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SIGNIFICANCE OF THE INTERACTION OF DRUGS WITH MELANIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Maria Margarita Salazar,
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(B.S. in Pharm.)
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The Ohio State University
1976

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I dedicate this dissertation to Dr. Antonio J. Muskus A., in recognition for his tireless effort for the professional and personal development of individuals; and for the further development of the Venezuelan university.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>x</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td><strong>I.  INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>Melanins</td>
<td>1</td>
</tr>
<tr>
<td>The Melanocyte System</td>
<td>5</td>
</tr>
<tr>
<td>Melanosomes</td>
<td>19</td>
</tr>
<tr>
<td>Chemical Composition of Melanosomes</td>
<td>24</td>
</tr>
<tr>
<td>Neuromelanin</td>
<td>29</td>
</tr>
<tr>
<td>Melanin Synthesis</td>
<td>37</td>
</tr>
<tr>
<td>Synthetic Melanins</td>
<td>46</td>
</tr>
<tr>
<td>Free Radical Properties of Melanin</td>
<td>50</td>
</tr>
<tr>
<td>Binding of Drugs to Melanin</td>
<td>60</td>
</tr>
<tr>
<td>Pathophysiological Significance of Natural Melanins</td>
<td>75</td>
</tr>
<tr>
<td>Statement of the Problem</td>
<td>81</td>
</tr>
<tr>
<td><strong>II.  METHODS AND MATERIALS</strong></td>
<td></td>
</tr>
<tr>
<td>Experimental Animals</td>
<td>84</td>
</tr>
<tr>
<td>Bovine Iris Melanin Granules</td>
<td>84</td>
</tr>
<tr>
<td><strong>vi</strong></td>
<td></td>
</tr>
</tbody>
</table>
III. RESULTS

*In vitro* Uptake of $^3$H-Atropine by Tissues... 111

Accumulation of $^3$H-Atropine by Human Irides and Pigment Epithelium... 115

*In vitro* Loss of $^3$H-Atropine from Tissues after Repeated Washings... 115

Ocular Penetration of $^3$H-Atropine... 119

Antimuscarinic Effects of Atropine in the Pigmented and Nonpigmented Rabbit Iris and Fundus Strips... 119

Recovery from Atropine Blockade... 124

*In vivo* Mydriatic Effects of Atropine... 129

Binding of $^{14}$C-Imipramine by Isolated Rabbit Iris... 133

*In vitro* Loss of $^{14}$C-Imipramine from Isolated Rabbit Iris... 140

Antimuscarinic Effects of Imipramine in the Pigmented and Nonpigmented Rabbit Iris... 143

Binding of $^{14}$C-Imipramine to Human Brain Homogenates... 143

Localization of $^{14}$C-Imipramine in Isolated Rabbit Iris by Discontinuous Sucrose Density Gradient Centrifugation... 143

Localization of $^3$H-Chlorpromazine in Isolated Rabbit Retina by Discontinuous Sucrose Density Gradient Centrifugation... 148
APPENDICES

A. *In vitro* Loss and Uptake of $^3$H-Atropine from Tissues .................. 226

B. Binding of $^{14}$C-Imipramine to Isolated Irides from 6-Hydroxydopamine Treated Albino and Nonalbino Rabbits .................. 234

BIBLIOGRAPHY. .................................................. 237
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Comparison of the Properties of Tyrosine Hydroxylase and Tyrosinase.</td>
<td>6</td>
</tr>
<tr>
<td>2. Formation of Melanosomes and Biosynthesis of Melanin</td>
<td>20</td>
</tr>
<tr>
<td>3. Ultrastructural, Histochemical, and Spectroscopic Characteristics of Neuromelanin, Cutaneous Melanin and Lipofuscin Granules</td>
<td>34</td>
</tr>
<tr>
<td>4. Preparation of Synthetic Melanins</td>
<td>101</td>
</tr>
<tr>
<td>5. Accumulation of $^3$H-Atropine (30 nCi/ml, $10^{-5}$M Incubated for 35 min) by Human Iris, Pigment Epithelium and Rabbit Iris</td>
<td>116</td>
</tr>
<tr>
<td>6. Sensitivity of Isolated Iris Sphincter to Carbachol from Atropine Treated (96 hours before) and Untreated Rabbits</td>
<td>132</td>
</tr>
<tr>
<td>7. Binding of Radiolabelled Drugs to Human Brain. Localization by Discontinuous Sucrose Density Gradient Centrifugation</td>
<td>162</td>
</tr>
<tr>
<td>8. Summary of the Binding Constants of $^3$H-Chlorpromazine to Bovine Iris Melanin Granules in the Absence and Presence of Other Drugs</td>
<td>170</td>
</tr>
<tr>
<td>9. Summary of the Linear Regression Parameters for the Langmuir Treatment of the Binding of $^3$H-Chlorpromazine to Bovine Iris Melanin Granules in the Absence and Presence of Other Drugs</td>
<td>176</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>10. Summary of the Binding Constants of $^3$H-Chlorpromazine to Synthetic L-Dopa Melanin in the Absence and Presence of Other Drugs</td>
<td>184</td>
</tr>
<tr>
<td>11. Summary of the Linear Regression Parameters for the Langmuir Treatment of the Binding of $^3$H-Chlorpromazine to Synthetic L-Dopa Melanin in the Absence and Presence of Other Drugs</td>
<td>185</td>
</tr>
<tr>
<td>12. Summary of Binding Constants of $^3$H-Chlorpromazine to Isolated Bovine Iris Melanin Granules Extracted with Organic Solvents</td>
<td>191</td>
</tr>
<tr>
<td>13. Summary of Linear Regression Parameters for the Langmuir Treatment of the Binding of $^3$H-Chlorpromazine to Isolated Bovine Iris Melanin Granules Extracted with Organic Solvents</td>
<td>192</td>
</tr>
<tr>
<td>14. In vitro, Accumulation of $^3$H-Atropine (30 nCi/ml, $10^{-5}$ M, Incubated for 35 min) by Irides from the Rabbits Treated Topically (24 hr before) with 0.1 ml of 4% Atropine</td>
<td>232</td>
</tr>
<tr>
<td>15. Comparative Blocking Effects of Atropine Against a Single Dose ED$_{50}$ of Carbachol on the Pigmented and Nonpigmented Iris Obtained from Atropinesterase Positive Rabbits</td>
<td>233</td>
</tr>
<tr>
<td>16. Effects of 6-Hydroxydopamine Pretreatment on the Binding of $^{14}$C-Imipramine to Isolated Pigmented and Nonpigmented Rabbit Iris</td>
<td>236</td>
</tr>
</tbody>
</table>
# List of Illustrations

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Human Melanocyte System</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Mechanisms of Hormonal Control of Pigmentation</td>
<td>17</td>
</tr>
<tr>
<td>3.</td>
<td>Schematic Representation of a Fully Developed Hair Melanosome</td>
<td>25</td>
</tr>
<tr>
<td>4.</td>
<td>The Raper-Mason Pathway of Melanin Synthesis</td>
<td>39</td>
</tr>
<tr>
<td>5.</td>
<td>The Site of Action of Catechol-O-methyltransferase (COMT) and Hydroxyindole-O-methyltransferase in the Conversion of Tyrosine to Melanin</td>
<td>42</td>
</tr>
<tr>
<td>6.</td>
<td>Pathway of Phaeomelanin Synthesis</td>
<td>44</td>
</tr>
<tr>
<td>7.</td>
<td>Electron Paramagnetic Spin Resonance Spectra of Natural and Synthetic Melanins</td>
<td>53</td>
</tr>
<tr>
<td>8.</td>
<td>Electron Paramagnetic Spin Resonance Spectra of Human Hair Melanin</td>
<td>55</td>
</tr>
<tr>
<td>9.</td>
<td>Charge-Transfer Interaction Model Reaction for Compounds Having Free Radical Properties</td>
<td>57</td>
</tr>
<tr>
<td>10.</td>
<td>Tissue Bath Assembly Used for Recording Tension Changes of Isolated Rabbit Iris</td>
<td>89</td>
</tr>
<tr>
<td>11.</td>
<td>In vitro Accumulation of $^3H$-Atropine (30 nCi/ml) by the Pigmented and Nonpigmented Rabbit Iris</td>
<td>112</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>12. The Rate of Disappearance of $^3$H-Atrpine from Irides and Stomach Fundus Strips Obtained from Albino and Nonalbino Rabbits.</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>13. In vivo Rate of Uptake of Radioactivity after the Topical Application of 0.1 ml of 2% Atropine Sulfate Solution, by the Aqueous Humor and Irides of Albino and Nonalbino Rabbits.</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>14. Dose-Response Curves of Carbachol Obtained in Isolated Pigmented and Nonpigmented Rabbit Iris and Fundus Strips Determined in the Presence and Absence (Control) of Atropine</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>15. Arunlakshana and Schild (1959) Plot of Log (DR-1) of the Agonist Carbachol Against Atropine Obtained from the Tissues of Albino and Nonalbino Rabbits.</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>16. In vitro Rate of Recovery from Atropine Blockade by the Pigmented and Nonpigmented Iris Obtained from Atropinesterase-positive Rabbits.</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>17. Onset and Duration of Atropine Mydriasis in Albino and Nonalbino Rabbits.</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>18. Duration of Atropine Mydriatic Effect After Instillation of 0.1 ml of 2% $^3$H-Atrpine (0.52 μCi total) in Atropinesterase-negative Rabbits.</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>19. Thin Layer Chromatography of Iris Extract from Pigmented Atropinesterase-negative Rabbits.</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>20. In vitro Accumulation of $^{14}$C-Imipramine by the Pigmented and Nonpigmented Rabbit Iris.</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>21. Rate of Disappearance of $^{14}$C-Imipramine from Isolated Pigmented and Nonpigmented Rabbit Iris</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>22. Dose-Response Curve of Carbachol Obtained in Isolated Pigmented and Nonpigmented Rabbit Iris Determined in the Presence and Absence (Control) of Imipramine</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>23. In vitro Accumulation of $^{14}$C-Imipramine by Homogenates of Human Substantia Nigra and Cerebral Cortex</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>24. Localization of $^{14}$C-Imipramine in Isolated Pigmented and Nonpigmented Rabbit Iris by Discontinuous Sucrose Density Gradient Centrifugation</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>25. Localization of $^3$H-Chlorpromazine in Isolated Pigmented and Nonpigmented Rabbit Retina by Discontinuous Sucrose Density Gradient Centrifugation</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>26. Localization of $^{14}$C-Imipramine in Human Brain by Discontinuous Sucrose Density Gradient Centrifugation</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>27. Localization of $^3$H-Haloperidol in Human Brain by Discontinuous Sucrose Density Gradient Centrifugation</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>28. Localization of $^3$H-Chlorpromazine in Human Brain by Discontinuous Sucrose Density Gradient Centrifugation</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>29. Determination of Equilibrium Time for $^{14}$C-Imipramine in Bovine Iris Melanin Granules</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>30. Binding of $^{14}$C-Imipramine to Bovine Iris Melanin Granules and Synthetic L-Dopa Melanin</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>31. Determination of Equilibrium Time for $^3$H-Chlorpromazine in Bovine Iris Melanin Granules</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>32. Binding of $^3$H-Chlorpromazine to Synthetic Melanins</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>33. Summary of Regression Lines Representing the Binding of $^3$H-Chlorpromazine to Synthetic Melanins</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>34. Summary of Regression Lines Representing the Binding of $^3$H-Chlorpromazine to Bovine Iris Melanin Granules in the Absence and Presence of Some Phenothiazines and Related Drugs.</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>35. Binding of $^3$H-Chlorpromazine to Bovine Iris Melanin Granules in the Absence and Presence of Some Phenothiazines and Related Drugs.</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>36. Binding of $^3$H-Chlorpromazine to Bovine Iris Melanin Granules in the Absence and Presence of Dopamine</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>37. Summary of Regression Lines Representing the Binding of $^3$H-Chlorpromazine to Synthetic L-Dopa Melanin in the Absence and Presence of Some Phenothiazines and Related Drugs.</td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>38. Binding of $^3$H-Chlorpromazine to Synthetic L-Dopa Melanin in the Absence and Presence of Some Phenothiazines and Related Drugs.</td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>39. Binding of $^3$H-Chlorpromazine to Bovine Iris Melanin Granules Unextracted and Extracted with Organic Solvents.</td>
<td>194</td>
<td></td>
</tr>
<tr>
<td>40. Schematic Representation which Explains the Effects of Atropine in the Pigmented and the Nonpigmented Eye.</td>
<td>202</td>
<td></td>
</tr>
<tr>
<td>41. Pathway of Melanin Synthesis Showing Different Precursors and Probable Intermediates Formed Corresponding to the Dopachrome of the L-Dopa Melanin Synthesis According to the Raper-Mason Pathway</td>
<td>213</td>
<td></td>
</tr>
<tr>
<td>42. In vitro Accumulation of $^3$H-Atropine by the Pigmented and Nonpigmented Iris of Atropinesterase-positive and Enzyme-unclassified Rabbits</td>
<td>227</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>43. Rate of Disappearance of $^3$H-Atropine from Iris and Stomach Fundus Strips Obtained from Atropinesterase-positive and Enzyme-unclassified Albino and Nonalbino Rabbits</td>
<td>231</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

Melanins

Melanins (μέλανας=black) are generally defined as black or brown pigments of high molecular weight formed by the enzymic oxidation of phenols. Melanins are widely distributed in animals and plants and usually occur as melanoproteins, the pigment being strongly bound to the protein. Melanin pigments possess definite chemical characteristics, including considerable physical and chemical stability. Melanins also possess free radical properties (Harley-Mason, 1965; Mason, 1953; Seiji, 1967; Swartz, 1972; Thomson, 1974).

Since numerous brown and black pigments such as uveal melanin, neuromelanin, rheomelanins and lipofuscin occur in tissues it is necessary to distinguish between melanins and other pigments. For example, melanin pigments resulting from oxidation of catechols in cells of the adrenal medulla could be different from the melanin found in skin and eyes.
The term eumelanin (ευ = good) covers brown to black pigment found in skin, hair, feathers, insect cuticles and melanomas. Phaeomelanin (φαι = dusky) covers a broad spectrum from violet to yellow. These pigments have been found only in feathers and mammalian hair, including human red hair. Both types of melanins are formed in melanocytes from oxidation of tyrosine by tyrosinase, but they differ in that phaeomelanins synthesis includes the incorporation of a molecule of cysteine. Phaeomelanins and eumelanins may occur together and are responsible for most mammalian coloring (Thomson, 1974).

Eumelanins are irregular, crosslinked polymers, containing 5,6-dihydroxyindole units that are conjugated with proteins.

Rheomelanins are soluble pigments of different colors which are naturally formed in the body from precursors present in the body as for example pigments formed in the cells of adrenal medulla from the oxidation of epinephrine and norepinephrine (Seiji, 1967). Formation of rheomelanins has also been demonstrated in human blood plasma in vitro from: (-)-epinephrine, (-)-norepinephrine, (-)-epinephrine methyl ether, L-dopa, dopamine and L-α-methyldopa (Hegedus and Altschule, 1970).

Neuromelanin is a pigment present in neurons of some areas of the central nervous system. It is believed
to be of different origin than that of other melanins.

Unfortunately, investigators working in the field of melanins have not used a consistent terminology. The following terms were proposed by Fitzpatrick et al. (1966) to define melanin containing cells, their precursors and related cells. Ontogenetic stages in the formation of melanin are also included.

**Melanocyte** (Greek: Melan = black; Kytos = cell): a cell which synthesizes a specialized melanin-containing organelle, the melanosome. This term includes differentiated cells which synthesize nonmelanized or partially melanized premelanosomes as terminal products. It is suggested that in albinism the melanocytes containing nonmelanized premelanosomes be called albino melanocytes.

**Melanophore** (Greek: Melan = black; Phore = bearing): a type of melanocyte which participates with other chromatophores in the rapid changes of color in animals by intracellular displacing (aggregation and dispersion) of melanosomes.

In human histology and pathology, a phagocytic cell that contains melanin but does not form the pigment (Stedman's Medical Dictionary). It should not be referred to as melanophore.

**Melanoblast** (Greek: Melan = black; Blastos = germ): a cell which serves at all stages of the life cycle as the precursor of the melanocyte (and/or melanophore).
**Langerhans Cell:** A distinctive cell of the mammalian epidermis and dermis presumed to belong to the "melanocyte" series. It is revealed by gold impregnation and contains distinctive nonmelanized disc-like organelles.

**Premelanosome:** All distinctive particulate stages in the maturation of melanosomes. Hence electron density is variable. It possesses an active tyrosinase system after the onset of melanin synthesis.

**Melanosome** (Greek: Melan=black; Some=body): A discrete melanin containing organelle in which melanization is complete; shown to be more or less electron-dense by electron microscopy; tyrosinase activity not usually demonstrable. Multiple melanosomes imbedded in supporting matrices, for example, as in the macrophages and malpighian cells of mammals may be designated as melanosome complex.

Accordingly, the terminology suggested by Fitzpatrick *et al.* (1966) will be used in this paper. However, since the terms melanin granules and melanosomes are commonly used as synonymous, for simplicity these terms will be used interchangeably in the text.

**Tyrosinase** (Mushroom, 1.10.3.1) also called polyphenol oxidase, o-diphenoloxidase and catecholase is a copper-containing enzyme which is localized in melanocytes and is responsible for the formation of melanin pigment.
It catalyzes two successive reactions.

\[
\text{Tyrosine} \rightarrow \text{Dopa} \rightarrow \text{Dopaquinone}
\]

Dopaquinone is polymerized to melanin nonenzymatically. L-dopa is the best substrate for the enzyme. Tyrosinase is also responsible for the synthesis of catecholamines in insects (Sekeres and Karlson, 1966) and in the banana plant (Nagatsu et al., 1972).

Before the discovery of tyrosine hydroxylase, tyrosinase was thought to be responsible for the synthesis of dopa from tyrosine in the biosynthesis of norepinephrine. A summary of the characteristics of both enzymes is presented in table 1.

The Melanocyte System

In mammals the melanocyte systems have different origins. The melanocytes of skin and hair are derived from melanoblasts which migrate from the embryonic neural crest early in development (Rawles, 1947). Melanocytes in the eye originate both from the optic cup (retinal melanocytes) and the neural crest (uveal melanocytes). There are indications that a small number of primary melanoblasts from the neural crest undergo proliferation and differentiation to color local regions of the skin (Mintz, 1967). The differentiation of melanoblasts into melanocytes starts with the synthesis of melanosomes. This
<table>
<thead>
<tr>
<th>Property</th>
<th>Tyrosine hydroxylase</th>
<th>Tyrosinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution Organs</td>
<td>Adrenal medulla, brain, sympathetically innervated organs (heart, spleen, salivary glands, vas deferens, etc.)</td>
<td>Skin, eye, melanoma</td>
</tr>
<tr>
<td>Cell type</td>
<td>Sympathetic nerve cell</td>
<td>Melanocyte</td>
</tr>
<tr>
<td>Cofactor</td>
<td>Tetrahydropterin (tetrahydrobiopterin?), Fe++</td>
<td>Dopa</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.0 (5.8-6.3)</td>
<td>7.0</td>
</tr>
<tr>
<td>Substrate</td>
<td>L-tyrosine ++</td>
<td>L-tyrosine +</td>
</tr>
<tr>
<td></td>
<td>L-phenylalanine ++</td>
<td>D-tyrosine +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyrine +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dopa +++</td>
</tr>
<tr>
<td>Km (M)</td>
<td>L-tyrosine (DMPH₄) 1 X 10⁻⁴</td>
<td>L-tyrosine 6 X 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>L-phenylalanine (DMPH₄) 3 X 10⁻⁴</td>
<td>D-tyrosine 8 X 10⁻³</td>
</tr>
<tr>
<td></td>
<td>DMPH₄ 5 X 10⁻⁴</td>
<td>L-dopa 5 X 10⁻³</td>
</tr>
<tr>
<td></td>
<td>L-tyrosine (tetrahydrobiopterin) 2 X 10⁻⁵</td>
<td>D-dopa 4 X 10⁻³</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>L-α-methyl-p-tyrosine +++</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dopa or other catechols +</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fe++ chelating agents, such as α,α',dipyridyl ++</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cu-chelating agent, such as diethylidithiocarbamate 0</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Melanin pigment formation 0</td>
<td>+</td>
</tr>
</tbody>
</table>

*Modified from Nagatsu (1973)*
cellular transformation is influenced by the genotype of the melanoblast which sets the minimal environment requirements necessary for differentiation as well as by the adequacy of the local tissue environment which may be subject to change during development (Quevedo, 1971).

**Melanocyte Characteristics**

Thin section electron microscopy studies show that melanocytes appear as distinctive cells. They are dendritic in nature possessing two or more dendritic processes, there is an absence of desmosomes and they contain some filaments. Generally, melanocytes are located above but in close proximity to the basement membrane. The nucleus is round, slightly indented and has a double nuclear membrane. The outer membrane is rough and it is separated from the inner membrane by a clear zone approximately 500Å wide. A nucleolus is also present and lacks a limiting membrane. Mitochondria are abundant and are dispersed throughout the cytoplasm. A well developed endoplasmic reticulum is located between the mitochondria and throughout the cytoplasm of the melanocyte with both rough and smooth surfaced types present. The rest of the cytoplasm is clear with a few electron dense particles dispersed throughout. The Golgi complex is easily recognized, is well developed and seems to be important in the formation of melanin granules (see below) (Zelickson, 1967).
The dendritic cells of human and guinea-pig epidermis have been divided into three types: type I contains melanin granules in different stages of maturity; these cells are true melanocytes; type II does not contain melanin granules but possesses characteristic rod-shape granules (Langerhans cell) and type III does not contain any granules. The functional significance of the third type of dendritic cells is unknown (Zelickson, 1967).

Melanocytes are found in the skin, eyes, inner ear and leptomeninges. Melanocytes in the skin are located at the epidermal-dermal junction. In the eyes they are present in the uveal tract, and in the central nervous system the cells are found at the junction of the pia and the arachnoid (fig. 1).

Under ordinary conditions melanocytes probably multiply at a slow rate. This belief seems probable because when these cells are destroyed by physical or chemical means permanent depigmentation results. When these cells undergo neoplastic change to become malignant melanoma they multiply very rapidly (Lerner and Fitzpatrick, 1953).

Several facts are in favor of the neural crest origin of the melanocytes: a) embryological studies in amphibia, birds, mice and human beings show that melanocytes are derived from the neural crest region; b) the
Figure 1. Human Melanocyte System (Modified from Brodthagen, 1972)
Figure 1
cells are dendritic in shape as are other nerve cells; c) the cells can be stained with silver, gold and methylene blue as can other nerve cells; these cells produce dopa which makes them related in function to the sympathetic nervous system cells producing norepinephrine; e) malignant melanomas (melanocyte tumors) do not respond to radiation therapy, resembling in this way most nerve cell tumors and f) abnormalities of pigmentation are frequently associated with abnormalities of the nervous system (neurofibromatosis) (Rawles, 1947).

Interestingly, although melanocytes have not been found in brain nuclei where the pigment neuromelanin is formed, an association between the pigmented areas and the presence of adrenergic system pathways have been found (Bazelon et al., 1967).

The interaction between melanocytes (also called pigment cells) and keratinocytes from the epidermis form a structural and functional unit called the epidermal melanin unit. This unit appears to be involved in several functions such as: formation of melanosomes, movement of melanosomes out into the melanocyte dendrites, attachment of melanocytes to keratinocytes and movement and breakdown of the melanosome complex within the keratinocyte (Wiskwo and Szábo, 1973).
Studies in frog skin, using cytochalasin B, suggest that a contractile microfilament system is involved in the movement of melanin granules in the melanophore (Malawista, 1971). McGuire and Moellman (1972) demonstrated in dermal and epidermal melanocytes of *Rana pipiens* that cytochalasin B prevents the dispersion of pigment granules by melanocyte stimulating hormone (MSH) and causes centripetal movement of pigment granules that have been dispersed by MSH. Microfilaments are abundant in the dendrites of epidermal melanocytes in which pigment granules have been dispersed by MSH and also, the number of microfilaments is reduced in the dendrites of melanocytes lightened with cytochalasin B.

McGuire and Moellman (1972) propose that dispersion of pigment granules is effected by way of the microfilaments; when microfilaments are destroyed, pigment granules move centripetally. Intact microtubules are required for pigment granule aggregation. More recently, Jimbow and Fitzpatrick (1975), studied the activity of cytoplasmic filaments in human melanocytes after UV irradiation. They found that human melanocytes *in vivo* contain 100 Å in diameter filaments. The filaments are primarily located in the
perinuclear area and endoplasmic regions. These 100-Å microfilaments appear to be involved in the elongation of the dendrites as well as in the movement and transfer of melanosomes. The melanocytic filaments appear also to be involved in the transfer of melanosomes from the melanocytes to the epithelial cells since there is an increase in the number of melanosomes transferred to the epithelial cells during the rapid translocation of the 100-Å filaments. These authors consider that microtubules are directly involved in the melanosome movement, although they may be involved in the elongation of the dendrites.

The process of translocation, dispersion and aggregation, of melanosomes has been thoroughly studied in lower vertebrates such as fish, amphibians and reptiles. In these animals the pigment cells are called melanophores. Like melanocytes, the melanophores are also of neural origin. Jacobowitz and Laties (1968) observed direct innervation of the pigment cell where they found catecholamine containing fibers in anatomic proximity to conjunctival and dermal melanocytes of a Teleost fish. In fish having directly innervated pigmented cells, electrical stimulation of a nerve to the skin produces localized blanching and cutting of the nerves produces immediate darkening (Lerner, 1971).
It must be pointed out, however, that melanophores in Teleost fish are substantially different from melanophores in mammals. Studies in normal mammalian skin (Falck and Rorsman, 1963), and of pigmented nevi and melanomas (Falck et al., 1965), by histofluoremetric technique failed to demonstrate adrenergic innervation to melanocytes.

Goldman and Hadley (1969), Gupta and Bhide (1967) and McGuire (1970), among others, demonstrated that melanocytes of all the animals they studied--fish, frogs, toads and lizards--have alpha- and/or beta-adrenergic receptors. Also it has been found that stimulation of alpha-receptors leads to aggregation of melanosomes and skin lightening. Stimulation of beta-receptors produces dispersion of melanosomes, therefore producing darkening of the skin.

Although some studies on the role of acetylcholine in the control of pigmentation in lower vertebrates have been done, the results have been inconsistent; acetylcholine darkens some fish but lightens about one of three Rana pipiens (Lerner, 1971). McGuire (1970) reported that epinephrine which has both alpha- and beta-adrenergic effects lightens skin previously darkened with MSH, and darkens lightened frog skin. On the other hand isoproterenol, a beta-receptor stimulant, produces only granule dispersion (darkening) whereas phenylephrine, an alpha-adrenergic agonist, causes only granule aggregation (lightening).
Melatonin produces lightening of *Rana pipiens* dermal melanocytes in concentrations less than those required for epinephrine or acetylcholine. Alpha- and beta-adrenergic blockers do not prevent the effects of melatonin. Melatonin does not have a direct effect on mammalian melanocytes. However, the possibility that melatonin may be acting centrally in the rat to prevent release of MSH from the pituitary has been suggested (Kastin and Schally, 1967).

Novales and Davis (1967) reported darkening of frog skin and melanin dispersion in salamander cultured melanophores by cyclic-AMP. Also, caffeine, a phosphodiesterase inhibitor, caused dispersion of pigment granules.

Abe et al. (1969a,b) found that in skin from *Rana pipiens* β-MSH, α-MSH, and ACTH increased cyclic AMP levels with the same potencies at which they stimulated darkening. On the other hand norepinephrine and melatonin inhibited the MSH-induced increase in cyclic AMP.

The dispersion and aggregation of melanosomes which occurs through relatively fixed channels in pigment cells of fish, amphibians, and reptiles in response to MSH, caffeine, catecholamines, acetylcholine and melatonin have not been shown in mammalian system. Nevertheless, MSH has a pronounced darkening effect on the skin or hair of man, guinea pigs, hamsters and mice. There is evidence that darkening is associated with an increase in tyrosinase
activity which is most likely due to an increase in synthesis and not to a simple activation of tyrosinase already present in the cell. The role of cyclic AMP in the induction of tyrosinase is still unknown, but since MSH increases cyclic AMP in melanomas it is possible that the nucleotide is responsible for initiating tyrosinase synthesis, although it is not known if increased tyrosinase synthesis leads to dispersion of melanosomes (Lerner, 1971). See fig. 2.

In mammalian pigment cells or melanocytes MSH increases cyclic AMP, induces tyrosinase synthesis and darkens skin. These steps may be related sequentially. Alpha- and beta-receptor sites have not been found in mammalian cells, but it is likely that the melanocytes are under neural control (Lerner, 1971).

Ehinger and Falck (1970) suggest that melanocytes of rat iris possess an adrenergic as well as a cholinergic innervation. This concept of an adrenergic innervation is supported by the fact that heterochromia at times accompanies Horner's Syndrome in humans (Duke-Elder and Wyber, 1958), that has been observed in cats sympathectomized a few years previously and that white spots appear in the iris of pigmented rabbits a few months after sympathectomy (Ehinger, Falck and Rosengren, 1969). Inhibition of pigmentation by sectioning of adrenergic nerves has been demonstrated (Bennet and Hausberger, 1938). Although a
Figure 2. Mechanisms of hormonal control of pigmentation.

A. MELANIN DISPERSION:

ACTH, α- and β-MSH and caffeine raise the intracellular levels of cyclic AMP in melanophores of amphibians, fish and reptiles to initiate dispersion of melanosomes.

B. MELANIN SYNTHESIS:

Injection of MSH into man, hamsters, mice and guinea-pigs increase tyrosinase activity, which in turn results in melanin formation. It is likely that these events are triggered by MSH, which raises cellular levels of cyclic AMP.

(From Lerner, 1971)
Figure 2

(A) MELANIN DISPERSION

(B) MELANIN SYNTHESIS

Figure 2
rapid mechanism for color changing is absent in mammals
the depigmentation occurring after sympathectomy suggests
some kind of slowly acting trophic nervous influence on the
melanocytes.

**Melanosomes**

Melanosomes are the final product of the synthesis
of melanin in the melanocyte (Fitzpatrick et al., 1966).
In order to understand the origin of melanin it is neces­sary to understand the process of formation of the melano­some which is illustrated in table 2. Toda and Fitzpatrick
(1971) outlined four stages in the development of the
melanosomes. Stage I is characterized as a spherical
membrane limited vesicle that contains tyrosinase and at
times some filaments but not melanin. In stage II (pre­melanosomes), the organelle is oval and shows numerous
membranous filaments, with or without cross linking, having
distinct periodicity. In stage III, the internal structure
of the stage II melanosome becomes partly obscured by the
deposition of melanin. In stage IV, the oval organelle is
electron opaque without a discernible structure.

It is speculated that the earliest form of pre­melanosomes in human black hair are large spherical vacu­oles. The inner structure of these vacuoles consists of
amorphous structures of protein matrices which transform
into a filamentous or lamellar structure with a regular
TABLE 2
FORMATION OF MELANOSOMES AND BIOSYNTHESIS OF MELANIN

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Organelle Morphology</th>
<th>Biochemical Composition</th>
<th>Probable Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosome</td>
<td>100-150 A</td>
<td>RNA + Protein</td>
<td>Biosynthesis of tyrosinase and formation of subunits containing tyrosinase</td>
</tr>
<tr>
<td>Golgi Complex</td>
<td>0.05 μ</td>
<td>Phospholipid + Protein</td>
<td>Assembly of subunits and formation of unit membrane</td>
</tr>
<tr>
<td>Intermediate Vesicle</td>
<td>0.5 μ</td>
<td>Phospholipid + Protein</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td></td>
<td></td>
<td>Cross-linking of subunits and arrangement in a final characteristic structural form. Final product in albino melanocyte</td>
</tr>
<tr>
<td>Premelanosome</td>
<td>0.7 X 0.3μ</td>
<td>Phospholipid + Protein</td>
<td>Beginning of melanin formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ Tyrosinase + Melanin</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>0.7 X 0.3μ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanosome</td>
<td>0.7 X 0.3μ</td>
<td>Melanin without measurable tyrosinase activity</td>
<td>Melanin without measurable tyrosinase activity. Final product of melanocyte</td>
</tr>
<tr>
<td>Stage IV</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Typical values for human brown melanin granules

*From Seiji, 1967; Witkop, 1971
striation pattern. The specific topographic relation between the large vacuoles and Golgi apparatus suggests that premelanosomes may be evolved from the Golgi apparatus. However it is not certain whether they arise from the enlargement of small vesicles that pinch off from the cisternae of Golgi system or are contained and develop within a tubular smooth endoplasmic reticulum which is connected with the Golgi apparatus during melanogenesis (Jimbow and Kukita, 1971).

In studying the relationship of the Golgi apparatus to the formation of melanosomes in human cellular blue nevus, Allegra (1974) suggests that the process of melanogenesis varies in relation to the maturity and differentiation of the melanocytes in which it occurs. Thus, small melanosomes incapable of maturing into melanin granules are prevalently seen in embryonal melanocytes in which, characteristically, the Golgi apparatus is not present; whereas, the adult melanocyte contains both Golgi areas and maturing melanin.

With the maturation process, the spherical vacuoles of the earliest premelanosomes become elongated and the inner structures arrange in a regular fashion. These morphological sequences are similar in various melanocytes of different origins (Jimbow and Kukita, 1971).

There is no agreement about the morphology of the inner structure of premelanosomes from different origins.
Jimbow and Kukita (1971) demonstrated that in human black hairs however, the longitudinal sectioning of premelanosomes shows two types of inner structure: a filamentous and a lamellar structure. Both structures present the same striation pattern. The filamentous structures generally run parallel to the long axis; the lamellar ones are generally present in the center. They then join at the poles forming an oval shaped structure. In cross section the inner structures are observed as concentrically arranged dark lines.

The nature of the striation patterns along the lamellae is interpreted as representing the alignment of macromolecules of proteins (Jimbow and Kukita, 1971). Seiji (1967) suggests that the protein molecules consist not only of tyrosinase but also various proteins including other enzymes and constitutional protein moieties.

Melanin synthesis begins with the deposition of granular substances on the protein matrix of the lamellar structure. As the process of melanization continues, the lamellar structures thicken and become electron dense. Melanin deposition seems to continue until the spaces between the inner structures become filled. Melanin granules, or fully developed melanosomes, are generally described as electron-dense, amorphous bodies. However, when prepared under cytochemical conditions, melanosomes
appear to have a characteristic inner structure (Drochmans, 1967).

In their study on human black hair, Jimbow and Kukita (1971) using ultrasectioning (300-400Å in thickness), have found that two components can be distinguished in the fully developed melanosomes: the dense cortical shell or region, and the less dense central core. The central core consists of regularly folded lamellar structures disposed in the amorphous matrix. The cortical shell is present below the outer membrane of melanosomes and encloses the central core. It is composed of fine granular osmiophilic substances.

In addition to those two structures these investigators also found round electron-lucent structures. Serial sectioning suggests that they are globular shaped and measure about 400Å in diameter. Most of them appear to be attached to the outer layer of the central core and are protruding into the cortical shell. These globular bodies are also found in the melanosomes passed into keratinocytes and very rarely in premelanosomes (Jimbow and Kukita, 1971). Mottaz and Zelickson (1969) reported that some melanosomes of very dark brown hair display some small amount of a "moth eaten" effect which is absent in lighter brown hairs. This effect and the globular bodies observed by Jimbow and Kukita (1971) seem to be identical. The
nature and significance of these structures needs to be elucidated. A schematic representation of a fully developed melanosome is presented in figure 3.

Chemical Composition of Melanosomes

At the present time, the information available on the chemical composition of melanosomes, or melanin granules, is very limited. This is probably due to the intrinsic characteristics of melanins. Because of their insolubility in most solvents melanins do not permit a detailed study of the chemical nature of the melanin granules, unless it is done under very drastic conditions which may jeopardize the validity of the experimental results obtained under such conditions.

The presence of succinic dehydrogenase and cytochrome oxidase has been demonstrated in both mitochondria and melanin granules. Stein (1955) studied the chemical composition of melanin granules obtained from ox choroid and compared it to that of mitochondria. He found that melanin granules contain far fewer proteins than mitochondria and the content of iron was much higher in melanin granules than in mitochondria. Higher content of zinc (Stein, 1955; Seiji et al. 1963) and of copper (Stein, 1955) in melanin granules of ox choroid and Harding-Passey and B-16 mouse melanoma have been found in comparison to the metal content of mitochondria. Tyrosinase was
Figure 3. Schematic Representation of a fully developed hair melanosome. Note the disposition of electronlucent, globular bodies. (From Jimbow and Kukita, 1971).
localized mostly in melanosomes and not in mitochondria. No tyrosinase activity could be demonstrated in the mitochondrial fraction of mouse melanoma. These results demonstrate than melanin granules are different from mitochondria.

Takahashi and Fitzpatrick (1966) reported the presence of large amounts of dopa in the melanosomal fraction of Harding-Passey melanoma. In B-16 mouse melanoma, the content of dopa was very low. This suggests genetic differences in the melanosomal proteins of Harding-Passey and B-16 melanoma which could account for the characteristic difference in color of these tumours (Takahashi and Fitzpatrick, 1966).

Recently, the chemical compositions of hair melanosomes has been elucidated. Borovanský and Duchůn (1974) analyzed the chemical composition of human hair melanosomes; Hall and Wolfram (1975) reported studies on poodle and human hair, and hair and squid melamins. Hair melanosomes were highly melanized with a content of approximately 60% melanin in contrast to 30% for melanomas. In both studies (Borovanský and Duchůn, 1974; Hall and Wolfram, 1975), hair melanosomes contained all 18 amino acids, although the content of arginine, glycine and proline was higher than that in melanoma melanosomes; however, dopa was not detected (Borovanský and Duchůn, 1974). Hall and
Wolfram (1975) also reports that the amino-acid composition of squid melanin protein is similar to the other melanin containing tissues. On the basis of these results they suggest the existence of a specific protein in melanin containing tissues. This hypothesis is considered a speculation at the present time.

Duchon et al. (1973) completed a chemical analysis of melanosomes isolated from centrifugation of: Harding-Passey, B-16, Cloudman S91 and "Stanford" mouse melanoma homogenates, human and horse melanoma homogenates, chicken embryos' retinal pigment epithelium and ox choroid homogenates, the squid Loligo pealii ink sac and the Sepia Officinalis ink sac homogenates. Using acid hydrolysis (6N HCl for 24 hours) they found that melanin forms 18-72% of the total weight of the melanosomes, with respect to their source. The amount of protein recovered varied from 5-61% expressed as the sum of all amino acids recovered. The amino acid analysis revealed that the protein component of the ten different melanosomes studied consist of all known amino acids present in proteins. Also, 0.2-0.8% of 3,4-dihydroxyphenylanine (dopa) was found in most, but not all, of the melanosomes. Taurine (2-amino-ethane-1-sulphonic acid) was found in Loligo pealii (squid) and Sepia officinalis melanosomes hydrolysate.
The chemical composition of melanosomes seems to vary according to species. Duchón et al. (1973) also found that melanosomes from *Loligo pelaii* (squid) and *Sepia officinalis* have a very much higher content of alanine and much lower content of lysine than the other melanosomes examined. Ox choroid and "Stanford" mouse melanoma melanosomes were found to have a very high content of glycine and proline and very low content of leucine and valine. The level of lysine seems to be inversely proportional to the content of melanin.

According to these authors, the chemical composition of melanosomes may reflect genetic and species differences in the composition of melanosomal proteins in the course of phylogenesis, organogenesis and ontogenesis.

**Neuromelanin**

In man neuromelanin is sharply localized in three areas of the brain: The substantia nigra, locus ceruleus, and the dorsal nucleus of the vagus nerve, Adler (1939). The greatest quantities of melanin in human brain are found in the substantia nigra where most of the pigmented neurons are in the pars compacta. Less pigment is present in nearly all cells of the locus ceruleus but only a small fraction of the cells in the dorsal motor nucleus of the vagus are pigmented.
Substantia nigra is made up of medium size multipolar neurons which in the adult human brain contain sufficient melanin pigment to give the nuclear group its characteristic black appearance and the name. The pigment lies within cells in the living brain but it is often extracellular in postmortem or pathological material. It is also present in albino humans (Foley and Baxter, 1956). The relationship of this pigment to the function of the substantia nigra is not well understood.

The substantia nigra extends throughout the whole length of the midbrain from the upper border of the pons fibers into the caudal end of the diencephalon. Bazelon et al. (1967) in serial sections from an adult human brain have demonstrated pigmented neurons in the substantia nigra and its associated nuclei as well as in a column extending the entire length of brainstem from the mesencephalon to the nucleus retroambigualis. Histologically the pigment was characterized as melanin.

Substantia nigra is regarded as being functionally related to the basal ganglia and, through the basal ganglia, to the cerebral cortex as part of the arc important in regulating tonus and in stabilizing voluntary movements. It is significant that certain degenerative involvement of the basal ganglia such as occurs in Parkinson's syndrome is frequently accompanied by degeneration of the neurons in the substantia nigra (Crosby et al., 1962).
The pigment granules are usually dispersed throughout the cytoplasm of the cell and extend into the axon hillock and initial portions of the axon and less commonly in the dendrites. Melanin granules have not been described in neuronal processes remote from cell bodies nor in synaptic endings and are not apparent in glial tissues in normal brains in man (Marsden, 1969).

There are no reports of nuclear distribution of neuronal melanin in the brains of non-primate mammals. However, pigmentation in certain species such as the gorilla (Adler, 1942), cat and dog (Brown, 1943), and horse (Gillilan, 1943) has been reported. Within the primates there is a progressive increase in the intensity of pigmentation as the phylogenetic relation to man becomes closer and the maximum intensity is seen in man. It also appears that the intensity of pigmentation is related to the degree of evolution of the brain (Marsden, 1969).

Pigmented ganglion cells have been observed throughout the amphibian brain and there is an apparent increase in melanin nerve cells of some amphibians at metamorphosis (Adler, 1939).

The age at which pigment first appears in human neurons is somewhat controversial. Mann and Yates (1974) reported similar results to those obtained by Foley and Baxter (1958) who suggested that most cells of the locus ceruleus become pigmented at or shortly after birth and
are regularly pigmented at 18 months of age. Nigral neurons are rarely pigmented at birth and at 18 months of age are only slightly pigmented. In both substantia nigra and locus ceruleus, therefore, the onset of pigmentation appears to be associated with the beginning of regular activity within each nerve cell type. The intensity of pigmentation increases in both the locus ceruleus and substantia nigra until adolescence. Both nuclei have been said to be equally and maximally pigmented (Foley and Baxter, 1958; Fenichel and Bazelon, 1968) at a level which remains fairly constant throughout adult life. Moses et al. (1966) noted that the pigment content and character in the substantia nigra and locus ceruleus of brains at 20-30 years was comparable to brains of 55-85 year-old patients.

Mann and Yates (1974) report that from birth to 60 years of age the mean degree of melanin pigmentation of both the substantia nigra and locus ceruleus is proportional to the age of the person, although the amount of melanin present in the individual cells of a group at a given age may vary considerably. The linearity of the relationship indicates that melanogenesis is not a random but rather a constant feature of those cells.

Melanin granules first appear in the cytoplasm adjacent to the nucleus (Foley and Baxter, 1956; 1958).
At this site there is usually an indentation and condensation of the nuclear membrane. There is no suggestion that the initial site of melanin deposition is related to the Golgi apparatus, in contrast to the melanocyte system where the association is clearly established.

The ultrastructure of human neuromelanin in the substantia nigra and locus ceruleus have been reported by D'Agostino and Luse (1964), Duffy and Tennyson (1965) and Moses et al. (1966). Neuromelanin granules are identified by their high electron density and are composed of: a lipid globule, a finely granular matrix of medium density, and an electron-dense coarsely granular material (Marsden, 1969).

Similarities and differences between neuromelanin, lipofuscin and cutaneous melanin have been described (D'Agostino and Luse, 1964, Duffy and Tennyson, 1965, Moses et al., 1966, Van Woert and Ambani, 1974). A summary of the ultrastructure, histochemistry and spectroscopic characteristics of neuromelanin granules, cutaneous melanin granules and lipofuscin is presented in table 3.

Van Woert et al. (1967) compared spectroscopically the pigment isolated from human substantia nigra, dopa melanin, melanin-like pigments prepared by the incubation of monoamineoxidase with various neurotropic amines. Also, a visceral melanin from the liver of an amphibian
### TABLE 3

**ULTRASTRUCTURAL, HISTOCHEMICAL AND SPECTROSCOPIC CHARACTERISTICS OF NEUROMELANIN, CUTANEOUS MELANIN AND LIPOFUSCIN GRANULES**

<table>
<thead>
<tr>
<th>Property</th>
<th>Neuromelanin Granule</th>
<th>Cutaneous Melanin Granule</th>
<th>Lipofuscin Granule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ultrastructure</strong></td>
<td>Acid phosphatase</td>
<td>Acid phosphatase</td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td></td>
<td>Small lipid globule</td>
<td>No lipid globule</td>
<td>Large lipid globule</td>
</tr>
<tr>
<td></td>
<td>High and low electron density melanin</td>
<td>High electron density melanin</td>
<td>Low electron density melanin</td>
</tr>
<tr>
<td><strong>Histochemistry</strong></td>
<td>Lipid stains-</td>
<td>Lipid stains-</td>
<td>Lipid stains+</td>
</tr>
<tr>
<td></td>
<td>PAS-</td>
<td>PAS-</td>
<td>PAS+</td>
</tr>
<tr>
<td></td>
<td>Acid fast-</td>
<td>Acid fast-</td>
<td>Acid fast+</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2$+</td>
<td>$\text{H}_2\text{O}_2$+</td>
<td>$\text{H}_2\text{O}_2$-</td>
</tr>
<tr>
<td></td>
<td>AgNO$_3$+</td>
<td>AgNO$_3$++</td>
<td>AgNO$_3$-</td>
</tr>
<tr>
<td></td>
<td>Thionine-yellow</td>
<td>Thionine-green</td>
<td>Nile blue-blue</td>
</tr>
<tr>
<td></td>
<td>Nile blue-green</td>
<td>Pyrroles+</td>
<td></td>
</tr>
<tr>
<td><strong>UV fluorescence</strong></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*a From Van Woert and Ambani, 1974*
(Amphiuma tridactylum, Amphiuma: amphibia genus including only congo snakes) and lipofuscin from human cardiac muscle were examined and compared. They demonstrated that neuromelanin is different from melanins found in other parts of the body. However the substantia nigra pigment is a melanin. Lipofuscin also contains a melanin component, but unlike substantia nigra granules it also contains a large fluorescent lipid fraction. The melanin component may explain the electron microscopic similarities of this pigment to substantia nigra (Duffy and Tennyson, 1965; Moses et al., 1966).

In contrast to the synthesis of cutaneous melanin, practically nothing is known about the synthesis of neuromelanin. Since cells of substantia nigra are rich in dopamine (Andén et al., 1965) and dopamine is oxidized to form melanin both by auto-oxidation and by several different enzymes, the amine has been suggested to be a precursor of neuromelanin (Van Woert and Ambani, 1974).

In experiments with human substantia nigra, dopa and dopamine were recovered in the hydrolysate of neuromelanin (Nordgren et al., 1971). Since after hydrolyzing three times, 7 hours each time, no dopamine was found in the hydrolisate, the authors suggest that the primary nucleus of neuromelanin formed is dopa melanin, while the dopamine melanin forms a shell on the melanin particles.
With the aid of infrared spectrometry, Maeda and Wegman (1969) compared brain melanin to melanin synthesized from dopa, dopamine, norepinephrine and epinephrine. The studies point to norepinephrine as a possible precursor of neuromelanin. Van der Wende and Sporlein (1963) demonstrated the oxidation of dopamine to a melanin by albino rat brain in vitro. Rodgers and Curzon (1975) obtained melanin formation in brain homogenates using dopamine, dopa, norepinephrine, epinephrine, and 5-OH-tryptamine as precursors. No melanin formation was observed using $^{14}$C-tyrosine. They also found that melanin formation was highest in the substantia nigra.

Van Woert and Ambani (1974) and Rodgers and Curzon (1975) were unable to demonstrate tyrosinase activity in human substantia nigra. On the other hand monoamineoxidase (MAO) is suggested as the enzyme responsible for the synthesis of neuromelanin (Van Woert et al., 1967). Evidence against a major role of MAO in neuromelanin formation has been presented by Rodgers and Curzon (1975). The authors used $^{14}$C-tranylcypromine, a MAO inhibitor, and in vitro conditions for the argument.

Evidence in favor of peroxidase as the enzyme responsible for neuromelanin synthesis has been presented by Van Woert and Ambani (1974), who found the highest peroxidase activity in substantia nigra and the lowest
activity in the temporal cortex of human brain. Peroxidase activities in the substantia nigra and caudate nucleus are reduced in brains from Parkinsonian patients. On the other hand, Rodgers and Curzon (1975) could not obtain melanin from tyrosine and peroxidase, nor could they increase melanin formation from dopamine when homogenates from substantia nigra and pons were used. The same authors suggest a non-enzymic process of melanin formation. They indicate however, that brain may contain an enzyme which loses its activity between death and autopsy. On the other hand, tissue obtained 2.5 hours after death did not show more activity for melanin formation than tissue obtained 3 days after death.

It is clear from the evidence presented so far that both the precursor and enzyme responsible for the synthesis of neuromelanin remain to be established.

**Melanin Synthesis**

Melanin synthesis takes place in the melanocyte, specifically in the premelanosome. Tyrosine is the precursor, tyrosinase being the enzyme catalyzing the first two steps of the reactions, which take place in the presence of molecular oxygen (Lerner and Fitzpatrick, 1950). Tyrosinase is inhibited by copper chelating agents such as diethyldithiocarbamate (table 1). Compounds such as 8-methoxypsoralen increase melanin formation
presumably by increasing the activity of the enzyme (Lerner and Fitzpatrick, 1953).

Fig. 4 shows the most accepted melanin synthesis pathway. The steps in this pathway are: a) in the presence of tyrosinase and molecular oxygen, tyrosine is oxidized to dopa. This reaction is usually slow at onset, but after an induction period it proceeds very rapidly. This is an irreversible reaction; b) dopa is enzymatically oxidized by a reversible reaction to dopaquinone (dopaquinone thus formed may be reduced to dopa when a reducing agent such as ascorbic acid is present in the reaction). Further stages of the reaction proceed relatively rapidly in the absence of the enzyme although the reaction rates are increased in the presence of the enzyme; c) dopaquinone undergoes a spontaneous irreversible and rapid intramolecular change in which the nitrogen of the side chain attaches itself to the 6-position of the benzene nucleus with the formation of 5,6-dihydroxyindole-2-carboxylic acid (leucodopachrome); d) leucodopachrome is readily oxidized by a reversible reaction to the corresponding quinone (dopachrome). Dopachrome is a red substance ($\lambda_{\text{max}} = 305 \text{ m}\mu$ and $475 \text{ m}\mu$) and it is the first visible product formed in the reaction; e) under physiological conditions dopachrome decarboxylates and undergoes a rearrangement to form 5,6-dihydroxyindole ($\lambda_{\text{max}} = 275 \text{ m}\mu$ and $298 \text{ m}\mu$); f) the indole compound is rapidly oxidized to
Figure 4. The Raper-Mason pathway of melanin synthesis (Modified from Blois, 1971).
Figure 4
the corresponding quinone; g) the indole-5,6-quinone then polymerizes to melanin through polyindole quinone with the consumption of approximately one atom of oxygen. Relatively little is known about the mechanism of polymerization (Seiji, 1967).

Some modifications have been introduced in the Raper-Mason melanin synthesis pathway. Nicolaus (1962) suggested the direct incorporation of dopaquinone, dopachrome and 5,6-indole quinone-2-carboxylic acid into melanin.

Axelrod and Lerner (1963) suggested O-methylation plays a role in the conversion of tyrosine to melanin in vivo. The melanin synthesis pathway as suggested by these authors is shown in figure 5. Since patients with melanomas excrete increased amounts of homovanillic acid (Duchon and Gregora, 1962), Axelrod and Lerner (1963) suggested the possibility that patients with melanomas excrete increased amounts of O-methyldopa and the methoxyhydroxyindoles.

Misuraca et al. (1969) indicate that phaeomelanins are formed in vivo by a deviation of the eumelanins pathway involving a reaction between cysteine and dopaquinone produced by the enzymatic oxidation of tyrosine. Misuraca et al. (1969) proposed the pathway as shown in figure 6.
Figure 5. The site of action of catechol-O-methyl-transferase (COMT) and hydroxyindole-O-methyltransferase in the conversion of tyrosine to melanin.

(From Axelrod and Lerner, 1963)
Figure 6. Pathway of phaeomelanin synthesis
(From Misuraca et al., 1969; modified according to Thomson, 1974).
Figure 6
This pathway is consistent with the fact that both tyrosinase and dopaoxidase* activities of the enzyme have been detected in melanic and phaeomelanin hair follicles and feather papillae. A change in the cysteine content of melanocytes could explain the mechanisms leading to eumelanin or phaeomelanin (Misuraca et al., 1969). In this respect, Cleffmann (1964) showed that the increase in sulfhydryl content of melanocytes is one of the initial events leading to the production of phaeomelanins instead of eumelanins.

The presence of tyrosinase seems to be necessary only for dopaquinone formation, as in eumelanins synthesis. The divergence from eumelanin pathway follows immediately after dopaquinone formation and it is the presence or absence of cysteine which determines whether melanocytes produce eumelanins and/or phaeomelanins. Very little is known about the synthesis of phaeomelanins beyond the dihydrobenzothiazine stage (Thomson, 1974).

**Synthetic Melanins**

Synthetic melanins, as most natural melanins, are amorphous brown to black substances, obtained either by an enzymatic process or by auto-oxidation. These synthetic

*Tyrosinase and dopaoxidase activities refer to hydroxylation of tyrosine and oxidation of dopa, respectively by the enzyme tyrosinase (Seiji, 1967).
melanins generally result from the oxidation of phenolic compounds.

Mushroom tyrosinase is the enzyme generally used for synthesis of melanin. In our laboratory we have used peroxidase (horseradish) for the synthesis of melanin from dopamine.

The model systems for synthesis of melanins in vitro are generally set up to understand the synthesis of natural melanins. Most studies of melanin formation in vitro have been oxidative processes, enzymic or otherwise, such as the oxidation of 5,6-hydroxyindole, 3,4-dihydroxyphenylalanine, or 3,4-dihydroxyphenylethylamine. In all of these cases, the intermediate formation of indole-5,6-quinone, has been assumed. Bu'Lock (1961) studied the nature of the reacting species, in particular the extension to which oxidation precedes, accompanies or follows the polymerization steps in the formation of melanin from adrenochrome.

Melanins derived from 3,4-dihydroxyphenylethylamine, 3,4-dihydroxyphenylalanine and 5,6-dihydroxyindole in vitro, either enzymically or auto-oxidation, as well as from tyrosine enzymically in vitro have been studied by Swan and associates to a great extent (Swan, 1963).

According to Swan (1973) if the Raper scheme of melanin synthesis were correct, it would be expected that the same melanin would be obtained from tyrosine, L-dopa,
dopamine, and 5,6-dihydroxyindole. However, this does not seem to be the case. From experiments on the decarboxylation of melanin derived from $^{14}$C-dopa, Swan and Wagott (1970) concluded that the melanin might contain not only indole 5,6-quinone, indole-5,6-quinone-2-carboxylic acid and indoline-5,6-quinone-2-carboxylic acid units, but also units which had not undergone cyclization. In dopamine melanin the proportion of uncyclized units (approximately one in three) appears to be greater than in dopa melanin (Binns et al., 1970). The authors concluded that in dopamine melanin there must be a considerable number of polymeric linkages at positions other than those thought to be involved in dopa melanin.

From these results, no conclusive evidence for the structure of melanin is available. Several authors (Blois, 1973) agree that the evidence available at the present time points to the probability that dopa-type melanins, natural or synthetic, are irregular polymers built up from several types of units. Mason (1967) defends the hypothesis of a regular polymer of melanin.

According to Binns et al. (1970), however, some physical and chemical differences between melanins derived from dopa, dopamine, and 5,6-hydroxyindole have been observed. Auto-oxidation of dopamine results in a black precipitate, soluble in dilute sodium hydroxide solution,
while dopa melanin remained in the solution. The i.r. spectra of these melanins did not show sharp peaks, but the peaks were not identical.

Thathachari (1973) studied structure of melanin, both synthetic and natural using X-rays diffraction techniques. He concluded that there is a short range order at the molecular level in all melanins. The high density of the melanins is responsible for the observed contrast in the electron micrographs of melanin containing tissues. McGinnes and Proctor (1973) suggest that melanin is black because absorbed light is not reradiated, but is converted to rotational and vibrational degrees of freedom (i.e. heat) and also that the relatively featureless spectrum of melanins from the far ultraviolet to the infrared means that such transition is available for any energy photon between these limits.

As in natural melanins, free radicals have been detected in dopa melanin (Adams, et al., 1958) by electron spin resonance. Hegedus and Altschule (1970) synthesized soluble rheomelanins from several catechols in plasma. All the rheomelanins obtained showed the presence of free radicals as expected.
Free Radical Properties of Melanin

A chemical substance that has an odd number of electrons and is therefore usually highly reactive and unstable and cannot be isolated by ordinary methods is generally referred to as a free radical. In contrast, most chemical compounds have an even number of electrons and are stable (Pryor, 1970).

All melanins known so far in their original state have free radical properties. They are considered stable free radicals. The chemical and biological significance of free radical development during the formation of natural and synthetic melanins is unknown (Mason et al., 1960).

Correlation between pigmentation and free radical content has been demonstrated in several species. Whole white Calliphora puparia (bottle fly larvae) have no free radical content, whereas the black puparia gives an electron spin resonance signal. Also, in human hair samples of varied colors an increasing order of free radical content was observed. Reduction of the free radicals with ascorbic acid or H2S produces a decrease in the free radical content (Mason et al., 1960).

Van Woert et al. (1967) studied neuromelanin, isolated neuromelanin pigment, Amphiuma (snake) melanin,
lipofuscin and several synthetic melanins by comparing them by electron paramagnetic resonance, infrared spectroscopy, ultraviolet spectroscopy and fluorescence microscopy. The electron paramagnetic resonance revealed that there is no paramagnetic resonance absorption in the non-pigmented tissue of substantia nigra; however, the pigment showed the presence of a stable free radical species with a G-value of 2.005 and a line width of 11.7. The signal amplitude increases when the pigment is irradiated with visible light. *Amphiuma* melanin, dopa melanin and other synthetic pigments demonstrated similar characteristics of the electron paramagnetic resonance spectra. The line widths of the signals are greater prior to the removal of the protein by digestion, which seems to indicate that the synthetic melanins combine with the enzyme protein in the preparations so that the protein interaction with the unpaired electrons resembles that seen with the natural melanoprotein pigments. The free radical properties of human melanoprotein from hair and melanoma tissue obtained by Bolt (1967) are similar to those reported by Mason et al. (1960) and Van Woert et al. (1967).

Lipofuscin also showed a similar spectra with a G-value of 2.005, due probably to the insoluble pigment residue and it is not present in the lipid fraction (Van Woert et al., 1967).
A summary of electronparamagnetic resonance spectra from several melanins is presented in figures 7 and 8. The free radical property of melanin seems to be a property of the pigment itself since, as reported by Mason et al. (1960), there is no evidence of any detectable free radical in the intermediate products between 3,4-dihydroxyphenylalanine and indole-5,6-quinone, or formation of radicals during the enzymic transformation. Mason et al. (1960) ascribe the free radical property of dopa melanin to a semiquinoid form,

\[
\begin{align*}
\text{HO} & \quad \text{N} \\
\text{O} & \quad \text{H}
\end{align*}
\]

which must be greatly stabilized by the possibility of resonance throughout the highly conjugated polymer and by steric restrictions in reactivity.

The importance of the characteristics of free radicals of melanins comes to light when one considers it as a possible mechanism of interaction of melanin with drugs which, per se, are electron donors.

The interaction of melanins with drugs is well documented especially the interaction with chlorpromazine.
Figure 7. Electron paramagnetic spin resonance spectra of natural and synthetic melanins.

(a) Van Woert and Ambani, 1974; (b) Borg, 1972; (c) Mason et al., 1960
Figure 8. Electron paramagnetic spin resonance spectra of human hair melanin before and after ultraviolet irradiation (From Mason et al., 1960).
Figure 8

77 GAUSS    UV

HUMAN HAIR (BLACK)

HUMAN HAIR (BLONDE)

77 GAUSS    UV
Figure 9. Charge-transfer interaction model reaction for compounds having free radical properties. An electron from the highest filled orbital of the donor supposedly goes to the lowest empty orbital of the acceptor.
CHLORPROMAZINE$^-$ + MELANIN$^-$ → CHLORPROMAZINE$^+$ + MELANIN$^-$

DONOR

ACCEPTOR

Figure 9
Karreman et al. (1959) postulate that due to the characteristics of electron donor of chlorpromazine it may interact with electron acceptors by a mechanism called "charge transfer." In "charge transfer" complexes an electron supposedly goes from the highest filled orbital of the donor to the lowest empty orbital of the acceptor (see figure 9). This transfer does not involve configurational change. Karreman et al. (1959), then support the hypothesis of a charge-transfer mechanism for drugs acting in the central nervous system since they also found that d-lysergic acid diethylamide and serotonin are very good electron donors. On the other hand, Borg (1965) studied the formation of free radicals of imipramine. His results led him to speculate that at least part of the pharmacological actions shared by phenothiazines and structurally related drugs may stem from their transformation to free radicals in vivo.

Bolt and Forrest (1968) suggest that, in vitro, melanoprotein accepts an electron from chlorpromazine to form a diamagnetic species; i.e., reduced melanoprotein, therefore melanoprotein participates in a complete charge-transfer reaction. These results are supported by Van Woert (1968) experiments demonstrating that NADH is oxidized in vitro in the presence of melanin. This NADH oxidation is inhibited by phenothiazines, which form
charge-transfer complexes with melanin, which indicates that this oxidation is most likely related to the free radical properties of melanin.

**Binding of Drugs to Melanin**

Potts (1962, 1964) was the first to observe binding of drugs to pigmented tissues. He found that phenothiazines were concentrated in the pigmented structures of the rabbit eye, with little or no retention in corresponding structures of the eye of albino animals (Potts, 1962).

Although Potts' experiments demonstrated binding of drugs to melanin in vivo and in vitro, the influence of pigmentation on the distribution and effects of drugs was first suggested by Chen and Poth (1929). They observed that racial differences influence the mydriatic action of several drugs. The initial mydriatic effect of cocaine, euphthalmine, \( l \)-ephe drine, \( dl \)-ephe drine, and \( d \)-pseudo- ephe drine was more marked in Caucasians, while the drugs were less effective in Negroes. These results were supported by Barbee and Smith (1957) who found ephedrine to be ineffective as a mydriatic in Negroes, while it was equally effective in Caucasians with blue or light brown eyes.
Angenent and Koelle (1952, 1953) assumed that the differences in drug effects in pigmented and nonpigmented eyes had an enzymatic basis since a tyrosinase system capable of transforming catecholamines to melanins is found in the nonalbino rabbit eye but not in the albino rabbit eye. They suggested that the continuous and rapid destruction of the adrenergic neurotransmitter by the enzyme would reduce the effectiveness of drugs such as ephedrine which produce their effects through the release of the chemical mediator.

Subsequently, Obianwu and Rand (1965) suggested that the poor mydriatic effect of ephedrine in pigmented eyes may be due to a lower content of norepinephrine in these irides, probably because of the diversion of catecholamine precursors to melanin synthesis. These authors also suggested that the adrenergic innervation in the pigmented eye is poorer than in the nonpigmented one.

Seidehamel et al. (1970) showed that nonpigmented guinea-pig irides are more sensitive to ephedrine mydriatic effects than the pigmented ones. Patil et al. (1974) found that (-)-$^{14}$C-ephrined accumulates in greater amounts in pigmented guinea-pig irides than in nonpigmented ones. The drug disappears faster from the nonpigmented than from the pigmented iris.
The differential binding of cocaine in pigmented and nonpigmented guinea-pig eyes was also observed. The binding of (±)-$^{14}$C-cocaine is inhibited in the presence of (-)-$^{14}$C-ephedrine, but it is not affected by chemical sympathectomy or reserpine (Patil, 1972).

Using autoradiographic studies, Lindquist (1973) demonstrated binding of epinephrine, dopamine, norepinephrine and serotonin to melanin granules in vitro, while L-dopa and L-tyrosine were not. $^{14}$C-labelled catecholamines and serotonin bound superficial cervical lymph nodes and melanin structures in the pigmented mouse in vitro, whereas no accumulation was observed in albino mice.

Jacquot et al. (1974), in studies on guinea-pigs found that forty hours after intravenous administration of (±)-ephedrine-$^{14}$C, intense accumulation of the drug could be seen autoradiographically in pigmented tissues such as choroid and skin. In vivo incorporation of (±)-amphetamine-$^{14}$C (Harrison et al., 1974a) and of L-dopa-$^{3}$H, L-$\alpha$-methyldopa-$^{3}$H and (±)-isoproterenol-$7^{3}$H (Harrison et al., 1974b) into melanin and nonmelanin components of pigmented and albino guinea-pig hair was studied. Incorporation of the drugs into melanin and nonmelanin fractions was demonstrated.

Wepierre et al. (1975) tried to correlate the mydriatic effect of several sympathomimetic amines to the pigmentation of the eye. In two different strains of
rats, using autoradiography, they found that the non-phenolic amines: (±)-ephedrine-$^{14}$C, (±)-norephedrine-methyl-$^{14}$C and (+)-amphetamine-7-$^{14}$C, had a high affinity for the pigmented tissues in pigmented rats. Tyramine, a phenolic compound, did not accumulate in the pigmented tissue. The nonpigmented eyes of albino rats did not accumulate any of the drugs. The authors suggest that in vivo, the accumulation of α-methylated sympathomimetic amines in pigmented tissues is related to the rate of metabolism of the drug. In in vitro experiments, Patil et al. (1974) demonstrated binding of tyramine to pigmented iris, but in this case a monoamineoxidase inhibitor was used.

The relationship between metals and melanin has been known for some time. Cotzias et al. (1964) and Cotzias (1974) have emphasized the importance of this relationship in the central nervous system. Potts and Au (1971) found that 129 hours after intramuscular administration of $^{204}$Thallium, the metal had accumulated in the uveal tissues of pigmented rabbits. No evidence of accumulation in cornea, aqueous, vitreous, sclera or blood was found. The accumulation seemed to increase with time, probably at the expense of other tissues.
There are very few reports on the binding of catecholamine pigmented tissues in the brain, in spite of the fact that the striking similarity of the distribution of neuronal melanin and that of the brain catecholamines (Bazelon, et al., 1967) indicates some relationship between neuromelanin and the presence of dopamine norepinephrine in the nerve cells. Yshii and Friede (1968) have reported accumulation of tritiated norepinephrine in pigmented nerve cells in human substantia nigra, in vitro. The authors concluded that the binding occurred at the surface of the cells since no binding was noted in the cytoplasm and on the melanin granules. In contrast to these results, Lindquist (1973) observed selective binding of $^3$H-dopamine and $^3$H-norepinephrine to melanin granules in the pigmented cells of human substantia nigra in vitro. No accumulation of radioactivity was seen in the cell membrane of pigmented cells. It is possible that differences in the thickness of the tissue sections used by each investigator may account for the differences in the results.

The antimalarial drugs, chloroquine and quinine, are well known for their ototoxic properties. Potts (1964) showed that quinine binds in vitro to uveal melanin granules. Lindquist and Ullberg (1972) found a high accumulation of $^{14}$C-chloroquine in the inner ear of fetuses from pregnant pigmented mice whereas no accumulation was found in the inner ear of albino fetuses following
administration of the drug by intravenous injection to the pregnant mouse.

The toxicity of aminoglycoside antibiotics to the inner ear has been the subject of numerous studies. The rate and extent of accumulation of these drugs in inner ear fluids (endolymph and perilymph) has been studied in guinea-pigs (Stupp et al., 1973). Evidence has been provided that the effect is due to the affinity of the antibiotics for melanin present in the cochlea, mainly in the stria vascularis, one of the sites where endolymph is thought to be produced (Dencker et al., 1973).

Dencker et al. (1973) have found serious damage of the stria vascularis of young pigmented guinea-pigs treated with kanamycin, but not in albino animals. In normal animals melanin granules are evenly distributed in the strial cells; in kanamycin treated animals the granules were aggregated.

In in vitro experiments using bovine iris melanin granules, Lindquist (1973) has shown that of the ototoxic drugs, kanamycin has the highest affinity for melanin, followed by chloroquine, quinine, dihydrostreptomycin, streptomycin and viomycin. Salicylic acid, a drug which may cause temporary hearing loss did not show affinity for melanin. Experiments conducted in rats and guinea-pigs to demonstrate the influence of pigmentation on the ototoxicity of kanamycin and neomycin indicate that pigmented
animals may be more likely to suffer hearing impairment following ototoxic drug administration, however this has not been confirmed so far (Harpur and D'Arcy, 1975).

The importance of the affinity of phenothiazines and 4-aminquinolines in the etiology of toxic retinopathy is well established. Potts (1962, 1964) was the first to demonstrate the binding of these as well as of several other polycyclic compounds to melanin in vitro. Potts (1962) studied the binding of phenothiazines in the eyes of pigmented and nonpigmented rabbits following systemic administration. He found selective accumulation of substituted phenothiazines in the uveal tract, the pigmentation being a very important factor for the accumulation. Interestingly no accumulation of the parent compound, phenothiazine, could be demonstrated.

In other experiments Potts (1964) demonstrated the binding of several polycyclic compounds to melanin granules in vitro. He also demonstrated that the accumulation is a function of melanin per se, because treatment of the granules with proteolytic enzymes did not modify the binding of the polycyclic compounds and also, binding of similar characteristics are observed with synthetic melanins. Changes in pH or salt concentration did not modify the binding of the drugs to melanin.
Blois (1965, 1968) demonstrated the binding of $^{35}$S-chlorpromazine and of iodoquine to melanin containing tissues in melanoma-bearing C3H mouse. $^{35}$S-chlorpromazine achieved tissue concentrations at equilibrium in proportion to the melanin content of the tissue. Blois and Taskovich (1969) determined the selectivity of the binding of drugs to melanins, comparing it to the binding to other bio-polymers using a dialysis procedure. The ratios (ratio of radioactivity remaining in the dialysis bag to that remaining in the control after 50 hours of dialysis) of binding of drugs to desoxyribonucleic acid, ribonucleic acid, albumin, lipoprotein and melanin were determined. Drug binding ratios were consistently higher for melanin than for the other polymers used.

Lindquist and Ullberg (1972) and Lindquist (1973) have reported the most complete studies on the binding of drugs to melanins. Lindquist and Ullberg (1972) using autoradiographic methods to study the melanin affinity of chloroquine and chlorpromazine in albino and nonalbino mice demonstrated that $^{35}$S-chlorpromazine was localized in several tissues including the melanin structures of the eye, Harder's gland, superficial cervical lymph nodes, skin, hair follicles, and brain. Accumulation in the brain decreased rapidly, but levels of the drug were high in melanin structures 90 days after injection. The drug was also accumulated in the fetal eye.
They showed that levels of $^{14}$C-chloroquine in melanin structures were constantly high 90 days after injection, and, surprisingly, the concentration of the drug in the uveal tract was high even one year after administration of the drug.

A leucocytic melanin transport through the body in normal Bantu negroes has been described (Wasserman, 1965). Satanove (1965) and Wasserman (1965) suggested that phenothiazine-induced general melanosis is caused by the leucocytic transport of melanin from the hyper-pigmented skin to the viscera. High accumulation of $^{35}$S-chlorpromazine, or $^{14}$C-chloroquine was found in the superficial cervical lymph nodes of pigmented mice after injection of the drugs. No accumulation was found in the corresponding lymph nodes of albino mice (Lindquist and Ullberg, 1972). The accumulated drugs in the lymph nodes seems to be located in melanosomes present in the nodes as demonstrated histologically and by electron microscopy by Lindquist (1973).

At the present time there is very little known about binding of drugs to pigmented tissues in the brain. The lack of information probably is due to the fact that neuromelanin is not found in small laboratory animals such as guinea-pig, mouse, rat or rabbit (Marsden, 1961). A very important finding in this respect was reported by
Christensen et al. (1970) who found degeneration of pigmented nerve cells in the substantia nigra of patients with phenothiazine-induced tardive dyskinesia.

Lindquist (1972, 1973) using autoradiography demonstrated in vitro uptake of $^{35}$S-chlorpromazine in the pigmented neurons of human substantia nigra and locus coeruleus. These results emphasize the importance of above noted finding (Christensen et al., 1970). However, Lindquist's experiments failed to show selectivity of the binding to the pigmented tissue since there was no comparison of the binding of the drug to nonpigmented tissues of the brain.

The affinity of phenothiazines and 4-aminoquinolines for melanins is generally accepted as the most important factor in the etiology of the toxic retinopathy caused by these drugs. Chloroquine and chlorpromazine induced pigmentation disorders of the skin have also been related to accumulation of drugs in skin melanin (Satanove, 1965; Stewart et al., 1968). Most of the ocular changes have been associated with the intake of NP-207, thioridazine, chlorpromazine and trifluoperazine (Sidall, 1967).

The first reports on retinotoxicity of a phenothiazine originated from a clinical test of piperidyl-chlorophenothiazine (NP-207) (Goar and Fletcher, 1957).
There are several reports on toxic chorioretinopathy in man after thioridazine treatment, (Scott, 1963; Sidall, 1966, Cameron et al., 1972). Although it is the most used phenothiazine, very few reports on chlorpromazine retinopathy have been published. However, chorioretinopathy in patients treated with chlorpromazine has been reported (Sidall, 1966). Investigations comparing the effects of chlorpromazine, thioridazine and NP-207 in the cat have demonstrated that although chlorpromazine content can be increased 15 times in the retina (relative to calculated equal distribution in the whole body), no retinotoxicity could be observed; NP-207 is highly retinotoxic with only a 10 times relative accumulation in the retina (Meier-Ruge et al., 1966). Legros et al. (1971) demonstrated chlorpromazine induced modifications of the electroretinogram and retinal histologic lesions in the dog. Electroretinogram changes with no histological lesions in albino rats and no changes in retina in pigmented rats were shown by Legros et al. (1973). The authors suggest that the ocular toxicity of chlorpromazine is not due to its affinity for melanin.

Many patients treated with phenothiazines develop what is called the eye-skin syndrome, which is described as a combination of pigment deposits in the eye and hyperpigmentation of the skin, localized mainly in the face, arms, hands and legs. The color of the affected areas is
described as diffuse violet to dark blue-black (Hays et al., 1964). The identity of the pigment has not been established, but the formation of melanin or a melanin-like substance has been suggested. Perry et al. (1964) indicated that the pigment may be a metabolite of chlorpromazine. Although Bolt and Forrest (1968) support the concept of chlorpromazine metabolism and hyperpigmentation of the skin, they consider that ocular opacities have a different but yet unexplained origin. Based on electron-microscopic studies, Zelickson (1965) reported that the pigment is not due to true melanin. He suggested that it is a drug metabolite, pseudomelanin, or some other form of pigment.

Howard et al. (1969) induced experimental cataracts with chlorpromazine in both albino and nonalbino guinea-pigs. The authors concluded that chlorpromazine cataracts do not appear to be related to the presence of melanin in the eye. The phenomenon according to them may represent a foci of denatured protein resulting from the interaction of light with the drug, a photosensitizing agent, and lens protein, or possible deposits of the drug within the lens. In chlorpromazine treated drugs, Tousimis and Barron (1970), demonstrated the presence of granules within the cytoplasm of corneal stromal cells that seem to be associated to the prolonged oral administration of the drug. Similar structures were not
found in untreated dogs.

In patients with chlorpromazine induced general melanosis the pigment has been found to be deposited throughout the reticuloendothelial system and viscera. The identity of the pigment has not been established. Greiner and Nicolson (1964) suggested that it is a melanin because of its histochemical reactions. Satanove (1965) suggested that the phenothiazine induced general melanosis is due to leucocytic transport of the pigment from the hyperpigmented skin to the viscera. This suggestion is supported by Wasserman's (1965) demonstration of a leucocytic melanin transport system which was found to be a normal phenomenon in Bantu negroes.

Arons et al. (1968) demonstrated lack of melanin pigmentation in the skin of an albino psychiatric patient treated with chlorpromazine in high doses. Dopa and tyrosinase activity were observed within the epidermis. Electron microscopic studies demonstrated that in epidermal melanocytes with nonpigmented melanosomes the response as well as the metabolism of the drug were normal. It is very interesting that in the study reported by Zidek and Janku (1971) albino mice were found to be less resistant than pigmented ones to the toxic effects of chlorpromazine and isoniazide.
It is a well recognized fact that the extrapyramidal disorders produced by neuroleptic drugs or encephalitic processes have in common the involvement of the pigmented centers or the midbrain, the neuromelanin of which has been altered within the cells or displaced from them (Forrest, 1974). When pigmented neurons degenerate, their melanin granules are released and may be seen lying free in the tissue or within phagocytes. Such changes occur irrespective of the cause and represents the outcome of cell death. The system of pigmented neurons appears to be specifically damaged in Parkinsonism (Marsden, 1969).

Decrease in the very dense component of melanin granules in substantia nigra (Duffy and Tennyson, 1965), decrease in the number and melanin content of cells in the substantia nigra (Pakkenberg and Brody, 1965), and quantitative reduction of melanin pigment in substantia nigra (Roy and Wolman, 1969) of Parkinsonian patients has been reported. Hornykiewicz (1973) demonstrated a reduction of dopamine concentration in the corpus striatum of Parkinson's disease patients. The change appears to result from destruction of the substantia nigra with loss of dopamine containing nigrostriatal fibers (Poirier and Sourkes, 1965). According to Marsden (1969) the appearance of Parkinson's syndrome during the treatment with
α-methyldopa, reserpine and phenothiazines relates to the ability of these drugs to interfere with adrenergic mechanisms in the brain.

As mentioned above, extrapyramidal disorders are known to be the most common adverse effects of phenothiazines. The main extrapyramidal disturbances caused by phenothiazines are akathisia, dyskinesia, and Parkinsonism. The mechanism responsible for the development of phenothiazine induced extrapyramidal symptoms is not known. Forrest et al. (1963) found depigmentation in substantia nigra and cell degeneration with neurophagia most pronounced in the putamen in the brain of a patient who had been treated for several years with trifluopromazine and chlorpromazine. Christensen et al. (1970) in a study of 28 brains from dyskinesia patients, of which 21 had neuroleptic drug induced dyskinetic symptoms, found cell degeneration in substantia nigra in 96% of the cases, whereas only 25% of the control cases showed histological changes.

In analyzing the origin of extrapyramidal diseases, Forrest (1974) concludes that neuroleptic drugs, and especially chlorpromazine, among the phenothiazines, have a special affinity and attach themselves to neuromelanin of the extrapyramidal neurons producing charge-transfer complexes. The resulting compounds are bioelectrically inert and lose the electron trapping function of melanin.
Histopathological autopsy findings on dyskinetic and drug treated patients seems to confirm the causative relationship between drug induced extrapyramidal disorders and the neuromelanin of the pigmented subcortical nuclei. Chlorpromazine-melanin complexes are eventually expelled as foreign bodies from the neuron, which itself may disintegrate in the process.

**Pathophysiological Significance of Natural Melanins**

Although melanin itself is considered by many as only a protecting agent against ultraviolet radiation, when one considers the abnormalities of pigmentation, melanin assumes a different dimension. Most of the disorders of pigmentation are genetically determined. Phenylketonuria and albinism are two of the most important biochemical disorders of pigmentation.

In phenylketonuria there is a reduction of phenylalanine hydroxylase, thus, the conversion of phenylalanine proceeds at a very low rate. The condition is inherited as a recessive trait, is characterized by decreased pigmentation of the hair and eyes and, if untreated, becomes associated with mental deficiency (Jervis, 1937). It is thought that phenylalanine, not being converted to tyrosine, may act as tyrosinase inhibitor causing a reduction of hair and eye pigmentation together with lessened
pigmentation in other locations such as the substantia nigra (Fitzpatrick et al., 1961). It has also been suggested that phenylalanine acts as repressor of tyrosinase synthesis and that the hypopigmentation of phenylketonuria is a combination of a repressor and a competitive inhibitor effect of phenylalanine (Riley, 1974). Changes in the activity of tyrosinase, however, do not explain the paradoxical depigmentation of the substantia nigra in phenylketonuria.

Albinism is due to a genetic defect in which tyrosinase is in an inactive form. The structural gene for tyrosinase appears to be located at the C locus, is responsible for the production of a tyrosinase with reduced activity (Coleman, 1962; Witkop, 1971). Hearing (1973) suggested that the C locus may be regarded as regulatory rather than a structural locus for tyrosinase.

There are two types of oculocutaneous albinism described so far. Tyrosinase-negative oculocutaneous albinism, characterized by the presence of nonmelanized melanosomes within melanocytes and lack of histochemically demonstrable tyrosinase activity. As in the albino mouse, human tyrosinase-negative oculocutaneous albinism appears to involve a defect in tyrosinase. On the other hand, tyrosinase-positive oculocutaneous albinism in man is defined by the existence of partially melanized melanosomes
within melanocytes and evidence of tyrosinase activity within hypomelanotic hair bulbs incubated in tyrosine-containing solutions. This type may result from limitations in the availability of tyrosine within melanosomes (Witkop, 1971).

Pathological disturbances of pigmentation may be classified as conditions associated with an increase in pigmentation and those in which there is a loss of pigmentation.

Hyperpigmentation includes: photosensitivity, acanthosis nigricans, urticaria pigmentosa; hyperpigmentation associated with hormonal disturbances involving ACTH and MSH, with mast cell tumors (mastocytosis) and with schizophrenia in patients not treated with phenothiazines (Riley, 1974).

According to Lerner and Fitzpatrick (1950) certain vitamins are involved in melanogenesis and skin pigmentation may result from deficiencies of vitamin A, nicotinamide and ascorbic acid. The hyperpigmentation may result from a decrease in sulfhydryl groups which, on the other hand, act as tyrosinase inhibitors (Seiji et al., 1969).

Hypomelanosis may result from: a reduction in the number of pigment producing cells; a reduction in the efficiency of melanin biosynthesis; a reduction in the transference of pigment from melanocytes to keratinocytes.
or a rapid loss of melanin from the epidermis (Riley, 1974). Depigmentation can be obtained by using chemical agents such as hydroquinone monobenzyl ether, p-hydroxy-anisole and other phenols.

Frenk et al. (1968) have reported data which suggests that mercaptoethylamines, 2-mercaptomethylamine (cysteimine) and 2-mercaptoethyl-diethylamine, destroy melanocytes. Although no explanation for the mechanism of this action has been offered, it is suggested (Riley, 1974) that these sulfhydryl compounds reduce the ability of the cell to inactivate potentially dangerous free radicals as a consequence of the inhibition of melanin synthesis since melanin seems to act as free radical scavenger because of its free radical properties.

Development of tumors in the melanocyte system is also well known. There are benign tumors such as lentigo, Hutchinson's freckle, cellular naevi and juvenile melanoma, and the malignant tumors, malignant melanoma and xeroderma pigmentosa. The discussion of nature and characteristics of these tumors is beyond the scope of this paper.

Other pathological problems related to melanin involve metals. The importance of metals in extrapyramidal areas is indicated by the association of extrapyramidal disturbances to abnormalities of copper
metabolism in Wilson's disease and manganism. In patients with Wilson's disease there is accumulation of copper in the liver. Also, Leu et al. (1970) described hyperpigmented lesions on the lower portion of the legs in patients with Wilson's disease. Histological studies showed that the lesions were due to excessive melanin deposition rather than to the presence of copper or iron. The etiology of the increase in melanin pigment is unknown. The deposition of copper in the brain in Wilson's disease (Cummings, 1968) and the therapeutic effects of chelating agents upon the extrapyramidal symptoms suggest that the extrapyramidal abnormalities are related to copper (Curzon, 1975). Neurological disturbances, mostly of Parkinsonian type have been described in chronic manganism (Mena et al., 1967).

Lack of wool pigmentation has been found in copper deficient sheep. The phenomenon has been attributed to the inhibition of tyrosinase activity resulting from copper deficiency (Underwood, 1971).

In relation to heavy metals, melanosis has been associated with prolonged use of medicaments containing arsenic, bismuth, gold or silver. The mechanism of melanosis seems to be related to binding of the metals to sulphhydryl groups, eliminating the inhibitory action of these groups on the activity of tyrosinase. An increase
in melanin within the basal cell layer and dermal melanocytes is generally observed (Lorincz, 1954). Irradiation seems to increase this pigmentation since dark areas are limited to the exposed skin (Molokhia and Portnoy, 1973).

Bleaching of the skin can be obtained by using cosmetic creams containing mercury. This is thought to be due to replacement of copper by mercury in tyrosinase and subsequent inactivation of the enzyme (Lerner, 1952). Hyperpigmentation however may result, probably as a consequence of binding of sulfhydryl groups by mercury (Burge and Winkelmann, 1970). A typical Parkinson's syndrome has occasionally been described in mercury poisoning (Cohen, 1962).

The preceding information reveals that melanin is not just a protecting shield against actinic radiation. Disturbances in melanin biochemistry result in phenylketonuria, for example, a serious problem that may cause mental retardation. The extrapyramidal syndrome as well as pigmentation disorders associated with drugs or metals are serious enough to deserve more attention not only from a clinical, but also from a pharmacological and toxicological point of view.
STATEMENT OF THE PROBLEM

It is obvious from the foregoing introduction that many aspects of melanin chemistry and biochemistry are still unexplained. There is very little information about the pharmacological and toxicological significance of the presence of melanins in the body.

Because of the broadness of the field it was decided to limit this study to only certain aspects of the interactions of drugs with melanins. These interactions may relate to the development of drug-induced side effects in melanin bearing tissues.

It has been known for many years that atropine and atropine-like drugs, when applied to heavily pigmented eyes, exhibit unusually slow onset of mydriatic and cycloplegic effects. Although atropine is classified as a competitive reversible blocker of the muscarinic receptor, there is no satisfactory explanation for the pigment-dependent initial slow effect and the long duration of its mydriatic and cycloplegic effects.

The appearance of the extrapyramidal syndrome in patients who have undergone prolonged treatment with phenothiazines and butyrophenones or who have been exposed to
toxic quantities of metals such as manganese is also well known. Although the possibility of neuromelanin involvement has been suggested, there is very little evidence reported so far, that the drugs or metals involved have affinity for neuromelanin.

On the other hand, the ability of phenothiazines and 4-amino-quinolines to bind melanins and, as a consequence, to produce hyperpigmentation of the skin and retinopathy are well recognized. However, the quantitative characteristics of the binding of drugs to pigmented tissues, as well as the possibility that other drugs with the same pharmacological activity but with less affinity for melanin could be used as substitutes for the most toxic ones have not been explored so far.

Therefore, the object of this study is to analyze some aspects of the binding of drugs to melanins in order to determine the following:

a) Relation of the prolonged mydriatic effects of atropine to pigmentation of the iris.

b) Character of binding of phenothiazines and related drugs to human brain pigmented and nonpigmented tissues in order to gain information about the selectivity of binding of drugs to brain tissue.

c) Capacity and affinity constants for binding of phenothiazines and related drugs to melanin granules
as compared to synthetic melanins, both in the absence and presence of fluphenazine, thioridazine, haloperidol and clozapine.

The results of these experiments may help to explain the prolonged atropine mydriatic effects and contribute to an understanding of the relationship between the presence of melanins in certain tissues and the localization and persistence of side effects produced by some drugs.
CHAPTER II

METHODS AND MATERIALS

Experimental Animals

Albino and nonalbino rabbits, of either sex, weighing 2.3 to 4.0 Kg were used. The strains used were New Zealand White rabbits (Kings Wheel Rabbitry, Centerburg, Ohio) and New Zealand Black rabbits (Three Springs Kennels, Zelinopoe, Ohio). The animals were housed in individual cages and were allowed free access to food (Purina rabbit chow) and water. A few days after arrival, the rabbits were classified according to the absence or presence of atropinesterase (atropinase).

Bovine Iris Melanin Granules

Bovine eyes were obtained on ice from a local slaughter house (Ohio Packing Co., Columbus, Ohio) and on the same day tissues were processed for the granules.

Human Brain

Post mortem adult samples of midbrain from adult humans containing substantia nigra and superior cerebellar peduncles and frontal cortex were obtained from the county morgue usually within 24 hours after death. The tissues
were carried on ice to the laboratory and kept frozen until used, usually for 5 to 10 days. Samples were taken from individuals with no apparent pathological brain lesion or record of drug medication at the time of death.

**Experimental Procedures**

**Qualitative test for atropinesterase**

Serum atropinesterase was determined by a slight modification of the method of Van Zuphten (1972). Blood was withdrawn from the medial ear artery and allowed to settle at room temperature; then it was stored in cold (3-5°C) up to 24 hours. The resulting serum was treated for atropinesterase activity in the following way.

Agar-cresol red media was prepared by adding 2.5 ml of 0.2% cresol red solution per each 100 ml of aqueous 1.5% solution of agar-agar (Bacto-Difco AgarR) at 65°C. The pH was adjusted to 8.0 with 0.1 N NaOH. The mixture was placed in Petri dishes and allowed to solidify. Wells of approximately 6 mm in diameter were cut with a cork borer and the agar plugs removed. A 2% atropine sulfate solution was prepared and pH adjusted to 7.5 with 0.1 N NaOH. Twenty five μl of serum were placed in each well followed by the addition of 25 μl of atropine sulfate solution. For the control experiments, 25 μl of serum heated at 65°C for one hour were used. The Petri dishes were then incubated at 37°C until a yellow color around the bottom of the well
appeared or for a maximum of four hours. Atropinesterase degrades atropine to tropine and tropic acid; the latter metabolite alters the alkaline pH of the medium to an acidic one. Thus, a change from red to yellow color around the bottom of the well was indicative of the presence of atropinesterase in the serum.

Binding of $^3$H-atropine by Tissues

Rabbits were killed by injecting air through the ear marginal vein and the irides were immediately dissected as described by Jacobowitz (1967). Tissues were rinsed twice in 5 ml each of cold physiological salt solution. Each iris was cut into two equal pieces and each piece was placed in an oxygenated physiological salt solution at 37°C. The composition of the solution in mM was: NaCl, 118; KCl, 4.7; CaCl$_2$, 2.5; MgCl$_2$, 0.54; NaH$_2$PO$_4$, 1.0; NaHCO$_3$, 25; Glucose, 11. The chemicals were dissolved in demineralized double distilled water.

After an initial equilibration period of 15 minutes, the tissue was incubated with known specific activity of $^3$H-atropine for a fixed time, then rinsed to remove excess radioactivity with two volumes of 5 ml each of cold physiological salt solution. The tissue was blotted on filter paper, weighed and homogenized twice in 1 ml each of 0.4 N perchloric acid. The combined homogenate was centrifuged at 43,500 Xg, 0-4°C, for 30 minutes in a Sorvall Model
RC2-B centrifuge. The radioactivity in the supernatant was counted in 12 ml of Aquasol (New England Nuclear) by liquid scintillation spectrometry in a Packard Tri-Carb Model 3375. Quenching was monitored by automatic external standardization. The counting efficiency for the tritium varied between 21 and 23%. The uptake of $^{3}$H-atropine by human iris and pigment epithelium was similarly studied.

When the binding of $^{14}$C-imipramine to rabbit iris was studied, the irides were divided in four pieces and each piece was incubated with the drug for a fixed time. Binding of the drug to the tissue was determined as described above.

Loss of radioactivity from tissues

The rate of loss of $^{3}$H-atropine from rabbit iris and stomach fundus strips and of $^{14}$C-imipramine from rabbit iris was determined by transferring the tissue every five minutes to a fresh oxygenated solution maintained at 37°C. At the end of the designated time the radioactivity retained by the tissue was determined as described above.

Ocular penetration of $^{3}$H-atropine

Rabbits were anesthetized with pentobarbital sodium, 30 mg/Kg, i.v., and 0.1 ml of 2% atropine solution containing $5.32 \times 10^{5}$ dpm/0.1 ml, was placed in the left eye. At the end of the designated period, the eye was blotted to remove excess radioactivity. The animals were
killed by injecting air through the ear marginal vein, the eye was quickly dissected and rinsed three times in volumes of 10 ml each of cold physiological salt solution for 5 minutes. The aqueous humor was withdrawn and the radioactivity was counted in Aquasol. The iris was isolated and the radioactivity in the tissue was determined as described above. The radioactivity in aqueous humor and iris from the contralateral eye was also determined for control purposes.

Pharmacologic experiments

An isolated iris was suspended in a 10 ml organ bath containing physiological salt solution (of the same composition as described above) at 37°C. A thread was passed through the pupillary margin and tied to a glass hook; the opposite end of the iris sphincter was connected by means of a silk thread to a force displacement transducer (FT-03) (see figure 10). A baseline tension of 150 mg was maintained throughout the experiment and the change in tension developed by the iris sphincter was recorded on a Grass Polygraph Model 7 (Grass Instruments, Quincy, Massachusetts).

After a 50 minute equilibration period, during which the tissue was washed at regular intervals, two cumulative dose-response curves to carbachol were obtained at 60 minute intervals. The tissue was thoroughly washed after the first dose-response curve and it was exposed
Figure 10. Tissue bath assembly used for recording tension changes of isolated rabbit iris.
Figure 10
to the antagonist for 60 minutes before recording the second dose-response curve, which was obtained in the presence of the antagonist. To check for changes in tissue sensitivity during the test period, the contralateral iris sphincter without the antagonist was simultaneously set up in a separate tissue bath (Furchgott, 1967). $pA_2$ values were calculated according to the method of Arunlakshana and Schild (1959). Dose ratio, which is defined as the ratio of $ED_{50}$ of the agonist with and without the antagonist, was used to obtain the $pA_2$ plots.

Rabbit fundus strips were prepared according to the procedure described by Furchgott and Bursztyn (1967).

In other experiments on iris, recovery of the muscarinic blockade was studied by testing the sensitivity of the tissue to a dose of carbachol at regular intervals. A control response to carbachol $ED_{90}$ ($1 \times 10^{-5}M$) was obtained and the tissue was thoroughly washed and incubated with a dose of atropine for 60 minutes. Sensitivity of the tissue to carbachol in the presence of atropine was ascertained. The tissue was washed three times within 5 minutes and 10 minutes after the last wash the tissue sensitivity to carbachol was tested. The procedure was repeated several times. The contralateral iris sphincter, without atropine treatment, was used to check the sensitivity changes to carbachol during the testing procedure.
In vivo experiments

Rabbits were classified according to the absence or presence of atropinesterase in the serum and they were trained in restraining cages. Pupillary diameter in the presence of light (Bausch and Lomb illuminator, setting #1, 12 cm from the eye) was measured. After the instillation of 0.1 ml of a 2% atropine sulfate solution at zero time, the onset of the mydriatic effect, determined as the change of sensitivity to light, was measured every fifteen minutes for two hours. Thereafter, the pupillary diameter was determined at regular intervals for 96 hours. In no case were the animals kept for more than four hours in the restraining cages. At the end of the 96th hour the animals were killed and the in vitro sensitivity of the iris sphincter to carbachol was tested as described above.

In a second series of experiments only atropinesterase negative rabbits were used. After instillation of 0.1 ml of a 2% atropine sulfate solution (5.2 μCi/ml ³H-atropine) the mydriatic effect was followed for 96 hours as described above. At the end of the 96th hour the animals were killed. The irides were isolated, weighed and homogenized with 2 ml of 0.4 N perchloric acid, and the homogenate was centrifuged at 43,5000 Xg, at 0-4°C, for 30 minutes. The total radioactivity in the supernatant was counted in 12 ml Aquasol by liquid scintillation
Spectrometry. Counting efficiency for tritium was 25%.

Thin layer chromatography

In two atropinesterase-negative nonalbino rabbits, 0.1 ml of a 2% atropine sulfate solution (46.29 μCi/0.1 ml ³H-atropine) was instilled in each eye. The higher radioactivity per instillation was necessary to detect sufficient counts in the tissue extract. The mydriatic effect of atropine was followed for 96 hours. At the end of the 96th hour the animals were killed and the irides isolated. The two irides of each animal were combined and homogenized in 2 ml of a 0.4 N perchloric acid solution and the homogenate was centrifuged at 43,500 Xg at 0-4°C, for 30 minutes. The precipitate was discarded and the supernatant was placed in 2.5 ml vials. This extract was subjected to evaporation under constant air current at room temperature. When the volume of the material was reduced to approximately 0.1 ml (after approx. 36 hr), the material was spotted on a silica gel plate. The plate was developed using a mixture of methylacetate, isopropanol, 25% ammonia (45:35:20) as solvent. The plate was divided into 1 cm segments and radioactivity was determined in each segment by liquid scintillation spectrometry as described above.
Binding of $^{14}$C-imipramine to human brain homogenates

The pigmented tissue of substantia nigra weighing 0.3 to 0.5 g, or an equal amount of cerebral cortex, was homogenized with physiological salt solution (1 ml/0.1 mg) in a 5 ml glass Teflon homogenizer (Arthur Thomas, Philadelphia, Pa.) at 600 rpm with 8 to 10 strokes. The homogenate was subjected to sonication on ice for five minutes, using a 20% relative output of a sonic 300 dismembrator (Artek Systems Corp., Farmingdale, N.Y.). Aliquots of the homogenate were incubated at 30°C under constant oxygenation. It was important that the oxygenating tubing did not come in close contact with the homogenate since it causes some frothing. The incubation volume was 2.5 ml. After an equilibration period of 30 minutes, $^{14}$C-imipramine was added to the preparation for a final concentration of 12.8 nCi/ml ($2.5 \times 10^{-6}$M). The incubation time with the drug was 30 minutes and the preparation was centrifuged at 43,500 g, 0-4°C, for 30 minutes. The radioactivity in the supernatant was determined and the radioactivity bound to the tissue was determined by difference from the initial amount in the incubation medium.
Localization of labelled drugs in tissues by discontinuous sucrose density gradient centrifugation

Rabbit iris

Albino and nonalbino rabbit irides were incubated in physiological salt solution with $^{14}$C-imipramine, 12.8 nCi/ml (2.5 x 10^{-6} M) for two hours, then rinsed twice in 5 ml of an isotonic (0.32 M) sucrose solution for three minutes. Excess radioactivity was blotted on a filter paper and the tissue homogenized in a glass Teflon homogenizer for 2 minutes with 4 ml of the isotonic sucrose solution. The total homogenate was layered on a discontinuous sucrose density gradient (1.0, 1.5, 2.0, and 2.5 M sucrose solution, 2.5 ml each). The gradient tubes were centrifuged in a swinging bucket (Beckman Rotor SW36) at 105,000 Xg for one hour in a Beckman Model L ultracentrifuge. The sucrose layers were carefully separated using Pasteur pipettes and the volumes of the fractions internally equalized using demineralized double distilled water. Proteins were precipitated by adding 1 ml of 10% trichloroacetic acid solution to each fraction and removed by centrifuging in a desk model centrifuge for ten minutes. The precipitate was subsequently washed twice with 0.5 ml of a 5% trichloroacetic acid solution. The supernatants were collected for determination of radioactivity. Protein content of the tissue was determined in the precipitate.
Rabbit Retina

Albino and nonalbino rabbits retinas were isolated and homogenized in 2.5 ml of cold physiological salt solution in a glass Teflon homogenizer, with 8 to 10 strokes, at 600 rpm. The homogenate was incubated with \(^{3}\text{H}\)-chlorpromazine, 244.2 nCi/ml (2.5 X 10\(^{-6}\)M) for one hour, under constant oxygenation at 37°C. After the incubation period the preparation was centrifuged at 43,500 Xg, at 0-4°C, for 30 minutes. The pellet was rinsed twice with 5 ml each of 0.32 M sucrose solution and was uniformly suspended in 4 ml of the isoosmotic sucrose solution. The suspension was layered on the top of a discontinuous sucrose density gradient prepared as described for rabbit iris. The rest of the procedure was similar to that described for rabbit iris.

Human Brain

Pigmented tissue of substantia nigra from two different samples, weighing 0.5 to 0.6 g, or equal amount of a secretion of superior cerebellar peduncles were homogenized in a 0.32 M sucrose-potassium phosphate buffer solution, pH 7.4, at 600 rpm, in a glass Teflon homogenizer, with 8 to 10 strokes. The concentration of the tissue in the homogenate was 0.1 g/ml. The homogenate was subjected to sonication on ice for five minutes, using a 20% relative output of a Sonic 300 dismembrator. Equal amounts
of homogenates from substantia nigra and cerebellar peduncles were incubated at 37°C, under constant oxygenation in a Dubnoff incubator shaking at 100 rpm. After a 30 minute incubation period equivalent concentrations of \(^{14}\text{C-}\text{imipramine}, \, ^{3}\text{H-haloperidol}, \, \text{or} \, ^{3}\text{H-chlorpromazine} \) were added to both preparations, and the incubation continued for an additional hour. The preparation was centrifuged at 43,500 Xg, 0-4°C, for 30 minutes. The pellet was rinsed twice with 5 ml each of 0.32 M sucrose solution and it was uniformly suspended in 5 ml of the same isoosmotic solution. The suspension was layered on a discontinuous sucrose density gradient (1.0, 1.5, and 2.0 M sucrose solutions; 3 ml each). The rest of the procedure was carried out as mentioned above for rabbit iris.

The concentration of the drugs used was 6.25 X \(10^{-6}\) M and the respective specific activity for each drug used was: \(^{14}\text{C-}\text{imipramine}, \, 56.3\text{ nCi/ml}; \, ^{3}\text{H-haloperidol}, \, 56.3\text{ nCi/ml}; \, \text{and} \, ^{3}\text{H-chlorpromazine}, \, 610.6\text{ nCi/ml}. \) Counting efficiency for \(^{14}\text{C}\)Carbon and tritium was 72-74% and 23-29%, respectively, as established by automatic external standardization.

Protein was determined in the different fractions of the sucrose density gradient according to the method of Lowry et al. (1951).
Preparation of bovine iris melanin granules

Bovine irides from ten eyes were isolated, weighed, cut in small pieces and placed in a 50 ml glass homogenizer container (Arthur Thomas Co., Philadelphia, Pa.) with a loosely fitted Teflon pestle. Twenty ml of 0.1 M potassium phosphate buffer, pH 7.4, were added. The Teflon pestle was used in brief strokes without disintegrating the tissue. The extract containing melanin granules was passed through a silk cloth (Mesh #10XX) into centrifuge tubes. The process was repeated until the iris stroma, originally brown to black in color, became gray and the supernatant did not take additional pigment. In order to remove large tissue particles the supernatant containing the pigment granules was centrifuged for five minutes at the lowest setting of an international model HN centrifuge. The supernatant from the low speed centrifugation was centrifuged at 5,900 Xg at 0°C for 10 minutes in a refrigerated RC2-B Sorvall centrifuge. The supernatant was discarded and the pellet resuspended in potassium phosphate buffer. This process was repeated ten times to obtain a clear supernatant. The weight of the residue was determined and a 250 mg/ml suspension of granules was prepared in potassium phosphate buffer. The purity of the material was examined by light microscopy and it mainly contained granules. The preparation was refrigerated and used after 24 hours. In
a given experiment, granules from 10 irides were used. The average weight of 10 irides was approximately 3.55 g and the average yield of granules from ten irides was 21%.

Preparation of synthetic melanins

L-dopa melanin

Synthetic L-dopa melanin was prepared according to a slight modification of the procedure described by Potts (1964) and Binns et al. (1970). Ten grams of L-dopa were dissolved in 3 L of 0.1 M potassium phosphate buffer (pH 7.4) and 66 mg of mushroom tyrosinase were added to the solution. The solution was continuously stirred in a water bath at 37°C for four hours. Then, it was gassed with 95% oxygen and 5% carbon dioxide at room temperature for 90 minutes. Stirring was continued at 37°C for four hours. The preparation was left overnight at room temperature with continuing bubbling of oxygen. The insoluble melanin formed was centrifuged at 19,000 rpm and the residue was washed several times with distilled water. The melanin residue was finally resuspended in distilled water and freeze dried. The synthetic melanin obtained was kept under refrigeration until used. Synthetic L-dopa melanin was prepared by Mr. Raman Baweja (Shimada et al., 1976).
Synthetic L-α-methyldopa melanin and D-α-methyldopa melanins

These melanins were prepared following a similar procedure to that described for L-dopa melanin, except 2.5 g of either isomer of α-methyldopa was dissolved in 400 ml of 0.1 M potassium phosphate buffer (pH 6.9, optimum pH for the enzyme). Twenty five mg of mushroom tyrosinase were added.

Synthetic dopamine melanin

Dopamine melanin was prepared by dissolving 2.5 g of dopamine HCl in 400 ml of 0.1 M potassium phosphate buffer (pH 6.9, optimum pH for peroxidase) and then adding 100 mg of horseradish peroxidase. The procedure followed was the same as that described for synthetic L-dopa melanin.

A summary of the yield of melanins is presented in table 3.

Binding of \( ^{14} \)C-imipramine and \( ^{3} \)H chlorpromazine to bovine iris melanin granules

Each 10 ml Erlenmeyer incubation flask containing 5, 10, 25 and 150 mg of granules in 2.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, was placed in a Dubnoff incubator shaking at 100 rpm, at 37°C. After a 30 minute incubation period, \( ^{14} \)C-imipramine, 24.7 nCi/ml (2.5 X \( 10^{-6} \)) was added and the incubation continued for an additional 60 minutes. The suspensions of granules were centrifuged at 43,500 Xg, at
<table>
<thead>
<tr>
<th>MELANIN</th>
<th>SUBSTRATE</th>
<th>g</th>
<th>ENZYME</th>
<th>g</th>
<th>YIELD</th>
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<tbody>
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<td>L-DOPA melanin(^a)</td>
<td>L-DOPA</td>
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<td>Tyrosinase</td>
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<td>14.1%</td>
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<tr>
<td>Dopamine melanin(^b)</td>
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<td>Peroxidase</td>
<td>0.100</td>
<td>26.0%</td>
</tr>
<tr>
<td>L-(\alpha)-methyl-DOPA melanin(^b)</td>
<td>L-(\alpha)-methyl-DOPA,</td>
<td>2.5</td>
<td>Tyrosinase</td>
<td>0.025</td>
<td>26.0%</td>
</tr>
<tr>
<td>D-(\alpha)-methyl-DOPA melanin(^b)</td>
<td>D-(\alpha)-methyl-DOPA,</td>
<td>2.5</td>
<td>Tyrosinase</td>
<td>0.025</td>
<td>15.6%</td>
</tr>
</tbody>
</table>

\(^a\)Prepared in Potassium Phosphate Buffer pH 7.4

\(^b\)Prepared in Potassium Phosphate Buffer pH 6.9
0-4°C, in a refrigerated RC2-B Sorvall centrifuge for 30 minutes. Radioactivity in the supernatant was determined by liquid scintillation spectrometry in a mixture of 1 ml of distilled water and 13 ml of liquid scintillation solution (ACS, Amersham/Searle). The amount of drug retained by the granules was determined by difference from a standard run under the same experimental conditions.

The procedure for \(^3\)H-chlorpromazine binding to melanin granules was similar to that described for imipramine except that 75 mg was the highest amount of granules used and the specific activity of \(^3\)H-chlorpromazine was 244.2 nCi/ml \((2.5 \times 10^{-6} \text{M})\). The equilibrium time was reduced to 30 minutes.

Affinity and capacity for binding were calculated as described by Shimada et al. (1976).

**Binding of \(^{14}\)C-imipramine and \(^3\)H-chlorpromazine to synthetic melanins**

Two, 4, 6, or 8 mg of synthetic L-dopa melanin were placed in each of four Erlenmeyer flasks containing 2.5 ml of potassium phosphate buffer, pH 7.4. After a 30 minute preincubation period \(^{14}\)C-imipramine was added to each flask for a final concentration of \(2.5 \times 10^{-6} \text{M}\) \((12.8 \text{ nCi/ml})\). Incubation was continued for an additional hour. The suspensions of melanin were centrifuged at 43,500 Xg,
0-4°C, for 30 minutes. Radioactivity was determined in 1 ml of the supernatant as described above for melanin granules. The amount of radioactivity retained by melanin was determined by difference from a standard run under the same experimental conditions.

$^3$H-Chlorpromazine binding to four synthetic melanins was studied. The synthetic melanins used were: L-dopa, dopamine, L-α-methyldopa, and D-α-methyldopa melanins. One, 2, 4, 6, and 8 mg of the melanins were placed in each of five Erlenmeyer flasks containing 2.5 ml of potassium phosphate buffer. After the preincubation period $^3$H-chlorpromazine, 244.2 nCi/ml (7.5 $\times$ 10$^{-5}$M) was added and the incubation continued for 30 additional minutes. After incubation, the melanin suspensions were centrifuged and radioactivity was determined in the supernatant. The amount of radioactivity retained by melanin was determined as described above.

Affinity and capacity for binding were calculated as described by Shimada et al. (1976).

Binding of $^3$H-chlorpromazine to melanin granules and synthetic L-dopa melanin in the presence of other drugs

The procedure followed was similar to that described above for binding of $^3$H-chlorpromazine to both melanin granules and synthetic melanins. After a 30 minute pre-incubation period, an appropriate amount of unlabelled
drugs was added to give a final concentration of $7.5 \times 10^{-5}$ M and incubation continued for 30 additional minutes. At this time, $^3$H-chlorpromazine, 244.2 nCi/ml ($2.5 \times 10^{-6}$ M) was added to the preparation and incubation with the labelled drug continued for an additional 30 minutes. The suspensions were centrifuged and radioactivity was determined in the supernatant. The amount of radioactivity retained by the granules was determined as described above. The proportion of $^3$H-chlorpromazine:unlabelled drug was 1:30.

For the study of the binding of $^3$H-chlorpromazine to L-dopa melanin an amount of the unlabelled drug to give a final concentration of $7.5 \times 10^{-4}$ M was added. After 30 minutes incubation with the unlabelled drug, $^3$H-chlorpromazine 244.2 nCi/ml ($7.5 \times 10^{-5}$ M) was added to the preparation and the incubation with the labelled drug continued for 30 minutes. The radioactivity retained by melanin was determined as described above. The proportion of $^3$H-chlorpromazine:unlabelled drug was 1:10.

Affinity and capacity for binding of $^3$H-chlorpromazine in the presence of unlabelled drugs were calculated according to Shimada et al. (1976).
Binding of $^3$H-chlorpromazine to melanin granules extracted with organic solvents

Bovine iris melanin granules were prepared as described above. The suspensions of granules originally obtained were divided into three equal portions. One portion was extracted with ether; a second portion was extracted with acetone and the third portion was used as control. The volume of each portion was generally 1-2 ml. The melanin granules suspensions were placed in 60 ml separation funnels and 50 ml of ether or acetone were added. The preparation was shaken for 15 to 20 minutes and was left in the organic solvent overnight. After approximately 15 hours, the preparation was shaken for 10 minutes and then centrifuged in a RC2-B Sorvall centrifuge at 5,900 Xg at 0-5°C, for ten minutes. The supernatant was discarded and the residue of granules was resuspended in 20 ml of potassium phosphate buffer, pH 7.4. The suspension was centrifuged again for 10 minutes. This process was repeated three times. The supernatant was discarded, the granule residue was weighed and kept in potassium phosphate buffer until used. An aliquot of unextracted material treated in the same fashion was used as a control for the binding studies.
Suspensions of melanin granules containing 2, 5, 10, 25 and 75 mg of granules total were prepared in potassium phosphate buffer. After a 30 minute preincubation period at 37°C, $^3$H-chlorpromazine was added to the preparation for a final concentration of $2.5 \times 10^{-6}$M (244.2 nCi/ml), and the incubation continued for 30 additional minutes. The preparation was centrifuged at 43,500 Xg, 0-4°C, for 30 minutes. Determination of radioactivity bound to the granules was done as described before.

**Procedure for calculating binding constants**

The analysis of the binding data assumed that the binding of drugs to melanin is analogous to the adsorption of a drug on a solid exemplified by the type I Langmuir Isotherm. The amount of drug bound per milligram of melanin, $r$, should be related to the concentration of free drug, $[D]_{\text{free}}$, by the equation

$$r = \frac{n k [D]_{\text{free}}}{1 + k [D]_{\text{free}}} \quad \text{Eq. 1}$$

where $n$ is the maximum number of binding sites in a class of mutually independent sites and $k$ is a constant related to the affinity of the drug for the binding sites.

Rearrangement of equation 1 leads to

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nk} \cdot \frac{1}{[D]_{\text{free}}} \quad \text{Eq. 2}$$
Equation 2 shows that a linear relationship should be obtained when $1/r$ is plotted as a function of $1/[D]_{\text{free}}$. The extrapolated y-axis intercept is $1/n$ and the slope is $1/nk$. Such a treatment of the binding data permitted the calculation of capacity, $n$ and $k$, affinity, for imipramine and chlorpromazine and, based on these values, a comparison of the binding of different drugs to melanin could be made since in the presence of an inhibitor of the binding, equation 1 is modified to

$$r = \frac{n k [D]}{1 + k[D] + k'[C]}$$

Eq. 3

where $k'$ is related to the affinity constant for the inhibitor and $[C]$ is the concentration of free inhibitor. Defining a term $k_{\text{app}}$ as

$$k_{\text{app}} = \frac{k}{1 + k'[C]}$$

and substituting into equation 3 one gets,

$$r = \frac{n k_{\text{app}} [D]_{\text{free}}}{1 + k_{\text{app}} [D]_{\text{free}}}$$

Eq. 4

or,

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nk_{\text{app}} [D]_{\text{free}}}$$

Eq. 5
Thus, if the reciprocal plots obtained for the data in the presence and absence of an inhibitor intersect at the same point on the y-axis, the nature of the binding is characterized as competitive. In the case of non-competitive inhibition, k remains the same as in equation 1 and n decreases; thus, reciprocal plots in the absence and presence of the inhibitor would have the same x-intercept, but different y-intercepts.

The regression line parameters (slopes and intercepts) of Eqs. 2 and 5 with their respective 95% confidence intervals and the F ratio (indicative of the variance of the regression line) were obtained via computer analysis. Computer generated intercepts and slopes yield capacity as 1/y-intercept and affinity values as y-intercept/slope.

For the other studies, comparisons between two means were done by using the student "t" test (Goldstein, 1964).

**Drugs**

The following drugs were used:

(a) Labelled drugs:

- $^3$H-Atropine sulfate (G) Amersham/Searle
  - S.A. 407 mCi/mM and Arlington Heights
  - 350 mCi/mM Illinois
  - Radiochemical Purity 98%
<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-Chlorpromazine ($^3$H-methyl)</td>
<td>CEA, IRE, SORIN (CIS)</td>
</tr>
<tr>
<td>S.A. 37 Ci/mM</td>
<td>France</td>
</tr>
<tr>
<td>Radiochemical Purity 99%</td>
<td></td>
</tr>
<tr>
<td>$^3$H-Haloperidol-(Chlorophenyl-3-$^3$H)</td>
<td>IRE, Belgium</td>
</tr>
<tr>
<td>S.A. 10.5 Ci/mM</td>
<td></td>
</tr>
<tr>
<td>Radiochemical Purity 97%</td>
<td></td>
</tr>
<tr>
<td>$^{14}$C-Imipramine</td>
<td>Amersham/Searle</td>
</tr>
<tr>
<td>S.A. 4.5 mCi/mM and 9.2 mCi/mM</td>
<td>Arlington, Virginia</td>
</tr>
</tbody>
</table>

(b) Unlabelled drugs:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine Sulfate</td>
<td>Mallinckrodt Chemical Works, St. Louis</td>
</tr>
<tr>
<td>L-$\alpha$-methyl-dopa</td>
<td>Merck Sharp &amp; Dohme</td>
</tr>
<tr>
<td>D-$\alpha$-methyl-dopa</td>
<td>Research Labs. West Point, Philadelphia</td>
</tr>
<tr>
<td>Carbamyl Choline Chloride</td>
<td>Aldrich Chemical Co.</td>
</tr>
<tr>
<td>Chlorpromazine. HCl</td>
<td>Smith Kline &amp; French Labs. Philadelphia</td>
</tr>
<tr>
<td>Clozapine (100-129)</td>
<td>Sandoz Pharmaceuticals</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>Merck Co.</td>
</tr>
<tr>
<td>Dopamine HCl</td>
<td>Regis Chemical Co.</td>
</tr>
<tr>
<td>6-OH-Dopamine. HBr</td>
<td>Morton Groves, Illinois</td>
</tr>
<tr>
<td>Fluphenazine HCl</td>
<td>Schering Corporation Bloomfield, New Jersey</td>
</tr>
</tbody>
</table>
The radiochemical purity of the labelled compounds was tested by thin layer chromatography at regular intervals and no degradation of the material was detected. Appropriate dilutions were prepared as needed and kept at 0°C until used. Freshly prepared solutions of the unlabelled compounds were always used. Due to the insolubility of the drug in most solvents and because lactic acid is recommended by McNeil Labs. Inc. as the best solvent for the drug, haloperidol was prepared in a 2.5% lactic acid solution. Clozapine was prepared in citrate buffer containing 1.2% (w/v) citric acid and 0.193% (w/v) sodium citrate dihydrate). All other solutions were prepared in saline.
CHAPTER III

RESULTS

In vitro uptake of $^3$H-atropine by tissues

Both pigmented and nonpigmented irides accumulated radioactivity when incubated with $^3$H-atropine; however, the amount accumulated by the pigmented iris exceeded that accumulated by the nonpigmented iris (fig. 11). The results could have been expressed as $^3$H-atropine dpm/iris; however, due to the differences in weight of the irides (20-23 mg, nonpigmented; 33-40 mg, pigmented iris), it was arbitrarily decided to normalize the results to common weight basis. Therefore, the results were expressed as $^3$H-atropine dpm/25 mg iris. Since a single radiochromatograph peak corresponding to authentic atropine was obtained from the radioactivity extract from the iris obtained from either albino or nonalbino atropinesterase-positive animals, it is highly probable that the accumulated radioactivity was essentially $^3$H-atropine. No differences in the accumulation of radioactivity by irides were noted when either atropinesterase-positive or enzyme-unclassified animals were used (see Appendix A).
Figure 11. A. In vitro accumulation of $^3$H-atropine (30 nCi/ml) by the pigmented and non-pigmented rabbit iris.

B. dpm per gram/dpm per milliliter (tissue/medium) ratio for the accumulation of the drug. Rabbits were atropinesterase-positive.

Each point represents an average of four to five observations with the S.E.M.
Figure 11
Although the proportion of atropinesterase-negative animals in nonalbino rabbits was high (23%), only a small percentage (8%) of albino rabbits were atropinesterase-negative. Thus, it was impractical to study all four types of animals all the time. Where essential, experiments were verified by examining a limited number of irides from atropinesterase-negative animals.

The accumulation of \(^3\text{H}-\text{atropine}\) (30 nCi/ml, \(10^{-5}\) M) by irides, when studied for 35 minutes in the atropinesterase-negative albino or nonalbino animals is shown in table 5: The values do not differ significantly (P > 0.05) from those irides of atropinesterase positive animals (table 5). At any given time, after a 10-minute equilibration of the drug, the ratio of radioactivity accumulated by the pigmented irides to those nonpigmented, varied between 3 and 4 fold. The uptake of \(^3\text{H}-\text{atropine}\) by the pigmented iris follows a complex pattern. The amount of drug bound after 60 minutes was much higher than expected on the basis of a single equilibration process. The tissue/medium ratios for the accumulation of drug were higher in the pigmented iris.
Accumulation of $^3$H-atropine by human irides and pigment epithelium

Human eyes with light brown to brown color obtained from the eye bank of The Ohio State University were stored in the cold and irides were studied within 36 to 48 hours after death. The accumulation of $^3$H-atropine by human iris or pigment epithelium compared favorably with that of the pigmented rabbit iris. Results are summarized in table 5.

In vitro loss of $^3$H-atropine from tissues after repeated washings

Figure 12 illustrates the loss of radioactivity when irides or stomach fundus strips were washed at 5-minute intervals. Little or no drug was lost after the repeated washing of the pigmented iris, whereas the radioactivity was rapidly lost from the nonpigmented iris and fundus strips. Although the fundus strips accumulated less drug per unit weight than the nonpigmented iris, the rate of loss of radioactivity by these nonpigmented tissues were identical. Half of the initial radioactivity from the nonpigmented tissues was lost in approximately 14 minutes.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>N</th>
<th>dpm/25 mg of Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iris (pigmented)(^a)</td>
<td>6</td>
<td>20948 ± 5672(^b)</td>
</tr>
<tr>
<td>Pigment epithelium(^a)</td>
<td>2</td>
<td>26894 ± 6</td>
</tr>
<tr>
<td><strong>Rabbit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigmented iris atropinesterase-negative</td>
<td>4</td>
<td>24908 ± 4193(^b)</td>
</tr>
<tr>
<td>Nonpigmented iris atropinesterase-negative</td>
<td>4</td>
<td>6356 ± 228</td>
</tr>
<tr>
<td>Pigmented iris atropinesterase-positive</td>
<td>4</td>
<td>16168 ± 2135(^b)</td>
</tr>
<tr>
<td>Nonpigmented iris atropinesterase-positive</td>
<td>4</td>
<td>5727 ± 376</td>
</tr>
</tbody>
</table>

\(^a\) Color - light brown to brown. The accumulation was studied as described for the rabbit iris (see Methods section). The eyes were stored in cold and uptake was studied within 36 to 48 h after death.

\(^b\) \(P > 0.05\)
Figure 12. The rate of disappearance of $^3$H-atropine from irides and stomach fundus strips obtained from albino and nonalbinic rabbits. Tissues were incubated with 30 nCi/ml of $^3$H-atropine ($10^{-5}$M) for 35 minutes and at regular intervals irides were transferred to the drug-free physiological solution at 37°C. Ordinate represents the amount of $^3$H-atropine bound per 25 mg of iris. Tissues were obtained from atropinesterase-positive animals only.
Pigmented Iris (atropinesterase positive)
n = 4-5

Nonpigmented Iris (atropinesterase positive)

Nonalbino rabbits

Albino rabbits

H^3-ATROPINE \text{ dpm} \times 10^4/25 \text{ mg TISSUE}

Figure 12
Ocular penetration of $^3$H-atropine

After topical application of $^3$H-atropine, absorption of the drug into the aqueous humor and the iris was studied up to 45 minutes (fig. 13). Only 0.3 and 0.2% of the total radioactivity was found in the aqueous humor of the nonpigmented and pigmented rabbit eye, respectively. Our results are consistent with similar findings of penetration of $^{14}$C-atropine in albino rabbit eye (James and Stiles, 1959). Either pigmented or nonpigmented iris accumulated only 0.16% of the drug. If it is assumed that the accumulated drug was not metabolized, only 3 ng of the drug were accumulated by either type of iris. At no time was the accumulation of the drug by the pigmented or nonpigmented iris significantly different. The drug transported across the corner (in aqueous humor and iris) of albino and nonalbino rabbits eye was approximately the same ($P > 0.05$). The aqueous humor and iris of the contralateral eye accumulated very small amounts of radioactivity.

Antimuscarinic effects of atropine in the pigmented and nonpigmented rabbit iris and fundus strips

Data obtained either from iris or fundus strips in the presence of increasing concentrations of atropine showed a parallel shift of the dose-response curves of the agonist carbachol to the right (fig. 14). In the pigmented iris a high dose of atropine produced significantly
Figure 13. In vivo rate of uptake of radioactivity after the topical application of 0.1 ml of 2\% atropine sulfate solution, by the aqueous humor and irides of albino and nonalbino rabbits. At any given time the total radioactivity found in the aqueous humor and iris was not significantly different in both types of animals. Each point corresponds to the mean of five observations with the S.E.M.
$^{3}$H-atropine SO$_4$, 0.1 ml 2% Solution

[0.1 ml = 5.32 x 10$^5$ dpm]

Aqueous humor from albino rabbits

Aqueous humor from nonalbino rabbits

Pigmented Iris

Nonpigmented Iris

Figure 13
Figure 14. Dose-response curves of carbachol obtained in isolated pigmented and nonpigmented rabbit iris and fundus strips determined in the presence and absence (control) of atropine.

A. Dose-response curves of carbachol obtained in isolated iris sphincter of albino and nonalbino rabbits. The tissue was incubated with atropine, $2 \times 10^{-8}$M, for 60 minutes and a second dose response curve to carbachol in the presence of the antagonist was obtained. Note the greater shift to right obtained with the nonpigmented rabbit iris.

B. Dose-response curves of carbachol obtained in isolated fundus strips of albino and nonalbino rabbits. The tissue was incubated with atropine, $2 \times 10^{-8}$M, for 60 minutes and a second dose-response curve to carbachol in the presence of the antagonist was obtained.
Figure 14

(A) % MAXIMUM CONTRACTION
- O NON-PIGMENTED IRIS
- • PIGMENTED IRIS
n = 5

WITH ATROPINE 2 x 10^{-8} M

CONTROL

(B) STOMACH STRIPS
- O ALBINO RABBIT
- • NON-ALBINO RABBIT
n = 5

WITH ATROPINE 2 x 10^{-8} M

CARBACHOL [M]
smaller dose-ratios than the ratio obtained in the non-pigmented iris. $\text{pA}_2$ plots are illustrated in fig. 15. $\text{ED}_{50}$ values of carbachol from the two types of irides were not significantly different ($P > 0.05$). The dose-response curves of carbachol when repeated in the contralateral iris, without the antagonist, were shifted to the right by approximately 0.2 log units. The dose ratios were corrected for the sensitivity changes. The slopes of the lines corresponding to the $\text{pA}_2$ plots obtained from non-pigmented iris or fundus strips were close to the theoretical value of 1 (Arunlakshana and Schild, 1959). However, the value from the pigmented irides did not give a straight line. When there are sites of loss, either for agonist or antagonist, unusual slopes for $\text{pA}_2$ plots are expected (Furchgott, 1972). On the various tissues examined, the $\text{pA}_2$ values of atropine varied from 8.88 to 8.58, and these values are approximately the same as those observed for chinchilla rabbit iris sphincter and guinea-pig ileum (Taylor, 1974; Arunlakshana and Schild, 1959).

Recovery from Atropine Blockade

The sensitivity to $\text{ED}_{90}$ concentration of carbachol from the nonpigmented iris was rapidly regained whereas that from the pigmented rabbit iris was very slowly recovered. Results are summarized in fig. 16. The sensitivity of the contralateral iris, which served as control,
Figure 15. Arunlakshana and Schild (1959) plot of log (DR-1) of the agonist carbachol against atropine obtained from the tissues of albino and non-albino rabbits. The slopes of the $pA_2$ plots from the nonpigmented iris, the fundus strip from albino rabbit and the fundus strip from nonalbino rabbit were 0.97, 0.95 and 1.02, respectively. (The slope of the regression line, if drawn, for the data from the pigmented iris is 0.84). As illustrated, all lines were drawn by unaided eye. Incubation of atropine with tissues was 60 minutes. P values compare the significance between two points at the same concentration of the antagonist.
Figure 15
Figure 16. In vitro rate of recovery from atropine blockade in the pigmented and nonpigmented iris obtained from atropinesterase-positive rabbits. The tissue was incubated with the antagonist for 60 minutes and the response to ED$_{90}$ dose of carbachol ($10^{-5}$M) was tested at regular intervals (See Methods for recording of the tension changes of the iris sphincter in vitro). Note that recovery of the blockade is very fast in the nonpigmented iris.
Figure 16
changed with time. In a series of experiments, the average
decline in sensitivity of the nonpigmented iris after four
repeated experiments was 20% ± 6%, whereas by the pigmented
iris was 32% ± 7%. Since these two values were not signif­
icantly different, no corrections were made for the changes
in sensitivity of the tissue during the course of these
experiments.

In vivo mydriatic effects of atropine

Although no differences in the onset of action of
atropine in the four types of rabbits could be observed,
differences in the duration of action were very clear
(fig. 17). Prior to the application of atropine, the
pupillary diameter in the albino and nonalbino animals
differed by 1.5 to 2.0 mm; the pigmented iris did not
constrict in response to light as much as the nonpigmented
iris. The duration of atropine mydriasis in the four types
of rabbits can be arranged as: nonalbino atropinesterase-
negative > albino atropinesterase-negative > nonalbino
atropinesterase-positive > albino atropinesterase-positive.
At the end of the 96th hour after the application of
atropine, the animals were killed and the sensitivity of
the irides to carbachol in vitro was tested. The results
are presented in table 6. Compared to control, only the
$ED_{50}$ of carbachol from the atropine treated, pigmented,
atropinesterase-negative animals was significantly shifted
Figure 17. Onset and duration of atropine mydriasis in albino and nonalbino rabbits. Atropine sulfate (2%, 0.1 ml) was instilled in the conjunctival sac at zero time and the response to light was measured at regular intervals. The time required for half of the maximal effect is indicated with the corresponding S.E.M. At the end of 96 hours the animals were killed and in vitro sensitivity of the iris sphincter to carbachol was tested (table 6). Note the longest duration of atropine mydriatic effect in atropinesterase-negative nonalbino rabbits.
RABBITS

Figure 17
<table>
<thead>
<tr>
<th>Type of Rabbit</th>
<th>Atropinesterase</th>
<th>Control</th>
<th>N</th>
<th>Atropine treated$^a$</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albino</td>
<td>positive</td>
<td>5.63 ± 0.13</td>
<td>4</td>
<td>5.76 ± 0.16</td>
<td>4</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Non-albino</td>
<td>positive</td>
<td>5.79 ± 0.06</td>
<td>5</td>
<td>5.96 ± 0.13</td>
<td>3$^c$</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Albino</td>
<td>negative</td>
<td>5.72 ± 0.16</td>
<td>5</td>
<td>5.59 ± 0.12</td>
<td>3$^c$</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Non-albino</td>
<td>negative</td>
<td>5.72 ± 0.07</td>
<td>9</td>
<td>5.06$^b$ ± 0.14</td>
<td>3$^c$</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

$^a$ 0.1 ml of 2% atropine was applied topically at 0 time and iris sphincter isolated 96 h after (refer to Fig. 17 for \textit{in vivo} effects).

$^b$ The value also differs significantly (P < 0.05) from other values obtained from atropine treated tissues.

$^c$ Because of some technical reasons only three out of four animal irides were tested.
to the right. The amount of drug retained by the tissue corresponds to a concentration of approximately $2 \times 10^{-8}$M.

Fig. 18 illustrates the results obtained when $^3$H-atropine was applied topically to the eyes of albino and nonalbino atropinesterase-negative rabbits. The duration of the atropine mydriatic effects is similar to that obtained with the first group of animals. The amount of $^3$H-atropine retained by the pigmented irides was significantly higher than that retained by the nonpigmented iris ($P > 0.05$), with the pigmented iris retaining about eight times more $^3$H-atropine than the nonpigmented irides. The total amount of atropine retained per iris 96 hours after topical application was 3.48 ng.

Data if fig. 19 indicates that the total radioactivity retained by the pigmented iris of atropinesterase-negative rabbits 96 hours after topical application is mainly $^3$H-atropine as determined by thin layer chromatography. In these experiments $^3$H-atropine was applied in both eyes. Half time of the atropine mydriatic effect was greater than 96 hours, as in the previous experiments in vivo.

**Binding of $^{14}$C-imipramine by isolated rabbit iris**

Both pigmented and nonpigmented rabbit irides accumulated radioactivity when incubated with $^{14}$C-imipramine. However, the amount of radioactivity accumulated by the pigmented iris (fig. 20) exceeded that accumulated by
Figure 18. Duration of atropine mydriatic effect after instillation of 0.1 ml of 2% $^3$H-atropine (0.52 μCi total) in atropinesterase-negative rabbits. Animals were killed at the 96th hour and the radioactivity remaining in the irides was determined. As compared to the non-pigmented iris, the pigmented iris retains a significantly higher ($P < 0.05$) amount of the drug.
Figure 18

Rabbits (Atropinesterase Negative)

- Nonalbino
  - $t_{1/2} > 96\text{h}$
  - $n=4$

- Albino
  - $t_{1/2} = 43.5 \pm 6.57$
  - $n=4$

Radioactivity in iris after 96 hrs

H-Atropine (2%)
Figure 19. Thin layer chromatography of iris extract from pigmented atropinesterase-negative rabbits treated 96 hours before with 0.1 ml of a 2% \( ^3 \)H-atropine solution (46.29 \( \mu \)Ci/0.1 ml \( ^3 \)H-atropine). Although the reference \( ^3 \)H-atropine contained a small amount of impurity corresponding to R\( _f \) 0.31, the iris extract gave only a single peak radioactivity corresponding to the main peak of atropine.
Solvent System
Methylacetate: 45
Isopropanol: 35
Ammonia (25%): 20

$^{3}H$ CPM $\times 10^{3}$

Distance From Origin (cm)
(Silica Gel Plate)

Figure 19
Figure 20. In vitro accumulation of $^{14}$C-imipramine by the pigmented and nonpigmented rabbit iris. The concentrations of $^{14}$C-imipramine used were: 1.28 nCi/ml ($2.5 \times 10^{-7}$M), 4.26 nCi/ml ($7.5 \times 10^{-7}$M) and 12.80 nCi/ml ($2.5 \times 10^{-6}$M). Each point represents the mean ± S.E.M. of 4-7 observations.
Figure 20
the nonpigmented one. Regardless of the concentration of 
$^{14}\text{C}\text{-imipramine}$ used, after 15 minutes of equilibration 
the ratio of radioactivity accumulated by the pigmented 
iris to the nonpigmented iris varied between 1.5 and 2.0. 
Equilibrium appears to be reached at 120 minutes, although 
the plateau is almost complete after 60 minutes of incubation. In both types of irides the tissue/medium (T/M) 
ratios were highest at the lowest concentration of imipra-
mine. When the concentration of the drug was increased 
the ratios decreased. In perfused lungs, a decrease in 
T/M ratios with increasing concentrations of imipramine 
were observed by Junod (1972). Thus, the process of 
accumulation appears to be a saturable one.

**In vitro loss of $^{14}\text{C}\text{-imipramine}$**
**from isolated rabbit iris**

Fig. 21 illustrates the loss of radioactivity from 
pigmented and nonpigmented rabbit irides when washed with 
drug-free physiological salt solution at 5-minute intervals. 
The loss of radioactivity from both types of irides seems 
to follow the same pattern; however, the amount of radio-
activity retained by the pigmented iris is, at all times, 
greater than that retained by the nonpigmented ones. The 
loss of radioactivity seems to stabilize between 60 and 
120 minutes. At these times, the radioactivity retained 
by the pigmented iris is approximately 2.5 times greater 
than that retained by the nonpigmented ones. Half of the
Figure 21. Rate of disappearance of $^{14}$C-imipramine from isolated pigmented and nonpigmented rabbit iris. Tissues were incubated with 12.8 nCi/ml ($2.5 \times 10^{-6}$M) of imipramine for one hour and at five-minute intervals the irides were transferred to drug-free physiological solution and the amount of drug remaining in the tissue was determined. The ordinate represents the amount of $^{14}$C-bound per iris.
Figure 21
initial radioactivity retained by the nonpigmented tissue is lost in approximately 14 minutes.

Antimuscarinic effects of imipramine in the pigmented and nonpigmented rabbit iris

The parallel shifts of the dose-response curves of the agonist by imipramine indicate a competitive blockade (fig. 22). Based on $K_B$ values, imipramine appears to be a more effective blocker in the nonpigmented iris than in the pigmented one, by a factor of 23.

Binding of $^{14}$C-imipramine to brain homogenates

When incubated with $^{14}$C-imipramine the substantia nigra homogenate accumulated more drug than the cortex homogenate. At higher amounts of tissue homogenates, the accumulation of labelled drug by the homogenate of substantia nigra was significantly higher than that from the cortex (fig. 23).

Localization of $^{14}$C-imipramine in isolated rabbit iris by discontinuous sucrose density gradient centrifugation

It was found that the heaviest melanin granules settle at the bottom of the density gradient. A small amount of lighter granules remains suspended in the 2.5 M sucrose layer. The distribution of radioactivity along the gradient follows different patterns in each type of
Figure 22. Dose-response curve of carbachol obtained in isolated pigmented and nonpigmented rabbit iris determined in the presence and absence (control) of imipramine.

The tissue was incubated with imipramine, $1 \times 10^{-5} \text{M}$, for 60 minutes and a second dose-response curve to carbachol was obtained in the presence of the drug. The dissociation constant ($K_d$) was calculated according to the method of Furchgott (1967).
CONTRACTION (%)

RABBIT IRIS SPHINCTER

PIGMENTED

100

n=5

With Imipramine $10^{-5}$ M

Dose-Ratio 3.6 (2-6)

$K_B = 3.8 \times 10^{-6} M$

CARBACHOL (M)

145

NONPIGMENTED

n=5

With Imipramine $10^{-5}$ M

Dose-Ratio 60 (44-83)

$K_B = 1.7 \times 10^{-7} M$

Figure 22
Figure 23. In vitro accumulation of $^{14}$C-imipramine by homogenates of human substantia nigra and cerebral cortex. Tissue homogenates were incubated with 12.8 nCi/ml ($2.5 \times 10^{-6}$M) of $^{14}$C-imipramine for 30 minutes.
Figure 23
iris (fig. 24). In the nonpigmented iris the greatest amount of $^{14}$C-imipramine is found in the lightest sucrose layers, 0.32 M and 1.0 M. Approximately 43% of the total amount of drug remains in the top gradients, whereas 29% separates in the 1.0 M sucrose layer. On the other hand, in the pigmented iris, the largest amount of drug (70% of the total bound) is found in the sediment containing melanin granules. The total amount of labelled drug present in the pigmented iris homogenate ($17,333 \pm 1811$ dpm) is significantly greater ($P < 0.05$) than that in the nonpigmented iris ($8918 \pm 1484$ dpm).

The protein content of the pigmented iris was always greater than that in the nonpigmented iris ($P < 0.05$). The distribution of proteins in the gradient follows a similar pattern in both types of irides. The largest amount of protein is found in the top gradients (0.32 M sucrose) and in the sediment at the bottom of the 2.5 M sucrose fraction (fig. 24).

Localization of $^3$H-chlorpromazine in isolated rabbit retina by discontinuous sucrose density gradient centrifugation

As in the rabbit iris the heaviest melanin granules of the rabbit retina settle at the bottom of the density gradient. However, one distinct band of pigmented material separates at the 2.0 M sucrose layer with the pigmented
Figure 24. Localization of $^{14}$C-imipramine in isolated pigmented and nonpigmented rabbit iris by discontinuous sucrose density gradient centrifugation.

Rabbit irides were incubated with 12.8 nCi/ml of $^{14}$C-imipramine ($2.5 \times 10^{-6}$M) for 120 minutes. Four milliliters of the iris homogenate were fractionated on a discontinuous sucrose density gradient (see Methods). In the pigmented iris, approximately seventy per cent of the total $^{14}$C-imipramine is accumulated in the pigmented fraction. Each point represents the mean ± S.E.M. of five observations.
RABBIT IRIS

DPM x 10^3 / fraction

Sucrose M

0.32
1.0
1.5
2.0
2.5

NONPIGMENTED

n=5

PIGMENTED

n=5

Protein mg / fraction

Figure 24
rabbit retina. A less pigmented band separated at the 1.5 M sucrose layer. The distribution pattern of radioactivity in each type of retina is different (fig. 25). In the albino rabbit retina the greatest amount of $^3$H-chlorpromazine is found in the lightest sucrose layers, 0.32 M and 1.0 M. Approximately 43% of the total bound drug remains in the top gradients, whereas 31% separates in the 1.0 M sucrose layer. In the pigmented rabbit retina, the largest amount of drug (41% of the total bound drug) is found in the sediment of melanin granules at the bottom of the gradient.

It was also found that the amount of $^3$H-chlorpromazine bound in the 2.0 M sucrose fraction (16% of the total drug bound) obtained from the pigmented tissue is significantly higher ($P < 0.05$) than that obtained from the corresponding fraction in the albino rabbit retina. The total amount of drug bound to the pigmented retina (3.55 ± 0.20 nM) is significantly greater ($P < 0.05$) than in the nonpigmented rabbit retina (2.82 ± 0.11 nM).

The subcellular distribution of proteins in various fractions of the tissue was similar ($P > 0.05$) in both albino and nonalbino rabbits retina, except in the granules sediment, which contained the largest amount of protein.
Figure 25. Localization of $^3$H-chlorpromazine in isolated pigmented and nonpigmented rabbit retina by discontinuous sucrose density gradient centrifugation.

The tissue was incubated in physiological salt solution with 244.2 nCi/ml ($2.5 \times 10^{-6}$M) of $^3$H-chlorpromazine for 60 minutes. Four milliliters of the retina homogenate were fractionated on a discontinuous sucrose density gradient (see Methods). In the pigmented retina, approximately 40 percent of the total $^3$H-chlorpromazine is accumulated in the pigment fraction which sediments at the bottom of the 2.5 M sucrose. The fraction of pigmented rabbit retina which separates at the 2.0 M sucrose solution accumulates significantly higher amounts of $^3$H-chlorpromazine than the corresponding fraction from the nonalbino rabbit retina ($P < 0.05$). Each point represents the mean of five observations ± S.E.M. $^3$H-Chlorpromazine bound is expressed as nanomoles per fraction.
**RABBIT RETINA**

<table>
<thead>
<tr>
<th>CPZ nM/FRACTION</th>
<th>PROTEIN mg/FRACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.75</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>0.50</td>
<td>0.32</td>
</tr>
<tr>
<td>2.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**SUCROSE M**

- 0.32
- 1.0
- 1.5
- 2.0
- 2.5

**PROTEIN CONTENT**

- --- *H-CHLORPROMAZINE (CPZ)*
- --- PROTEIN CONTENT

*Figure 25*
Localization of $^{14}\text{C}$-imipramine, $^3\text{H}$-haloperidol and $^3\text{H}$-chlorpromazine in human brain by discontinuous sucrose density gradient centrifugation

In the human brain, the distribution of radioactivity follows the same pattern as protein content. A distinct pigmented band, presumably of neuromelanin, consistently separated at the 1.5 M sucrose fraction, in the interface with the 2.0 M sucrose fraction.

The largest amount of drug was retained in the top gradients (0.32 M sucrose). The amount of $^{14}\text{C}$-imipramine, for example, bound separated in the 1.5 M sucrose fraction of substantia nigra was found to be significantly higher ($P < 0.05$) than in the corresponding fraction from the nonpigmented brain tissue. This pattern of binding of drug was similar for both $^3\text{H}$-haloperidol and $^3\text{H}$-chlorpromazine (fig. 26, 27, and 28).

Although the protein content in the 1.5 M sucrose fraction did not differ in both substantia nigra and superior cerebellar peduncles, $^3\text{H}$-chlorpromazine binds 1.78 times more than $^{14}\text{C}$-imipramine and 2.3 times more than $^3\text{H}$-haloperidol in this fraction. When the drug/protein ratios in the 1.5 M sucrose fraction from the superior cerebellar peduncles were compared, no differences in the binding among the drug were found ($P > 0.05$).

In some of the samples a very small amount of pigment was observed to separate at the bottom of the
Figure 26. Localization of $^{14}\text{C}$-imipramine in human brain by discontinuous sucrose density gradient centrifugation.

Homogenates of substantia nigra and superior cerebellar peduncles were incubated with 56.3 nCi/ml (6.25 X 10^{-6}M) of $^{14}\text{C}$-imipramine for 60 minutes. Five milliliters of the tissues homogenates were fractionated on a discontinuous sucrose density gradient (see Methods). The fraction of substantia nigra homogenate separating in the 1.5 M sucrose solution binds significantly higher ($P < 0.05$) amounts of $^{14}\text{C}$-imipramine than the corresponding fraction in the nonpigmented superior cerebellar peduncles. Each point represents the mean of five observations ± S.E.M. $^{3}\text{H}$-Chlorpromazine bound is expressed as nanomoles per fraction.
Figure 27. Localization of $^3$H-haloperidol in human brain by discontinuous sucrose density gradient centrifugation.

Homogenates of substantia nigra and superior cerebellar peduncles were incubated with 56.3 nCi/ml ($6.25 \times 10^{-6}$M) of $^3$H-haloperidol for 60 minutes. Five milliliters of the tissue homogenates were fractionated on a discontinuous sucrose density gradient (see Methods). The fraction of substantia nigra homogenate separating in the 1.5 M sucrose solution binds significantly higher ($P < 0.05$) amounts of $^3$H-haloperidol than the corresponding fraction of the nonpigmented superior cerebellar peduncles. Each point represents the mean of five observations ± S.E.M. $^3$H-Chlorpromazine bound is expressed as nanomoles per fraction.
HUMAN BRAIN

nM/FRACTION

PROTEIN mg/FRACTION

SUCROSE

nM

0.32

1.0

1.5

2.0

SUPERIOR CEREBELLAR PEDUNCLE

n = 5

--- \(^3\)H-HALOPERIDOL

--- PROTEIN CONTENT

SUBSTANTIA NIGRA

PROTEIN mg/FRACTION

Figure 27
Figure 28. Localization of $^3$H-chlorpromazine in human brain by discontinuous sucrose density gradient centrifugation.

Homogenates of substantia nigra and superior cerebellar peduncles were incubated with 610.6 nCi/ml ($6.25 \times 10^{-6}$M) of $^3$H-chlorpromazine for 60 minutes. Five milliliters of the tissues homogenates were fractionated on a discontinuous sucrose density gradient (see Methods). The fraction of substantia nigra homogenate separating in the 1.5 M sucrose solution binds significantly higher ($P < 0.05$) amounts of $^3$H-chlorpromazine than the corresponding fraction of the nonpigmented superior cerebellar peduncles. Each point represents the mean of five observations $\pm$ S.E.M. $^3$H-Chlorpromazine bound is expressed as nanomoles per fraction.
Figure 28
gradient. However, this was not a consistent finding. A detailed summary of these results is presented in table 7.

**Binding of $^{14}$C-imipramine to bovine iris melanin granules and synthetic L-dopa melanin**

Fig. 29 shows the equilibrium time determination for the binding of $^{14}$C-imipramine to different concentrations of bovine iris melanin granules. Equilibrium is reached in approximately 60 minutes.

Data on the affinity, capacity and the linear regression parameters upon which they are based for $^{14}$C-imipramine binding to melanin granules and synthetic L-dopa melanin are shown in fig. 30.

The capacity of melanin granules to bind $^{14}$C-imipramine is about 1/50th that of synthetic L-dopa melanin, but, on the other hand, the affinity of the drug for the binding sites in the granules is higher than that for the synthetic melanin by a factor of 250.

**Binding of $^3$H-chlorpromazine to bovine iris melanin granules and synthetic melanin**

Fig. 31 shows the equilibrium time determination for the binding of $^3$H-chlorpromazine to different concentrations of melanin granules. Equilibrium reaches very fast. Equilibrium time was considered to be 30 minutes.

When the data on binding of $^3$H-chlorpromazine to melanin granules was treated according to the method described by Shimada et al. (1976), a regression line with slope 1.41 and y-axis intercept of 0.55 were obtained
### TABLE 7

BINDING OF RADIOLABELLED DRUGS TO HUMAN BRAIN. LOCALIZATION BY DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION

<table>
<thead>
<tr>
<th>Sucrose Fraction (M)</th>
<th>¹⁴C-Imipramine (nMoles)</th>
<th>³H-Haloperidol (nMoles)</th>
<th>³H-Chlorpromazine (nMoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. Peduncles</td>
<td>S. Nigra</td>
<td>C. Peduncles</td>
</tr>
<tr>
<td>0.32</td>
<td>4.760b</td>
<td>3.280</td>
<td>4.130b</td>
</tr>
<tr>
<td>± 0.200</td>
<td>± 0.370</td>
<td>± 0.170</td>
<td>± 0.250</td>
</tr>
<tr>
<td>1.00</td>
<td>1.690±0.300</td>
<td>3.061c</td>
<td>1.229±0.201</td>
</tr>
<tr>
<td>1.50</td>
<td>0.120±0.020</td>
<td>0.363c</td>
<td>0.086±0.019</td>
</tr>
<tr>
<td>2.00</td>
<td>0.034±0.005</td>
<td>0.026</td>
<td>0.006±0.002</td>
</tr>
</tbody>
</table>

*Each value represents the mean of five observations ± S.E.M.

*Statistically greater than the binding of drug in the corresponding fraction of substantia nigra (P < 0.05).

*Statistically greater than the binding in the corresponding fraction of superior cerebellar peduncles (P < 0.05).

*Statistically greater than the binding in the corresponding fraction of superior cerebellar peduncles (P < 0.01).

*Statistically different from the binding of ¹⁴C-imipramine, and ³H-haloperidol to Fraction III (1.5 M sucrose) of the substantia nigra (P < 0.05).
Figure 29. Determination of equilibrium time for $^{14}$C-imipramine binding bovine iris melanin granules. Increasing concentration of melanin granules were incubated with $2.5 \times 10^{-6}$M (27.2 nCi/ml) $^{14}$C-imipramine. At fixed times radioactivity accumulated by the granules was determined. Maximum binding usually occurs in 60 minutes.
MELANIN GRANULES

$^{14}$C-IMIPRAMINE DPM x 10$^3$

TIME (min)

Figure 29
Figure 30. Binding of $^{14}$C-imipramine to bovine iris melanin granules and synthetic L-dopa melanin.

A. The regression line represents binding of $^{14}$C-imipramine to bovine iris melanin granules which were incubated with 24.7 nCi/ml ($2.5 \times 10^{-6}$M) of $^{14}$C-imipramine for 60 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).

B. The regression line represents binding of $^{14}$C-imipramine to synthetic melanin which was incubated with 12.8 nCi/ml ($2.5 \times 10^{-6}$M) of $^{14}$C-imipramine for 60 minutes.
Figure 30

I/M DRUG BOUND PER mg × 10^6

MELANIN GRANULES

SYNTHETIC L-DOPA MELANIN

I/M FREE DRUG × 10^6

I/M FREE DRUG × 10^8

I/M FREE DRUG × 10^9

n = 5
Affinity = 3.84 × 10^3 M^-1
Capacity = 1.11 × 10^-8 Moles/mg

n = 4
Affinity = 9.85 × 10^5 M^-1
Capacity = 2.5 × 10^-10 Moles/mg
Figure 31. Determination of equilibrium for $^3$H-chlorpromazine bovine iris melanin granules. Increasing concentrations of melanin granules were incubated with $2.5 \times 10^{-6}$M (244.2 nCi/ml, $^3$H-chlorpromazine. At fixed times radioactivity accumulated by the granules was determined. Maximum binding usually occurs in 30 minutes.
Figure 31

MELANIN GRANULES

n=4

100 mg
25 mg
10 mg
5 mg

TIME (min)

$^3$H-CHLORPROMAZINE DPM x 10^5
Data related to the affinity and capacity for \(^3\text{H}\)-chlorpromazine are shown in table 8.

When compared with synthetic melanins, the capacity of melanin granules to bind \(^3\text{H}\)-chlorpromazine is about 1/60th, 1/90th, 1/120th and 1/180th that of dopamine melanin, L-dopa melanin, L-\(\alpha\)-methyldopa melanin and D-\(\alpha\)-methyldopa melanin, respectively.

The order of capacity of synthetic melanins to bind \(^3\text{H}\)-chlorpromazine, accordingly was: D-\(\alpha\)-methyldopa melanin > L-\(\alpha\)-methyldopa melanin > L-dopa melanin > dopamine melanin (fig. 32).

On the other hand, the affinity of \(^3\text{H}\)-chlorpromazine for the binding sites in melanin granules is greater than that for L-dopa melanin by a factor of 15, and greater than the affinity for dopamine, L-\(\alpha\)-methyldopa and D-\(\alpha\)-methyldopa melanins by a factor of 6, 12, and 24, respectively. The order of affinity of \(^3\text{H}\)-chlorpromazine for synthetic melanins being: Dopamine melanin > L-\(\alpha\)-methyldopa melanin > L-dopa melanin > D-\(\alpha\)-methyldopa melanin (fig. 32).

The data also shows that the affinity of \(^3\text{H}\)-chlorpromazine for L-\(\alpha\)-methyldopa melanin is two times greater than for D-\(\alpha\)-methyldopa melanin. In all cases the slope of the regression line obtained for binding of \(^3\text{H}\)-chlorpromazine to L-dopa melanin was greater than those obtained with the other synthetic melanins. A summary of the regression lines corresponding to the binding of \(^3\text{H}\)-chlorpromazine to synthetic melanins is shown in figure 33.
TABLE 8

SUMMARY OF THE BINDING CONSTANTS OF $^3$H-CHLORPROMAZINE TO BOVINE IRIS MELANIN GRANULES IN THE ABSENCE AND PRESENCE OF OTHER DRUGS

<table>
<thead>
<tr>
<th>DRUG</th>
<th>AFFINITY M$^{-1}$</th>
<th>CAPACITY Moles/mg granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-Chlorpromazine</td>
<td>$3.88 \times 10^8$</td>
<td>$1.82 \times 10^{-9}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Fluphenazine (1:30)</td>
<td>$8.03 \times 10^8$</td>
<td>$5.01 \times 10^{-10}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Thioridazine (1:30)</td>
<td>$1.04 \times 10^9$</td>
<td>$4.14 \times 10^{-10}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Haloperidol (1:30)</td>
<td>$6.94 \times 10^8$</td>
<td>$4.92 \times 10^{-9}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Clozapine (1:30)</td>
<td>$4.73 \times 10^8$</td>
<td>$7.07 \times 10^{-10}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Dopamine (1:30)</td>
<td>$2.98 \times 10^8$</td>
<td>$1.42 \times 10^{-9}$</td>
</tr>
</tbody>
</table>
Figure 32. Binding of $^3\text{H}$-chlorpromazine to synthetic melanins.

The regression lines represent the binding of $^3\text{H}$-chlorpromazine to synthetic melanins. Increasing amounts of synthetic melanins were incubated with 244.2 nCi/ml ($7.5 \times 10^{-5}$M) of $^3\text{H}$-chlorpromazine for 30 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).
Figure 32

(A) L-Dopa melanin
CAPACITY = 1.56 x 10^7 moles/mg
AFFINITY = 2.54 x 10^9 M
SLOPE = 0.24 (0.23 - 0.27)

(B) Dopamine-melanin
CAPACITY = 1.2 x 10^7 moles/mg
AFFINITY = 5.99 x 10^7 M
SLOPE = 0.14 (0.12 - 0.16)

(C) L-α-methyl-dopa melanin
CAPACITY = 2.17 x 10^7 moles/mg
AFFINITY = 3.23 x 10^7 M
SLOPE = 0.19 (0.17 - 0.22)

(D) D-α-methyl-dopa melanin
CAPACITY = 3.27 x 10^7 moles/mg
AFFINITY = 1.62 x 10^7 M
SLOPE = 0.14 (0.13 - 0.16)
Figure 33. Summary of regression lines representing the binding of $^3$H-chlorpromazine to synthetic melanins. Suspensions of synthetic melanins were incubated with 244.2 nCi/ml ($7.5 \times 10^{-5}$M) $^3$H-chlorpromazine for 30 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).
Figure 33
Binding of $^3$H-chlorpromazine to bovine iris melanin granules in the presence of some phenothiazines and other related drugs

Table 8 shows the affinity and capacity values for $^3$H-chlorpromazine binding to bovine iris melanin granules in the absence and presence of drugs. Table 9 shows the linear regression parameters obtained from the binding data, with the corresponding 95% confidence intervals.

When drugs were introduced as inhibitors of $^3$H-chlorpromazine binding to melanin granules, the analysis of the data showed that the y-axis intercept of the regression lines obtained from experiments using fluphenazine and thioridazine (1:30) are statistically different ($P > 0.05$) from that obtained with $^3$H-chlorpromazine alone. The intercept values, with the respective confidence intervals, obtained from experiments using haloperidol, clozapine or dopamine (1:30) fell within the range of that obtained with $^3$H-chlorpromazine alone.

In all cases the slope of the lines obtained with $^3$H-chlorpromazine in the presence of any of the other drugs was always greater than that obtained from the binding of $^3$H-chlorpromazine per se (table 9). Haloperidol and clozapine seem to be more effective inhibitors of the binding of $^3$H-chlorpromazine than fluphenazine, thioridazine or dopamine (fig. 34).
## TABLE 9

SUMMARY OF THE LINEAR REGRESSION PARAMETERS FOR THE LANGMUIR TREATMENT OF THE BINDING OF $^3$H-CHLORPROMAZINE TO BOVINE IRIS MELANIN GRANULES IN THE ABSENCE AND PRESENCE OF OTHER DRUGS

<table>
<thead>
<tr>
<th>DRUG</th>
<th>SLOPE (C.I.)$^a$</th>
<th>INTERCEPT $\times 10^{-9}$ (C.I.)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-Chlorpromazine</td>
<td>1.41 (1.28 - 1.55)</td>
<td>0.55 (-0.23 - 1.33)</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Fluphenazine (1:30)</td>
<td>2.48$^b$ (2.26 - 2.70)</td>
<td>1.99$^c$ (1.34 - 2.65)</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Thioridazine (1:30)</td>
<td>2.33$^b$ (2.06 - 2.59)</td>
<td>2.42$^c$ (1.68 - 3.16)</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Haloperidol (1:30)</td>
<td>2.93$^b$ (2.57 - 3.28)</td>
<td>2.03 (0.96 - 3.12)</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Clozapine (1:30)</td>
<td>2.99$^b$ (2.62 - 3.36)</td>
<td>1.41 (0.25 - 2.57)</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Dopamine (1:30)</td>
<td>2.37$^b$ (2.03 - 2.71)</td>
<td>0.71 (-0.63 - 2.04)</td>
</tr>
</tbody>
</table>

$^a$ C.I.: 95% confidence intervals.

$^b$ Statistically different from $^3$H-Chlorpromazine binding regression line slope, $P < 0.05$.

$^c$ Statistically different from $^3$H-Chlorpromazine binding regression line intercept, $P < 0.05$. 
Figure 34. Summary of regression lines representing the binding of $^3H$-chlorpromazine to bovine iris melanin granules in the absence and presence of some phenothiazines and related drugs. Suspensions of melanin granules were incubated with 244.2 nCi/ml ($2.5 \times 10^{-6}$M) of $^3H$-chlorpromazine in the absence and presence of unlabelled drugs in a $^3H$-chlorpromazine:drug proportion of 1:30.
Figure 34

MELANIN GRANULES

WITH CLOZAPINE
WITH HALOPERIDOL
WITH FLUPHENAZINE
WITH THIORIDAZINE
WITH DOPAMINE

CONTROL

$^3$H-CPZ: Inhibitor 1:30
A comparison of the binding of $^3$H-chlorpromazine to melanin granules both in the absence and presence of other drugs is shown in figures 35 and 36.

**Binding of $^3$H-chlorpromazine to synthetic L-dopa melanin in the presence of some phenothiazines and other related drugs**

Table 10 summarizes affinity and capacity values for the binding of $^3$H-chlorpromazine to the melanin in the absence and presence of some phenothiazines and related drugs. Although the ratio chlorpromazine:drug is smaller than in the case of melanin granules, the concentrations used were higher. The concentrations of $^3$H-chlorpromazine and the inhibitors were $7.5 \times 10^{-5}$M and $7.5 \times 10^{-4}$M respectively. This change in the concentration of $^3$H-chlorpromazine in the incubation medium was necessary due to the high capacity of binding of L-dopa melanin. When a final concentration of $^3$H-chlorpromazine of $2.5 \times 10^{-6}$M was used at the lowest concentration of synthetic melanin, 2 mg, approximately 95% of the drug added to the medium was already bound.

Table 11 shows the linear regression parameters obtained from the binding data with the corresponding 95% confidence intervals.

Of the four drugs introduced as inhibitors of the $^3$H-chlorpromazine binding to synthetic L-dopa melanin, the comparison of the y-axis intercepts of the regression lines
Figure 35. Binding of $^3$H-chlorpromazine to bovine iris melanin granules in the absence and presence of some phenothiazines and related drugs.

Regression lines represent the binding of $^3$H-chlorpromazine to bovine iris melanin granules in the absence and presence of some phenothiazines and related drugs in a $^3$H-chlorpromazine:drug proportion of 1:30. Melanin granules were incubated with $^3$H-chlorpromazine 244.2 nCi/ml (2.5 X 10^-6M) for 30 minutes, after incubation with the unlabelled drug for 30 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).
Figure 35
Figure 36. Binding of $^3$H-chlorpromazine to bovine iris melanin granules in the absence and presence of dopamine.

Regression lines represent the binding of $^3$H-chlorpromazine to bovine iris melanin granules in the absence and presence of dopamine in a proportion of $^3$H-chlorpromazine:dopamine of 1:30. Melanin granules were incubated with $^3$H-chlorpromazine 244.2 nCi/ml (2.5 X $10^{-6}$M) for 30 minutes, after incubation with unlabelled drug for 30 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).
MELANIN GRANULES

$[^3\text{H}]-\text{CPZ}:\text{DOPAMINE}$

$1:30$

$n=5$

$[^3\text{H}]-\text{CPZ}$

$n=6$

Figure 36
### TABLE 10

**SUMMARY OF THE BINDING CONSTANTS OF $^3$H-CHLORPROMAZINE TO SYNTHETIC L-DOPA MELANIN IN THE ABSENCE AND PRESENCE OF OTHER DRUGS**

<table>
<thead>
<tr>
<th>DRUG</th>
<th>AFFINITY $M^{-1}$</th>
<th>CAPACITY Moles/mg Melanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-Chlorpromazine</td>
<td>$2.55 \times 10^7$</td>
<td>$1.60 \times 10^{-7}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Fluphenazine (1:10)</td>
<td>$2.16 \times 10^7$</td>
<td>$1.11 \times 10^{-7}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Thioridazine (1:10)</td>
<td>$2.33 \times 10^7$</td>
<td>$0.98 \times 10^{-7}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Haloperidol (1:10)</td>
<td>$1.22 \times 10^7$</td>
<td>$1.62 \times 10^{-7}$</td>
</tr>
<tr>
<td>$^3$H-Chlorpromazine + Clozapine (1:10)</td>
<td>$1.44 \times 10^7$</td>
<td>$1.86 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
### TABLE 11

SUMMARY OF THE LINEAR REGRESSION PARAMETERS FOR THE LANGMUIR TREATMENT OF THE BINDING OF \(^3\text{H}\)-CHLORPROMAZINE TO SYNTHETIC L-DOPA MELANIN IN THE ABSENCE AND PRESENCE OF OTHER DRUGS

<table>
<thead>
<tr>
<th>DRUG</th>
<th>SLOPE (C.I.(^a))</th>
<th>INTERCEPT X 10(^{-7}) (C.I.(^a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^3\text{H})-Chlorpromazine</td>
<td>0.25 (0.23 - 0.27)</td>
<td>6.26 (4.38 - 8.14)</td>
</tr>
<tr>
<td>(^3\text{H})-Chlorpromazine +</td>
<td>0.40(^b) (0.37 - 0.43)</td>
<td>8.45 (6.48 - 10.42)</td>
</tr>
<tr>
<td>Fluphenazine (1:10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^3\text{H})-Chlorpromazine +</td>
<td>0.44(^b) (0.41 - 0.46)</td>
<td>10.18(^c) (8.84 - 11.53)</td>
</tr>
<tr>
<td>Thioridazine (1:10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^3\text{H})-Chlorpromazine +</td>
<td>0.51(^b) (0.47 - 0.55)</td>
<td>6.19 (3.87 - 8.52)</td>
</tr>
<tr>
<td>Haloperidol (1:10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^3\text{H})-Chlorpromazine + Clozapine (1:10)</td>
<td>0.37(^b) (0.35 - 0.40)</td>
<td>5.38 (3.80 - 6.95)</td>
</tr>
</tbody>
</table>

\(^a\)C.I.: 95\% Confidence Intervals

\(^b\)Statistically different from \(^3\text{H}\)-Chlorpromazine binding regression line slope, \(P < 0.05\)

\(^c\)Statistically different from \(^3\text{H}\)-chlorpromazine binding regression line intercept, \(P < 0.05\).
corresponding to haloperidol, clozapine and fluphenazine show no statistical differences among them; therefore the capacity of synthetic melanin to bind $^3$H-chlorpromazine is not affected in the presence of these other drugs. However, a statistically different intercept was obtained in the presence of thioridazine. This indicates that this drug modifies the capacity of synthetic L-dopa melanin to bind $^3$H-chlorpromazine. The slope of the regression lines obtained in the presence of the binding inhibitors was always significantly greater ($P < 0.05$) than that obtained with $^3$H-chlorpromazine in the absence of the other drugs (table 11). Haloperidol appears to be a more effective inhibitor of the binding of $^3$H-chlorpromazine to synthetic L-dopa melanin than fluphenazine, thioridazine or clozapine (fig. 37). A comparison of the binding of $^3$H-chlorpromazine to synthetic L-dopa melanin both in the absence and presence of the other drugs is shown in figure 38.

**Binding of $^3$H-chlorpromazine to bovine iris melanin granules extracted with organic solvents**

Data on the affinity and capacity of melanin granules extracted with ether or acetone is shown in table 12. The corresponding linear regression parameters are shown in table 13. When compared to control granules processed in the same fashion, the intercept of the regression line
Figure 37. Summary of regression lines representing the binding of $^3$H-chlorpromazine to synthetic L-dopa melanin in the absence and presence of some phenothiazines and related drugs. Synthetic L-dopa melanin was incubated with 244.2 nCi/ml ($7.5 \times 10^{-5}$M) of $^3$H-chlorpromazine in the absence and presence of unlabelled drugs in a $^3$H-chlorpromazine:drug proportion of 1:10.
L-DOPA MELANIN

WITH HALOPERIDOL
WITH THIORIDAZINE
WITH FLUPHENAZINE
WITH CLOZAPINE

\[
\frac{[I/M \text{ CPZ per mg MELANIN}]}{[I/M \text{ FREE CPZ}] \times 10^{-8}}
\]

\[\text{CONTROL }^{3}\text{H-CPZ}\]

\[^{3}\text{H-CPZ: Inhibitor 1:10}\]
Figure 38. Binding of $^3$H-chlorpromazine to synthetic L-dopa melanin in the absence and presence of some phenothiazines and related drugs.

A. Regression lines represent the binding of $^3$H-chlorpromazine to synthetic L-dopa melanin in the absence and presence of some phenothiazines and related drugs in a proportion of $^3$H-chlorpromazine:drug of 1:10. Synthetic melanin was incubated with $^3$H-chlorpromazine, 244.2 nCi/ml ($7.5 \times 10^{-5}$M) for 30 minutes after incubation with the unlabelled drug for 30 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).
Figure 38
<table>
<thead>
<tr>
<th>TYPE OF GRANULES</th>
<th>Affinity $\text{M}^{-1}$</th>
<th>Capacity Moles/mg granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Unextracted)</td>
<td>$1.46 \times 10^8$</td>
<td>$2.31 \times 10^{-9}$</td>
</tr>
<tr>
<td>Ether extracted</td>
<td>$0.98 \times 10^8$</td>
<td>$3.85 \times 10^{-9}$</td>
</tr>
<tr>
<td>Control (Unextracted)</td>
<td>$0.39 \times 10^8$</td>
<td>$8.45 \times 10^{-9}$</td>
</tr>
<tr>
<td>Acetone extracted</td>
<td>$1.71 \times 10^8$</td>
<td>$1.80 \times 10^{-9}$</td>
</tr>
</tbody>
</table>
### TABLE 13

**SUMMARY OF LINEAR REGRESSION PARAMETERS FOR THE LANGMUIR TREATMENT OF THE BINDING OF $^3$H-CHLORPROMAZINE TO ISOLATED BOVINE IRIS MELANIN GRANULES EXTRACTED WITH ORGANIC SOLVENTS**

<table>
<thead>
<tr>
<th>Type of granules</th>
<th>SLOPE (C.I.)$^a$</th>
<th>INTERCEPT X 10$^{-9}$ (C.I.)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Unextracted)</td>
<td>2.96 (2.70-3.23)</td>
<td>0.43 (-0.29-1.16)</td>
</tr>
<tr>
<td>Ether extracted</td>
<td>2.64 (2.39-2.89)</td>
<td>0.26 (-0.52-1.04)</td>
</tr>
<tr>
<td>Control (Unextracted)</td>
<td>2.89 (2.63-3.16)</td>
<td>0.11 (-0.73-0.95)</td>
</tr>
<tr>
<td>Acetone extracted</td>
<td>3.27 (2.65-3.89)</td>
<td>0.56 (-0.02-0.22)</td>
</tr>
</tbody>
</table>

$^a$(C.I.), 95% Confidence Intervals
obtained using either ether or acetone extracted granules did not differ from the control values (P > 0.05).

Although there appear to be differences in the affinity of $^3$H-chlorpromazine for the acetone extracted granules, as compared to the controls, no statistically significant differences were found between the regression parameters (table 13). This is probably due to experimental variability, as shown by the dispersion of the points around the regression lines (fig. 39).
Figure 39. Binding of $^3$H-chlorpromazine to bovine iris melanin granules unextracted and extracted with organic solvents.

A. Regression lines represent the binding of $^3$H-chlorpromazine to bovine iris melanin granules unextracted and extracted with acetone (See Methods). Melanin granules were incubated with $^3$H-chlorpromazine 244.2 nCi/ml ($2.5 \times 10^{-6}$M) for 30 minutes. Affinity was calculated as intercept/slope and capacity as the reciprocal of the y-axis intercept (Shimada et al., 1976).

B. Regression lines represent the binding of $^3$H-chlorpromazine to bovine iris melanin granules unextracted and extracted with ether (see Methods). The granules were incubated with $^3$H-chlorpromazine 244.2 nCi/ml ($2.5 \times 10^{-6}$M) for 30 minutes.
Figure 39

(A) MELANIN GRANULES

- ACETONE EXTRACTED
  - n = 5
- CONTROL
  - n = 5

(B) MELANIN GRANULES

- ETHER EXTRACTED
  - n = 5
- CONTROL
  - n = 5

\[
\left[\text{I/M CPZ per mg GRANULES}\right] \times 10^{-8}
\]

\[
\left[\text{I/M FREE CPZ}\right] \times 10^{-8}
\]
CHAPTER IV

DISCUSSION

Differential Mydriatic Effects of Atropine

When compared to the nonpigmented iris, the marked accumulation of $^3$H-atropine by the pigmented iris is very similar to that observed with other liposoluble drugs like chlorpromazine (Potts, 1964), cocaine (Patil, 1972), ephedrine (Patil et al., 1974) and β-phenethyamine (Patil and Jacobowitz, 1974). However, at high concentration of $^3$H-atropine, the equilibrium profile for the accumulation in pigmented iris differed from the above drugs. A rapid equilibrium is reached within 30 min and when the incubation is continued there is an increased accumulation. Since irides from atropinesterase positive and the enzyme unclassified animals show similar accumulation patterns, the unusual uptake equilibration profile in vitro may not be enzyme dependent.

Many structurally unrelated drugs are accumulated by melanin granules (Potts, 1964; Patil, 1972; Patil et al., 1974) and synthetic L-dopa melanin (Shimada et al., 1973). Hence, the accumulation of drugs by the pigmented iris is
attributed to the binding of drugs to the melanin. Shimada et al. (1973) studied the binding of \(^3\)H-atropine to synthetic L-dopa melanin. The binding is a saturable process, with the affinity and the capacity of the drug for the binding being \(0.2 \times 10^5 \text{M}^{-1}\) and \(17.9 \times 10^{-9}\) moles/mg, respectively.

In vitro, the rate of loss of \(^3\)H-atropine by the two types of irides is also similar to that observed with cocaine and ephedrine (Patil, 1972; Patil et al., 1974). In repeated washing, drugs are rapidly lost from the non-pigmented iris. The \(t_\frac{1}{2}\) for the loss of radioactivity from the nonpigmented iris incubated with \(^14\)C-cocaine, (-)-\(^14\)C-ephedrine and \(^3\)H-atropine were 10 min, 12 min and 14 min, respectively. Despite the fact that the values for \(t_\frac{1}{2}\) are approximately equal, the loss of the former two drugs from the tissue follows a single exponential decline while that of the latter is quite complex. The drugs accumulated by the pigmented iris, on the other hand, are retained and only a fraction of the total drug is lost in washings. This suggests that the interaction between the drug and melanin may not be a simple, reversible process; but the binding appears to be irreversible.

Previously, several theories were proposed and discussed to explain the well-known iris color-dependent, mydriatic effects of drugs (Chen and Poth, 1929; Angenent
and Koelle, 1952, 1953; Obianwu and Rand, 1965; Seidehamel et al., 1970). The unequal effects of mydriatics are not race dependent because albino Africans respond more like Caucasians with light color iris (Emiru, 1971). An atypical muscarinic receptor of the pigmented iris sphincter could explain the observed initial slow onset of mydriasis. This explanation is less likely because (i) the unequal mydriatic effects in humans or in guinea pigs are also observed with nonmuscarinic drugs like cocaine and ephedrine (Chen and Poth, 1927; Seidehamel et al., 1970); and (ii) in vitro, the sensitivity of the pigmented or the nonpigmented iris sphincter to the quaternary, nonliposoluble muscarinic agonist carbachol in the two types of irides is identical (table 6). If the ED₅₀ reflects the affinity of the drug to the muscarinic receptors, it can be concluded that the receptors in the two types of irides are similar.

In the case of the pigmented and non-pigmented iris no differences in the pA₂ values were obtained. The characteristics of the line obtained for the pigmented iris can be explained on the basis that at low concentrations of the antagonist, the gradient of the drug to the pigment is very small, whereas at higher concentrations the loss of drug to the pigment makes it a less effective blocker in the pigmented iris. This observation should
explain the slow onset of action of the drug in the heavily pigmented eye. Equal $pA_2$ values of atropine on the stomach fundus strips either obtained from albino or nonalbino rabbits emphasize the pigment-dependent effects of the drug. In vivo, however, the topical application of atropine to the pigmented and the nonpigmented rabbit eye failed to show the differences in the onset of action. For several reasons the accurate comparisons of in vivo data in rabbits with that in humans could not be made. (i) The rabbit cornea is only half as thick as the human cornea and, therefore, relative to that seen in humans, the rate of penetration of drugs through rabbit cornea will be faster. The fast initial drug interaction kinetics could obscure the differences in the onset of action in the two types of rabbits. (ii) The initial pupillary diameter of the pigmented and the nonpigmented eye differ. Although equal amounts of the drug are transported across the corner in both types of rabbits, the resting position of the iris sphincter could affect the initial drug effects. In humans, this factor does not exist; the initial pupillary diameter of the albino and the nonalbino Africans are equal (Emiru, 1971). (iii) In vitro the drug effects were accurately quantitated, while such a quantitation in vivo had a limitation. If the small differences in onset of action of atropine in vivo in the pigmented and the nonpigmented eye do exist, it
could be easily obscured by the measurement error.

The data on the duration of atropine mydriasis are particularly interesting (fig. 17). The shortest duration of action in albino atropinesterase positive animals could be explained by the rapid destruction of the drug by the enzyme (Werner, 1965; Kalow, 1962; Ecobichon and Comeau, 1970). In the nonalbino, atropinesterase positive animals, the enzyme will limit the action but a part of the drug which is not destroyed by the enzyme could still be stored by the pigment. The stored drug could be released to produce the muscarinic block, but again the enzyme will limit the free concentration of the drug (fig. 17). In albino atropinesterase negative animals, a relatively prolonged effect of the drug is understandable as the enzyme will not destroy the drug. These results in albino atropinesterase negative and positive animals are identical to those reported before (Werner, 1965; Kalow, 1962). Since the longest duration of action was observed in nonalbino atropinesterase-negative animals, this indicates the role of both the enzyme and the pigment in atropine mydriasis. Data from experiments using albino and nonalbino atropinesterase negative rabbits indicated that when 2% $^3$H-atropine was applied topically, at the end of the 96th hour a significant amount of radioactivity was found in the pigmented
iris, whereas the nonpigmented iris retained little or no radioactivity. This radioactivity was demonstrated to be authentic atropine in a separate series of experiments on atropinase-negative nonalbino rabbits. Thus, the long duration of the mydriatic effect of the drug is explained.

The accumulation of $^3$H-atropine by the pigmented human iris is very similar to that accumulated by pigmented iris obtained from atropinesterase-negative rabbit (table 5). Since anatomically the pigment cells and the smooth muscle cells of the sphincter lie in very close proximity to each other (Walls, 1967), it seems logical that the drug could be slowly released from the pigment cell to produce a prolonged muscarinic block. Thus, the long duration of mydriatic effects of the drug is explained. These prolonged effects in nonalbino atropinesterase-negative animals are very similar to the prolonged effects seen in nonalbino humans who lack the enzyme (Goodman and Gilman, 1971; Havener, 1966). In vitro studies on the recovery of the muscarinic block by atropine from the pigmented and the nonpigmented iris also support the role of pigment in the recovery of the antimuscarinic effect (fig. 16).

Our interpretation of differential mydriatic effects of atropine in albino and nonalbino rabbits is summarized in figure 40. Since the initial rate of
Figure 40. Schematic representation which explains the effects of atropine in the pigmented and the nonpigmented eye. The transport of atropine across the cornea in both types of eye appears to be equal. The transported drug could be partly destroyed by the atropinesterase in aqueous humour, or partly stored in the pigment cell and its constituents and only a fraction of the transported drug will block the muscarinic receptor. The duration (t½) of drug effect of atropine in four types of rabbits is albino atropinesterase-positive, 3.8 h; nonalbino atropinesterase-positive, 12.4 h; albino atropinesterase-negative, 29.7 h; nonalbino atropinesterase-negative ≥ 96 h. Thus, the relative contribution of the enzyme and the pigment in the duration of atropine mydriasis is quite clear. The drug stored by the pigment could be slowly released to produce a prolonged block in nonalbino atropinesterase negative animals. The duration of action of atropine in nonalbino atropinesterase-negative animals is similar to that seen in human eye which contains melanin and lacks the enzyme.
Figure 40
atropine transport across the cornea (fig. 13) is the same in albino and nonalbino rabbits, when atropine is applied topically in the eye, after absorption the following may happen. In the albino rabbits, the atropine will interact with muscarinic receptors, but it will also be metabolized by atropinesterase in the aqueous humor or the serum after it enters the circulation. In the nonalbino rabbit, on the other hand, melanin becomes an additional factor in determining the fate of atropine. After the drug is bound to melanin it may be slowly released, prolonging in this way the blockade of the muscarinic receptors. In the absence of enzymatic degradation, in the nonalbino rabbit, a significant amount of drug is retained in the pigmented tissue. Since the pigment cells and iris muscles lie in close proximity (Walls, 1967), slow release of the drug from the pigment may permit a more prolonged blockade of the muscarinic receptors of the iris, leading, therefore, to a longer duration of the mydriatic and cycloplegic effects.

Although the role of atropinesterase in selecting the rabbit for research has been emphasized previously and in this study, more importantly we wish to emphasize the value of selecting nonalbino animals for ocular research because of the far greater frequency of individuals with pigmented eyes in the population at large.
Binding of $^{14}$C-imipramine to pigmented and nonpigmented tissues

The results clearly indicate that, as compared to nonpigmented iris, the pigmented one accumulates large amounts of $^{14}$C-imipramine. Although several tissue components may contribute to the total accumulation of drug in the iris, data from the discontinuous sucrose density gradient experiments indicate that $^{14}$C-imipramine is not randomly accumulated by tissue fractions. In the nonpigmented iris, which lacks melanin, a relatively large percent of the drug is bound by low sucrose density fractions where synaptosomes separate (Whittaker et al., 1964). Since imipramine is known to interact with the transport site in the neurone, the high accumulation by low density-sucrose fractions is expected. In the pigmented iris, the drug is accumulated by both and the low density sucrose fraction accumulates about 70% of the total drug bound by the iris. Since synthetic L-dopa melanin and melanin granules accumulate $^{14}$C-imipramine, the observed binding in pigmented tissue must largely be to the endogenous melanin.

On repeated washings very little drug is lost from the pigmented iris, while half of the total drug was lost from the nonpigmented tissues in 14 min. The retention of the high amount of drug by the pigmented iris is very similar to the other liposoluble drugs like cocaine,
ephedrine and 8-phenethy1amine (Patil, 1972; Patil and Jacobowitz, 1974; Patil et al., 1974). However, the rate of loss of $^{14}$C-imipramine from the nonpigmented iris is unlike the drugs which are lost from the nonpigmented iris at a single exponential rate. Considering the various tissue components with which $^{14}$C-imipramine is postulated to interact (Roth and Gillis, 1974; Hunt et al., 1975; Bickel and Börner, 1974), a complex rate of loss of the drug from the nonpigmented tissue is understandable.

The affinity constant values obtained in our experiment for binding of imipramine to melanin granules are comparable to those obtained in rat liver fractions (mitochondria, microsomes, etc.) (Bickel and Steele, 1974), to whole blood, red blood cells and red blood cell membranes (Bickel, 1975) and human serum albumin (Sharples, 1975). On the other hand melanin granules showed to have a capacity to bind imipramine similar to that of rat liver but it is about $10^4$ times greater than that of blood elements. This fact should be considered in the light of the kinetics of imipramine in the body in relation to the onset of appearance and duration of imipramine effects.

The relevancy of unequal drug binding by pigmented and nonpigmented irides in relation to the pharmacological effect was examined in the in vitro iris sphincter preparation. The antimuscarinic effect of imipramine was used
as a test parameter. Results indicate that imipramine is apparently a weaker antimuscarinic substance in the pigmented iris. If the drug is rapidly bound by the pigment, there should be a drop in the free concentration of the drug to interact with the receptor and thus the degree of receptor blockade will be small. The converse should occur in the nonpigmented eye to produce a greater degree of the receptor blockade. These results obtained with imipramine are in agreement with those obtained in our laboratory with atropine (see above).

Melanins obtained from a variety of sources, including neuromelanin from the human substantia nigra, show characteristic electron spin resonance signals which indicate the presence of free radicals in the polymer (Van Woert et al., 1967). These free radicals of melanins probably interact with and retain many substances (Karreman et al., 1959; Mason et al., 1960; Potts, 1964; Blois, 1971). In fact, Borg (1965) demonstrated the formation of imipramine free radicals. He suggests that these free radicals, as for chlorpromazine, may be related to the pharmacological actions of these drugs. On this basis a selective accumulation of imipramine in the substantia nigra was anticipated. Results from the tissue homogenate (fig. 23) indicate a significantly higher accumulation of the drug by the substantia nigra.
Lindquist (1972) used autoradiographic technique to demonstrate the localization of $^{35}$S-chlorpromazine by the human brain neuromelanin. It was implied that the accumulation may be important in understanding the extrapyramidal side effects produced by tricyclic psychotropic drugs. However, the accumulation does not necessarily mean a toxicity, which is determined by as yet unknown mechanisms. In any case, imipramine is known to produce mild extrapyramidal symptoms resembling those of chlorpromazine. After medication of imipramine, if the drug is accumulated by neuromelanin and released to produce weaker blockade of dopamine receptors of the caudate nucleus, it may explain the symptoms produced by the drug.

Although no reference to prolonged mydriatic effects of imipramine has been made, the fact that imipramine preferentially accumulates in melanin emphasizes our contention of the relevance of the pigment for the distribution, and therefore for the effects of drugs.

**Localization of labelled drugs in human brain and rabbit retina by discontinuous sucrose density gradient centrifugation**

$^{14}$C-Imipramine, $^3$H-haloperidol and $^3$H-chlorpromazine were found to selectively bind the pigmented fraction of substantia nigra which separates at the 1.5 M sucrose layer of the density gradient. However, no differences in
binding of $^{14}\text{C}$-imipramine and $^{3}\text{H}$-haloperidol to this fraction were found. $^{3}\text{H}$-chlorpromazine was found to bind the pigmented fraction significantly more than haloperidol and imipramine. However, the differences in binding obtained in our experiments do not correlate with the reported severity of extrapyramidal syndrome produced by these drugs.

Although imipramine has been reported to produce only very mild extrapyramidal symptoms, haloperidol and chlorpromazine are well known for inducing Parkinson's syndrome after prolonged use. An inverse relationship between antimuscarinic effects in the central nervous system and ability to produce Parkinson's syndrome among neuroleptic drugs has been suggested (Snyder et al., 1974). Haloperidol is known to produce the most serious extrapyramidal disturbances. Haloperidol has also been reported to be an excellent blocker of dopamine receptors in the central nervous system (Carlsson and Lindquist, 1963). Snyder et al. (1974) have suggested that blockade of dopamine receptors by phenothiazines and butyrophenones may explain the prominent extrapyramidal side effects of these drugs. By blocking the dopamine receptors in the corpus striatum these drugs produce a functional deficiency of dopamine. It is suggested that the Parkinsonian side-effects of phenothiazines are the result of blockade of
dopamine receptors in the striatum, whereas the antischizophrenic action may be related to dopamine receptors in other areas of the brain.

In light of the evidence presented so far on binding of phenothiazines to brain tissue (Lindquist, 1972, 1973) and degeneration of substantia nigra in patients with prolonged treatment with phenothiazines (Forrest et al., 1964; Christensen et al., 1970) and based on our experimental results, we suggest that accumulation of the drugs in neuromelanin may be a prerequisite to the appearance of the extrapyramidal symptoms. Accumulation of the drug, accompanied by "inactivation" of neuromelanin by phenothiazines followed by degeneration of the neuron have been suggested by Forrest (1974) as part of the mechanism underlying the depigmentation of substantia nigra found in patients with drug induced dyskinesia.

Our results on the differential binding of $^3$H-chlorpromazine in pigmented and nonpigmented rabbit retina and the selective accumulation of the drug in the pigmented fraction of the tissues indicate that drug induced retinopathies are not the result of a random accumulation of drug in the eye. Since albino retinas do not accumulate polycyclic compounds and do not develop retinal damage (Meier-Ruge et al., 1966; Meier-Ruge and Cerletti, 1968), drug-induced retinotoxicity in the pigmented eye appears
to depend on the melanin content of the uvea.

**Binding of $^3$H-chlorpromazine to bovine iris melanin granules and synthetic melanins**

Our results on binding of $^3$H-chlorpromazine to bovine iris melanin granules and synthetic melanins demonstrated that the drug has more affinity for the binding sites in melanin granules than in synthetic melanins; however, the capacity of synthetic melanins to bind $^3$H-chlorpromazine is much greater than that of melanin granules. The affinity of $^3$H-chlorpromazine to bind melanin granules is approximately 1,000 times greater than that for rat liver cell fractions (Bickel and Steele, 1974) and human serum albumin (Sharples, 1975). The nature of the two systems may account for the differences, but does not explain them. It may be suggested that there are more binding sites in the synthetic melanins or that they are more readily accessible to the drug if one assumes that synthetic melanins are just melanin polymers, without the probable complexity of the melanin granules. It would not be proper, however, to consider synthetic melanins just simple polymers. Our experiments on binding of $^3$H-chlorpromazine to synthetic melanins suggest more than that.
Although one would expect that melanins synthesized from L-dopa, dopamine, L-α-methyldopa or D-α-methyl-dopa might be structurally different polymers, our results suggest that the nature of the polymer may have great influence on its ability to bind other compounds. For example, the capacity of dopamine melanin to bind $^3$H-chlorpromazine is smaller than that of the other melanins. Dopamine differs from the other precursors in lacking the carboxyl and the α-methyl groups, respectively. On the other hand, the affinity of $^3$H-chlorpromazine for binding sites in dopamine melanin is greater than for the other synthetic melanins, and the affinity of the drug for L-α-methyldopa melanin is two times greater than for the melanin obtained from the D-isomer. In looking at the structure of the precursors used to synthesize the melanins and the corresponding intermediate compound, one could speculate that the presence and stereochemistry of the carboxyl group, as well as the presence of substituents in the side chain may determine, in part, the number or availability of binding sites in the synthetic melanins polymer (fig. 41). These speculations would agree with the hypothesis of an irregular melanin polymer formed from the polymerization of intermediates, including the indole-5,6-quinone, of the melanin synthesis.
Figure 41. Pathway of melanin synthesis showing different precursors and probable intermediates formed corresponding to the dopachrome of the L-dopa melanin synthesis according to the Raper-Mason pathway.
L-dopa → dopachrome → Melanin₁

dopamine

L-α-methyldopa → L-α-methyldopachrome (?)

D-α-methyldopa → D-α-methyldopachrome (?)
Lack of knowledge of the structure of melanin makes an analysis of binding characteristics and differences between natural and synthetic melamins on the basis of chemical structure impossible at the present time. However, a detailed study of the binding of drugs to synthetic melamins may help to understand the mechanisms of binding of drugs to natural melamins.

One could assume that if the binding of drugs to melanin granules only takes place in the melanin moiety one could expect differences in the binding when one compares the binding of $^3$H-chlorpromazine to intact melanin granules with that to melanin granules which have been subjected to extraction with organic solvents. Our results on the binding of $^3$H-chlorpromazine to intact granules and granules extracted with organic solvents do not confirm these assumptions. No differences in binding between both kinds of granules were obtained. The very high liposolubility of the drug may account for the rapidity with which the binding equilibrium with chlorpromazine is reached in melanin granules (fig. 31), but it does not explain the lack of differences in binding when the granules are extracted with organic solvents.

It has been demonstrated that phenothiazines affect membrane permeability in various biological systems (Guth and Spirtes, 1964; Domino et al., 1968; Van Woert, 1970). These drugs reduce surface activity and they
accumulate and cause lysis of membranes similar to the action of detergents. If chlorpromazine produces this detergent effect in intact melanin granules, promoting its own penetration and binding, one would expect the same results when other agents such as organic solvents are used. Therefore, in granules extracted with organic solvents, binding of chlorpromazine is expected to be similar to that in intact granules as demonstrated by our results.

The analysis of the data on binding of $^3$H-chlorpromazine to melanin granules revealed that the phenothiazine fluphenazine and thioridazine significantly decrease the capacity of melanin granules to bind $^3$H-chlorpromazine ($P < 0.05$) and also the apparent affinity constant of the drug. This is suggestive that the fluphenazine and thioridazine mechanism of inhibition of chlorpromazine binding is very complex. A combination of competitive and noncompetitive inhibition seems to be taking place. Although an explanation on the basis of structure-activity relationship is attractive, our experimental design and results do not allow us to account for it.

On the other hand, clozapine, haloperidol, and dopamine are demonstrated to be competitive inhibitors of $^3$H-chlorpromazine binding. Haloperidol and clozapine were better inhibitors than the other drugs. Because of the different chemical structure of the compounds, generalizations about the nature of the binding are not allowed.
All the drugs showed ability to bind the melanin granules; however, the fact that the ability to inhibit $^3$H-chlorpromazine differs from one drug to another indicates that other factors more than their affinity for melanin are involved in the binding of these drugs. Physicochemical characteristics of the drugs seem to be very important factors in determining the binding of the drugs.

Binding of $^3$H-chlorpromazine to synthetic L-dopa melanin is competitively inhibited by haloperidol, clozapine, and fluphenazine. Thioridazine appears to inhibit the binding of $^3$H-chlorpromazine to synthetic L-dopa melanin by a combination of competitive-noncompetitive inhibition. Haloperidol seemed to be a better inhibitor than the other drugs. It is interesting that clozapine does not seem to be as good an inhibitor of chlorpromazine binding to synthetic L-dopa melanin as it showed to be for binding of chlorpromazine to melanin granules.

These findings on the binding of $^3$H-chlorpromazine to melanin granules and synthetic melanins in the absence and presence of other drugs emphasizes the idea that the binding of drugs to melanin involves more than the simple affinity of the drugs for melanin. However, although the differences in binding among drugs and between the two systems do not seem important enough to object to synthetic melanins as suitable models for the study of binding of drugs to melanins in the body, it points out the problems
of extrapolating results obtained in studies with synthetic melanins to natural melanins.

All of the results obtained in these studies point to the fact that melanins are not inert compounds with no specific role in the body. Due to the drastic conditions necessary in most cases for the demonstration of the formation of free radicals, the electron paramagnetic properties of many drugs are not known. However, the fact that the stable free radical properties of melanins have been demonstrated (Mason et al., 1960; Van Woert et al., 1967) and drugs such as phenothiazines have been demonstrated to form free radicals and interact with melanin forming charge-transfer complexes suggests that the formation of free radicals may be a factor determining the interaction of drugs with melanin. In fact, free radicals have been demonstrated for several drugs, imipramine, salicylic acid, opiates, etc. (Borg, 1965; 1972).

These facts, together with the evidence on binding of drugs to melanin presented so far, indicate that the binding to melanins may be an important element of the pharmacokinetics of drugs. Therefore, due to the consequences that the binding of drugs to melanin may provoke, and due to the fact that the population at large have melanin, we recommend that studies on the binding of drugs be conducted in pigmented animals and in pigmented tissues for drugs with possible therapeutic use.
CHAPTER V

SUMMARY

1. In studying the differential mydriatic effects of atropine in albino and nonalbino rabbits it was found that:

In vitro experiments:

a. In irides from albino and nonalbino atropinesterase positive rabbits, the pigmented iris accumulated larger amounts of $^3$H-atropine than the nonpigmented ones. On repeated washings little or no radioactivity was lost from the pigmented iris, whereas in the nonpigmented iris radioactivity was rapidly lost ($t_{1\%}$ 14 min).

b. Pigmented human irides accumulated $^3$H-atropine in an amount comparable to that accumulated by pigmented iris obtained from atropinesterase-negative rabbits.

c. After topical application of a 2% solution of the drug, the rate of penetration of $^3$H-atropine through the cornea was similar in both albino and nonalbino rabbits, and after 40 minutes only 0.1% of the drug was bound.

219
d. $pA_2$ values from nonpigmented iris and fundus strips varied between 8.58 and 8.88 with slope values close to the theoretical value of 1. The $pA_2$ value of atropine in the pigmented iris was 8.82, but at higher concentrations atropine was a less effective blocker than that observed in the nonpigmented iris. The unusual $pA_2$ plot obtained from the pigmented iris is attributed to loss of the drug to the pigment.

e. Recovery of nonpigmented iris from atropine blockade was faster than that of pigmented iris. The degree of atropine blockade was evaluated by introducing an $ED_{90}$ dose of catechol every fifteen minutes.

In vivo experiments were conducted in atropinesterase positive or negative albino and nonalbino rabbits. It was found that:

a. The order of half-times of atropine mydriasis in the four types of rabbits was: Nonalbino atropinesterase-negative > albino atropinesterase-negative > nonalbino atropinesterase-positive > albino atropinesterase-positive. In the nonalbino atropinesterase-negative rabbits, the half-time of duration of action of atropine was 96 hours. This value is comparable to the half-time mydriatic effect of atropine in humans.
b. In atropinesterase negative rabbits, $^3$H-atropine retained in the iris 96 hours after topical application was higher in the pigmented iris than in the nonpigmented ones. With the aid of thin layer chromatography it was confirmed that the radioactivity retained in the tissue was atropine.

c. The prolonged duration of atropine effects in non-albino rabbits is explained on the basis of accumulation of the drug in the eye pigment from which it is slowly released onto the muscarinic receptors producing a prolonged local blockade.

2. In in vitro experiments using $^{14}$C-imipramine:

   a. Both pigmented and nonpigmented rabbit irides accumulated $^{14}$C-imipramine. The ratio of radioactivity accumulated by the pigmented iris to the nonpigmented one varied between 1.5 and 2.0. The loss of radioactivity from the pigmented iris is faster than from the pigmented one, but the loss of radioactivity from the nonpigmented iris does not follow a simple exponential process.

   b. Imipramine is a less effective blocker of muscarinic receptors in the pigmented rabbit iris than in the nonpigmented iris.
c. When homogenates of human substantia nigra were incubated with $2 \times 10^{-5}$ M (12.8 nCi/ml) of $^{14}$C-imipramine they accumulated significantly higher amounts of the drug than cerebral cortex homogenates.

d. $^{14}$C-imipramine accumulated by the pigmented iris was mainly localized in the heavy melanin granules fraction separated by discontinuous sucrose density gradient centrifugation.

e. The capacity of bovine iris melanin granules to bind $^{14}$C-imipramine is about 1/50th that of synthetic L-dopa melanin, whereas the affinity of the drug for the binding sites in the melanin granules is higher than that for the synthetic melanin by a factor of 250.

3. Albino and nonalbino rabbits retinas were incubated with $2.5 \times 10^{-6}$ M (244.2 nCi/ml) $^{3}$H-chlorpromazine. When fractionated on a discontinuous sucrose density gradient two pigmented bands were separated and the largest amount of drug (41% of total bound) in the pigmented retina, was found in the sediment of melanin granules at the bottom of the gradient. Little or no drug was found in the corresponding nonpigmented fraction of the albino rabbit retina.
4. In *in vitro* studies on binding of drugs to human brain, $^{14}\text{C}$.imipramine, $^{3}\text{H}$.haloperidol and $^{3}\text{H}$.chlorpromazine were incubated with homogenates of substantia nigra and superior cerebellar peduncles and the homogenates were fractionated on a discontinuous sucrose density gradient. In these experiments:

   a. A distinct pigmented band, presumably of neuro-melanin, consistently separated at the 1.5 M sucrose fraction (at the interface with the 2.0 M sucrose fraction).

   b. The amount of $^{14}\text{C}$.imipramine, $^{3}\text{H}$.haloperidol or $^{3}\text{H}$.chlorpromazine separated at the 1.5 M sucrose fraction was found to be significantly higher ($P < 0.05$) than in the corresponding fraction from the nonpigmented superior cerebellar peduncles homogenates. Presumably the melanin fraction binds more drug than the corresponding fraction of nonpigmented brain tissue.

   d. The amount of $^{3}\text{H}$.chlorpromazine bound to the 1.5 M sucrose fraction from substantia nigra was significantly higher ($P < 0.05$) than the amount of $^{14}\text{C}$.imipramine or $^{3}\text{H}$.haloperidol bound to the same fraction of substantia nigra. No differences in the binding of the drugs to the 1.5 M sucrose fraction of superior cerebellar peduncles homogenate were found.
The binding of $^3$H-chlorpromazine to bovine iris melanin granules and to synthetic L-dopa, dopamine, L-α-methyl-dopa and D-α-methyldopa melanins was compared. In these experiments:

a. Compared to synthetic melanins the capacity of bovine iris melanin granules to bind $^3$H-chlorpromazine was 1/60th, 1/90th, 1/120th, and 1/180th that of dopamine melanin, L-dopa melanin, L-α-methyldopa melanin and D-α-methyldopa melanin, respectively. The affinity of $^3$H-chlorpromazine for the binding sites in the melanin granules is greater than for any of the synthetic melanins used.

b. The capacity of synthetic melanins to bind $^3$H-chlorpromazine was as follows: D-α-methyldopa melanin > L-α-methyldopa melanin > L-dopa melanin > dopamine melanin. The affinity of $^3$H-chlorpromazine for synthetic melanins was: dopamine melanin > L-α-methyldopa melanin > L-dopa melanin > D-α-methyldopa melanin.

c. Haloperidol and clozapine were more effective inhibitors of the binding of $^3$H-chlorpromazine to melanin granules than fluphenazine or thioridazine or dopamine. Haloperidol, clozapine and dopamine behaved as competitive inhibitors of
the binding, whereas fluphenazine and thioridazine showed a combination of competitive-noncompetitive inhibition.

d. Haloperidol, clozapine and fluphenazine behaved as competitive inhibitors of the binding of $^3$H-chlorpromazine to synthetic L-dopa melanin. Thioridazine showed a combination of competitive-noncompetitive inhibition. Haloperidol was demonstrated to be the most effective inhibitor and clozapine the least effective.

e. Treatment of melanin granules with organic solvents, such as ether and acetone, did not affect the binding of $^3$H-chlorpromazine to the granules. The detergent effect of phenothiazines, probably already affecting the normal granules may account for the lack of differences in binding obtained in granules extracted with organic solvents as compared to control granules.
APPENDIX A

**In vitro Uptake and Loss of $^3$H-Atropine from Tissues**

Uptake and loss of atropine from tissues was studied in isolated irides and stomach fundus strips from albino and nonalbino rabbits unclassified for atropinesterase. The irides were incubated with increasing concentrations of the drug. The procedure followed was the same as described in Methods for atropinesterase-positive rabbits.

At all concentrations of $^3$H-atropine used, pigmented irides accumulated significantly larger amounts of drug than the nonpigmented ones. No differences were observed when the radioactivity accumulated by the unclassified rabbit irides was compared to that accumulated by the atropinesterase-positive rabbits for both albino and nonalbino strains (fig. 42).

Figure 43 illustrates the loss of radioactivity from albino and nonalbino rabbit iris stomach strips. The rate of loss of radioactivity from unclassified pigmented and nonpigmented rabbit iris was found to be similar to that in the atropinesterase-positive rabbits. Little or no drug was lost from the pigmented irides, whereas in the nonpigmented iris radioactivity disappeared very rapidly.
Figure 42. *In vitro* accumulation of $^3$H-atropine by the pigmented and nonpigmented iris of atropinesterase-positive and enzyme-unclassified rabbits. The accumulation of the drug by irides obtained from serum atropinesterase-positive rabbits were not significantly different than that selected at random on unclassified animals. Each point represents an average of 4 to 5 different observations with S.E.M.
Figure 42

- Pigmented Iris
- Nonpigmented Iris
- Atropinesterase positive
- Unclassified

n = 4 - 5
(t½, 14 min). The loss of radioactivity from stomach fundus strips shown in this figure corresponds to atropinesterase-positive rabbits.

Accumulation of ³H-atropine by irides obtained from atropine pretreated rabbit eyes

Atropine sulfate, 0.1 ml of 4% solution, was applied to one eye of both albino and nonalbino rabbits unclassified for atropinesterase. Twenty four hours after the application, the animals were killed and the irides isolated, and incubated with ³H-atropine. The procedure followed was the same as described in the Methods section for accumulation of ³H-atropine by tissues. The contralateral iris was used as a control.

The results from this series of experiments are summarized in table 14. It can be noted that ³H-atropine accumulation was reduced only in the pigmented iris.

Antimuscarinic effects of atropine on pigmented and nonpigmented rabbit iris

The blocking effects of a 2 X 10⁻⁸ M atropine dose were tested against a single ED₉₀ dose of carbachol in both pigmented and nonpigmented rabbit irides. The procedure followed was the same as described in Methods (Functional experiments).

The results are summarized in table 15.
Figure 43. Rate of disappearance of $^{3}$H-atropine from iris and stomach fundus strips obtained from atropinesterase-positive and enzyme-unclassified albino and nonalbino rabbits.

Tissues were incubated with 30 nCi/ml of $^{3}$H-atropine ($10^{-5}$M) for 35 min and at regular intervals it was transferred to the drug-free physiological salt solution at 37°C. Irides obtained from atropinesterase-unclassified rabbits were selected at random. Stomach fundus strips were obtained from atropinesterase-positive animals only. Each point on the graph represents the mean of 4 to 5 observations with S.E.M.
TABLE 14

IN VITRO, ACCUMULATION OF $^{3}$H-ATROPINE (30 nc/ml, $10^{-5}$M, incubated for 35 min) BY IRIDES FROM RABBITS TREATED TOPICALLY (24 hr before) WITH 0.1 ml OF 4% ATROPINE

<table>
<thead>
<tr>
<th>Tissue</th>
<th>dpm/25 mg iris</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Atropine treated</td>
</tr>
<tr>
<td>Nonpigmented iris</td>
<td>7456 ± 1214</td>
<td>7228 ± 437</td>
</tr>
<tr>
<td>(N = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigmented iris</td>
<td>28623 ± 3102</td>
<td>20334 ± 2940</td>
</tr>
<tr>
<td>(N = 6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aContralateral iris from the same animal without the atropine treatment.
TABLE 15

COMPARATIVE BLOCKING EFFECTS OF ATROPINE AGAINST A SINGLE DOSE ED₉₀ OF CARBACHOL ON THE PIGMENTED AND NON-PIGMENTED IRIS OBTAINED FROM ATROPINESTERASE POSITIVE RABBITS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean tension (in mg with SEM) after carbachol (10⁻⁵M)</th>
<th>With atropineb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controla</td>
<td>2 X 10⁻⁸M</td>
</tr>
<tr>
<td>Nonpigmented iris</td>
<td>239 ± 42</td>
<td>50 ± 6</td>
</tr>
<tr>
<td></td>
<td>N = 6</td>
<td></td>
</tr>
<tr>
<td>Pigmented iris</td>
<td>240 ± 50</td>
<td>190 ± 55c</td>
</tr>
<tr>
<td></td>
<td>N = 5</td>
<td></td>
</tr>
</tbody>
</table>

aSensitivity of contralateral control iris (to carbachol) during the testing procedure was increased by 106% (±8) and 115% (±5) for the nonpigmented and pigmented iris, respectively.

bIncubation time = 60 min.

cP < 0.05 when compared with that from nonpigmented iris.
APPENDIX B

Binding of $^{14}$C-Imipramine to Isolated Irides from 6-Hydroxydopamine Treated Albino and Nonalbino Rabbits

Rabbits were given two doses, 15 mg/Kg each, of 6-hydroxydopamine (Regis Chemical Co.) at 24 hours intervals. Each dose was infused during a two hour period in the ear marginal vein, using a polyethylene tubing connected to a syringe. The 6-hydroxydopamine solutions were prepared in 0.1% sodium metabisulfite. The syringe cylinder was tightly wrapped with aluminum foil as additional protection of the 6-hydroxydopamine solution from photooxidation. At the end of each injection period, 0.1 ml of a 2% solution of 6-hydroxydopamine was topically applied to each eye. At the end of 48 hours the animals were killed. The irides were isolated as described by Jacobowitz (1967). The irides were incubated with $2.5 \times 10^{-6} M$ (12.8 nCi/ml) of $^{14}$C-imipramine for 60 minutes and the loss of radioactivity from the tissue was determined as described above in the Methods section.

The results are summarized in table 16. When compared to normal rabbit iris, the amount of drug retained
by the pretreated rabbit iris, no significant differences were found except for the nonpigmented rabbit iris after 15 minutes of washing. In this particular instance, the amount of drug retained by the 6-hydroxydopamine treated rabbit iris was significantly higher than in the normal rabbits (P < 0.05).

At 120 minutes after washings, the amount of drug retained by both pigmented and nonpigmented iris from pretreated rabbits was similar to the corresponding iris in the normal group of rabbits. An explanation for the increase in retention of drug in nonpigmented iris from 6-hydroxydopamine treated rabbits after 15 minutes of washings is not available at the present time.
### TABLE 16

**EFFECTS OF 6-OH DOPAMINE PRETREATMENT ON THE BINDING OF $^{14}$C-IMIPRAMINE TO ISOLATED PIGMENTED AND NONPIGMENTED RABBIT IRIS**

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$C-imipramine DPM/iris</th>
<th>5 min</th>
<th>15 min</th>
<th>35 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonpigmented iris</td>
<td>n = 5</td>
<td>13056 ± 721</td>
<td>9542 ± 1054</td>
<td>7668 ± 660</td>
<td>7127 ± 712</td>
<td>6817 ± 1392</td>
</tr>
<tr>
<td>Pigmented iris</td>
<td>n = 5</td>
<td>25786 ± 3450</td>
<td>21310 ± 1210</td>
<td>--</td>
<td>18977 ± 2933</td>
<td>17317 ± 3036</td>
</tr>
<tr>
<td><strong>6-OH-dopamine</strong></td>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonpigmented iris</td>
<td>n = 5</td>
<td>13081 ± 607</td>
<td>20308 ± 3238a</td>
<td>11927 ± 2825</td>
<td>6778 ± 901</td>
<td>6228 ± 1518</td>
</tr>
<tr>
<td>Pigmented iris</td>
<td>n = 5</td>
<td>23528 ± 5706</td>
<td>30630 ± 3504</td>
<td>24021 ± 1815</td>
<td>15705 ± 1758</td>
<td>18246 ± 2306</td>
</tr>
</tbody>
</table>

>aStatistically different from normal rabbit iris (P<0.05).
BIBLIOGRAPHY


237


