to the postsynaptic muscle cell membrane, a greater number of grains would be expected to occur in the direction of the muscle cell (as seen for the ACh receptor, Porter and Barnard, 1975). Concentration dependence studies indicated the amount of toxin necessary to saturate the sites in the nerve-muscle preparation used. The number of sites per \(\mu m^2\) of membrane for \(^{125}\text{I-BoNT}\) types A and B could then be determined and was found to be \(152\pm31\) and \(627\pm131\) respectively; there are therefore
approximately 4 times as many B sites as A sites. The density of acceptors suggests that it is unlikely that toxin types bind to a site within the active zones. The number of sites, which appear uniformly distributed on the terminal membrane, far exceeds the estimated number of 500 active zones per nerve terminal (Heuser, 1978). Nevertheless, the possibility that a small number of high affinity sites lie within the active zones (although this is difficult to reconcile with other data discussed here and previously in Chapters 1 and 3) or that toxin molecules interact with active zones intracellularly, cannot be excluded.

In the next Chapter, the nature of the acceptor sites for BoNT type A at the motor nerve terminal, as well as the mechanism of uptake of molecules into the cytosol, is investigated using electron-microscope autoradiography.
CHAPTER V

NATURE OF THE BOTULINUM NEUROTOXIN BINDING

AND INTERNALISATION STEPS AT THE MURINE MOTOR NERVE TERMINAL
5.1 INTRODUCTION

Binding of BoNT molecules to acceptor sites on the motor nerve terminal.

The 'nicked' botulinum neurotoxin molecule consists of two heterologous subunits linked by a disulphide bond (DasGupta and Sugiyama, 1976) (see General Introduction). When the disulphide bond is reduced, the chains can separate and are referred to, in the literature, as the heavy (H; \( M_r \approx 100,000 \)) and the light (L; \( M_r \approx 50,000 \)) chain.

A number of bacterial and plant toxins (i.e. cholera toxin, diphtheria toxin, tetanus toxin, abrin and ricin) as well as glycoprotein hormones (thyroid-stimulating hormone, luteinizing hormone, follicle stimulating hormone and human chorionic gonadotropin) which are known to act intra-cellularly, are remarkably similar in overall structure to BoNT types. They all consist of two heterologous components and appear to have a common strategy of approach to target cells (Neville and Chang, 1978; Van Heyningen, 1982): one of the subunits is thought to bind to an acceptor on the external surface of the cell, while the other enters the cell and carries out the biological activity of the molecule. It has been suggested for types A and B BoNT that binding to acceptors on rat brain synaptosomes is mediated by the heavy subunit (Kozaki, 1979; Williams et al., 1983). Interestingly, tetanus toxin, which like BoNT is thought to bind to acceptors in the central nervous system by its larger subunit (Matsuda and Yoneda, 1975; Van Heyningen, 1976), is prevented from binding to the neuromuscular junction by Fragment C of the toxin (part of the large subunit) (Simpson, 1984). However, at present there is no evidence that the heavy subunit of BoNT mediates its binding to the motor nerve terminal.
Nature of the acceptor sites for BoNT type A.

This has been studied at the neuromuscular junction and in the central nervous system (Simpson and Rapport, 1971; Van Heyningen and Mellanby, 1973; Mellanby et al., 1973; Habermann, 1974; Habermann and Heller, 1975; Kitamura et al., 1980; Williams et al., 1983). However, the results are contradictory and therefore inconclusive. Most of these studies have focused on gangliosides as possible acceptors for the toxin. Gangliosides are unique acidic glycolipids that are selectively concentrated in the plasma membrane of cells; they have a structure that makes them well suited to the role of membrane acceptor (Fishman and Brady, 1976). Being comparatively small, they can move freely in the plane of the membrane. Their hydrophobic tails are anchored in the lipid membrane, leaving their negatively charged carbohydrate portion exposed to the external environment. This hydrophobic part of the molecule varies greatly, therefore offering many possibilities for ligand interactions. It is well known that cholera toxin interacts with the monosialoganglioside GM$_1$ (King and Van Heyningen, 1973) and there is considerable evidence that specific gangliosides can inhibit the actions of tetanus toxin (Van Heyningen, 1973), serotonin (Woolley and Gommi, 1965) and interferon (Besancon and Ankel, 1974). Two reports indicated that trisialogangliosides inactivate botulinum toxin type A (Simpson and Rapport, 1971; Kitamura et al., 1980) but other studies failed to show any interaction of the toxin with these lipids (Van Heyningen and Mellanby, 1973; Mellanby et al., 1973; Habermann, 1974; Habermann and Heller, 1975). Although treatment with neuraminidase significantly reduced the binding of botulinum neurotoxin type A to rat brain synaptosomes (Habermann and Heller, 1975; Williams et al., 1983), it did not prevent the inhibitory action of toxin on neurotransmitter release in brain preparations (Bigalke et al., 1981; Habermann et al., 1981) or at the neuromuscular junction (Habermann and Heller, 1975).
Moreover, it has been shown that proteolysis with trypsin and proteinase K, or heat treatment, reduce the binding of $^{125}$I-BoNT types A and B to synaptosomes (Williams et al., 1983; Williams, 1984) suggesting that the acceptor may be a protein. Clearly, further studies on the nature of the acceptor for BoNT at the nerve terminal are required.

Possible antagonists of toxin binding.

It has been suggested that tetanus toxin [another clostridial neurotoxin which has many similarities, both in molecular structure and pharmacological action, to botulinum toxin (Habermann, 1981; Sugiyama, 1980; Mellanby and Green, 1981)] shares similar acceptor sites with BoNT type A on nerve endings in the central nervous system (Williams et al., 1983). However, Habermann (1981) failed to show any interaction in the binding of these two clostridial neurotoxins and, more recently, Simpson (1984a) suggested that, at least at the neuromuscular junction, the acceptors are distinct. The binding fragment of tetanus toxin (Fragment C), which inhibits the action of the native toxin, failed to prevent BoNT type A-induced inhibition of neurotransmitter release (although it does appear to antagonise the action of types C and E BoNT; Simpson, 1984b). However, the effect of tetanus toxin on the binding (rather than the action of BoNT) at the motor nerve terminal has not been studied to date.

Another condition which involves a presynaptic defect in neuromuscular transmission with very similar electromyographic characteristics to those produced by BoNT, is Lambert-Eaton myasthenic syndrome (LEMS) (Lambert and Elmqvist, 1971). The main symptoms in this disorder, which is frequently associated with carcinoma of the lung, are weakness and easy fatigability of proximal muscles of the limbs, with respiratory insufficiency in very severe cases. As in botulinum toxin
intoxication, there is no evidence of abnormality of function of the peripheral nerve or muscle fibres themselves (Lambert and Elmqvist, 1971), and presynaptic stores of ACh are normal (Molenaar, 1982). In myasthenic syndrome, there is a pronounced depression of the muscle response to a supraximal stimulus applied to the motor nerve (Lambert and Elmqvist, 1971) as seen in botulinused muscles: stimulation of nerves innervating paralysed muscles results in end-plate potentials of small amplitude (0.5-2.0mV) and high failure rate (Cull-Candy et al., 1976; Dolly et al., 1981; Thesleff, 1981). In both botulism and myasthenic syndrome, the defect in neuromuscular transmission is due to a decrease in the number of ACh quanta released from the motor nerve terminal in response to a nerve impulse. The cause of the defective release of transmitter is unknown in both cases. It is thought that, in myasthenic syndrome, an autoimmune antibody (Lang et al., 1981) interacts with sites (Heuser, 1976) involved in the release of ACh at the active zones. Recent freeze-fracture electron-microscope studies of the LEMS neuromuscular junction, in humans and mice (Fukunaga et al., 1982; Fukunaga et al., in press), have revealed a selective depletion of the active zones and active zone particles, but not of any other membrane particles, in these preparations. This is accompanied by the appearance of large membrane particles grouped into clusters, which may be aggregates of active zone components produced by a cross-linking antibody (Fukunaga et al., in press). These components are thought to be voltage-sensitive Ca++ channels (Llinas et al., 1976; Pumplin et al., 1981). Thus, the diminished quantal release induced by the nerve impulse in LEMS could be the result of reduced entry of Ca++ into the nerve terminal (Fukunaga et al., in press). In the case of botulinum toxin, it has been suggested that paralysis is the result of reduced sensitivity of the release machinery to Ca++. Accordingly, both conditions are temporarily reversed by administration of 4-aminopyridine
or guanidine (Lang et al.; 1981; Habermann et al., 1980) or, in in vitro systems, by increasing the levels of extracellular Ca$^{++}$ (in the case of BoNT, in the presence of ionophores)(Lang et al., 1981; Cull Candy et al., 1976).

The autoimmune origin of the disease has been suggested by a number of workers (Guttmann et al., 1972; Lang et al., 1981; 1983). The physiological abnormality can be passively transferred to mice by injection of the IgG fraction of plasma from patients with myasthenic syndrome (Lang et al., 1981; 1983). This strongly supports the proposal that an autoantibody, binding to the nerve terminal, may be implicated in this disorder of neuromuscular transmission.

In view of the notable similarities in the characteristics of these conditions, it seemed worthwhile to establish whether the putative antibody and BoNT compete for the same site(s) at motor nerve terminals. Nerve muscle preparations from mice to which the syndrome has been passively transferred could be used as models for the study of this interaction.

Nature of the uptake process.

The strategy of approach of the toxin molecule to target cells involves a second step following binding to cell surface acceptors; the translocation step. A very large energy barrier must be overcome in order to pull a large hydrophilic protein such as the BoNT molecule across the hydrophobic lipid bilayer surrounding the nerve terminal. Several specialised processes have been proposed for the uptake of toxin; these include 1) direct entry into the cytosol by means of an active mechanism inherent in the toxin molecule (e.g. enzymatic activity), 2) an active mechanism of the cell membrane such as endocytosis, and 3) a combination of the two. To date, there is no evidence that the BoNT molecule possesses enzymatic activity (Simpson, 1972;
suggest that the process of acceptor-mediated endocytosis is responsible for entry of BoNT into the cytosol (see General Introduction for a description of this process which is almost universal among cells).

In order to test the validity of this proposal, the effect of incubation conditions and drugs that inhibit the process was studied. Ca++ deprivation has been shown, in a number of cases, to prevent certain steps in the endocytic pathway (Davies et al., 1980). Likewise, lysosomotropic agents such as chloroquine, ammonium chloride and methylamine interfere with the process of by raising the intravacuolar pH of lysosomes and other acidic compartments, thus preventing ligand-acceptor dissociation and/or lysosomal degradation (de Duve et al., 1974; Leppla et al., 1980). These agents are known to inhibit the action of diphtheria toxin (Leppla et al., 1981), modeccin (Sandvig et al., 1979), certain viruses (Helenius et al., 1980) and many other substances that act intracellularly. Ca++ deprivation and lysosomotropic agents delay the onset of toxin-induced blockade of neurotransmission in phrenic nerve-hemidiaphragm preparations (Simpson, 1980; 1982; 1983) and maintain the toxin molecules at a site sensitive to antitoxin. To date, the location of this site has not been demonstrated by histological or autoradiographic techniques.

Simpson (1974) has also shown that toxin, added to isolated nerve-muscle preparations at low temperatures, can be inactivated by antitoxin antibodies if they are added at the same time as the temperature is raised to 37°C and nerve stimulation is applied. Whether the antitoxin prevents endocytic uptake of the toxin into the nerve terminal is not known. The fungal metabolite cytochalasin B causes disassembly of
microfilaments in the cytoskeleton (Pollard, 1976) and has been shown to affect cellular movements such as pinocytosis, phagocytosis, and pseudopod extension (Weihing, 1976).

In this Chapter, the binding and internalisation of BoNT intoxication are studied at the murine motor nerve terminal using $^{125}\text{I-BoNT}$ type A. An attempt is made to determine whether the neurotoxin interacts with its target cells in a manner analogous to that of other bacterial toxins and hormones of similar structure that also act intracellularly. The process by which toxin is internalised is investigated by observing the effects of drugs that are known to interfere with the proposed uptake mechanism and, also, with BoNT-induced blockade of neurotransmission.

5.2 MATERIALS AND METHODS

The isolated larger subunit of BoNT type A ($M_r 97,000$) was kindly provided by R. Williams in this laboratory. The toxin's subunits were separated by QAE-Sephadex anion-exchange chromatography in the presence of dithiothreitol and urea (Kozaki et al., 1981). Each was dialysed against 0.02M sodium phosphate buffer pH 7.2 for 48 hours prior to use in competition experiments with $^{125}\text{I-BoNT}$ type A. The nature of the acceptor for the toxin type A at the motor nerve terminal was investigated by pre-treating diaphragm tissue with the enzyme neuraminidase from Clostridium perfringens Type X (0.6 units/ml pre-incubation medium). One unit of the enzyme (purchased from Sigma) will liberate 1 μm of N-acetyl neuraminic acid per minute at pH 5.0 at 37°C using N-acetyl-neuraminyl-lactose as substrate. A preparation of pure tetanus neurotoxin for competition studies with $^{125}\text{I-BoNT}$ type A was kindly provided by Dr. P.D. Walker.
As mentioned previously (Section 5.1), myasthenic syndrome can be passively transferred to mice by injection of the IgG fraction of plasma from patients with the disease. Diaphragm tissue used in studies with $^{125}$I-BoNT type A was provided by Dr. Bethan Lang (Department of Neurological Science and Pharmacology, Royal Free Hospital School of Medicine). The mice from which the tissue was taken showed definite signs of the disease as demonstrated electrophysiologically. The IgG fraction injected was purified from plasma obtained at plasma exchange (which was undertaken with a Haemonetics 30 cell separator) using the Rivanol ammonium sulphate method (Newsom-Davies, 1979). 10mg IgG was administered daily for 58 days to a BKTO mouse. Cyclophosphamide (300mg/kg) was injected on day 1 to suppress the response to foreign immunoglobulin (see Lang et al., 1981; 1983). Control samples were taken from a mouse injected with pooled normal plasma for 58 days.

The lysosomotropic agents chloroquine and methylamine were purchased from Sigma Chemicals Co. and used in localisation experiments at a concentration known to prevent BoNT-induced blockade of neurotransmission (Simpson, 1982; 1983). Cytochalasin B was also purchased from Sigma. A stock solution (0.125% w/v) was prepared by dissolving 5mg cytochalasin B in absolute ethanol (4ml). The protein-A purified rabbit antitoxin antibodies were kindly provided by Mr. A. Ashton working in this laboratory.

5.3 RESULTS

PART A: BINDING

5.3.1 Effect of the large subunit of BoNT type A on the binding of intact $^{125}$I-BoNT type A.

The larger subunit ($M_r$ 97,000) of BoNT, which retained only 0.1% of the toxicity of the native protein (Williams et al., 1983), was tested for its ability to compete with the binding of $^{125}$I-BoNT type
A. A 50-fold excess of this larger fragment of the toxin (0.5μM) (lower concentrations were not tested) was effective in totally preventing the binding of 125I-BoNT both in the presence and absence of 15mM sodium azide (Table 5.1, Fig. 5.1). It also inhibited the internalisation of toxin in untreated samples, emphasising the need for binding prior to translocation of the toxin into the nerve terminal. This complete inhibition of binding of radiolabelled toxin by the larger subunit suggests that the latter is involved directly with the binding of toxin to the nerve terminal membrane. As this fragment is non-toxic when injected into mice (cf. above), it may be responsible for mediating the translocation of the smaller subunit (Mr 55,000) across the plasma membrane to its intracellular target site, by directing the toxin to its acceptor. This is consistent with the multi-step process of intoxication suggested by Simpson (1980).

5.3.2 Effect of neuraminidase on the binding and internalisation of 125I-BoNT at the motor nerve terminal.

Neuraminidase is an enzyme which splits off N-acetyl neuraminic acid (sialic acid) from a variety of glycoproteins and glycolipids found in the membranes of animal tissues. A sialoglycoprotein has been implicated in the binding of BoNT to rat cerebrocortical synaptosomes (Williams et al., 1983), but reports on the existence of such an acceptor site at the neuromuscular junction are contradictory (Simpson and Rapport, 1971; Mellanby et al., 1973; Van Heyningen and Mellanby, 1973). A qualitative appraisal of the results obtained in one experiment (Fig. 5.2) indicated that neuraminidase does not prevent the binding (or internalisation) of 125I-BoNT at the neuromuscular junction (Figs. 5.2b,c). Protease inhibitors were included in the incubation medium of one test sample to prevent inactivation of toxin or acceptor molecules by proteases that might be contaminating the pure
Table 5.1

Mouse nerve-muscle preparations were treated as described in Figs. 5.1-5.6. The data obtained from electron-microscope autoradiograms was analysed quantitatively as follows: photographs were taken of at least 20-30 end-plates from each preparation examined at a magnification of 3,600x using the 35 mm camera in a Hitachi-600 electron microscope. Prints were then prepared at a magnification of 19,600x, and the nerve terminal membrane lengths were determined by digitisation. The grains detectable on the plasma membrane and within the terminal cytoplasm were counted and expressed per length of membrane measured. The extent of labelling in test and control specimens could thus be compared.

In order to quantitate the extent of internalisation of radio-labelled toxin molecules under different conditions, silver grains on, or within 70nm, of the membrane and grains in the cytoplasm were counted separately and expressed as a percentage of the total associated with the nerve terminal.
Table 5.1 Effects of various agents on the binding and internalisation of 125I-BoNT (type A) at the murine nerve terminal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative number of grains/µm plasma membrane (%)</th>
<th>Distribution of grains with respect to the plasma membrane (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>on</td>
<td>within</td>
</tr>
<tr>
<td>10nM 125I-BoNT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>100</td>
<td>65 (±26, n=12)</td>
</tr>
<tr>
<td>BoNT large subunit (0.5µM)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Na Azide (15mM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Na Azide (15mM) + BoNT large subunit (0.5µM)</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>15nM 125I-BoNT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>100</td>
<td>59 (±16, n=18)</td>
</tr>
<tr>
<td>Neuraminidase (0.6 units/ml)</td>
<td>100</td>
<td>59 (±24, n=12)</td>
</tr>
<tr>
<td>Neuraminidase (0.6 units/ml) + Inhibitor (1.0mM)</td>
<td>94</td>
<td>53 (±20, n=18)</td>
</tr>
<tr>
<td>11nM 125I-BoNT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>100</td>
<td>61 (±17, n=22)</td>
</tr>
<tr>
<td>Tetanus toxin (0.9µM)</td>
<td>31</td>
<td>61 (±25, n=23)</td>
</tr>
<tr>
<td>10nM 125I-BoNT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>100</td>
<td>56 (±27, n=10)</td>
</tr>
<tr>
<td>Tetanus toxin (1.0µM)</td>
<td>41</td>
<td>59 (±34, n=14)</td>
</tr>
<tr>
<td>21nM 125I-BoNT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>100</td>
<td>62 (±22, n=19)</td>
</tr>
<tr>
<td>2.1µM BoNT</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>IgG from myasthenic patients</td>
<td>96</td>
<td>63 (±16, n=18)</td>
</tr>
<tr>
<td>IgG from myasthenic patients + 2.1µM BoNT</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 5.1 Effect of the large subunit of BoNT type A on the binding and internalisation of intact $^{125}\text{I-BoNT}$.

Mouse diaphragm tissue was incubated with 0.5ml Krebs Ringer containing $^{125}\text{I-BoNT}$ type A (10nM) and a 50-fold excess (0.5μM) of the large subunit of the toxin in the presence and absence of Na azide (15mM). After 90 minutes at 22°C, the sections of tissue were washed in the appropriate incubation buffer (6 times, 5 minutes each) and fixed in 2% glutaraldehyde. Control sections were treated similarly but in the absence of the large subunit of the toxin.

a. Two nerve terminals treated with $^{125}\text{I-BoNT}$ type A in the presence of the large subunit of the toxin. Absence of silver grains on the membrane and in the cytoplasm suggests that the large subunit mediates binding to specific acceptors and is responsible for delivery of toxin to the cytosol.

b. Control sample.

c. Nerve terminal pretreated with Na azide (30 minutes) and then exposed to $^{125}\text{I-BoNT}$ type A in the presence of the larger subunit and of the metabolic inhibitor. Binding of the radiolabelled molecules was inhibited completely by the large subunit of the toxin.

d. Control sample treated with $^{125}\text{I-BoNT}$ type A in the presence of Na azide.
Fig. 5.2 Effect of neuraminidase on the binding and internalisation of $^{125}$I-BoNT at the murine motor nerve terminal: a qualitative analysis.

A test sample of mouse diaphragm tissue was preincubated (for 30 minutes at 22°C) in 0.5ml Krebs Ringer containing EGTA (1.3mM) (to bind divalent cations such as Ca$^{++}$ and Mg$^{++}$ which appear to inhibit the action of the enzyme neuraminidase), the protease inhibitors PMSF ($5 \times 10^{-4}$M), bacitracin (0.1%), trypsin inhibitor (0.002%), and benzamidine ($10^{-4}$M), and a pure preparation of the enzyme neuraminidase from Clostridium perfringens (0.6 units/ml; 9μg/unit) (taken from a stock solution known to inhibit the binding of $^{125}$I-BoNT type A to rat cerebrocortical synaptosomes, Williams et al., 1983). The tissue was then washed extensively (for 30 minutes at 22°C) and incubated with 0.5ml Krebs Ringer including 34nM $^{125}$I-BoNT type A (a concentration known to saturate all the acceptor sites at the motor nerve terminal); after 75 minutes at 22°C the tissue was washed with Ringer solution. A second test preparation was incubated as above but the preincubation medium contained only the enzyme neuraminidase (0.6 units/ml). A control sample was treated similarly but was preincubated in Krebs Ringer without the enzyme. Saturability of binding was tested by addition of a 100-fold excess of native neurotoxin type A to the incubation medium (data not shown).

a. Control sample treated with $^{125}$I-BoNT type A (34nM) after preincubation in Krebs Ringer for 30 minutes at 22°C.

b. Nerve terminals pretreated with neuraminidase in the presence of protease inhibitors and EGTA before incubation with $^{125}$I-BoNT type A (34nM).

c. Nerve terminals pretreated with neuraminidase and then incubated with $^{125}$I-BoNT type A.
neuraminidase preparation used. It appears that no such enzymes were present, as the extent of toxin binding was unchanged in the absence of protease inhibitors (Fig. 5.2c). The binding of $^{125}$I-BoNT to nerve membranes in the presence of neuraminidase was saturable as it was prevented by excess native neurotoxin (data not shown).

Quantitative analysis of the results from a second experiment (Fig. 5.3) showed that neuraminidase did not affect the binding of toxin to any detectable extent relative to untreated samples (Table 5.1, Figs. 5.3a,b,c). The slight decrease in the amount of binding seen in the preparation treated with neuraminidase and the inhibitor 2,3-dehydro-2-deoxy-NANA (94% of the binding seen in control and neuraminidase-treated samples) can be attributed to experimental error rather than any effect of the inhibitor or enzyme-inhibitor complex.

The presence of neuraminidase did not affect the translocation of toxin across the nerve terminal membrane (Table 5.1, Figs. 5.2b,c; 5.3a).

5.3.3 Effect of tetanus toxin on the binding and internalisation of $^{125}$I-BoNT.

The overall effects of BoNT and tetanus toxin on neurotransmission are qualitatively very similar, although BoNT is 1000 times more potent than tetanus toxin in the periphery (Habermann et al., 1980). A 5-fold excess of tetanus toxin had no effect on the binding of $^{125}$I-BoNT (A) to nerve terminal membranes (data not shown). However, a large excess (80-100 fold) inhibited the binding by 60-70% (Table 5.1, Figs. 5.4a,b). The low efficacy of tetanus toxin in antagonising the binding of $^{125}$I-BoNT to terminal membranes suggests that its interaction with motor nerve terminals, at least with sites which enable it to exert this inhibition, is of lower affinity relative to that of $^{125}$I-BoNT. This does not mean, however, that high affinity sites for tetanus toxin are
Fig 5.3 Effect of neuraminidase on the binding and internalisation of 125I-BoNT type A at the motor nerve terminal: a quantitative analysis.

a. A test tissue sample was preincubated with 0.5ml Krebs Ringer containing the enzyme neuraminidase (0.6 units/ml) for 1 hour at 22°C followed by extensive washing (30 minutes at 22°C). It was then incubated with 125I-BoNT type A (15nM) for 75 minutes at 22°C, washed, fixed and processed for autoradiography.

b. A second sample was preincubated with 0.5ml Krebs Ringer/neuraminidase (0.6 units/ml) containing 2,3-dehydro-2-deoxy-NANA, a specific inhibitor of neuraminidase (previously mixed together for 15 minutes) for 1 hour, prior to extensive washing and incubation with radiolabelled toxin as above.

c. A control sample was preincubated with Krebs Ringer solution (0.5ml) for 1 hour followed by incubation with toxin as above.

Large numbers of end-plates were photographed and the extent and distribution of the labelling in each preparation was quantitated as described in Section 4.2.2. Quantitative results are shown in Table 5.1.
Fig. 5.4 Effect of tetanus toxin on the binding and internalisation of 
$^{125}$I-BoNT.

a. Nerve terminals treated with $^{125}$I-BoNT in the presence of excess 
tetanus toxin. A sample of tissue was preincubated with 0.5ml 
Krebs Ringer containing 0.9µM tetanus toxin (isolated from 
Clostridium tetani) for one hour at room temperature. $^{125}$I-
BoNT was then added (11nM) and the incubation was continued for a 
further 2 hours. The tissue was then washed, fixed, and prepared 
for electron-microscope autoradiography as described in Chapter 
2.

b. Terminals from a control sample preincubated with Krebs Ringer for 
1 hour at room temperature prior to the addition of $^{125}$I-BoNT 
as above. [This experiment was repeated using 1µM tetanus and 
10nM $^{125}$I-BoNT (from a different radio-iodination) (data shown 
in Table 5.1).]

All results were quantitated as described in Section 4.2.2 (see 
Table 5.1). Note the significant reduction in the extent of labelling 
seen at terminals preincubated with tetanus toxin.
not found at the motor nerve terminal. As mentioned previously, tetanus toxin inhibited $^{125}$I-BoNT binding only partially, suggesting possible heterogeneity of the BoNT binding sites at the motor nerve terminal. The pattern of $^{125}$I-BoNT binding in the presence of tetanus toxin was the same as that seen in untreated samples; for example, tetanus toxin does not interact solely with sites located away from the active zones or along the axonal plasma membrane (Fig. 5.4a). The ratio of membrane-bound to internalised grains was also unaffected by tetanus toxin (Table 5.1), indicating that it does not interact with a population of sites responsible for mediating BoNT translocation across the membrane. Collectively, the results suggest that tetanus toxin interacts with some of the acceptors for BoNT, either directly or indirectly by means of steric interference. The physiological significance of this interaction is questionable, however, as nerve-muscle preparations pre-incubated with the binding fragment (fragment C) of tetanus toxin (1μM) were still paralysed by low concentrations of BoNT type A (0.1nM) (Simpson, 1984). This concentration of fragment C, however, totally prevented tetanus toxin-induced blockade of neurotransmission (concentration of intact toxin: 3nM).

5.3.4 Binding of $^{125}$I-BoNT to diaphragms from mice treated with IgG from myasthenic (Lambert-Eaton syndrome) patients.

Injection of BoNT or the IgG fraction of plasma from patients with myasthenic syndrome produces strikingly similar symptoms in mice (Lang et al., 1981; 1983). Nerve-muscle preparations treated with these substances show comparable electrophysiological responses suggesting that toxin and putative autoantibody interact with the same, or similar, membrane components at the motor nerve terminal and/or interfere with common intracellular pathways. In an attempt to determine the validity of such a proposal, diaphragms from mice treated with IgG from
myasthenic patients (and showing abnormal neuromuscular transmission as a result of reduced quantal content of the end plate potential; Lang et al., 1981) were exposed to $^{125}$I-BoNT at low concentrations (0.5nM). Light microscope autoradiography of treated and untreated muscles showed that the interaction of $^{125}$I-BoNT with the neuromuscular junction was not prevented by the presence of IgG from myasthenic patients (Fig. 5.5). This interaction was specific and saturable in test (IgG-treated) and control (treated with normal plasma) samples. Clusters of silver grains were detectable only at the neuromuscular junction (shown by staining for acetylcholinesterase) and were absent in sections treated with labelled toxin in the presence of excess native BoNT. Thus, binding of $^{125}$I-BoNT in test and control samples was qualitatively indistinguishable.

Quantitative analysis of these results was carried out at the ultrastructural level (Table 5.1, Fig. 5.6). The number of grains associated with the nerve terminal in treated and untreated samples (expressed as number of grains per micron of plasma membrane) was the same (treated preparations showed 95% of the binding observed in controls). Likewise, the presence of putative antibody or an antibody-induced change in the presynaptic membrane did not affect translocation of labelled toxin across the terminal membrane. The results suggest that, although the physiological effects produced in the disease and in the intoxication may be similar, the membrane components with which antibody and toxin interact at the motor nerve terminal are distinct. This does not preclude a common action; both BoNT and the autoantibody may interfere with a common intracellular target, the membrane site acting merely to deliver active substance to the cytoplasm.
Fig. 5.5 Binding of $^{125}$I-BoNT to diaphragms from myasthenic (Lambert-Eaton syndrome passively transferred) mice: qualitative analysis by light-microscope autoradiography.

Diaphragm tissue from a mouse showing definite symptoms of myasthenic syndrome was incubated with 0.5ml Krebs Ringer containing 0.5nM $^{125}$I-BoNT type A for 90 minutes at 22°C. The tissue was then washed with Krebs Ringer at 4°C (for 30 minutes) and processed for light-microscope autoradiography. Control samples were taken from a mouse injected with pooled normal plasma and were treated similarly.

a. Binding of $^{125}$I-BoNT type A to the neuromuscular junction of a mouse injected with the IgG fraction of plasma from patients with myasthenic syndrome.

b. Control samples from a mouse treated with normal plasma.
Fig. 5.6 Binding and internalisation of $^{125}$I-BoNT type A to the motor nerve terminal of mice with myasthenic syndrome (passively transferred): a quantitative, ultrastructural study.

A small piece of tissue from the diaphragm of a mouse treated with IgG from myasthenic patients was incubated with 0.5 ml Krebs Ringer/21nM $^{125}$I-BoNT type A for 90 minutes at 22°C before washing and processing for electron-microscope autoradiography. A control sample from a mouse injected with normal plasma was treated similarly.

a. Nerve terminals from the test sample.
b. Control.

The results obtained were quantitated as detailed in Section 4.22 (see Table 5.1).
PART B: INTERNALISATION

5.3.5 Effect of the absence of Ca\[^{++}\] on the internalisation and binding of \(^{125}\text{I-BoNT}\) at the motor nerve terminal.

Calcium deprivation has been reported to prolong the time to blockade of neuromuscular transmission by botulinum toxin type A in rat hemidiaphragm preparations (Simpson, 1980). In an attempt to clarify the nature of this effect, nerve-muscle preparations were exposed to \(^{125}\text{I-BoNT}\) in the absence of Ca\[^{++}\] (+2mM EGTA). Quantitative analysis of the binding of labelled toxin to the nerve terminal in the presence and absence of the divalent cation was carried out at the ultra-structural level. The number of silver grains observed at end-plates in which toxin binding had occurred in Ca\[^{++}\]-free medium was the same as that in samples incubated under physiological conditions (quantitative analysis of the extent of binding showed that in test sections there was 106% of the labelling seen in controls). This suggests that the protective effect of Ca\[^{++}\] deprivation cannot be attributed to inhibition of the binding step. Surprisingly, the internalisation of \(^{125}\text{I-BoNT}\) was also unaffected in preparations incubated in Ca\[^{++}\]-free medium (Table 5.2, Fig 5.7a,b). The proportion of silver grains in the cytosol was the same in test and control preparations. However, it is impossible to determine whether these silver grains represented toxin molecules confined to endocytic vesicles and thus unable to enter the cytoplasm, or in a position which allowed access to the lytic site. Whatever their location, Ca\[^{++}\] must be essential for an event in the multistep process of intoxication which occurs after binding and uptake. Of course, the possibility that only a very small population of sites (undetectable by the autoradiographic technique) is responsible for delivery of active toxin to the cytosol and that these are affected by Ca\[^{++}\] deprivation in such a way that the toxin remains on the cell surface (or is unable to bind to it), cannot be excluded.
Table 5.2

Mouse nerve-muscle preparations were treated with $^{125}$I-BoNT under the conditions described in Figs. 5.7-5.11. Large numbers of end-plates were then photographed and the distribution of silver grains with respect to the plasma membrane was determined. Silver grains on or near the membrane (to a cut-off distance of 70nM) and within the terminal cytoplasm were counted separately and expressed as a percentage of the total associated with the nerve ending.
Table 5.2 Effect of various agents on the internalisation of $^{125}$I-BoNT at the nerve terminal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Distribution of grains with respect to the plasma membrane (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15nM $^{125}$I-BoNT</strong></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>57 ($\pm$26, n=21)</td>
</tr>
<tr>
<td>$-\text{Ca}^{++}$ (+2mM EGTA)</td>
<td>54 ($\pm$14, n=16)</td>
</tr>
<tr>
<td><strong>28nM $^{125}$I-BoNT</strong></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>62 ($\pm$16, n=15)</td>
</tr>
<tr>
<td>$-\text{Ca}^{++}$ (+2mM EGTA)</td>
<td>61 ($\pm$24, n=21)</td>
</tr>
<tr>
<td><strong>Lysosomotropic agents:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>20nM $^{125}$I-BoNT</strong></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>59 ($\pm$17, n=21)</td>
</tr>
<tr>
<td>Chloroquine (50μM)</td>
<td>50 ($\pm$12, n=26)</td>
</tr>
<tr>
<td>Methylamine (6mM)</td>
<td>73 ($\pm$21, n=25)</td>
</tr>
<tr>
<td>Ammonium chloride (6mM)</td>
<td>74 ($\pm$20, n=20)</td>
</tr>
<tr>
<td>BoNT (2μM)</td>
<td>-</td>
</tr>
<tr>
<td><strong>11nM $^{125}$I-BoNT</strong></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>61 ($\pm$17, n=22)</td>
</tr>
<tr>
<td>Cytochalasin B (5μg/ml)</td>
<td>52 ($\pm$7, n=9)</td>
</tr>
<tr>
<td><strong>11nM $^{125}$I-BoNT</strong></td>
<td></td>
</tr>
<tr>
<td>$4^\circ$C</td>
<td>100</td>
</tr>
<tr>
<td>1.1μM BoNT ($4^\circ$C)</td>
<td>-</td>
</tr>
<tr>
<td>$4^\circ$C, warmed to RT</td>
<td>71 ($\pm$17, n=15)</td>
</tr>
<tr>
<td>$4^\circ$C, warmed to RT in the presence of antitoxin</td>
<td>72 ($\pm$25, n=17)</td>
</tr>
</tbody>
</table>
Fig 5.7 Effect of Ca\(^{++}\) deprivation on the internalisation and binding of \(^{125}\)I-BoNT type A at the motor nerve terminal.

a. A small piece of mouse diaphragm was pre-incubated in Ca\(^{++}\)-free Krebs Ringer containing 2mM EGTA for approximately 15 minutes at 22°C. This was followed by incubation in 0.5ml of the same buffer containing 15nM \(^{125}\)I-BoNT for 90 minutes at 22°C. Extensive washing was carried out using Ca\(^{++}\)-free Krebs Ringer (6 times, 5 minutes each) prior to fixation in 2% glutaraldehyde and preparation of the tissue for electron-microscope autoradiography.

b. Control samples were treated in the same way except that normal Ringer was used instead of Ca\(^{++}\)-free medium containing EGTA.

(This experiment was also carried out using 28nM \(^{125}\)I-BoNT from a different radiiodination. Results from both experiments were quantitated as described in Section 4.2.2; see Table 5.2.)
The binding seen in the absence of Ca\textsuperscript{++} was saturable as excess native BoNT prevented totally the binding of $^{125}$I-BoNT to the nerve terminal (data not shown). The interaction of labelled toxin with samples in the presence and absence of Ca\textsuperscript{++} was therefore qualitatively and quantitatively indistinguishable.

5.3.6 Effect of lysosomotropic agents on the internalisation of $^{125}$I-BoNT at the murine nerve terminal.

Chloroquine, ammonium chloride and methylamine, are known to delay the onset of effects of several pharmacologically active substances that exert their actions within the cytoplasm of target cells (Goldstein et al., 1979; Pastan and Willingham, 1981). In a recent study, chloroquine (50\mu M) was shown to delay the onset of neuromuscular blockade caused by type A botulinum neurotoxin (Simpson, 1982). Similarly, ammonium chloride and methylamine were found to antagonise the toxin's paralytic action (Simpson, 1983). According to pharmacological measurements, these agents did not prevent toxin binding but trapped the molecules at an antitoxin-sensitive site. In order to localise this site at the ultrastructural level, mouse diaphragm preparations were pre-treated with chloroquine, ammonium chloride or methylamine, and then exposed to $^{125}$I-BoNT type A in the presence of these lysosomotropic agents at concentrations known to interfere with the toxin's neuroparalytic effects. Sections from each of the preparations were processed for electron-microscope autoradiography and, after three weeks, silver grains were detectable at nerve endings in all cases (Fig. 5.8). Qualitative analysis of the results suggested that binding of toxin to the terminal membrane was unaffected by these agents. Although internalisation of labelled toxin was not prevented fully by any of these drugs, changes in the ratio of membrane-bound to internalised grains were apparent (Table 5.2). In chloroquine (50\mu M)-treated
Fig. 5.8 Effect of lysosomotropic agents on the internalisation of $^{125}$I-BoNT at the murine motor nerve terminal.

The concentration of lysosomotropic drugs used are known to antagonise the onset of neuromuscular blockade caused by BoNT type A without inactivating toxin molecules or producing irreversible changes in tissue function (Simpson, 1980).

a. Control sample preincubated in physiological Ringer solution for 10 minutes and incubated with $^{125}$I-BoNT (20nM) for 90 minutes at 22°C, prior to washing (30 minutes) and processing for autoradiography.

b. 1. Nerve terminal from diaphragm tissue preincubated in Krebs Ringer/50µM chloroquine for 10 minutes and incubated with Krebs Ringer/20nM $^{125}$I-BoNT/50µM chloroquine for 90 minutes at 20°C. Note the increase in internalised radioactivity relative to controls.

2. Nerve terminal from tissue preincubated with 2mM chloroquine for 45 minutes and then incubated with $^{125}$I-BoNT (25nM) in the presence of the drug. Note extensive vacuolation in the terminal cytoplasm and absence of silver grains in areas free of vesicular structures (arrow).

c. Nerve ending from a sample of tissue preincubated with 6mM ammonium chloride for 10 minutes and incubated with $^{125}$I-BoNT (20nM) in the presence of ammonium chloride. Note extensive vacuolation of the tissue and reduced internalisation of toxin. Internalised silver grains are associated with vesicular structures (arrows) emphasising that uptake occurs by an endocytic mechanism.
d. Synaptic profile from tissue pretreated with 6mM methylamine for 10 minutes and incubated with $^{125}$I-BoNT type A (20nM) in the presence of the drug. Most of the silver grains are located on the membrane and those found in the cytosol are usually associated with vesicular structures (arrows).

These results were quantitated as described in Section 4.4.2, see Table 5.2.
samples, there were slightly more (~20%) internalised grains relative to control untreated preparations. The grains were distributed throughout the cytoplasm and were quite often associated with vacuole-like structures (Fig. 5.8b2). In the presence of 2mM chloroquine (a concentration at which the drug itself causes neuromuscular blockade; Simpson, 1982), extensive vacuolation was detected in the cytoplasm of the nerve ending (Fig. 5.8b2). Within the limitations of resolution in the autoradiographic technique, it could be said that the internalised silver grains were associated almost exclusively with vesicular structures; areas devoid of vacuoles were unlabelled.

In the presence of ammonium chloride, however, the proportion of membrane-bound to internalised silver grains was 74% : 26%; thus, relative to control preparations, endocytosis of toxin molecules was reduced by approximately 35% when the drug was included in the incubation medium (Table 5.2). Vacuolation was extensive in the terminal cytoplasm (Fig. 5.8c) and the majority of internalised silver grains were associated with vesicles in close proximity to the plasma membrane. The central areas of the nerve ending were often almost devoid of grains.

Similar results were obtained in samples treated with methylamine (Fig. 5.8d, Table 5.2). The extent of endocytosis of toxin molecules was reduced by approximately 33% relative to controls (only 27% of the grains associated with the nerve terminal were found in the cytoplasm). As seen in ammonium chloride-treated samples, most internalised grains were associated with endocytic vesicles near the cell surface (with very few grains towards the middle of the synaptic bouton) (Fig. 5.8d). Although the short chain amines ammonium chloride and methylamine (which are known to inhibit uptake of α₂-macroglobulin and insulin into the target cells) reduced internalisation of BoNT molecules, they did not prevent this process to the extent seen in azide-treated samples (where
almost 100% of the silver grains were membrane-bound. In all samples treated with lysosomotropic agents, the extent of retrograde intra-axonal transport of toxin appeared greater than that routinely observed in untreated samples (Fig. 5.9).

The observations made by Simpson from electrophysiological studies, and the results obtained from autoradiographic experiments described in this Chapter, strengthen the proposal that toxin uptake into the nerve terminal occurs via the process of acceptor-mediated endocytosis. Chloroquine probably prevents exit of toxin molecules from endocytic vesicles into the terminal cytoplasm; the increased proportion of intracellular radioactivity can be attributed to inhibition of toxin degradation by the drug. This agent does not appear to interfere with the endocytic process itself, unlike ammonium chloride and methylamine. These drugs probably act by inhibiting uptake of BoNT molecules important in causing paralysis both from the cell surface into an intraterminal compartment and/or from the latter into the cytosol.

5.3.7 Effect of cytochalasin B on the uptake of $^{125}$I-BoNT into the nerve terminal.

In an attempt to elucidate further the mechanism of uptake of $^{125}$I-BoNT type A molecules into the motor nerve terminal, cytochalasin B was included in the incubation medium of test samples. This fungal metabolite interferes with microfilaments, the actin-like components of the cytoskeleton found in all cells including neurons. However, in the presence of cytochalasin B at a concentration known to produce disassembly of microfilaments (Pollard, 1976) without being toxic to cells, uptake of $^{125}$I-BoNT type A into the nerve ending was slightly increased, rather than reduced, relative to control samples (Table 5.2, Fig. 5.10). In treated samples, 48% of the grains associated with nerve terminals were located within the cytoplasm, whereas in control
Fig. 5.9 Retrograde intra-axonal transport in the presence of lysosomotropic agents.

Tissue samples were treated with chloroquine, ammonium chloride and methylamine and then incubated with $^{125}$I-BoNT Type A (20nM) as described in Fig. 5.8. Silver grains were often associated with the axoplasm of nerve fibres (to a greater extent than normally seen in controls by qualitative analysis of the results).

Transverse section through unmyelinated axons: a. control, b. treated with chloroquine, c. ammonium chloride, d. methylamine.
Fig. 5.10 Effect of cytochalasin B on the internalisation of $^{125}\text{I-BoNT}$ at the motor nerve terminal.

Nerve ending from tissue pre-treated with Krebs Ringer containing cytochalasin B (5µg/ml) for 35 minutes and then incubated with $^{125}\text{I-BoNT}$ type A (11nM) in 0.5ml Krebs Ringer/cytochalasin B for 90 minutes at 22°C.

(A control sample was treated similarly but in the absence of cytochalasin B. The results were quantitated as detailed in Section 4.2.2 and are shown in Table 5.2.)
preparations only 39% of the radioactivity had been internalised. This result suggests that the uptake process is not dependent on an intact cytoskeleton and that other mechanisms are involved.

5.3.8 Effect of temperature and antitoxin antibodies on the internalisation of $^{125}\text{I}\text{-BoNT}$ at motor nerve terminals.

As shown in Chapter 4 (Section 4.3.3), the internalisation of $^{125}\text{I}\text{-BoNT}$ type A was prevented totally by incubation at low temperatures ($4^\circ\text{C}$). Under these conditions, the majority of silver grains were found on the nerve terminal membrane (Table 5.2, Fig. 5.11a). However, if the tissue was allowed to warm to room temperature ($\sim 22^\circ\text{C}$) following incubation with $^{125}\text{I}\text{-BoNT}$ type A and washing at $4^\circ\text{C}$ (as described in this Chapter), the process of endocytosis occurred and silver grains appeared in the cytosol (Fig. 5.11b). The fraction of internalised grains (29% of the total) was lower than that routinely observed in tissue in which binding as well as internalisation had taken place at room temperature ($\sim 40\%$). This can be attributed to a time lag required for re-activation of the endocytic machinery. In most cases, internalised grains were located in the vicinity of the plasma membrane (Fig. 5.11b) indicating that incubation at low temperature imposed partial synchrony on the action of the toxin; under these conditions binding was allowed to go to completion while onset of subsequent steps was delayed. Simpson (1980; 1982; 1983) has reported that antitoxin prevented the paralytic effect of BoNT bound to the nerve terminal membrane under conditions that prevented or delayed internalisation. If the antibody was added at the same time as conditions were altered to allow the toxin to exert its lytic action, complete protection was achieved. In order to determine whether antitoxin trapped the toxin at an extracellular site, or whether it prevented the action of the latter once it had been taken up into the nerve terminal, a concentration of
Fig. 5.11 Effect of temperature and antitoxin antibodies on the internalisation of $^{125}$I-BoNT type A at motor nerve terminals.

Tissue samples were preincubated with ice-cold Krebs Ringer for 10 minutes.

a. Nerve terminals from a control preparation treated with $^{125}$I-BoNT type A (11nM) in 0.5ml Krebs Ringer at 4°C for 2 hours, prior to washing for 30 minutes at 4°C.

b. Nerve ending from a preparation treated with toxin as described in (a). After incubation, the medium was replaced with 0.5ml Krebs Ringer (4 °C) for 45 minutes, after which the tissue was warmed to 22°C and incubation was continued for a further 90 minutes. Note silver grains in the cytoplasm of the nerve terminal.

c. Test specimen treated with toxin as described in (a). The medium was then removed and replaced with 0.5ml anti-BoNT antibody (a quantity known to precipitate all the toxin added in the incubation medium). After incubation with antibody at 4°C for 45 minutes, the tissue was warmed to 22°C and incubation was continued for a further 90 minutes. Note that antibodies to the toxin did not prevent its uptake into the nerve terminal cytoplasm.

These results are expressed quantitatively in Table 5.2.
rabbit anti-BoNT IgG known to precipitate all the BoNT molecules to which the tissue was exposed at 4°C (large excess; Ashton et al., 1984) was added. Incubation was continued for 45 minutes at 4°C and for 90 minutes at room temperature. The extent of toxin internalisation that occurred during this treatment was comparable to that seen when the tissue was warmed to room temperature in the absence of antitoxin (28% of the silver grains associated with the nerve terminal were found in the cytoplasm) (Table 5.2, Fig. 5.11c). This suggests that the whole antibody-toxin complex was subsequently taken up, probably in endocytic vesicles (Fig. 5.11c). Thus, expression of toxicity was prevented even after toxin had been internalised.

The validity of this experiment can be discussed by comparing it with an interesting study recently carried out by Simpson (1983). Mouse phrenic nerve-hemidiaphragm preparations were exposed to BoNT type A at low temperature for 40 min (required for maximum binding at the concentration used); the tissue was then washed, and the incubation medium was replaced with toxin-free solution at 36°C. Phrenic nerves were then stimulated and the paralysis times were monitored. The addition of antitoxin at the same time as stimulation was initially applied produced substantial inactivation of the toxin, as shown by prolonged paralysis times. If an interval was left between the onset of nerve stimulation and the addition of antitoxin, the neutralising effect of the latter was diminished. BoNT became insensitive to the action of antibody within 20-25 minutes. In the experiment described in this Chapter, antitoxin was added before the tissue was warmed. Under the conditions used, the antibodies (M_r ~160,000) should have been able to reach the toxin molecules before they became inaccessible as a result of internalisation. Problems of diffusion into the tissue at the temperature of incubation should not have had a significant effect as the toxin, which is comparable in size to an antibody molecule, was shown to penetrate the tissue under the conditions used.
5.4 DISCUSSION

5.4.1 The binding step

BoNT binds specifically to the plasma membrane of motor nerve terminals and is subsequently taken up into the cytoplasm (Chapters 3 and 4) where it exerts its inhibitory action on the process of neurotransmitter release (Simpson, 1980). The binding to cell surface acceptor sites is mediated by the heavy subunit of the toxin as a preparation, treated with $^{125}$I-BoNT type A in the presence of an excess of the heavier subunit, showed no deposition of silver grains at the nerve terminal. This result agrees with the reports of Kozak (1979) and Williams et al., (1983) which suggest that the larger fragments of types A and B botulinum toxin derivatives mediate binding to synaptosomal membranes, the lighter fragment having little (Kozaki, 1979) or no effect (Williams, 1983) on the binding of radiolabelled toxin. The slight inhibitory effect of the smaller fragment observed by Kozaki can be attributed to a small amount of contamination by native neurotoxin in the preparation used.

In the presence of excess heavy subunit, no internalised silver grains were detectable at nerve endings. Thus, it appears that binding of BoNT via the heavy subunit is required for its uptake and is, therefore, probably a pre-requisite for expression of its lytic action under physiological conditions, as seen with other bacterial and plant toxins. It has been shown that binding of diphtheria toxin to its glycoprotein acceptor is necessary for expression of its cytotoxicity; insensitive cell lines lack the acceptor (Boquet and Pappenheimer, 1976). Likewise, binding to carbohydrates containing galactose residues is required for the action of abrin and ricin as cells can be partially protected by the addition of galactose to the medium (Olsnes et al., 1978).
Integrity of the BoNT molecule appears essential for expression of its toxicity (Simpson, 1973) as reduction of its disulphide bonds leads to inactivation (DasGupta, 1981; Simpson and DasGupta, 1983). Moreover, the isolated heavy subunit is non-toxic when injected into mice (Williams, 1984) indicating that the binding step is not directly responsible for toxicity as suggested previously (Burgen et al., 1949; Simpson, 1980). These results and the striking structural similarities between BoNT and other toxins in which the smaller fragment is responsible for biological activity, suggest by analogy that the neurotoxin's lighter subunit may be directly responsible for inhibition of neurotransmitter release at nerve terminals.

The nature of the acceptor for the larger subunit of BoNT type A at the motor nerve terminal is still unknown. The high concentration of gangliosides in neuronal tissue makes these glycolipids strong candidates for probes that interact specifically with components of the nervous system. Although several workers have sought a ganglioside acceptor for botulinum toxin, it is unlikely that these lipids are involved in 'effective' binding of the toxin in the peripheral nervous system as neuraminidase had no effect on the interaction of 125I-BoNT type A with the motor nerve terminal membrane. The number of silver grains associated with nerve endings was the same in samples treated with the enzyme and untreated controls. Simpson and Rapport (1971), however, reported that, of numerous membrane components tested, only ganglioside, especially GT1, could reduce the neurotoxicity of the toxin sample. Their approach was indirect as it involved incubation of toxin (botulinum toxin-haemagglutinin complex) with the acceptor candidate followed by assay for toxicity to determine whether this had been decreased. It is possible that the toxin's interaction with ganglioside involved the lighter subunit, causing a secondary conformational change in the heavy subunit, thus preventing binding to
Its physiological acceptor on the membrane. The protective effect can also be attributed to a non-specific detergent action of the ganglioside as discussed by Van Heyninghen and Mellanby (1973). At high ganglioside concentrations, the toxin-ganglioside complexes would have been present as large micelles (King et al., 1976) (micellar weight of the order of \(10^6\) Daltons); under these conditions, the toxin would have been unable to gain physical access to its site on the membrane. Inactivation of BoNT type A by gangliosides was also reported, more recently, by Kitamura et al. (1980). These investigators found that the trisialo-ganglioside GT\(_{1b}\) prevented the binding of \(^{125}\)I-botulinum toxin derivative (type A) to monkey brain synaptosomes. However, their observation that detoxified toxin also formed a complex with GT\(_{1b}\) makes the specificity of the interaction questionable. Other workers have been unsuccessful in their attempts to reproduce the inactivation by ganglioside (Van Heyninghen and Mellanby; 1973; Mellanby et al., 1973; Habermann and Heller, 1975; Williams et al., 1983). Although Habermann and Heller (1975) and Williams et al., (1983) showed that incubation of cerebrocortical synaptosomes with neuraminidase significantly decreased the binding of BoNT type A, the presence of exogenous sialic acid (Williams et al., 1983) or a mixture of gangliosides (Williams, 1984; Habermann and Heller, 1975) in the assay mixture had no appreciable effect on toxin binding. Likewise, pre-incubation of synaptosomes with gangliosides (Williams, 1984) failed to enhance \(^{125}\)I-BoNT type A binding. It has been suggested that the acceptor site on synaptosomes has a proteinaceous nature as binding can be prevented by proteolysis with proteinase K and trypsin, or by heating (Williams et al., 1983). Collectively, these results suggest that, at least in the brain, sialic acid residues may be critical in maintaining the conformation of other components more directly responsible for binding. However, the specificity of the interaction with ganglioside
synaptosomes with ganglioside produced no shift in the dose-response curve for the toxin on $^{14}\text{C}]\text{ACh}$ release, and neuraminidase had no effect on the toxin's action on transmitter uptake or release in brain preparations (Bigalke et al., 1981; Habermann et al., 1981). Similarly, pretreatment of rat isolated diaphragm preparations with neuraminidase did not prevent the inhibitory effect of BoNT type A on neurotransmitter release (Habermann and Heller, 1975; Habermann et al., 1981). Van Heyningen and Mellanby (1973) found that if they repeated the experiments of Simpson and Rapport (1971) including protective colloid in the toxin-ganglioside mixture, botulinum toxin still produced neuromuscular blockade in phrenic nerve-diaphragm preparations. These results are consistent with those obtained herein by electron-microscope autoradiography. The neuraminidase preparation used was shown to be active in synaptosome preparations where it significantly reduced the binding of $^{125}\text{I} \text{-BoNT}$ type A. Lack of penetration of the enzyme ($M_r \approx 90,000$) into the tissue, although a possibility, is unlikely as $^{125}\text{I} \text{-BoNT}$ ($M_r 150,000$) was shown to penetrate the synaptic cleft within less than 20 minutes incubation. Although the data are not clear cut, it appears that sialic acid-containing molecules are not important in mediating binding or toxicity of BoNT in target cells of the peripheral nervous system.

It is interesting to note that tetanus toxin, which was the first protein to be shown to bind to a ganglioside (Van Heyningen, 1974) can exert its intracellular toxic effect on neuro-transmission after target cells have been treated with neuraminidase (Mellanby and Green, 1981; Zimmermann and Piffaretti, 1977; Bizzini, 1979). This clostridial neurotoxin is similar to BoNT both in structure and pharmacological action. It interacts with motor nerve terminals via its heavy subunit (Fragment C) (Simpson, 1984) and, in addition to producing spastic
paralysis by blocking the release of inhibitory transmitters in the central nervous system, it induces blockade of neurotransmission (flaccid paralysis) in the periphery. Three sequential steps, namely binding, translocation and paralysis are involved in its action at the neuromuscular junction (Schmitt et al., 1981). Its overall effect on neurotransmission is similar to that of BoNT except that 500-1000-fold higher concentrations of tetanus are required to produce equivalent effects with both toxins (Habermann, 1981). It interacts with gangliosides GD₁b and GT₁, (Dimfel et al., 1977; Ledley et al., 1977), the specificity for binding being in the carbohydrate part of the molecule. The binding is of high affinity: half saturation occurs at about 5 x 10⁻⁸M ganglioside (Helting et al., 1977); however, the affinity of ¹²⁵I-BoNT for some of its sites on synaptosomes is higher (K₀ of 6 x 10⁻¹⁰M). Gangliosides GD₁b and GT₁ occur almost exclusively in neuronal cells; tetanus toxin has thus become a marker for neuronal tissue (Mirsky et al., 1978; Raff et al., 1979). However, there is still no direct evidence that binding of tetanus toxin to membranes or gangliosides is a necessary step in its action. Zimmermann and Piffaretti (1977) proposed that there are at least two binding sites on the toxin molecule, corresponding to 'effective' and 'ineffective' sites on the surface of the membrane. 'Ineffective' binding is sensitive to neuraminidase and β-galactosidase whereas 'effective' binding, which leads to a visible biological effect, is insensitive to this treatment. A toxin molecule can bind to either of these sites but not to both simultaneously.

The nature of the binding site for tetanus toxin at the neuromuscular junction is unknown. Simpson (1984a,b) reported that the binding fragment of tetanus toxin (fragment C) did not prevent the action of botulinum toxin type A derivative in phrenic nerve-hemidiaphragm preparations. However, using electron-microscope autoradio-
graphy, it was shown in this study that pre-treatment of this tissue with excess toxin inhibits the binding of $^{125}$I-BoNT type A to the motor nerve terminal by 60-70%. This result supports the proposal that tetanus toxin binds to the motor nerve terminal. It does not necessarily contradict the suggestion that BoNT does not interact with gangliosides at this site as tetanus toxin may not bind solely to these glycolipids in the peripheral nervous system (Zimmermann and Piffaretti, 1977). Furthermore, the inhibition could be the result of steric interference [as two large molecules ($M_r \approx 150,000$) are involved in the effect] rather than direct interaction with a common site. Nevertheless, it is clear that the sites for BoNT with which tetanus toxin interacts are not necessary for the expression of the former toxin's paralytic action (Simpson, 1984a). This suggests that BoNT may also have 'effective' and 'ineffective' binding sites at the motor nerve terminal, analogous to those proposed for tetanus toxin. The difference in potency of the toxins at the neuromuscular junction could be attributed to different mechanisms of delivery of active molecules to the cytosol as a result of binding to different 'effective' sites (see General Discussion). Binding of tetanus toxin at the nerve terminal could be of relatively low affinity; the uptake mechanism for the flaccid paralysis may be non-specific. It must be emphasised that although the overall effect of the action of these toxins at the neuromuscular junction is similar, recent studies suggest that they act at different stages in the chain of events leading to transmitter release (Dreyer and Schmitt, 1983). Lastly, treatment with tetanus toxin did not affect the ratio of membrane-bound to internalised silver grains. This indicates that tetanus toxin did not interact exclusively with a population of sites which is not internalised and, likewise, did not selectively prevent $^{125}$I-BoNT binding to sites involved in its delivery to the cytosol.
As mentioned previously, the clinical and electromyographic characteristics of the condition known as myasthenic syndrome are strikingly similar to those produced by BoNT. It is thought that antibodies to determinants [possibly Ca\(^{++}\) channels at the active zones (Fukunaga et al., 1982)] on the motor nerve terminal membrane cause abnormality of function of the transmitter release mechanism in this condition (Lang et al., 1981; 1983). Pronounced depression of the muscle response to a supramaximal stimulus applied to the motor nerve is seen in patients with the syndrome (Lambert and Elmqvist, 1971) and can be attributed to defective release of neurotransmitter. Despite the similarities in the effects produced by BoNT and the putative autoantibody in myasthenic syndrome, these molecules do not share binding sites at the motor nerve terminal, nor does the antibody induce a change in the presynaptic membrane which impedes BoNT binding. \(^{125}\)I-BoNT type A was found to bind to terminals pretreated with IgG from patients with myasthenic syndrome; the number of grains was unchanged relative to controls treated with normal plasma. This suggests that either the mechanism of neuromuscular blockade or of access to target sites is different in each case. As shown by Simpson (1980), BoNT molecules, or a fragment thereof, must be internalised to exert their paralytic effect. It is possible for an antibody molecule to be taken up in vesicles and survive lysosomal processing (Mellman et al., 1984; Schneider and Trouet, 1981). However, there is no evidence to suggest that antibodies are delivered to the cytosol of target cells by this mechanism. Moreover, it appears that the putative antibodies in myasthenic syndrome disrupt membrane components within the active zones (cf.above) and it is likely that this is induced at the cell surface by the cross-linking activity of the antibodies. Therefore, it would seem that BoNT and the autoantibodies affect the process of neurotransmitter release in different ways and at different sites at the nerve terminal.
5.4.2. The internalisation step.

Using electron-microscope autoradiography, it has been shown directly that $^{125}$I-BoNT type A is taken up into the nerve terminal by an acceptor-mediated process. The importance of the internalisation step is emphasised by a number of studies in which it was shown (1) that there is a latent period between irreversible binding of toxin and onset of paralysis (Burgen et al., 1949), (2) that paralysis occurs more rapidly when nerves are stimulated frequently (Hughes and Whaler, 1962), and (3) that antitoxin antibodies do not antagonise toxin exerting its paralytic effect but will inactivate toxin bound to the nerve membrane (Simpson, 1974). Collectively, these findings suggest that the toxin is internalised before it produces blockade of neurotransmitter release. As was suggested in Chapter 3, a likely mechanism for toxin uptake into the nerve terminal cytoplasm is acceptor-mediated endocytosis. In this process, the acceptors serve to concentrate ligands present in the extracellular fluid (even if they occur in very low amounts with a vast excess of unrelated molecules) on the plasma membrane so that uptake takes place in a very efficient manner compared to bulk fluid pinocytosis. The high affinity, specific acceptor sites for BoNT at the nerve terminal could account for the efficacy of the toxin even at very low concentrations ($10^{-11}$M).

It has now been suggested that a number of other toxins that have a similar structure to that of BoNT enter their target cells by acceptor-mediated endocytosis (Nicholson, 1974; Olsnes et al., 1974; Refsnes et al., 1974). This proposal is based partly on electron-microscope studies showing extensive endocytosis of ferritin-labelled ricin in toxin-sensitive cells, and partly on the finding that abrin and ricin are not toxic to reticulocytes, although the toxins are readily bound to these cells which have very little endocytic activity. It is also based on the finding that the action of numerous protein toxins and
peptide hormones is antagonised by 'lysosomotropic agents', which are substances known to affect the endocytic pathway (Goldstein et al., 1979; Leppla et al., 1980; Olsnes and Sandvig, 1983). The action of cholera toxin, which is believed to penetrate target cells directly from the cell surface by translocation of its A-fragment across the membrane, is not affected by chloroquine (Gill et al., 1981). Likewise, the action of the snake toxin β-bungarotoxin, which is not internalised is not affected by lysosomotropic agents (Simpson, 1982; 1984). The discovery that the endocytic pathway involves a pre-lysosomal compartment, the receptosome or endosome, which has a low pH but few or no degradative enzymes, suggests that internalised ligands can avoid lysosomal processing and enter the cytosol in an active form. That it is from this compartment and not from lysosomes that toxin enters the cytosol is suggested by the report that unnicked diphtheria toxin (and possibly botulinum toxin) is not cytotoxic. Lysosomal enzymes would be expected to 'nick' and thus activate the toxin (Olsnes and Sandvig, 1983). Finally, the most direct evidence for involvement of endocytosis in toxin uptake is that diphtheria toxin (Sandvig and Olsnes, 1980) and certain viruses such as Semliki Forest virus (Helenius et al., 1980; Marsh et al., 1983) and influenza virus (Matlin et al., 1981) require low pH to enter the cytoplasm of target cells. Under artificial conditions in which the pH of the extracellular fluid is lowered, these toxins enter target cells directly from the cell surface (Sandvig and Olsnes, 1980). Under physiological conditions, however, the acid endosome could provide the required environment to allow access of these substances to the cytosol. It has been suggested that a change in pH, or in the concentration of ions such as Ca++ may provide the signal to open up pores in the vesicle membrane through which proteins may pass into the cytosol (Olsnes and Sandvig, 1983).
The toxic effect of BoNT can be antagonised by the lysosomotropic agents chloroquine, ammonium chloride and methyamine, and by Ca\textsuperscript{++} deprivation (Simpson, 1980; 1982; 1983). As lysomotropic agents such as chloroquine antagonise a variety of pharmacological substances that go through the sequence of cell surface binding, internalisation and expression of biological activity, sensitivity to these drugs is viewed as suggestive evidence that the toxin in question is internalised. Lysosomotropic, or acidotropic, agents are weak bases which accumulate in acidic compartments of cells (de Duve et al., 1974; de Duve, 1983). The plasma membrane, as well as the endosomal and lysosomal membranes, are highly permeable to the neutral form of weak bases allowing rapid diffusion of these drugs into lysosomes and endosomes. Because of the acidity of these compartments, the bases become protonated and are trapped within the vesicles as the membrane is much less permeable to the protonated form. The concentration of intra-endosomal and lysosomal base goes up resulting in osmotic influx of water making the vesicles swell. The membrane needed to accommodate swelling vacuoles may be supplied by vesicle fusion or recruited from the plasma membrane. The presence of these drugs may raise the pH from \( \approx 5.5 \) in endosomes and \( 4.7-4.8 \) in lysosomes to values that may be as high as 6.5 (Ohkuma and Poole, 1978; 1981).

In the presence of chloroquine, BoNT remains at an antitoxin-sensitive site (Simpson, 1982). Autoradiograms of motor nerve endings incubated with toxin in the presence of chloroquine showed that, under these conditions, the toxin still binds to the membrane and is subsequently internalised. Silver grains within the cytosol were associated almost exclusively with vacuolar structures. The extensive vacuolation seen in these endings showed that the drug had exerted its effect. Therefore, the vesicular pool with which the toxin was associated must be accessible to antibodies. This could occur in two
ways: antibodies could penetrate the same vesicles from the extracellular medium (Gordon et al., 1965) and/or the toxin could be re-exposed to the surface of the cell. Given the known effects of chloroquine in other systems, a possible explanation for events in the presence of the drug is the following: the toxin still binds to extracellular acceptors and is taken up into endosomes [this also occurs with diphtheria toxin; chloroquine protects cells from its action but the initial rate of binding is unchanged (Leppla and Dorland, 1981) and there is no measurable effect on toxin uptake (Middlebrook and Dorland, 1981)]. There, because of the rise in pH as a result of the presence of the dibasic compound, the toxin does not undergo the conformational change required for entry into the cytoplasm. The acceptor-toxin complex is therefore returned to the cell surface intact where it is inactivated by antitoxin (see Fig. 5.12). It has been shown that lack of detachment of a ligand from its acceptor in the endosomal compartment may cause it to be returned to the cell surface with recycling membrane patches, thereby resulting in severe inhibition of net endocytic uptake (Schneider and Trouet, 1981). That the BoNT acceptor is recycled was suggested by results in Chapter 4; more silver grains were associated with nerve endings in the absence of metabolic inhibitors (and thus inhibitors of the energy-requiring recycling process) than in the presence of such agents.

In many cases of acceptor-mediated endocytosis, two parallel uptake mechanisms occur. In studying the entry of BoNT into target neuronal cells, it was necessary to include high numbers of molecules in the incubation medium in order to satisfy the demands of adequate structural preservation of the tissue and the limits of detection by the autoradiographic technique. These molecules may enter cells by several different routes (see Introduction, Chapter 1). One is a highly efficient route which mediates expression of biological activity,
a. Acceptor-mediated endocytosis in the presence of the lysosomotropic agent chloroquine. Owing to the raised pH in the endosome, ligand-acceptor complexes cannot dissociate; ligand is thus recycled back to the cell surface and re-exposed to the external environment.

b. Non-specific endocytic pathway. Ligand is taken up into endocytic vesicles delivered directly to lysosomes where, because of the raised pH induced by chloroquine, degradation cannot take place.
whereas the other(s) is a non-specific adsorptive route which leads directly to degradation within lysosomes (see Fig. 5.12b). If such a scheme applies for BoNT [as it does for diphtheria toxin (Dorland et al., 1981), modeccin (Olsnes and Sandvig, 1981), α₂-macroglobulin (Steinman et al., 1983)], then the slight increase in the proportion of intracellular grains seen in the presence of chloroquine could be due to undegraded, non-productive toxin molecules taken up by a non-specific route and delivered to lysosomes. It is well known that chloroquine prevents lysosomal degradation of exogenous and endogenous proteins (Middlebrook and Dorland, 1981).

The short chain amines NH₄Cl and methylamine also antagonise the onset of neuromuscular blockade by BoNT (types A, B and C). In the presence of these compounds, the toxin also remains at an antitoxin sensitive site (Simpson, 1983). In addition to raising endosomal/lysosomal pH and thus preventing ligand-acceptor dissociation and lysosomal processing (de Duve, 1974), these drugs are thought to prevent initial uptake of ligands into endocytic compartments (Maxfield et al., 1979). Simpson concluded that, in the presence of these drugs, the toxin remained on the cell surface as has been suggested for α₂-macroglobulin, epidermal growth factor (Maxfield et al., 1979), and diphtheria toxin (Sandvig et al., 1979). Autoradiograms of nerve terminals treated with ¹²⁵I-BoNT type A in the presence of NH₄Cl or methylamine showed that binding was not affected but the uptake process was somewhat reduced (by about 35% relative to controls treated under physiological conditions, in both cases). Extensive vacuolation occurred in treated nerve endings indicating that the drugs had exerted their effect; most internalised grains were associated with these vesicles. Maxfield et al., (1979) showed that methylamine prevents clustering of α₂-macroglobulin and epidermal growth factor acceptor-ligand complexes in coated pits and suggested that the action of the
enzyme transglutaminase is inhibited in the presence of the amine.

Transglutaminase is an intracellular enzyme found in many types of cells which covalently cross-links proteins by forming -(E-glutaminyl)-lysine cross bridges in the presence of Ca$^{++}$. Davies et al. (1980) suggested that binding of ligands induces a change in the acceptors diffusely distributed on the membrane so that they form reversible aggregates in coated pits; they proposed that transglutaminase then cross-links the proteins in the complex, i.e. acceptors might be cross-linked to intrinsic membrane proteins or coupled to each other. This would make the aggregation process irreversible and would result in progressive accumulation of ligand-acceptor complexes in the coated pit. Ultimately, the pit would invaginate and pinch off to form an intracellular vesicle. However, a number of observations casts doubt on the universal existence of such a process in cells. Low density lipoprotein acceptors cluster in the absence of ligand binding (Anderson et al., 1977), the cytotoxicity of diphtheria toxin is not prevented by inhibitors of transglutaminase (Dorland et al., 1981), and Ca$^{++}$ is not required for uptake of $^{125}$I-epidermal growth factor (King and Cautrecasas, 1983) (or for BoNT as shown here). Pastan and Willingham suggest that 'there must be a biochemical difference between how different acceptors get into the same cells, even though the morphological pathway is the same'.

Whatever the mechanism involved in the action of these amines, it appears that they do inhibit the initial uptake of proteins by the 'effective' pathway (Maxfield et al., 1979). It has been shown that they do not affect non-specific adsorptive pinocytosis (Sandvig et al., 1979; Davies et al., 1980; Dorland et al., 1981); it is therefore possible that, in the presence of these drugs, the 'effective' and 'ineffective' routes of uptake are distinguished. Internalised silver grains could be attributed to non-specific adsorptive pinocytosis with
direct delivery to lysosomes where processing does not occur due to the elevated pH of these drug-filled compartments. It must be pointed out that some uptake into endocytic vesicles may still be taking place, with re-exposure of undissociated toxin to the surface of the cell as seen with chloroquine. In a well-designed experiment, Draper and Simon (1980) showed that although diphtheria toxin remains at an antitoxin-sensitive site in the presence of NH$_4$Cl, the effect is only seen at 37°C. Thus, if the protective amine was washed out and antitoxin added immediately at 37°C, cells were protected from diphtheria toxin. If, however, cells preincubated with toxin in the presence of NH$_4$Cl were exposed to antitoxin at 4°C, then washed to relieve the NH$_4$Cl inhibition and warmed to 37°C, no protective effect was seen. It was concluded that the antigenic determinants of the toxin were probably sheltered within endocytic vesicles; at 37°C the membrane patches containing toxin-acceptor complexes were recycled back to the surface and thus exposed to antitoxin (Mellman et al., 1984). Alternatively, antitoxin was delivered to the same compartment by fluid phase endocytosis (Gordon et al., 1965). If this is the case with BoNT (i.e. that molecules are taken up into a vesicular pool which is in equilibrium with the cell surface), less uptake must take place than in the presence of chloroquine, as the proportion of internalised grains was significantly reduced in the presence of NH$_4$Cl and methylamine relative to chloroquine-treated samples. Collectively, the effects of lysomotrophic agents on the action of botulinum toxin, as well as on the distribution of molecules at the nerve terminal, compared with their known actions in other systems, provide strong evidence for the involvement of the process of acceptor-mediated endocytosis in BoNT uptake.

Simpson (1980) also reported that antitoxin antibodies added (at 37°C) to preparations which had previously been incubated with toxin at low temperatures had a protective effect. Autoradiograms of nerve
terminals treated with $^{125}$I-BoNT type A at 4°C followed by incubation with antibody at 4°C and then at room temperature, showed that BoNT molecules were still taken up into the nerve terminal under these conditions. Antibody must therefore inactivate the toxin at an intracellular site, possibly by preventing exit of the lytic fragment from an endocytic vesicle into the cytosol. Uptake of antibodies into the cell has been reported in a number of studies. The Fc receptor on macrophages is internalised and recycled rapidly (~7 min, too rapid to have included a lysosomal step) (Mellman et al., 1984). In the presence of multivalent IgG-containing immune complexes against the Fc receptor, ligand-acceptor complexes are internalised and delivered rapidly to lysosomes (Mellman and Plutner, 1984), both being degraded therein. The antibody does not dissociate in the endosomal compartment, and is also not recycled. This raises the possibility that the state of receptor aggregation (cross-linking) on the endosomal membrane dictates whether a particular acceptor is recycled to the cell surface or is removed from the recycling pathway and transferred to lysosomes. Receptor aggregation probably prevents inclusion of receptor-ligand complexes into nascent recycling vesicles that continuously leave CURL bound for the plasma membrane. This effect of polyclonal antibody on receptor recycling is seen with low-density lipoprotein (Anderson et al., 1982) and epidermal growth factor (Schreiber et al., 1983). However, in the presence of monoclonal antibodies to the Fc receptor (which do not induce receptor cross-linking), ligand-receptor complexes are recycled rapidly back to the cell surface; as in the case of multivalent immune complexes, the monoclonal antibodies do not dissociate from their receptors in the endosomal compartment (Mellman et al., 1984). In the case of BoNT, it is possible that the antitoxin exerts its protective effect by forming BoNT-antibody complexes which are internalised and subsequently delivered to lysosomes.
In another study, Simpson (1980) showed that Ca\(^{++}\) deprivation prevented the toxicity of botulinum toxin and arrested the toxin molecules at a site accessible to antibodies. Autoradiograms of nerve endings treated with \(^{125}\)I-BoNT in the absence of Ca\(^{++}\) showed that the binding of toxin is not affected by Ca\(^{++}\) deprivation and that internalisation is also unchanged. A number of toxins known to enter cells by acceptor-mediated endocytosis require Ca\(^{++}\) for expression of toxicity. The lectins abrin and modeccin do not inhibit protein synthesis in the absence of Ca\(^{++}\), and ricin is less effective under these conditions (Olsnes and Sandvig, 1983). The protective effect of Ca\(^{++}\) deprivation in these cases is not due to reduced binding of the toxins to the cell surface or to inhibition of internalisation. The results obtained are compatible with the possibility that Ca\(^{++}\) is required for the transport of the toxin, or a lytic fragment, from endocytic vesicles to the cytoplasm (Sandvig and Olsnes, 1982a). A possible explanation for the lack of toxicity of BoNT in the absence of Ca\(^{++}\) is the following: BoNT binds to its acceptor and is taken up into endocytic vesicles. There, although the pH is low, the toxin cannot cross the endosomal membrane and enter the cytosol due to the absence of Ca\(^{++}\) [it has been suggested that Ca\(^{++}\) flux into cells through physiological Ca\(^{++}\) channels may be required for transport of some toxins into the cytosol (Olsnes and Sandvig, 1983). Ca\(^{++}\) influx can provide considerable amounts of energy, as there is normally a very steep concentration gradient of Ca\(^{++}\) across the membrane]. It remains bound to its acceptor and returns to the cell surface where it is exposed to antitoxin. Alternatively, antitoxin may enter the vesicle and inactivate the toxin therein (Gordon et al., 1965). Williams (1984) has shown that the extent of binding of \(^{125}\)I-BoNT type A to synaptosomes is not appreciably different at pH's between 5 and 7, so toxin could remain bound to its acceptor sites within the acidic endosomes.
It was more difficult to determine the source of the internalised silver grains in preparations incubated in the absence of Ca\(^{++}\) as these conditions do not induce the extensive enlargement of intracellular compartments seen with lysosomotropic agents. The resolution of the autoradiographic technique does not permit localisation to such small vesicles. It must be noted that Ca\(^{++}\) is also required for the action of the enzyme transglutaminase (Davies et al., 1980). Although the toxin does not appear to remain bound to the plasma membrane in the absence of Ca\(^{++}\), the possibility that only a minute population of 'effective' sites, undetectable by the autoradiographic method, is affected by Ca\(^{++}\) deprivation cannot be excluded.

Finally, cytochalasin B, known to inhibit pinocytosis and phagocytosis (Weihing, 1976), did not affect uptake of \(^{125}\)I-BoNT into the nerve terminal. It appears to partially protect cells from diphtheria toxin, but has no effect on the toxicity induced by Pseudomonas aeruginosa exotoxin A (Middlebrook and Dorland, 1977). These results emphasise that variations in the pathway of acceptor-mediated endocytosis do occur with different substances.

The studies mentioned above and described in this Chapter must be interpreted with caution. Although some of the effects of the substances used are known, there may be secondary interactions which affect various ligands in different ways and may interfere with the endocytic pathway at a stage that differs from that envisaged originally. Taken collectively, the results discussed herein allow the following model to be proposed for the toxin's delivery to its lytic site: the heavy subunit of the BoNT molecule binds to an acceptor (apparently neuraminidase-insensitive) on the nerve terminal membrane and the acceptor-ligand complex is then internalised in an endocytic vesicle or endosome. It appears that the acid environment is required for entry of the toxin into the cytosol, as no toxicity was expressed in the presence
of chloroquine or short chain amines. The low pH probably causes a conformational change in the toxin molecule allowing it to cross the membrane, possibly through a channel created by the heavy subunit, as suggested for tetanus and diphtheria toxins (Donovan et al., 1981), a step which also requires Ca$^{++}$. The free acceptor is then recycled to the cell surface in the tubular portions of CURL. Meanwhile, the small subunit exerts its lytic effect in the cytosol. It has been shown for diphtheria toxin that very small amounts of toxin reach the cytosol in this way (Pappenheimer and Moynihan, 1981); there is probably also a pathway of non-specific adsorption in which the toxin bound to the same or a different acceptor goes in by a different route and is delivered directly to lysosomes. This may also occur with BoNT at the nerve terminal.

In the next Chapter, the interaction of $^{125}$I-BoNT with different synapses in the central and peripheral nervous systems is studied in an attempt to explain the lack of toxicity of BoNT at some of these sites.
CHAPTER VI

INTERACTION OF $^{125}$I-BONT WITH NERVE TERMINALS IN THE
CENTRAL AND PERIPHERAL NERVOUS SYSTEMS OF DIFFERENT SPECIES.
6.1 INTRODUCTION

Although the action of botulinum toxin has been studied mainly at the mammalian neuromuscular junction, its effects have also been investigated in the central nervous system, at a number of other peripheral synapses (i.e. adrenergic and nonadrenergic-noncholinergic), and in non-mammalian tissue (such as the frog neuromuscular junction and Electrophorus electric organ). To date, no pathological effects of the neurotoxin in the brain have been observed in vivo after systemic administration, presumably because the toxin cannot cross the blood/brain barrier. Other possible reasons include hindered diffusion of toxin in brain tissue and/or absence of the specific mechanism necessary for delivery of active toxin molecules to the cytosol. Acceptors for botulinum neurotoxins types A, B and E have been observed in rat brain preparations in vitro (Habermann, 1974; Kitamura, 1976; Kozaki, 1979; Kozaki and Sakaguchi, 1982; Williams et al., 1983; Williams, 1984). Toxin binding data have shown that $^{125}$I-BoNT type A binds preferentially to the synaptic plasma membranes of subfractionated synaptosomes (Tse, 1981; Williams, 1984), and that these membranes contain heterogeneous populations of acceptors for types A and B $^{125}$I-BoNT (Williams et al., 1983; Williams, 1984). The acceptors exhibit either high or low affinity for the toxin and are similar in nature. Using a double-sandwich immunocytochemical technique, Hirokawa and Kitamura (1979) demonstrated binding of botulinum neurotoxin type A to the presynaptic membrane of large synaptosomes from cerebellar and cerebral cortices of rats at the ultrastructural level. Binding was observed at junctional and extra junctional areas of the presynaptic membranes and appeared as patches of various sizes. However, no immune complexes were observed on the postsynaptic membranes. Collectively, these findings suggest that absence of acceptors for the toxin in brain tissue is un-
likely to account for the lack of central effects in vivo. It must be emphasised that some reports have suggested a central action for the haemagglutinin-toxin complex when administered directly into the brain or peripherally (Davies et al., 1953; Polley et al., 1965; Wiegand and Wellhoner, 1977). However, as crude toxin-haemagglutinin complexes were used in these studies, the effects observed are thought to be due to some contaminant or dissociation product of the toxin (Simpson, 1981). When homogeneous toxin was used, no effects were observed (Simpson, 1968). Intraventricular injections of high doses of BoNT type A (up to 5,000MLD$_{50}$) in this laboratory (Williams et al., 1983) produced no detectable neurological symptoms until slow escape of the neurotoxin from the brain into the blood caused death by paralysis of the skeletal musculature. However, this lack of effect of BoNT on central synapses was not observed in vitro. A decrease in evoked and spontaneous release of acetylcholine from neurotoxin-treated brain synaptosome preparations has been reported (Bigalke et al., 1981; Dolly et al., 1981). It has been shown that the toxin does not directly affect choline uptake (Bigalke et al., 1978; Gundersen and Howard, 1978; Wonnacott, 1980), acetylation of choline by inhibition of choline acetyltransferase (Wonnacott and Marchbanks, 1976) or levels of endogenous acetylcholine (Gundersen and Howard, 1978) in synaptosomes. It is likely, therefore, that the release mechanism is directly affected as observed at the neuromuscular junction. However, large concentrations were required to produce this effect, suggesting a non-specific mechanism. The effect of botulinum toxin on central synapses has, therefore, not been unequivocally established; localisation studies using intact rather than particulate tissue are necessary to ascertain whether the toxin binds to central synapses and is internalised in a similar manner to that observed at the neuromuscular junction.
It is generally believed that botulinum toxin is specific for cholinergic synapses in the peripheral nervous system. Toxin-induced blockade has been shown to occur at autonomic ganglia, postganglionic parasympathetic nerve terminals and postganglionic sympathetic nerve terminals that release acetylcholine. Muscarinic cholinergic nerve endings in the myenteric plexus are affected by both botulinum toxin-haemagglutinin complex (Bigalke and Habermann, 1980) and purified BoNT (Mackenzie et al., 1982). Some reports suggest that the toxin complex may act to inhibit release of neurotransmitter from adrenergic nerve terminals (Rand and Whaler, 1965; Westwood and Whaler, 1968, Holman and Spitzer, 1973). Results are inconclusive as other investigators have found no such effects using either the complex (Ambache, 1951; Vincenzi, 1967) or the purified neurotoxin (Habermann, 1980; Mackenzie et al., 1982). Blockade of neurotransmission was only seen with high concentrations of toxin, suggesting that the toxin may be taken up by a non-specific mechanism. Localisation studies are necessary to establish whether specific binding occurs to these synapses. No effect of botulinum toxin has been observed at inhibitory nonadrenergic-noncholinergic synapses of the guinea-pig fundic strip (Paul and Cook, 1980) and guinea-pig taenia coli (Mackenzie et al., 1982). However, BoNT was found to block transmission completely at excitatory nonadrenergic-noncholinergic terminals of the guinea-pig urinary bladder. It is thought that these terminals co-release ACh and a non-cholinergic excitatory neurotransmitter such as ATP or polypeptides (Mackenzie et al., 1982). It would be interesting to establish whether absence of the acceptor, or of some other step in the process of intoxication, is responsible for the lack of toxicity of BoNT at some of these terminals.

Non-mammalian synapses are also known to be affected. The frog neuromuscular junction is highly susceptible to the action of botulinum toxin-haemagglutinin complex (Harris and Miledi, 1971; Pumplin and
Reese, 1977) and to the purified neurotoxin (Boroff et al., 1973; Hirokawa and Heuser, 1977). Toxin-induced blockade of transmission in the Electrophorus electric organ has been claimed (Ambache and Ferreira, 1952) although, to date, there is no evidence for a binding site on this tissue (R. Williams and A. Ashton of this laboratory, unpublished data). No histological data exist to support reports of the interaction of the toxin with these non-mammalian tissues.

Neuroblastoma or pheochromocytoma (PC12) cell lines could be useful as models for study of the mechanisms involved in the action of BoNT since release of transmitter and Ca\(^{++}\) flux in these cells can be quantified readily. To date, specific binding of BoNT to cell lines has not been reported and experiments in this laboratory have proved unsuccessful (Ashton, personal communication), although ability of the cells to release ACh was measurable.

In this Chapter, light-microscope and ultrastructural autoradiographic localisation of \(^{125}\)I-BoNT types A and B was carried out in the central nervous system and at different terminals of the autonomic nervous system, i.e. cholinergic, adrenergic and nonadrenergic-noncholinergic. The interaction of radiolabelled BoNT with non-mammalian tissue and malignant cell lines of the nervous system was also investigated. Resultant localisation data are used in an attempt to explain the lack of toxicity of BoNT at some of these synapses.

6.2 MATERIALS AND METHODS

Part A: The Central Nervous System

6.2.1 Acceptors for BoNT types A and B in rat brain.

6.2.1.1 Effect of paraformaldehyde on the binding of \(^{125}\)I-BoNT type A to synaptosomal membranes. In order to ensure that the acceptor(s) for \(^{125}\)I-BoNT type A on synaptosomal membranes
(synaptosomes, prepared as described in Chapter 2, Section 2.10.1, lysed by freeze-thawing) was not altered by fixation with paraformaldehyde. Binding studies were carried out on membranes prefixed with varying concentrations of the cross-linking agent. Synaptosomal suspensions [0.5 - 0.7 mg/ml phosphate-buffered saline (PBS)] were pre-incubated in 0.1% or 3% paraformaldehyde for 30 minutes at room temperature. This was followed by extensive washing (3 times in PBS at 22°C) by centrifugation (9,000 x g, 2 min) to remove the paraformaldehyde and incubation with 2 nM 125I-BoNT type A in PBS/BSA (1 mg/ml) for 40 minutes at 22°C. Control preparations were not pre-treated with fixative. Binding was terminated by dilution with ice-cold PBS/BSA and centrifugation. The pellets were washed twice in an ice-cold buffer (containing albumin) before being submitted to γ-radiation counting. Non-specific binding was determined by incubation in the presence of a 100-fold molar excess of native BoNT type A.

6.2.1.2 Binding of 125I-BoNT types A and B to cryostat sections of rat brain.

Rat brain tissue was prepared for cryostat sectioning as described in Chapter 2. Serial sections (10μm thick) were cut using a Bright cryostat microtome (Model OTF/AS/M/V) at 300μm intervals through the brain and cerebellum, and thaw-mounted in pairs on gelatin-coated glass microscope slides. Three slides were prepared at each interval (1 test, 1 control, 1 for histological staining). The sections were stored at -20°C for 48 hours to allow hardening of the tissue.

For studies on binding of 125I-BoNT type A, the slides were brought to room temperature and placed in humidifiers (6 tests and 6 control slides per box). All incubations and washes were carried out in 50mM Tris-buffer containing 190mM sucrose and
1mg/ml BSA (pH 7.4) at room temperature. Test sections were incubated with 1nM 125I-BoNT type A (200μl). Control sections (to determine non-specific binding) were pre-incubated in 1μM native BoNT type A (100μl) for 20 minutes prior to addition of 125I-BoNT type A (100μl) to a final concentration of 1nM (a concentration known to label the high affinity sites preferentially; Williams et al., 1983). Binding was terminated after 40 minutes by draining excess ligand onto tissue paper and immersing the slides (in groups of 12 in slide racks) into 300ml Tris buffer/BSA at 22°C for 15 minutes. Two more washes (15 minutes each) in 300ml fresh Tris buffer were carried out prior to rinsing in distilled water and fixation in 3% paraformaldehyde (15 min) to inactivate unbound toxin. The extensive washing was necessary to remove toxin associated non-specifically with myelinated areas of the brain. The slides were then rinsed in a large volume of distilled water, drained on tissue paper, and dried with a hair dryer (at 30°C). They were left overnight in a desiccator in uncovered slide boxes containing packets of silica gel. Preparation for autoradiography (using LKB 3H-ultrafilm) was carried out as described in Chapter II (Section 2.8.4). The film was developed after 2 days exposure at 4°C. The sections prepared for histological staining were processed as described in Chapter II (Section 2.8.4.3).

For 125I-BoNT type B binding studies, cerebellar sections were prepared and treated with radiolabelled type B toxin as described for type A.

6.2.2 Ultrastructural localisation of 125I-BoNT type A in rat cerebellum in vitro.

Hand-cut surface slices of adult rat cerebellum were prepared as
described in Chapter II, Section 2.8.1, and pre-incubated in Krebs solution (pH 7.4) for 15 minutes at 37°C. The medium was gassed continuously with 95% O₂/5% CO₂. The test slices were then incubated in 3ml Krebs solution containing 30nM ¹²⁵I-BoNT type A (450 Ci/mmol) for 1 hr at 37°C with gentle shaking and continuous gassing. Controls were incubated with 30nM ¹²⁵I-BoNT type A and a 100-fold molar excess of native BoNT. Following incubation, the slices were washed in fresh medium at 37°C (5 times, 5 minutes each), and then fixed, osmicated and processed for electron-microscope autoradiography as described in Chapter II.

6.2.3 Binding of ¹²⁵I-BoNT type A to rat cerebrocortical synaptosomes in the presence and absence of sodium azide.

Rat cerebrocortical synaptosomes were prepared by Ficoll density-gradient centrifugation as described in Chapter II, Section 2.10.1. All incubations and washes were carried out in Krebs Ringer containing BSA (1mg/ml). Synaptosomal suspensions (0.5mg in 0.6ml) were incubated with 1nM or 10nM ¹²⁵I-BoNT type A in the presence or absence of 15mM Na azide for 40 minutes at 37°C. Binding was terminated by dilution in ice-cold Krebs Ringer/BSA and centrifugation. Control samples were incubated with type A ¹²⁵I-BoNT under the conditions described above in the presence of a 100-fold molar excess of native BoNT type A. After washing twice in ice-cold buffer, the synaptosomal pellets were processed for electron-microscope autoradiography as described in Section 2.10.2.
Part B: The Peripheral Nervous System

6.2.4 Cholinergic nerve terminals in non-mammalian tissue: binding of $^{125}$I-BoNT type A to electroplaques from Electrophorus electricus and to the frog neuromuscular junction.

Like a striated muscle fibre, the electroplaque from Electrophorus electricus is a giant syncytium which contains approximately 5,000 to 6,000 nuclei distributed at random beneath the cell surface. It is highly asymmetrical and receives nerve terminals (which are cholinergic in nature) exclusively on its caudal or 'innervated' face. After extensive branching, the nerve fibres lose their myelin sheath and make multiple synaptic contacts with the cell surface. These contacts are characterised by a gutter-like structure and local thickening of the postsynaptic plasma membrane (see below, Fig. 6.8).

In order to study the binding of $^{125}$I-BoNT type A to the electric organ, eels were sacrificed by exposure to low temperature (ice cold water). Single electroplaques were dissected from Sachs' organ and pre-incubated in eel Ringer [containing 160mM NaCl, 5mM KCl, 2mM CaCl$_2$, 2mM MgCl$_2$, 1.5mM phosphate buffer, pH 7.0, 1.5% (w/v) glucose] (Bourgeois et al., 1978) prior to incubation with $^{125}$I-BoNT type A.

For localisation of $^{125}$I-BoNT type A target sites in amphibian tissue, cutaneous pectoris muscles from the frog were used as the fibres are innervated by cholinergic neurons (Matthews-Bellinger and Salpeter, 1978). The muscles were dissected from an adult frog, and pre-incubated in frog Ringer containing 116mM NaCl, 2mM KCl, 2mM CaCl$_2$, 0.5mM NaH$_2$PO$_4$, 3mM glucose, 5mM Hepes, pH 7.2, prior to incubation with $^{125}$I-BoNT type A in frog Ringer.
6.2.5 Adrenergic, muscarinic and inhibitory nonadrenergic-noncholinergic nerve terminals: binding of $^{125}$I-BoNT type A to nerve endings in the mouse vas deferens and ileum.

The mouse vas deferens is rich in adrenergic innervation and was, therefore, used as a model for investigating the interaction of $^{125}$I-BoNT type A with adrenergic nerve terminals. It was dissected from beneath the epididymal fat pads in the male mouse and transferred to pre-gassed Krebs Ringer, pH 7.4, at 22°C. The well-developed serosal coat which envelops the vas deferens and may be a barrier to toxin penetration was removed by gentle pulling. Each preparation was pinned out on wax.

The interaction of $^{125}$I-BoNT type A with cholinergic muscarinic and nonadrenergic-noncholinergic (e.g. purinergic and peptidergic) nerve terminals was investigated in the mouse ileum. A section of ileum was dissected and the contents were gently flushed out with Krebs Ringer (using a Pasteur pipette). Small segments were tied at one end with cotton and pinned out on wax.

The protocol used for fixation of the vas deferens and ileum was slightly different to that used for the mouse diaphragm. The fixative used was 3% glutaraldehyde/2% paraformaldehyde in 0.1M Na Cacodylate buffer, pH 7.4. After incubation with $^{125}$I-BoNT type A, the vas deferens tissue was washed, stretched to its maximum length and held in place with pins. It was fixed for 90 minutes at 22°C and then cut transversely into small segments which were washed in 5% sucrose/0.1M Na Cacodylate buffer (two changes, 10 minutes each). The tissue was then post-fixed in 2% OsO$_4$ in the latter buffer for 1 hour at 22°C and washed in 5% sucrose/0.1M Na Cacodylate buffer, prior to dehydration and embedding as described in Chapter II.

In the case of the ileum, the open end of the small segments previously incubated with toxin was tied with cotton and fixative was
then gently injected into the lumen. More fixative was added to submerge the tissue and, after 30 minutes, the segments were cut into rings and fixed for a further hour. The tissue rings were then processed as described for the small segments of vas deferens. This fixation method was used as it ensures maximum preservation of dense core vesicles (which facilitate identification of the adrenergic terminals) and also gives good fixation of intestinal tissue (Gordon-Weeks, personal communication).

Part C: Neuroblastoma and Pheochromocytoma (PC12) cell lines.

6.2.6 Binding of $^{125}$I-BoNT type A to neuroblastoma and to pheochromocytoma (PC12) cell lines.

Cell suspensions of the neuroblastoma cell lines CHP100, MR32, NB212, Lanl and TR14 (kindly provided by Dr. John Kemshead, Imperial Cancer Research Fund) were incubated in 0.5ml growth medium or Krebs Ringer BSA (1mg/ml) containing 8nM $^{125}$I-BoNT type A for 90 minutes at 22°C. The leukaemic cell line HL60 was used as a control. Binding was terminated by dilution in ice-cold buffer (growth medium or Krebs/BSA) and centrifugation. The cells were washed three times at 4°C, fixed in 2% glutaraldehyde before being subjected to $\gamma$-radiation counting. Some of the pellets were processed for autoradiography as described for synaptosomes (Section 2.10.2). Control sampes were treated similarly except that an excess of native BoNT type A (100-fold) was included in the incubation medium.

PC12 cells (kindly provided by Mr. Anthony Ashton) were suspended in DMEM growth medium (0.5ml) containing 25nM $^{125}$I-BoNT type A for 60 minutes at 22°C. Controls were incubated with 25nM $^{125}$I-BoNT type A in the presence of a 100-fold excess of native (A) BoNT. After incubation, the cells were washed in ice-cold DMEM (3 times at 4°C) and processed as described above.
6.3 RESULTS

6.3.1 Acceptors for $^{125}$I-BoNT in the central nervous system of the
rat.

6.3.1.1 Effect of tissue fixation on the binding of $^{125}$I-BoNT to
brain synaptosomal membranes

A useful technique for localisation of specific acceptors in
the brain involves preparation of cryostat sections from pre­
fixed tissue. In order to assess the validity of such a technique
for localisation of BoNT type A acceptor sites in the central
nervous system, the effect of pre-treatment of brain tissue with
fixatives on the high affinity binding of labelled toxin was
determined. As shown in Table 6.1, specific binding of $^{125}$I-BoNT
type A to synaptosomal membranes treated with varying
concentrations of paraformaldehyde was unaltered relative to
control (untreated) membranes. The binding was saturable as the
presence of excess native BoNT type A reduced binding of labelled
toxin by approximately 75-80% in all cases. Levels of non­
specific binding were not appreciably changed by fixation of the
tissue. In addition, freeze-thawing of synaptosomes does not
affect the binding site for $^{125}$I-BoNT type A (Williams, 1984).

Taken collectively, these result show that cryostat sections of
fixed brain tissue do provide a valid system for localisation of
$^{125}$I-BoNT type A binding sites in the central nervous system.

6.3.1.2 Binding of $^{125}$I-BoNT types A and B to different areas of
the rat forebrain and cerebellum.

Autoradiograms of cryostat sections from different brain
areas incubated with $^{125}$I-BoNT types A and B showed
concentration of label in synaptic regions, particularly in the
hippocampal formation and the cerebellum. Acceptors with high
Table 6.1 Effect of treatment with paraformaldehyde on the acceptors for $^{125}\text{-BoNT type A}$ on synaptosomal membranes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative specific binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (total binding)</td>
<td>100</td>
</tr>
<tr>
<td>0.1% paraformaldehyde</td>
<td>90</td>
</tr>
<tr>
<td>3.0% paraformaldehyde</td>
<td>98</td>
</tr>
</tbody>
</table>

Synaptosomal suspensions (0.5-0.7 mg/ml PBS) were preincubated with 0.1% or 3% paraformaldehyde for 30 mins at 22°C. Following extensive washing, the membranes were incubated with 2nM $^{125}\text{-BoNT type A}$ for 40 minutes at 22°C. Control preparations were not pre-treated with fixative. Binding was terminated by dilution with ice-cold PBS/1% BSA and centrifugation. The pellets were washed twice in ice-cold buffer before being submitted to $\gamma$-radiation counting.
affinity for the toxin should have been labelled preferentially at the toxin concentration used (1nM).

The six layers of the cerebral cortex appeared uniformly labelled with $^{125}$I-BoNT type A (Figs. 6.1, 6.2) although, in some autoradiograms, a higher density of silver grains was detected in lamina I. Absence of this band of higher grain density can be attributed to loss or damage of the outer layer of the cortex during dissection or freezing of the tissue. Binding in this area was saturable as controls showed few silver grains in all layers of the cortex (Fig. 6.1). The caudate putamen was also labelled (Fig. 6.1a); however, the distribution of grains therein was not uniform as numerous patches were totally devoid of silver grains. The nucleus accumbens showed particularly high levels of radioactivity (Fig. 6.1a), but myelin tracts, i.e. the corpus callosum and anterior commissures, were totally unlabelled.

A schematic representation of the general shape and location of the hippocampal formation in the rat brain is given in Fig. 6.2a. In addition, diagrams of the different layers in the hippocampus and dentate gyrus seen in serial sections are shown to facilitate identification of the labelled areas in autoradiograms (Figs. 6.2b1, c1, d1, e1) (for a detailed description of the hippocamal formation, see Storm-Mathisen, 1981). Silver grains were located in all synaptic areas (Figs. 6.2b,c,d,e), particularly in the stratum oriens (fields CA2 and CA3). High levels of radioactivity were also detectable in the molecular layer and hilar region of the dentate gyrus, and the stratum radiatum was labelled but to a lesser extent. Areas containing cell bodies (i.e. pyramidal and granule cell layers) showed few grains and the fimbria was unlabelled. The binding seen in the
Fig. 6.1 Specific binding of $^{125}\text{I}$-BoNT to cryostat sections of rat forebrain: caudate nucleus, putamen and neocortex.

Cryostat sections (10µm thick) of fixed rat forebrain (areas anterior to the hippocampal formation) were incubated with 1nM $^{125}\text{I}$-BoNT type A in 200µl 50mM Tris-buffer (pH 7.4)/190mM sucrose/1%BSA for 40 minutes at 22°C. Control sections (to determine non-specific binding) were preincubated with 1µm native neurotoxin type A (100µl) for 20 minutes prior to addition of $^{125}\text{I}$-BoNT (100µl) to a final concentration of 1nM. Binding was terminated by tipping off excess ligand onto tissue paper and washing the slides in Tris buffer/BSA (3 changes, 15 minutes each). The slide-mounted tissue was then rinsed in distilled water, fixed in 3% paraformaldehyde (15 minutes) to inactivate unbound toxin and rinsed again. When the tissue was thoroughly dry, the slides were arranged in a light-tight X-ray cassette and a sheet of LKB Ul trofilm was placed over them (emulsion side down). The cassette was then clamped shut, compressing the film onto the tissue sections. After 2-5 days exposure, the film was developed with Kodak D-19. The autoradiographic images, essentially negative images of the tissue, were cut out and placed in a photographic enlarger for printing. The tissue sections were stained with 0.2% safranin and compared with the autoradiograms.

Symbols: c, cerebral cortex; cc, corpus callosum; cpu, caudate putamen; na, nucleus accumbens; ac, anterior commissure.

a. Localisation of $^{125}\text{I}$-BoNT type A sites in the cortex (c) and caudate putamen (cpu) of the forebrain. Note the absence of silver grains in the corpus callosum (cc) and anterior commissures (ac) (myelin tracts).

b. Control.
Fig. 6.2 Specific binding of $^{125}$I-BoNT type A in the rat hippocampal formation.

Cryostat sections of the rat hippocampus were treated with $^{125}$I-BoNT type A as described in Fig. 6.1 and prepared for autoradiography. After exposure and development, prints were made of the autoradiographic images produced. The tissue sections were stained histologically, placed in the enlarger and printed; diagrams of the different cell layers were then prepared.

Symbols:  
c, cerebral cortex;  
cI, lamina I of the cerebral cortex;  
cc, corpus callosum;  
h, hippocampus;  
hf, hippocampal fissure;  
or, stratum oriens;  
p, stratum pyramidale;  
r, stratum radiatum;  
1m, stratum lacunosum-moleculare;  
dg, dentate gyrus;  
g, granular layer of the dentate gyrus;  
m, molecular layer of the dentate gyrus;  
f, fimbria;  
a, alveus;  
s, subiculum;  
lT, lamina terminalis;  
CA1, field of CA1 of Ammon's horn;  
CA2, field CA2 of Ammon's horn;  
CA3 field CA3 of Ammon's horn;  
CA4, field CA4 of Ammon's horn or hilus of the dentate gyrus;  
th, thalamic nuclei;  
mhb, medial habenular nucleus;  
zi, zona incerta;  
am, amygdala;  
lh, lateral hypothalamic area;  
IB1, inner blade of the dentate gyrus;  
OBI, outer blade of the dentate gyrus;  
IPN, interpeduncular nucleus.

a. Diagram of the medial aspect of the cerebral hemisphere in rodents (hippocampus shown in black) (adapted from Sarnat and Netsky, 1974).  
b, c, d, e correspond approximately to areas in the autoradiograms.

b. Localisation of $^{125}$I-BoNT type A binding sites in the hippocampus (h), medial habenular nucleus (mhb), lamina I of the cerebral cortex (cI), lateral hypothalamus (lh) and zona incerta (zi).

b.1 Schematic drawing of the hippocampal formation.
c. Acceptor sites for $^{125}$I-BoNT type A in the hippocampus. Most of the label is seen over the stratum oriens (or) especially in CA2 and CA3 (arrow). The molecular layer of the dentate gyrus is also labelled and, to a lesser extent, the stratum radiatum. The hilus of the dentate gyrus shows some labelling but the pyramidal (p) and granule cell (g) layers are totally devoid of silver grains. Note the labelling in lamina I of the cerebral cortex (cl), the zona incerta (zi), the amygdala (am) and in the medial habenular nuclei (mhb). No silver grains were detected in the fimbria (fi).

d. Schematic drawing of the different layers of the hippocampus for comparison with the autoradiograph.

d.1 Schematic drawing of the posterior hippocampal formation.

e. Most of the acceptor sites for $^{125}$I-BoNT type A in this area of the forebrain are found in the stratum oriens of the hippocampus (or).

e.1 Schematic drawing of the hippocampus (posterior areas).

f. Control specimen showing saturability of binding of $^{125}$I-BoNT type A in the hippocampus.
hippocampal layers was saturable as controls showed few silver grains (Fig. 6.2f).

Pockets of acceptor sites were demonstrated in the medial habenular nuclei (Figs. 6.2b,c), the thalamic nuclei (Fig. 6.2b), the zona incerta and the amygdala (Fig. 6.2b,c,d).

High affinity acceptor sites for $^{125}$I-BoNT types A and B were also found in the cerebellum. The cerebellar cortex displays a uniform and simple structure (Palay and Chan-Palay, 1974). It consists of a highly folded sheet of cells and their processes which are arranged into three layers: the granular layer (which derives its name from the presence in it of numerous small nerve cells, which gave it a uniform granular texture in early preparations), the molecular layer (which is rich in synapses, dendrites and unmyelinated axons), and the Purkinje cell layer (one cell thick) which divides the two. Each trilaminar folium (Fig. 6.3a) encloses a thin lamella of white matter composed of the myelinated afferent and efferent fibres connecting the cortex with other centres of the central nervous system. Both radio-labelled toxin types were found to bind saturably to the molecular layer of the cerebellum and, although to a much lesser extent, to the granule cell layer (Figs. 6.3b,c,d,e). Myelinated areas, however, were almost devoid of silver grains.

6.3.2 Ultrastructural localisation of $^{125}$I-BoNT type A in slices of rat cerebellum.

Using light-microscope autoradiography, it was shown that $^{125}$I-BoNT types A and B bind saturably to the molecular layer of the cerebellum and, to a lesser extent, to the granule cell layer, but do not interact with myelin (Section 6.3.1). In order to localise the acceptor sites and establish whether bound toxin molecules are taken up into the
Fig. 6.3 Binding of $^{125}\text{I-BoNT}$ types A and B to areas of the rat cerebellum.  

Cryostat sections of rat cerebellum were treated with $^{125}\text{I-BoNT}$ type A or type B and processed for autoradiography using $^3\text{H-Ultrofilm}$ as described in Figs. 6.1 and 6.2.

Symbols: ce, cerebellum; g, granule cell layer of the cerebellum; m, molecular layer of the cerebellum; w, white matter of the cerebellum.

a. Diagrammatic representation of the cell layers in the cerebellum.

b. Localisation of acceptor sites for $^{125}\text{I-BoNT}$ type A in the cerebellum. Note heavy labelling of the molecule layer (m), slight labelling of the granule cell layer (g) and absence of silver grains in white matter (w).

c. Control specimen indicating the saturability of binding of $^{125}\text{I-BoNT}$ type A to acceptor sites in the cerebellum.

d. Localisation of acceptor sites for $^{125}\text{I-BoNT}$ type B in the cerebellum. Silver grains are detected mainly over the molecular layer (m); some label is present on the granule cell layer (g) but white matter (w) is totally devoid of silver grains as seen with $^{125}\text{I-BoNT}$ type A.

e. Control section showing saturability of binding of $^{125}\text{I-BoNT}$ type B to the cerebellum.
nerve terminal cytoplasm of central neurons (as occurs at the motor nerve terminal in the periphery) ultrastructural studies using unfixed tissue were carried out (using $^{125}$I-BoNT type A). Brain tissue is very difficult to preserve; the cells readily become anoxic and the intricate network of neuronal processes is easily destroyed when the tissue is removed from the animal and incubated in vitro. Thus, some of the sections processed for autoradiography showed poor tissue preservation. Interestingly, labelling was only observed in tissue which had been damaged during incubation and washes. It seemed that the toxin had been unable to penetrate intact tissue (no silver grains were seen in these areas) and only reached its target sites when the close-knit network of cells had been disrupted. The molecular layer of the cerebellum consists mainly of nerve endings, dendrites and unmyelinated axons (Figs. 6.4, 6.5a). Labelling of nerve endings was observed in test sections (Fig. 6.5a) but controls treated with excess native neurotoxin showed only a few randomly distributed silver grains (data not shown). Not all endings were labelled (Fig. 6.5a), but observed silver grains were almost exclusively associated with these structures. Although most of the silver grains were located on the membrane of the terminals, the resolution of the autoradiographic technique does not allow the possibility of internalisation to be ruled out.

Labelling was also observed in the granule cell layer. Silver grains were detected on the endings of mossy fibres and possibly on vesicle-containing varicosities of golgi axons (Fig. 6.5bl). No grains were seen associated with these structures in control preparations treated with excess BoNT type A indicating that the binding is saturable (Fig. 6.3b2). Very little internalised radioactivity could be detected at these terminals relative to that observed at the motor nerve terminal; however, this could be due to cell death rather than absence of a specific uptake mechanism for toxin molecules in central neurons.
Fig. 6.4 The neurons of the cerebellar cortex.

Beginning from the left, the boundaries and layers of the cortex are indicated: the pial surface, molecular layer, Purkinje cell layer, granular layer, and white matter. Four granule cells (g) are shown with their ascending axons bifurcating like a T. The resultant branches travel parallel with the long axis of the folium in the transverse plane. Two Purkinje cells are shown in the sagittal plane (black, PC, and grey). A basket cell (B) with its axon running in the sagittal plane gives off collaterals to the Purkinje cell body. A stellate cell (S) and a Lugaro cell (L) are also shown. The second Purkinje cell (grey) is shown with the terminal arborization of a single climbing fibre (CF). The Golgi cell (GC) occupying the corner position in this slab of cortex has a three-dimensional axonal and dendritic plexus. The axons of the Golgi cell participate with the mossy fibre (MF, red) and the dendrites of granule cells in the construction of glomeruli. Three neurons, a Purkinje cell (PC), a basket cell (B) and a stellate cell (S) display their characteristic profiles in the transverse plane. The axons of two Purkinje cells are shown extending to the cerebellar nuclei (From Palay and Chan-Palay, 1974).
Fig. 6.5 Localisation of $^{125}\text{I-BoNT}$ type A binding in the cerebellum.

Brain slices (400µm) thick were prepared as described in Chapter 2 (Section 2.9.1) and incubated with 30nM $^{125}\text{I-BoNT}$ for 60 minutes at 37°C. Control slices were treated similarly with the addition of excess unlabelled toxin to the incubation medium.

a. Binding of $^{125}\text{I-BoNT}$ to the molecular layer of the cerebellum. Note that silver grains are always located on or near the membrane of nerve terminals (identified by the presence of synaptic vesicles) (arrowheads).

b. Binding of $^{125}\text{I-BoNT}$ to the granular layer of the cerebellum.
1. Test specimen showing a labelled mossy fibre terminal (mf) and two Golgi axons (g). Note absence of grains on myelin (m). Mossy fibre terminals can be identified as large structures containing numerous mitochondria and synaptic vesicles. Golgi axons abut against the mossy fibre endings.

2. Control sample.
Silver grains were absent from myelin and the cell bodies of granule cells (Fig. 6.5b).

Collectively, these results suggest that $^{125}$I-BoNT type A binds selectively and saturably to nerve terminals in the molecular and granule cell layers of the cerebellum and that uptake of toxin appears minimal at these terminals.

6.3.3 Binding of $^{125}$I-BoNT type A to rat cerebrocortical synaptosomes.

Specific labelling of isolated nerve terminals from rat cerebral cortex was observed both in the presence and absence of sodium azide (15mM) (Figs. 6.6, 6.7). This suggests that, as shown by light-microscope autoradiography (Section 6.3.1.2), acceptors for $^{125}$I-BoNT type A are present in the cerebral cortex. In sections treated with either 1nM or 10nM toxin in the absence of azide, silver grains were associated almost exclusively with the nerve terminal membrane and were not restricted to areas opposite the postsynaptic densities (Figs. 6.6a, 6.7a). However, owing to the limits in resolution of the technique, it cannot be said that the toxin is not taken up into the nerve endings. In the absence of metabolic inhibitors, silver grains were sometimes (although very rarely) associated with cytoplasmic vacuolar structures. There was considerably more labelling at 10nM $^{125}$I-BoNT than at 1nM indicating that all the acceptor sites had not been occupied at the lower concentration, as shown previously by kinetic methods (Williams et al., 1983).

Part B: The Peripheral Nervous System.

6.3.4 Cholinergic nerve terminals in non-mammalian tissue.

The electric organ of Electrophorus electricus is an anatomical
Fig. 6.6. Interaction of $^{125}$I-BoNT type A with rat brain synaptosomes.

Synaptosomes, prepared by Ficoll density centrifugation as described in chapter 2, were treated with 1nM or 10nM $^{125}$I-BoNT for 40 minutes at room temperature and processed for autoradiography.

a) Treated with 1nM $^{125}$I-BoNT type A. Note the silver grains on the plasma membrane which are not restricted to areas opposite postsynaptic densities.

b) Treated with 10nM $^{125}$I-BoNT type A. Note increased levels of binding compared with (a) and the presence of some (although rare) internalised silver grains.

c) Treated with 10nM $^{125}$I-BoNT type A in the presence of 500nM native BoNT.
Fig. 6.7. Interaction of $^{125}$I-BoNT type A with rat brain synaptosomes in the presence of azide.

Rat cerebrocortical synaptosomes, prepared by Ficoll density centrifugation as described in Chapter 2, were treated with 1nM $^{125}$I-BoNT type A in the presence of 15mM sodium azide (40 minutes at 22$^\circ$C).

a) Treated with 1nM $^{125}$I-BoNT type A. Note silver grains on the plasma membrane not restricted to areas opposite the postsynaptic densities.

b) Treated with 10nM $^{125}$I-BoNT type A. Note the increased binding with the same pattern as in (a).
analogue of the neuromuscular junction; it is innervated by cholinergic neurons which form a number of contacts along the caudal face of the electroplaques. Acetylcholine receptors are observed on the innervated membrane both between the synapses and under the terminals, but the density of sites is approximately 100 times higher at the synapse than in extra-junctional areas (Bourgeois et al., 1978). An early study by Ambache and Ferreira (1952) showed that injection of botulinum toxin blocks the electrical discharge of *Electrophorus*. Despite the similarities between the mammalian neuromuscular junction and the electroplaque, no specific binding of $^{125}$I-BoNT type A to the nerve terminal, or to any other area of the electroplaque, was detectable even at high toxin concentrations (30nM) (Fig. 6.8). Test and control terminals were completely devoid of grains. Low levels of background were present in the sections, indicating that barriers to toxin penetration of the tissue do not account for the absence of silver grains at nerve terminals. Ambache and Ferreria used toxin- haemagglutinin complex in their study; a re-examination of their results using purified neurotoxin is necessary before any conclusions can be drawn regarding binding and the action of the toxin in the electric organ. The apparent lack of a high affinity acceptor site therein suggests that effects observed were the result of a non-specific interaction of the toxin complex with the organ or the nerves that activate it. The possibility that BoNT binds to a low affinity site in this tissue, and is then washed out, cannot be excluded.

Several investigators have shown that botulinum toxin type A interacts with cholinergic nerve terminals in the frog and causes progressive blockade of neuromuscular transmission (Boroff et al., 1974; Pumplin and del Castillo, 1975; Pumplin and Reese, 1977; Gundersen et al., 1981). The toxin's effects have been studied using freeze-fracture techniques and transmission electron microscopy (Pumplin and Reese,
Fig. 6.8 Interaction of $^{125}$I-BoNT type A with electric organ of *Electrophorus electricus*.

Single electroplaques were dissected from Sachs' organ and pre-incubated in eel Ringer. Test cells were incubated with 30mM $^{125}$I-BoNT type A in 0.5ml physiological Ringer for 2.5 hours at 22°C with occasional agitation. They were then washed extensively (5 times, 6 minutes each) in eel Ringer at 4°C, fixed for 2 hours in 2% glutaraldehyde, and processed for electron-microscope autoradiography. No specific labelling was detectable in any area of the sections, including the cholinergic nerve terminals that innervate the organ (see arrow).
1977; Hirokawa and Heuser, 1981). However, localisation of acceptor sites by histological or autoradiographic techniques has not been reported. In an attempt to visualise the interaction of BoNT type A with cholinergic nerve terminals in the frog, cutaneous pectoris muscles (known to be paralysed by botulinum toxin type A; Pumpul and Reese, 1977) were exposed to labelled neurotoxin and processed for electron-microscope autoradiography. Silver grains were detected at nerve terminals after three weeks exposure (Fig. 6.9a), although the extent of labelling was less pronounced than that observed at murine motor nerve endings using equivalent toxin concentrations. Unmyelinated terminal areas were also labelled (data not shown). Binding was saturable, as control terminals incubated with excess native BoNT type A were devoid of silver grains (Fig. 6.9b). It was also specific for nerve terminals as no grains were detectable on muscle fibres, Schwann cells, myelin, collagen or blood vessels. The pattern of labelling was similar to that seen at mammalian nerve terminals: grains were found on the plasma membrane and also within the cytosol (Fig. 6.9a). It is interesting that, in the freeze-fracture studies carried out by Pumpul and Reese (1977), $3 \times 10^6$ MLD$_{50}$ of crystalline type A toxin injected in the dorsal lymph sac were required to produce paralysis within 18-24 hours, whereas a mouse would be killed in the same time with about 5MLD$_{50}$ and $3 \times 10^6$ MLD$_{50}$ would cause death within 2 hours or less (unpublished observations). The apparent lower sensitivity of frogs to BoNT type A could possibly be explained by the presence of fewer acceptors on frog neuronal membranes.

6.3.5 Adrenergic, cholinergic muscarinic and nonadrenergic noncholinergic nerve terminals.

The time course of blockade of transmission from postganglionic nerves to smooth muscle (in isolated preparations of mouse vas deferens)
Fig. 6.9 Binding and internalisation of $^{125}$I-BoNT type A at the frog neuromuscular junction.

Cutaneous pectoris tissue from a frog was incubated with 15nM $^{125}$I-BoNT type A in 0.5ml frog Ringer solution for 90 minutes at 22°C prior to extensive washing for 30 minutes. The tissue was then fixed and end-plate regions were located visually, dissected out and processed for electron-microscope autoradiography.

a. Nerve terminal labelled with $^{125}$I-BoNT type A. The pattern of labelling is similar to that seen at the mammalian motor nerve terminal; i.e. silver grains are seen on the presynaptic membrane and in the terminal cytoplasm.

b. Control specimen treated as described above with the addition of a 100-fold excess of native neurotoxin. The absence of silver grains indicates saturability of the acceptor sites.
was 2 to 6 times slower than that observed with the same concentration of the same batch of toxin complex (type A) on the rat diaphragm (Holman and Spitzer, 1973). The vas deferens is rich in adrenergic innervation and is, therefore, an ideal model for studies on the binding of BoNT to adrenergic nerve terminals. In an attempt to explain why junctions in the vas deferens are so resistant to blockade compared with the skeletal neuromuscular junction, small pieces of the tissue were exposed to relatively high concentrations of $^{125}$I-BoNT type A in the presence and absence of native (A) BoNT. After 3-6 weeks exposure, no silver grains were detectable at adrenergic nerve terminals (identified by the presence of granular vesicles) or elsewhere in the preparation (Fig. 6.10). Control samples were also devoid of grains (data not shown). It is unlikely that the toxin was unable to penetrate the tissue as the thick serosal coat that protects it was removed prior to incubation with toxin. This result suggests that the acceptor for BoNT type A is absent from adrenergic nerve terminals or present in such small amounts as to be undetectable by the autoradiographic technique. Both these possibilities may account for the resistance of adrenergic nerve terminals to the effects of BoNT.

Pharmacological studies clearly show that several neurotransmitters are released from intramural neurons of the gut. In addition to adrenalin and acetylcholine, other substances which may play the role of neurotransmitters in the ileum include serotonin, adenosine triphosphate (ATP), and several peptides such as substance P, vasoactive intestinal peptide, somatostatin and enkephalin (Gabella, 1981). Nerve terminals containing the different transmitters can be identified morphologically. Cholinergic endings are (Fig. 6.11c) recognised in the electron microscope as varicosities containing predominantly small agranular vesicles (about 50nm in diameter) and an occasional larger vesicle (80 to 110nm) with a granule of medium electron density. Adrenergic nerve endings can
Fig. 6.10 Binding of $^{125}$I-BoNT type A to adrenergic nerve terminals in the vas deferens of the mouse.

Tissue from the mouse vas deferens was incubated with 22nM $^{125}$I-BoNT type A in 0.5ml Krebs Ringer solution for 90 minutes at 22°C. It was then washed for 30 minutes, fixed with 3% glutaraldehyde/2% paraformaldehyde and processed for electron-microscope autoradiography. Adrenergic nerve terminals were identified by the presence of dense core vesicles in the cytoplasm (arrow). No silver grains were observed in any area of the sections, including the adrenergic endings, which suggests that the latter do not have membrane acceptors for $^{125}$I-BoNT type A.
Fig. 6.11 Interaction of $^{125}$I-BoNT type A with nerve endings in the mouse ileum.

A small segment of mouse ileum, tied at one end with cotton, was incubated with 20nM $^{125}$I-BoNT type A in 0.5ml Krebs Ringer for 90 minutes at 22°C. It was then washed with Krebs Ringer for 30 minutes, the open end was tied, and fixative (3% glutaraldehyde/2% paraformaldehyde) was carefully injected into the lumen. The tissue was immersed in fixative for 30 minutes when it was cut into rings and fixed for a further 60 minutes. The tissue rings were then washed and processed for electron-microscope autoradiography. Several different nerve endings were seen in the sections: peptidergic (p), purinergic (Pu), adrenergic, and cholinergic (c). Only cholinergic endings and some axons were labelled with $^{125}$I-BoNT type A.

a. Autoradiogram showing two labelled cholinergic endings (c) and an unlabelled 'p' type ending. Note the small agranular vesicles in (c) profiles (arrow) and large granular vesicles in (p) endings (arrowhead). $x_{24100}$.

b. No silver grains are detectable on purinergic (pu) varicosities (see Uehara et al. 1976 for comparison) but possible cholinergic terminals are labelled. $x_{30200}$.

c. Transverse section through a cholinergic nerve terminal (c) containing many small agranular vesicles. Note the internalised silver grains. $x_{43000}$.

d. Nerve axons (ax) (shown here in transverse section) are also sometimes labelled. $x_{24500}$. 
be identified as nerve profiles with a predominance of granular vesicles (40 to 60nm in diameter). Nonadrenergic-noncholinergic (purinergic) inhibitory neurons have endings that mainly display large vesicles (80 to 200nm in diameter) with an electron-dense granule separated from the vesicle membrane by an electron-lucent halo (large opaque vesicles) (Fig. 6.11b). The inhibitory transmitter in these neurons is thought to be ATP (Burnstock et al., 1970; Crowe, 1980). Endings containing a few agranular vesicles and a number of larger vesicles (85 to 160nm in diameter) with a large granule of medium electron-density are labelled p-type varicosities, where 'p' refers to polypeptide (Fig. 6.11a). It must be emphasised that the classification and identification of nerve endings in the electron microscope on the basis of the size, shape, electron density and distribution of the synaptic vesicles is an uncertain one. In a more elaborate classification, eight or more different endings were identified morphologically in the myenteric plexus of the guinea pig (Cook and Burnstock, 1976). It is thought that more than one transmitter may co-exist in some nerve endings. There are also endings with flattened vesicles and varicosities in which, in addition to the predominantly agranular vesicles (especially in the proximity of the prejunctional membrane), there is a substantial number of large granular vesicles. Adrenergic endings are often difficult to identify as the dense core vesicles are not easily preserved; when they are not present, an adrenergic ending may look similar to a cholinergic one. For a review on the innervation of the gut, see Gabella (1981).

Keeping in mind the difficulties in identifying the nerve endings in sections of mouse ileum, a number of conclusions can be drawn regarding the interaction of $^{125}\text{I-BoNT}$ type A with nerve terminals containing different neurotransmitters. The toxin does not bind to nonadrenergic-noncholinergic (purinergic) nerve endings (Fig. 6.11b) or to peptidergic endings (Fig. 6.11a); silver grains were never found
associated with these structures. Varicosities which could be identified as adrenergic were also always unlabelled (data not shown, see Fig. 6.10) as were smooth muscle cells, glial processes, neuron cell bodies, collagen fibrils and most of the less easily identifiable nerve endings and axons in the myenteric and sub-mucosal plexuses (Fig. 6.11). However, silver grains were often seen associated with nerve terminals characterised by small agranular vesicles and perhaps a single larger granular vesicle which are thought to be cholinergic (Figs. 6.11a,b,c). In a few cases, axons were found to be labelled (Fig. 6.11d). The silver grains at cholinergic terminals were located on the plasma membrane (Figs. 6.11a,b) and also in the cytoplasm (Figs. 6.11a,b,c) suggesting that the toxin is internalised therein. The acetylcholine receptors in the gastrointestinal tract are muscarinic; it appears, therefore, that BoNT type A interacts with nicotinic and muscarinic cholinergic synapses. Control sections were totally devoid of silver grains indicating that the binding to cholinergic synapses was saturable.

6.3.6 Binding of $^{125}$I-BoNT type A to neuroblastoma and pheochromocytoma (PC12) cell lines.

No specific labelling of the neuroblastoma cell lines CHP100, MR32, NB212, Lan 1, or TR14 (thought to be cholinergic; Tom Rupniak, personal communication) was observed. Likewise, PC12 cells incubated with $^{125}$I-BoNT type A and processed for electron-microscope autoradiography, were totally devoid of silver grains (data not shown). The acceptor(s) for $^{125}$I-BoNT type A appears to be absent from these cells; the membrane component may have been lost when the cells became malignant, or the cell lines may have been derived from cells that did not possess the sites (e.g. non-cholinergic nerves). High concentrations (100mM-1µM) of toxin were required to cause inhibition of
transmitter (ACh) release in PC12 cells (A. Ashton, unpublished results), emphasizing the importance of the acceptor for efficient delivery of the toxin to its target site in the cytosol. The effect observed can probably be attributed to inefficient entry by a non-specific mechanism.

6.4 DISCUSSION

6.4.1 BoNT as a marker for cholinergic nerve terminals.

Acceptors for $^{125}$I-BoNT type A (which are unaffected by fixation with paraformaldehyde) were observed autoradiographically in several areas of rat brain, particularly in synaptic regions of the hippocampus and cerebellum. Although botulinum toxin is thought to affect primarily cholinergic nerve terminals in the peripheral nervous system (see Simpson, 1981), evidence for such selectivity in the brain is inconclusive. Relatively high toxin concentrations (compared with those required for toxicity at the neuromuscular junction) have been shown to cause inhibition of ACh release from rat brain synaptosomes (Wonnacott, 1980; Dolly et al., 1981; Habermann, 1981). Release of other transmitters (i.e. nonadrenaline, glycine, $\gamma$-aminobutyric acid) was shown not to be affected (Wonnacott and Marchbanks, 1976) or to be affected with considerably lower efficiency relative to the effect on release of ACh (Dolly et al., 1981; Haberman, 1981). Autoradiographic localisation studies described in this Chapter show a striking correlation between areas of high grain density and areas of cholinergic innervation (for review see Fibiger, 1982) (although it must be stressed that other neurotransmitters are also found in these regions). Until recently, the distribution of central cholinergic systems was essentially unknown due to the absence of unequivocal anatomical methods for identifying central cholinergic neurons and their projections. Early studies (Shute and
Lewis, 1965, 1967; Lewis and Shute, 1967) were carried out using acetylcholinesterase histochemistry. Subsequently, it was shown that acetylcholinesterase is contained in axons and terminals of neurons that are not cholinergic (Lehmann and Fibiger, 1978; Kan et al., 1980) and is therefore unreliable as a marker for central cholinergic pathways. Recently, improved methods for identification of cholinergic neurons have been developed; these include (1) successful purification of choline acetyltransferase (ChAT) (generally acknowledged as a reliable marker for cholinergic neurons) with subsequent production of antibodies to ChAT for use in immunochemistry (Kimura et al., 1980) and (2) autoradiographic identification of nicotinic and muscarinic ACh receptors using labelled ligands, i.e. α-bungarotoxin (Hunt and Schmidt, 1978), propylbenzylcholine mustard (Rotter et al., 1979) and 5-quinuclidinyl benzilate (QNB) (Kuhar and Yamamura, 1976).

Using these methods, it has been shown that important afferents of the hippocampal formation are cholinergic (nicotinic or muscarinic) in nature (Kuhar and Yamamura, 1976; Hunt and Schmidt, 1978). The source of this innervation is a group of AChE-intense neurons in the medial septal nucleus and adjacent vertical limb of the diagonal band (Daltz and Powell, 1954). The fibres pass through the fimbria and dorsal fornix and project to several regions of the hippocampus; particularly to fields CA2 and CA3 and to the hilar region of the dentate gyrus. In fields CA2 and CA3, the stratum oriens appears to receive a particularly dense innervation, whereas the stratum radiatum shows somewhat less, although still significant, input from these fibres (Rose et al., 1976). The molecular layer and hilus of the dentate gyrus also have relatively high levels of cholinergic innervation (higher than the stratum radiatum) (Mellgren and Srebro, 1973). Autoradiograms of the binding of $^{125}$I-BoNT type A showed high grain density over the stratum oriens (especially in fields CA2 and CA3) and also, although to a slightly
observed in the molecular layer and hilar region of the dentate gyrus. The distribution of the silver grains in the hippocampus, therefore, corresponds closely to areas of dense cholinergic innervation. It has been shown that muscarinic ACh receptors are present in similar amounts both in the stratum oriens and stratum radiatum (Kuhar and Yamamura, 1976; Rotter et al., 1979) of the hippocampus proper and in the molecular layer of the dentate gyrus, with about half as many in the hilar region. Cholinergic nicotinic receptors, however, are found mainly in the stratum oriens and hilar region, but not in the stratum radiatum or molecular layer (Hunt and Schmidt, 1978). Pharmacological studies of the toxin's action in the peripheral nervous system (Habermann et al., 1980; Simpson, 1981) combined with ultrastructural localisation of radiolabelled derivatives (described in this Chapter) suggest that the toxin causes inhibition of acetylcholine release at both muscarinic and nicotinic synapses. It is likely, therefore, that 125I-BoNT type A interacts with muscarinic and nicotinic synapses in the central nervous system as well and this may account, in part, for the higher grain density observed in the stratum oriens (where both types of ACh receptors are present in significant amounts). The distribution of silver grains in the hippocampal formation may, therefore, also reflect the distribution of both types of ACh synapses therein.

Other areas of the forebrain labelled by BoNT type A include the cerebral cortex, the caudate putamen, the zona incerta of the subthalamus, the medial habenular nucleus, the thalamic nuclei and the amygdala. It is thought that the major cholinergic projection to the cortex has as its source a group of magnocellular neurons in the basal forebrain (Shute and Lewis, 1967); however, the distribution of the cholinergic terminals in the six laminae of the cortex is not well
understood (Fibiger, 1982). The only generalisations that can be made are that all the layers consist of at least some cholinergic activity and that lamina I has high levels of ChAT activity and α-bungarotoxin binding. Acceptor sites for $^{125}$I-BoNT type A, demonstrated autoradiographically, appeared uniformly distributed over the cortical regions, although in some cases higher grain density was observed over lamina I. The fact that labelling was observed in the cerebral cortex is consistent with biochemical (Williams et al., 1983) and ultrastructural (Hirokawa and Kitamura, 1979; data described in this Chapter) studies which showed that high affinity acceptor sites ($K_D$ 0.6nM) for $^{125}$I-BoNT are present on the membrane of rat cerebrocortical synaptosomes.

The caudate putamen shows high levels of intrinsic cholinergic activity (Fibiger, 1982) possibly found in interneurons. Although this area contains few nicotinic ACh receptors (Hunt and Schmidt, 1978), it contains high levels of muscarinic receptors (Kuhar and Yamamura, 1976) and of ChAT, AChE and ACh activities. Studies of the distribution of muscarinic receptors in the caudate putamen by autoradiographic techniques (Kuhar and Yamamura, 1976) showed regional variation in density of the label ($[^3H]$-QNB), with many areas almost totally devoid of silver grains. These regions appeared to contain myelinated fibres of the internal capsule. Acceptors for $^{125}$I-BoNT type A in the caudate putamen also showed this patchy distribution (Fig. 6.1A1), suggesting the possibility that the toxin interacts with muscarinic synapses in this area. Kuhar and Yamamura (1976) also observed relatively dense labelling by $[^3H]$-QNB of the nucleus accumbens, another region labelled by BoNT (Fig. 6.1).

The zona incerta contains significant levels of muscarinic and nicotinic ACh receptors (Rotter et al., 1979; Hunt and Schmidt, 1978) and, accordingly, this area showed relatively high grain density in
autoradiograms of brain sections treated with $^{125}$I-BoNT. Finally the medial habenular nucleus, the amygdala and the thalamic nuclei, all of which contain high levels of ChAT activity (Fibiger, 1982), were labelled by the toxin. However, the interpeduncular nucleus which contains significant levels of cholinergic activity, showed relatively sparse deposition of specific silver grains.

Taking into account the limits in resolution of the autoradiographic technique used and accuracy in the identification of labelled areas, these results collectively suggest that the distribution of high affinity acceptors for $^{125}$I-BoNT type A in the forebrain of the rat corresponds closely with the distribution of cholinergic nerve terminals therein. This, however, does not preclude binding to nerve terminals containing other neurotransmitters (see section on the cerebellum, below). It must be noted, however, that the distribution of such transmitters does not coincide as closely with BoNT binding. For example, glutamate and aspartate occur mainly in the stratum radiatum of the hippocampus (rather than the stratum oriens) and ε-aminobutyric acid is found preferentially in the granule and pyramidal cell layers (Storm-Mathisen, 1981) with low levels occurring in synaptic regions. In addition, the pattern of labelling of hippocampal layers seen autoradiographically with other presynaptic neurotoxins, i.e. B-bungarotoxin and dendrotoxin, is not identical to that seen with BoNT. Neither of these toxins interacts selectively with cholinergic neurons; accordingly, B-bungarotoxin binds preferentially to the molecular layer of the dentate gyrus while other synaptic regions of the hippocampal formation are uniformly labelled by the toxin (Othman et al., 1983) and dendrotoxin shows a similar pattern with binding to myelinated areas as well (A. Pelchen-Matthews, unpublished results).

The possible selectivity of toxin binding in the forebrain is not observed in the cerebellum. To date, cholinergic activity has been
found only in the granule cell layer, exclusively in the mossy fibres (Kan et al., 1978; 1980; Woodward et al., 1982). It has been suggested that the Golgi cell, an interneuron, might also be cholinergic (Kasa and Silver, 1969) but the evidence is inconclusive. Although ultrastructural autoradiographic studies showed deposition of silver grains on mossy fibre terminals and Golgi cell axons, studies at the light and electron-microscope level showed higher grain densities in the molecular layer (where nerve terminals were preferentially labelled). This suggests that, unless cholinergic innervation of this area exists but is yet to be identified, acceptors for $^{125}\text{I}}$-BoNT types A and B are also found on nerve terminals containing different neurotransmitters (at least in the cerebellum).

The usefulness of BoNT as a marker for cholinergic nerve terminals in the central nervous system is, therefore, yet to be ascertained conclusively. In the peripheral nervous system, however, the situation is less complex. Specific acceptors can be detected with radiolabelled BoNT at nicotinic (i.e. neuromuscular junction) and muscarinic cholinergic synapses (in smooth muscle), and these are known to be important sites of action for the toxin (Burgen et al., 1949; Simpson, 1981; Habermann et al., 1980; MacKenzie et al., 1982). Adrenergic and inhibitory nonadrenergic-noncholinergic nerve endings, however, are not labelled by the toxin. Absence of acceptors at these terminals is consistent with results obtained pharmacologically; adrenergic nerve terminals in the vas deferens (Holman and Spitzer, 1973) and anococcygeus muscle (MacKenzie et al., 1982) are only affected by high toxin concentrations (suggesting a non-specific mechanism of uptake, see below and Chapter 7) and inhibitory nonadrenergic-noncholinergic transmission in the guinea-pig taenia coli is not inhibited by the toxin.

Botulinum toxin is also known to affect cholinergic transmission in non-mammalian tissue. Accordingly, specific acceptors have been
located for $^{125}$I-BoNT type A at the motor nerve terminal in frog cutaneous pectoris muscles (see Section 6.3.4). However, no acceptors were detected at cholinergic endings in Electrophorus electric organ. The effects seen in an early study (Ambache and Ferreria, 1952) could be attributed to non-specific interactions with the tissue of the haemagglutinin moiety of the toxin, or of contaminants in the crude preparation used. Since this early report, no further evidence for the action of the toxin in electric tissue has been obtained (Simpson, unpublished results) and attempts in this laboratory to detect an acceptor using biochemical methods have been unsuccessful (R. Williams and A. Ashton, unpublished results). These findings suggest that acceptors for BoNT may not be present at cholinergic nerve terminals of all species.

6.4.2 Why is BoNT ineffective at certain synapses?

Results described in previous chapters and elsewhere (see Simpson, 1981) suggest that effective toxin-induced blockade of neurotransmission at a particular synapse depends on the presence therein of two relevant cellular features: the first of these is the existence of specific acceptors for the toxin on the nerve terminal membrane which serve to concentrate toxin molecules on the cell surface, and the second is the presence of a specific uptake mechanism for delivery of toxin molecules to the cytosol in a biologically active form. Nerve terminals in the central nervous system possess high affinity membrane acceptors for the toxin as shown biochemically (Williams et al., 1983), immunohisto- logically (Hirokawa and Kitamura, 1979) and autoradiographically. There is some evidence to suggest, however, that the specific uptake mechanism required to allow the toxin access to the cytoplasm is absent from these endings (see Tse, 1981; Williams, 1984, section on subfractionation studies which show that the toxin is found mainly in the membrane
fraction; also autoradiographic studies presented herein). High toxin concentrations in the incubation medium may lead to entry of a few molecules by a non-specific mechanism; this could account for the observed inhibition of ACh release from rat brain synaptosomes (Wonnacott, 1980; Dolly et al., 1981). Intraventricular injections of BoNT type A carried out in this laboratory probably failed to induce toxicity because the toxin was unable to penetrate the fine network of cell processes in brain tissue in sufficient amounts to enter target cells by a non-specific pathway.

Adrenergic and inhibitory nonadrenergic-noncholinergic terminals in the peripheral nervous system are also only affected (in vitro) if the toxin is applied at high concentrations for prolonged periods of time (Holman and Spitzer, 1973; MacKenzie et al., 1982). These terminals do not possess the acceptor so, again, non-specific and thus inefficient mechanisms must be involved. Central nerve terminals containing different neurotransmitters (e.g. γ-aminobutyric acid, glycine, adrenaline) may also only be affected (Habermann, 1981; Dolly et al., 1981) by the toxin as a result of uptake by an inefficient route.

Finally, although a specific acceptor and uptake mechanism are required for efficient action of the toxin at a nerve terminal, the fact that the toxin is able to affect the release of different neurotransmitters (after somehow overcoming the problems of access) suggests interference with a common target in the release process, probably found in nerve endings of all types.
CHAPTER VII

CONCLUSIONS AND PERSPECTIVES.
Ultrastructural autoradiographic localisation studies have shown conclusively that BoNT types A and B interact specifically and saturably with membrane components of cholinergic nerve terminals in skeletal muscle, and are subsequently internalised by an energy-requiring process. These results are consistent with the neurotoxin's unique potency at the neuromuscular junction, as demonstrated electrophysiologically (Harris and Miledi, 1971; Cull-Candy et al., 1976; Thesleff, 1981). Moreover, they can be reconciled with observations made previously, using indirect methods, that there is a lag between the time when the toxin becomes irreversibly bound to the nerve terminal and the onset of neuromuscular blockade (Burgen et al., 1949), that toxin causing paralysis is inaccessible to antibody (Simpson, 1974), and that nerve stimulation accelerates expression of toxicity (Rand and Whaler, 1962). Although the studies presented herein are in close agreement with the three-phase model of intoxication proposed by Simpson (1980), the question still remains as to whether all, or only a fraction of, the silver grains observed in autoradiograms of nerve terminals treated with 125I-BoNT types A or B [at relatively high concentrations (e.g. 10nM) relative to those required to induce neuromuscular blockade (0.1nM or lower)] represent biologically active and pharmacologically significant molecules. One way to approach this problem is to compare the extent and distribution of label at nerve terminals of different types with reported observations on the toxin's actions at these synapses. Correspondence between autoradiographic data and electrophysiological finding; in vivo and in vitro, would strengthen the proposal that a significant proportion of the silver grains seen at motor nerve terminals represent active toxin molecules.

As mentioned previously, when botulinum toxin is administered to susceptible animals in vivo, its potent effects on the neuromuscular...
junction lead to death as a result of respiratory paralysis, while autonomic cholinergic nerves are largely unaffected (Ambache, 1951a). However, it has been shown that these nerves are also vulnerable to the actions of the toxin, both in vivo, (Ambache, 1949: 1951b) and in vitro (Bigalke and Habermann, 1980; MacKenzie et al., 1982), although not to the same extent as somatic cholinergic nerve endings. In accordance with these observations, autoradiographic studies have shown that 125I-BoNT type A also interacts saturably with cholinergic nerve endings of the autonomic nervous system; silver grains were observed on the membrane and within the cytoplasm of these varicosities. Moreover, the density of label over these structures was appreciably lower than that seen at motor nerve terminals and this could explain the reduced effectiveness of the toxin at these sites. This correspondence between density of label over nerve terminal areas and sensitivity to the neuro-paralytic action of the toxin is also evident at the frog neuromuscular junction. Although frogs are susceptible to the toxin, higher doses are required to cause paralysis in vivo relative to those necessary to kill a mouse (e.g. Pumplin and Reese, 1977) and, accordingly, exposure of nerve-muscle preparations from each species to equivalent concentrations of the same preparation of radiolabeled BoNT type A resulted in considerably higher grain densities over mammalian motor nerve terminals. This finding cannot be attributed to hindered diffusion of the toxin into amphibian tissue as the preparation used for localisation studies (cutaneous pectoris muscle) is even thinner than mouse diaphragm muscle.

Pharmacological studies have indicated that adrenergic nerve terminals (Holman and Spitzer, 1973; MacKenzie et al.; 1982) and inhibitory nonadrenergic-noncholinergic endings (Paul and Cook, 1980; MacKenzie et al.; 1982) are highly resistant to the actions of the toxin-haemagglutinin complex and the purified neurotoxin. No acceptors
were detected in autoradiograms of these terminals treated with $^{125}$I-BoNT type A, even at high concentrations (see Chapter 6); these results are therefore consistent with reported pharmacological data. Autoradiographic data could also be reconciled with biological activity when the toxin was localised in preparations stimulated electrically until complete neuromuscular blockade was observed. Nerve stimulation is known to accelerate the onset of paralysis and, accordingly, the proportion of silver grains seen within the nerve terminals was considerably greater than in unstimulated controls (see Chapter 3). More internalised toxin molecules could account for the increased toxicity under these conditions.

Collectively, the results obtained from localisation studies at different nerve endings, examined in relation to reports on the action of the toxins at these synapses, suggest strongly that an appreciable proportion of the silver grains seen at cholinergic nerve terminals must represent biologically significant molecules. In addition, these findings emphasise the importance of specific acceptor sites in the process of toxin-induced blockade of neurotransmission. It appears that the susceptibility of a nerve terminal to the toxin is governed by the availability of acceptors for the toxin therein. This dependence, however, is not absolute as very high concentrations of toxin may induce blockade of neurotransmission at nerve terminals that lack the acceptor [as seen at adrenergic nerve terminals, Holman and Spitzer, 1973; MacKenzie et al., 1982]]. In this case, the importance of the uptake mechanism is emphasised. At highly susceptible nerve terminals (such as the motor nerve terminal), the toxin must be delivered to the cytoplasm by a very efficient route. As shown by autoradiographic studies, toxin uptake is acceptor-mediated, energy-requiring and temperature-dependent and may involve acceptor-mediated endocytosis. However, other routes of entry (i.e. pinocytosis or non-specific adsorptive endocytosis, see
General Introduction), may also allow access of the toxin to its intraterminal target site, albeit with much lower efficiency. This proposal could explain the susceptibility of adrenergic nerve terminals to the toxin at high concentrations. The fact that inhibitory nonadrenergic-noncholinergic terminals are not paralysed even at high toxin concentrations may reflect the fact that, in addition to lacking the acceptor, these cells have deficient uptake mechanisms. The endocytic process is known to occur at different rates in different cells (Steinman, et al., 1983). Target cells that are resistant to diphtheria toxin, abrin or ricin and that endocytose at dramatically reduced rates (Olsnes and Sandvig, 1983) have been identified. That the presence of high affinity acceptors for BoNT may not be sufficient for expression of toxicity has also been suggested: this may be the case in the central nervous system (see Chapter 6). The central acceptors (Williams et al., 1983; Williams, 1984) may be unable to cluster in coated pits, or the toxin may not be transported across the membrane of endocytic vesicles into the cytosol (as has been suggested for abrin, ricin and modeccin in certain toxin-resistant cells; Olsnes and Sandvig, 1983).

In conclusion, these studies suggest that susceptibility of nerve endings to botulinum neurotoxin types is determined by both the number of acceptors present and efficiency of the uptake mechanism involved. The fact that certain toxin types may be effective in some species but not others can thus be explained. Moreover, the results obtained suggest that release of different neurotransmitters may involve a common step, affected by BoNT types. Further studies using the toxins may reveal that the steps involved in neurotransmitter release are universal among nerve endings of all types.

Another interesting conclusion that may be drawn from autoradiographic studies regards reports of toxin-induced postsynaptic effects (Sellin, 1981) at the neuromuscular junction. As no toxin binding was
detected on muscle cells, these effects (e.g. increase in the number and
distribution of acetylcholine receptors; Guyton and MacDonald, 1947) on
the muscle cell surface must be due to the toxin's ability to interfere
in an indirect way with the neurotrophic effects that the nerve normally
exerts on the muscle. Moreover, localisation studies also appear to
support the proposal that BoNT interacts mainly with cholinergic nerve
terminals in the mammalian peripheral nervous system (cf. Simpson,
1981). This suggests that nerve endings that release particular types
of transmitters have unique membrane determinants and implies that the
toxins could be used as histological markers to localise cholinergic
nerve endings, as affinity ligands to isolate cholinergic synaptosomes,
and to extract and characterise specific membrane determinants.

7.2 THE MECHANISM OF ACTION OF BOTULINUM NEUROTOXIN.

The three phases of botulinum neurotoxin intoxication at the
murine neuromuscular junction will now be discussed in light of studies
on the action of other bacterial and plant toxins of similar overall
structure. Diphtheria toxin, tetanus toxin, abrin, ricin and modeccin
all consist of two heterologous subunits, linked by a disulphide bond,
and all have an intracellular site of action. Although there is danger
in discussing data solely by analogy, the similarities between the
toxins and BoNT are too striking to be overlooked.

Diphtheria toxin (from Corynebacterium diphtheria) is a protein of
Mₚ 62,000, which is synthesised by a bacteriophage as a single poly-
peptide and subsequently nicked by proteases to form a dichain molecule
linked by a disulphide bond (reviewed by Pappenheimer, 1977). The sub-
units are known as fragment A (Mₚ 24,000) and B (Mₚ 38,000); fragment B (the heavier subunit) is thought to be responsible for
binding to a glycoprotein acceptor (Proia et al., 1979) while fragment A
is found to inhibit protein synthesis in cell-free extracts by
inactivating elongation factor 2 (Strauss and Hendee, 1959). Tetanus toxin, as described previously (see Chapter 5), is also produced as a single-chain molecule ($M_r \approx 150,000$) which may be nicked by endogenous protease(s) to form a dichain. The subunits ($M_r 100,000$ and $50,000$) are linked by a disulphide bond (reviewed by Mellanby and Green, 1981; Wellhoner, 1982) and the larger chain is responsible for binding to neuronal acceptors (Simpson, 1984) which may be gangliosides. Abrin, ricin and modeccin are plant toxins ($M_r \approx 65,000$) which consist of a binding fragment (B) and an enzymatic fragment (A) of approximately equal size. They bind to carbohydrates containing terminal galactose residues (Olsnes et al., 1978) and the A chains inhibit protein synthesis by inactivation of the 60S ribosomal subunit. Interestingly, inhibition of protein synthesis only occurs when fragment A is in a free state (cf. Olsnes and Sandvig, 1983) suggesting the possibility that only the smaller fragment is internalised for activity. It is interesting that internal disulphide bonds are absent from the enzymatic (or smaller) subunits of all these toxins, including BoNT. whereas they appear to be common in the binding fragments (the binding chains of diphtheria toxin, abrin, tetanus toxin and botulinum neurotoxin have one internal disulphide bond and that of abrin has four). Another common characteristic of these toxins (i.e. diphtheria toxin, abrin, ricin and modeccin) is that the A chain is resistant to denaturation by SDS (Gill, 1978); the enzymatic fragment of diphtheria toxin will even tolerate boiling and extreme pH. In all toxins that have been studied, hydrophobic stretches occur either in the A-chain or in the B-chain, or in both. All A chains (except those of tetanus toxin and BoNT for which this possibility is yet to be excluded) have enzymatic activity and one molecule is enough to kill a cell. The acceptors for these toxins, however, are different and, as suggested for BoNT type A, susceptibility to a particular toxin is dependent on the number of specific acceptors found on the target cell (Olsnes and Sandvig, 1983).
7.2.1 The binding step.

Studies with the bacterial and plant toxins described above have shown that the presence of high affinity membrane acceptors ensures that toxin added to the medium is concentrated on the cell surface. To secure a high local concentration of toxin appears to be the main role of these acceptors in the process of intoxication. They may also be involved in directing the toxin to a particular vesicular compartment, or in the formation of channels in the membrane for transport of toxin into the cytosol of target cells. This also appears to be the case with BoNT. In light of the results obtained using electron-microscope autoradiography, the 'pipe and valve' model of Hanig and Lamanna (1979), which suggests that the interaction of BoNT with surface acceptors is directly responsible for toxicity, does not seem a viable proposition. The acceptors for $^{125}$I-BoNT types A and B at the motor nerve terminal, which are mainly distinct, are not restricted solely to the active zone areas; it is, therefore, unlikely that these toxins act by blocking the release sites. Moreover, this proposal is difficult to reconcile with the apparent requirement for toxin internalisation prior to expression of neurotoxicity, and with the action of other toxins of similar overall structure. Another model that has been proposed suggests that the toxin acts as a Ca$^{++}$ channel blocker (Hirokawa and Heuser, 1981). Although the density of sites for botulinum neurotoxin types (see Chapter 4) could be consistent with the number of Ca$^{++}$ channels thought to exist at the motor nerve terminal (see Simpson, 1981), it cannot be reconciled with the number of toxin molecules required for paralysis. The autoradiographic data appears to dismiss totally a 'one hit' model for the mechanism of action of BoNT types. For such a model to be acceptable, it must satisfy at least two criteria: the susceptible membrane component must be present in numbers equal to or less than the minimum number of toxin molecules required to cause paralysis, and it must have
a slow turnover rate to explain the long duration of toxin action. Clearly, the first of these criteria is not satisfied: the $LD_{50}$ for a mouse is approximately $1.2 \times 10^{-11}$g, or $8 \times 10^{-17}$ moles or $5 \times 10^7$ molecules (for BoNT type A). From this $LD_{50}$, it can be calculated that $10^2$ to $10^3$ molecules are required to induce blockade of neurotransmission at each nerve terminal. Quantitation studies showed that there are $\sim 150$ and $630$ sites per $\mu m^2$ of membrane for $^{125}$I-BoNT types A and B respectively, so there is a definite discrepancy between the calculated density of acceptors and the number of molecules needed to produce paralysis. The possibility that there are heterogeneous populations of sites for these toxins at the nerve terminal, and that only a fraction of these are involved in the toxin's action, cannot be excluded. However, although types A and B BoNT have a similar overall pharmacological action at the neuromuscular juncion, most of their acceptor sites are distinct (since their competition for binding sites was not mutual, it is likely that the partial inhibition exerted by type A on type B binding is due to steric interactions); this would seem to be strong evidence against a 'one hit' mechanism of toxin action.

The proposal that botulinum toxin is a Ca$^{++}$ channel blocker is attractive as this would account for its ability to block both impulse-evoked and spontaneous release of acetylcholine. The possibility that the toxin occludes Ca$^{++}$ channels intracellularly cannot be excluded. However, there are other reasons to suggest that this is not the case. Wonnacott et al., (1978); Dolly et al., (1981) and Gundersen et al., (1982) have reported that Ca$^{++}$ flux into the terminal is not affected in synaptosomes or at the neuromuscular junction. A second point relates to the use of Ca$^{++}$ ionophores; Kao et al. (1976) showed that the Ca$^{++}$ ionophore X537A dramatically increased the frequency of mepps in control preparations, but was ineffective in poisoned preparations. Cull-Candy et al., (1976) found that Ca$^{++}$ ionophores only had an
effect in the presence of high extracellular Ca\(^{++}\) concentrations. Thus, it appears that the toxin does not block Ca\(^{++}\) channels, but rather diminishes the effectiveness of intracellular Ca\(^{++}\) in promoting neurotransmitter release. Accordingly, procedures that evoke displacement of intracellular Ca\(^{++}\) stores had no effect in poisoned muscles (Cull-Candy et al., 1976). However, agents that enhance Ca\(^{++}\) influx (e.g. 4-aminopyridine) are able to reverse the effects, albeit temporarily, of toxin-induced paralysis. The evidence is, therefore, strong for an intracellular site of action for BoNT types which does not involved the Ca\(^{++}\) channel. Moreover, these experiments indicate that the release mechanism is not destroyed by the toxin.

Although it seems clear that the specific acceptors for BoNT at the motor nerve terminal are not directly involved in neurotransmitter release, this does not preclude a functional role for these membrane components. The nature of the acceptors, at least at the neuromuscular junction, remains unknown but, as mentioned previously, the botulinum neurotoxins may recognise determinants unique to cholinergic nerve terminals and may thus prove to be powerful tools for studies on the nature of these terminals. Questions such as whether membranes of cholinergic nerve endings differ in any fundamental way from membranes of other nerve endings and whether there are distinct populations of cholinergic nerve membranes (i.e. species differences) could be investigated. The fact that these determinants appear to occur at different densities at somatic and autonomic cholinergic nerve terminals may be significant. It is interesting that recent localisation studies at the light microscope level have shown that \(\alpha\)-latrotoxin (from the venom of the black widow spider) also binds specifically and with high affinity to the plasma membrane of nerve terminals, although it does not appear to be a selective marker for any particular type of synapse (Valtorta et al., 1984). This suggests that there may be several neuro-
toxins that could be used to investigate the topological specialisation of the unmyelinated nerve terminal plasma membrane with respect to the myelinated axonal membrane. In addition, the cholinergic pathways in the brain have still not been identified fully and 'a selective toxin for cholinergic nerve endings would be of great utility but such a compound has not been discovered' (Flbiger, 1982). BoNT may be such a compound but, as it appears to be ineffective in the central nervous system (see Chapter 6), possibly due to deficient uptake mechanisms, a more profound knowledge of the uptake process may be useful for such studies and this will be discussed below.

7.2.2 The internalisation step.

This acceptor-mediated step can be divided into two phases: an endocytic phase that involves uptake of acceptor-toxin complexes into endosomes or lysosomes, and a translocation phase in which the toxin, or a fragment thereof, is transported through the vesicular membrane into the cytosol. The suggestion that the toxin is delivered to the terminal cytoplasm by acceptor-mediated endocytosis is viable in so far as the structures involved in this process are found at motor nerve terminals (see Fig. 7.1). Coated pits, coated vesicles, endosomes and lysosomes are all present, although the latter are less plentiful than in the cell soma (Holtzman, 1971). In addition, agents known to affect this pathway of uptake (i.e. lysosomotropic agents) have been reported to prevent delivery of toxin molecules to their intracellular target site at the motor nerve terminal (Simpson, 1983; present results). That the acceptor-ligand complexes cluster in coated pits prior to internalisation (a process that could not be detected directly in autoradiograms due to the limitations in resolution of the autoradiographic technique) can be inferred by the ability of methylamine and ammonium chloride to partially inhibit the uptake of $^{125}\text{I-BoNT}$ type A into the motor nerve
Fig. 7.1 Structures involved in the process of acceptor-mediated endocytosis.

Coated pits (a), coated vesicles (b), endosomes and lysosomes (c) are present at cholinergic nerve terminals suggesting that the process of acceptor-mediated endocytosis takes place in these cells.
terminal. These amines have been found to inhibit clustering of complexes of α2-macroglobulin, epidermal growth factor, and human choriogonadotropin with their respective receptors (Maxfield et al., 1979). That the toxin is taken up into an acidic compartment, from which it gains access to the cytosol, is suggested by the fact that weak bases such as chloroquine, which raise the pH of endo-lysosomal compartments (de Duve, 1983), delay the onset of toxin-induced blockade of neurotransmission at motor nerve terminals (Simpson, 1982). Analogies with other toxins may be useful in this context. It now appears most likely that diphtheria toxin, abrin, ricin, and modeccin all enter the cytosol from endocytic vesicles (Sandvig and Olsnes, 1983). The best evidence that endocytosis is involved is the finding that diphtheria toxin requires low pH to enter the cytosol. Under artificial conditions when the pH of the extracellular medium is reduced to 4.5, diphtheria toxin apparently enters directly through the surface of the membrane (Sandvig and Olsnes, 1981). Under normal conditions, however, such low pH only occurs in intracellular vesicles like lysosomes and endosomes (Tycko and Maxfield, 1982). Therefore, it is likely that transfer across the membrane occurs from these vesicles. Moreover, lysosomotropic agents have been found to protect cells against the actions of diphtheria toxin (Leppla et al., 1980) and modeccin (Sandvig et al., 1979). A process in which the low pH environment could be involved is channel formation (discussed below). Although there is no direct evidence to suggest that the toxin enters the cytosol from an endosome rather than a lysosome, an interesting observation may support this proposal indirectly. Both the botulinum neurotoxin and diphtheria toxin molecules, and also viruses (e.g. Sendai virus), must be nicked if toxicity is to be expressed. In the case of Sendai virus, the fusion protein must be proteolytically nicked to be active, and the two fragments obtained are linked by a disulphide bond. The rich supply of
proteolytic enzymes in lysosomes would lead one to expect that, if toxin entry involved the lysosomal compartment, unnicked toxin would also be active; both nicked and unnicked forms of the molecule should thus be equally potent. Since this is not the case, it appears more likely that toxin molecules enter the cytoplasm from the endosome, from which the acceptor may be recycled rapidly back to the cell surface (Melman et al., 1984) without exposure to the degradative enzymes in the lysosomes.

In the case of other toxins and viruses, it has been suggested that low pH is required to induce a conformational change in the protein which, in turn, promotes some kind of interaction with the endosomal membrane, resulting in transfer of the enzymatic fragment into the cytosol. Most ligands dissociate from their acceptors at low pH. However, BoNT type A appears to bind with high affinity to its acceptor sites in the central nervous system at pHs between 5. and 7 (Williams, 1984); this may be relevant as many of the characteristics of central acceptors are similar to those of the acceptors in the periphery (Williams, 1984). Another problem that may be envisaged is that of dissociation of the toxin from its acceptor during its journey through this complicated pathway. It has been shown, however, that the toxin binds to central acceptors with high affinity and the dissociation rate is slow (about 4 hours at 4°C). Diphtheria toxin attaches to its glycoprotein acceptor with a $K_D$ of approximately 10nM, which is an order of magnitude higher than that of BoNT. It must be emphasised that the possibility that only a few molecules of toxin gain access to the cytosol in this manner, with the majority being delivered to lysosomes for degradation, cannot be excluded. This has been shown to be the case with diphtheria toxin. The effectiveness of these intracellular toxins and viruses is thought to be controlled by the competing processes of cytoplasmic entry and lysosomal processing (Gonzales-Noriega et al., 1984). As these toxins are so potent (in most
Endocytosis may prove to be a common process for internalisation of macromolecules destined for the cytosol. Physiologically important molecules, such as certain protein hormones, must reach the cytosol to exert their effect and entry from an endocytic vesicle may be advantageous to the cell. Transfer of a large molecule directly through the plasma membrane may induce transient leakiness, as has been shown to occur during virus entry (Fernandes Puentes and Carrasco, 1980). Therefore, if entry occurred from the cell surface, concentration gradients across the plasmalemma could be dissipated. However, if the macromolecules are taken up into endocytic vesicles first, with transfer across the membrane only after the vesicle has been sealed off from the surface, the vesicle would act as a lock protecting the cell from entry of undesirable solutes.

The second phase of toxin entry will now be discussed. An understanding of the mechanism by which the toxin molecule is transferred across the membrane of the endocytic vesicle into the cytosol is essential. Again analogies with other toxins may be useful. It has been suggested that release of toxin molecules occurs by vesicle rupture (Nicolson, 1974). However, this is unlikely as, for example, cell lines which are resistant to one toxin are not necessarily resistant to others internalised in the same vesicles. Selective resistance to one toxin would be difficult if entry involved non-specific rupture of vesicular structures. Moreover, this phenomenon does not occur frequently enough to account for intoxication (Pappenheimer, 1977). The effects of toxins causing inhibition of protein synthesis in target cells can be studied in cell-free systems. It has been reported that diphtheria toxin and the plant toxins induce toxic effects via their enzymatic (A) subunit; in fact, abrin, ricin (cf. Olsnes and Sandvig, 1983) and diphtheria
toxin exert their action more efficiently if the subunits are separated. This finding suggests the possibility that only the small subunit of the toxin is transported across the membrane. Evidence supporting this proposal has also been obtained from experiments with planar lipid bilayer membranes. It has been shown that, under appropriate conditions, diphtheria toxin (Donovan et al., 1981; 1982; Kagan et al., 1981), cholera toxin (Tosteson and Tosteson, 1978), and tetanus toxin (Boquet and Duflot, 1982) can form discrete ion-conducting channels in the membrane. These are formed when a positive potential is applied across the membrane and the pH is low (~4.5) in the toxin-containing chamber. The channels only open when the membrane potential is positive on the same side as the toxin and, in the case of diphtheria toxin, the presence of phosphatidylinositol in the membrane strongly increases the extent of channel formation (Donovan et al., 1982). The channels appear to span the membrane (Kagan et al., 1981) and are permeable to solutes with molecular weight up to 1500. Such permeability indicates a diameter of 1.8 nm which is just enough to accommodate the A fragment of diphtheria toxin (M r 24000) in extended form (interestingly, it is also the size of normally occurring pores such as gap junctions). The fact that the toxin molecules contain hydrophobic regions, suggests that they are able to enter membranes (Blobel and Dobberstein, 1975). It appears that low pH induces changes in the structure of the toxin molecules which result in exposure of hydrophobic regions. For example, at neutral pH, native diphtheria toxin does not bind Triton X-100 whereas high amounts are bound if the pH is lowered to 4.4 (and also if the toxin is first denatured with SDS) (Sandvig and Olsnes, 1981). It has been suggested that proteolytic cleavage of the inserted protein is a possible mechanism by which the hydrophobic part is separated from the enzymatic part (Pappenheimer, 1977).

The size of the channel formed in the membrane (Kagan et al.,
1981), combined with the fact that fragment A of diphtheria toxin is very stable and can recover activity after denaturing conditions (suggesting that unfolding and refolding can take place), has prompted the proposal that the enzymatic fragment of diphtheria toxin is transported in a manner similar to that observed with proteins synthesised in the cell for export. In this case the protein is transferred across the membrane of the endoplasmic reticulum into the lumen as an extended polypeptide chain (Walter and Blobel, 1981) (Fig. 7.2). In the lumen, the protein is folded into its three dimensional structure and various post-translational modifications are carried out such as glycosylation, hydroxylation or disulphide bond formation (Rothman and Lodish, 1977; Freedman, 1979). The entry of toxin A chains into the cytosol may to some extent be considered as a reversal of this mechanism. An analogous process may take place to transport proteins from the cytoplasm into mitochondria. It appears that the transfer of diphtheria toxin across the membrane is an energy-requiring process and Olsnes and Sandvig (1983) suggest that the potential across the membrane may be the driving force allowing the A fragment to enter the cytoplasm. In their hypothetical scheme, at pH below 5, the carboxyl groups of the side chains of glutamic and aspartic acids are protonated and the toxin will have a net positive charge. The positively charged groups will be attracted to the negatively charged cell interior and this could initiate transfer through the channel formed by the B fragment of the toxin. As the protonated carboxyl groups reach the cytosolic side of the membrane, where the pH is close to neutrality, they lose their protons and form bonds with the amino groups of the protein. This initiates refolding of the A fragment which in itself could provide additional energy to pull the A fragment through the channel (see Fig. 7.3). Olsnes and Sandvig also suggest that the fact that at neutral pH the N-terminal two thirds of the A chain has approximately the same number of positively and
The translocation of secretory proteins from the cytoplasm into the lumen of the endoplasmic reticulum (ER) is thought to be a receptor-mediated process. It is now clear that the protein is transferred across the membrane as an extended polypeptide chain. The messenger RNA for such a protein is first attached to free ribosomes in the cytoplasm. The polysome complex becomes attached to the ER membrane as soon as the first part of the polypeptide is synthesised because this part of the chain, the signal sequence, has affinity for a 'signal recognition protein' on the cytosolic side of the ER. The ribosome also interacts with membrane receptors. The peptide chain is then transferred in its extended form through pores in the membrane into the lumen, where it is folded into its three dimensional structure and various post-translational modifications are carried out (at some point the signal sequence is cleaved off by a signal peptidase) (adapted from Walter and Blobel, 1981).
Fig. 7.3 Hypothetical scheme of the transfer of diphtheria toxin fragment A across membranes. (adapted from Olsnes and Sandvig, 1983)
negatively charged groups, whereas the C-terminal third has an excess of negatively charged groups, may be important in this process. Other sources of energy for transfer of A moieties across the membrane include linkage to certain ion transport systems (e.g. Ca\(^{++}\)).

Another possibility for the transfer of diphtheria toxin A fragment was suggested by Boquet et al., (1976). As indicated above, the hydrophobic domain of the B fragment (exposed by change in pH) becomes inserted into the lipid bilayer, where it may form a channel either by itself or in association with a part of the membrane-bound acceptor. Since fragment A is attached to the hydrophobic part of the B fragment, A may be pulled through the channel as it is being formed until the disulphide bridge and the short exposed loop that link the two fragments together reach the inner surface of the membrane. There nicking and reduction take place allowing the A fragment to enter cytoplasm. Because A readily renatures after drastic denaturing conditions, any channel in the membrane need only be large enough to accommodate A in its unfolded form.

It is interesting, that the heavier subunit of BoNT types also appears to form 1.8nm channels in asolectin membranes (Simpson and Finkelstein, personal communication). As the amino acid sequence of the light subunit has not been determined to date, it cannot be said that it would be able to pass through these channels in extended form. Moreover, the heavy and light chains of BoNT are difficult to separate (requiring treatment with reducing agents and 2M urea) so mechanisms must be proposed to overcome this problem. However, the possibility that enzymatic cleavage of the heavy and light chains does occur in the endosome, and that the strong hydrophobic interactions between the chains are weakened by interactions with the endosomal membrane in such a way as to allow the small subunit to pass through a channel in the membrane, cannot be excluded. This may be the reason why nicking is
essential for toxicity. It is interesting that the fragments of diphtheria toxin are also difficult to separate. After treatment with trypsin in the presence of thiol, fragments A and B remain associated through noncovalent forces and can be separated using 0.1% SDS and 0.01% $\alpha$-mercaptoethanol (Boquet et al., 1976) or with 0.01% $\alpha$-mercaptoethanol and 7.5M urea (Boquet, 1979). On the other hand, it is also possible that the entire BoNT molecule inserts through the membrane (both the heavy and light subunits have hydrophobic regions) and enters the cytosol as the intact molecule. Further studies are required to resolve this problem.

One aspect of the uptake process that cannot be overlooked is that toxin molecules, bound to the membrane, may be taken up by non-specific mechanisms and delivered directly to lysosomes. In addition, some molecules taken up by the 'effective' pathway may also be transferred to lysosomes. Keeping this in mind, a comparison can be made between the actions of tetanus toxin and botulinum neurotoxin in susceptible animals in vivo. Both these toxins cause flaccid paralysis by inhibition of neurotransmitter (ACh) release at the motor nerve terminal. Tetanus toxin also causes spastic paralysis by blocking inhibition (almost certainly by blocking inhibitory synapses where the transmitter involved in probably glycine) acting on spinal cord motoneurons (Mellanby and Green, 1981). This powerful effect usually masks the symptoms of flaccid paralysis also produced. Tetanus toxin reaches its target sites in the central nervous system by fast retrograde axonal transport and transsynaptic migration (Wellhoner, 1982). BoNT, however, has no central effects in vivo. Tetanus toxin is about 500-1000 times less potent than BoNT in producing flaccid paralysis at the neuromuscular junction (Habermann et al., 1981), whereas BoNT is 1000 times less potent than tetanus toxin in the central nervous system. A possible explanation for these effects is the following: in order to reach its
target in the central nervous system, tetanus toxin makes use of a pre-existing physiological mechanism that normally transfers endogenous macromolecules from one neuron to another and may be responsible for some trans-neuronal trophic interactions (cf. Grafstein and Forman, 1980). It has been localised autoradiographically within vesicles and tubules of smooth endoplasmic reticulum which are structural components of fast retrograde axonal transport (cf. Wellhoner, 1982). After binding to the motor nerve terminal, tetanus toxin is probably taken up by this postulated mechanism and delivered rapidly and efficiently to its target site for spastic paralysis in the spinal cord. For its action at the neuromuscular junction, however, tetanus toxin is probably taken up by a non-specific adsorptive route and this may explain the need for higher doses of this toxin to produce flaccid paralysis. The action of tetanus toxin at the neuromuscular junction is not affected by chloroquine (Simpson, 1982) which suggests that tetanus toxin and BoNT are internalised in different ways at this synapse. BoNT molecules appear to be taken up efficiently to produce flaccid paralysis but material transported up the axon probably represents degradation products (and thus inactive toxin) carried to the cell soma for further processing.

Thus, a complex mechanism of uptake appears to be responsible for translocation of BoNT into the nerve terminal. In the course of evolution, all these toxins have acquired a structure which has enabled them to make use of a pre-existing mechanism for internalisation. Furthermore, they may exploit forces and transport mechanisms across the membrane which must have been developed for other purposes. Further studies are required to establish whether physiological macromolecules make use of similar mechanisms to reach targets in the cytosol. Toxins such as BoNT may prove useful as probes to study steps involved in acceptor-mediated endocytosis. The uptake mechanism could be studied
using immunocytochemical techniques. Markers such as colloidal gold (e.g. 5-8 nm in diameter) would provide better resolution than silver grains; antibodies, linked to this marker, have been used successfully to localise various ligands (i.e. α-macroglobulin, asialoglycoproteins) in coated pits, coated vesicles, endosomes and lysosomes (Geuze et al., 1983). Furthermore, localisation studies combined with histochemical staining for lysosomal enzymes would enable identification of the compartment from which the toxin, or a fragment thereof, gains access to the cytosol. Antibodies against each of the toxin's subunits could be used in immunocytochemical localisation studies to establish whether one or both of these fragments enter the cytosol. These studies may also prove useful in elucidating the mechanism of entry of other toxins into target cells.

7.2.3 The lytic step.

This phase is the most obscure. It undoubtedly occurs intracellularly, after a lag time required for toxin entry into the cytosol (Simpson, 1980). The exact mechanism by which BoNT induces blockade of neurotransmitter release is yet to be determined, but some conclusions can be made based on findings obtained from pharmacological and autoradiographic studies. The toxin appears to diminish the sensitivity of a step in the release process to the actions of Ca\(^{++}\) (Cull-Candy et al., 1976; Thesleff, 1981). Simpson (1981) proposed that it affects the process by which vesicles fuse with the plasmalemma and thus eject their contents (ACh) into the synaptic cleft. It has been suggested that the toxin affects the vesicular membrane by interacting with it directly (cf. Simpson, 1981). However, the fact that there are approximately 2 to 5 \(\times\) 10\(^5\) vesicles in a motor nerve terminal, and only 10\(^2\) to 10\(^3\) molecules of toxin are required for paralysis at each nerve ending, suggest that a 'one hit' mechanism is not applicable.
in this case. Another aspect that must be emphasised is that BoNT has a prolonged action at the nerve terminal extending to weeks or months. Given the lengthy duration of toxin action and the small number of molecules necessary for paralysis, it appears that the toxin could not interact directly with any molecule that has a rapid turnover; moreover, there may not be a component(s) in the nerve terminal present in small enough quantities to be reconciled with the number of molecules needed for expression of toxicity at this site. A more acceptable proposition is that a single toxin molecule can exert its action in a multiplicative fashion; the data suggest that botulinum toxin molecules could act in small numbers to produce sustained paralysis if they had enzymatic activity and acted on a substrate with a slow turnover rate. This would be consistent with the action of a number of other bacterial and plant toxins (Van Heyningen, 1982); in fact there are so many bacterial toxins that are enzymes that this seems to be the rule rather than the exception. No enzymatic activity has been reported for the toxin to date but efforts are being made to this end in several laboratories.

The concept of 'spare' or 'redundant' acceptors can now be discussed. A toxin concentration of 15-20nM was required to saturate the binding sites for BoNT type A at the motor nerve terminal (in a 90-minute incubation period), and this is two orders of magnitude greater than that required to saturate the binding step (in the same time period), in pharmacological studies (0.1nM; Simpson, 1980). Thus, under conditions in which maximum toxicity was expressed, the membrane acceptors available for the toxin were not fully occupied. There are two possible explanations for the discrepancy: the biologically significant sites could represent only a small proportion of those observed autoradiographically, the majority being 'redundant' sites which are not involved in the toxin's action. Alternatively, toxicity may not be directly related to occupancy of membrane acceptors, but
rather to that of an intracellular lytic site which is saturated at toxin concentrations orders of magnitude lower than that required for saturation of binding sites on the cell surface. Pharmacological methods detect saturation of the lytic site; if this occurs when only a small fraction of the membrane acceptors have been occupied, further 'binding' would not be observed. Thus, there could be 'spare' biologically significant sites for BoNT at the motor nerve terminal; the presence of large numbers of these specific sites could contribute to the unique potency of the toxin at the synapse. It is also possible that a combination of these two models exists, with both 'spare' and 'redundant' sites present on the terminal membrane. The possibility of heterogeneity of the acceptor sites for BoNT types A and B was suggested by autoradiographic studies (Chapters 4 and 5) but this could reflect a single set of sites in different microenvironments. It should also be noted that the fact that a concentration of 15-20nM was required to saturate the sites for BoNT type A does not preclude the possibility that the acceptors exhibit a high affinity for BoNT, as even higher concentrations (~1μM) of α-bungarotoxin (K₀ ~10⁻¹¹M) are likewise required to overcome diffusion barriers in this tissue (Dolly et al., 1977), within a similar incubation period.

It is clear from these studies that not only is further investigation required to determine the mechanism of action of the toxin (i.e. its effects on the process of neurotransmitter release), but also to establish the relationship between the acceptor sites and expression of toxicity.
It has become apparent during the course of research on the site of action and biological effects of botulinum neurotoxin that this potent pharmacological agent can be used to expand present knowledge in at least three different fields of study. It may be useful as a probe to investigate the process of neurotransmitter release, the steps involved in acceptor-mediated endocytosis, and the specialisation of neuronal membranes. The scope for research with BoNT could be expanded even further by use of the isolated subunits. The non-toxic binding fragment could be used to tissue-target and deliver other agents (e.g. antibodies) to the cytosol of cholinergic nerve endings. The lytic process could be studied by linking the small fragment to agents recognising markers on different neuronal membranes. It is clear that a deeper understanding of the toxin molecule itself (its structure, pharmacological action, etc.) would be useful for realising its full potential as a powerful tool for research in neurobiology and other disciplines.
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