Further studies concerning the accumulation of polycyclic compounds on uveal melanin

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Results are presented which show that chlorpromazine and chloroquine may be recovered from uveal pigment in vitro without change. Chlorpromazine given to living rabbits is recovered from the uvea partly as the sulfoxide. When acridine orange is used to wash uveal pigment granules on which chlorpromazine has been adsorbed, a small portion of the drug is removed. When chlorpromazine is offered to uveal pigment on which acridine orange has been adsorbed, much less drug is taken up than by untreated pigment granules. Similarly, acridine orange given to the living rabbit inhibits subsequent uptake of chlorpromazine. The possible prophylactic and therapeutic use of chlorpromazine against toxic phenothiazines and chloroquine compounds is discussed.

Previous reports from this laboratory which described the accumulation of melanin in the uveal tract of pigmented animals and which demonstrated the accumulation to be direct adsorption on uveal melanin have given rise to two additional, remotely related questions. The first question concerns the nature of the compound adsorbed on the uveal pigment in the living animal. Particularly when one uses an isotopic label to follow storage in and loss from the uvea, it is of some significance to know whether the substance in question is in the same chemical form as when administered to the animal, or whether one could be following only a small fragment which happens to bear the labeled atom.

The second question concerns the possible competition between compounds for the available sites on the melanin. Recently, several polycyclic compounds have been shown to cause choroidopathy or retinopathy. These compounds all have desirable therapeutic properties, but potential toxicity restricts dosage or entirely prevents their use. If the adsorptive sites on uveal melanin could be pre-empted by a strongly adsorbed compound known to be harmless, the potentially toxic substance might then be able to be used freely. The following experiments were performed in an attempt to answer these questions.

Experimental

1. Recovery of chlorpromazine and chloroquine from Bovine choroidal pigment. Samples of chlorpromazine and chloroquine (2.5 µmole) were reacted with 10 mg quantities of choroidal pigment granules, as described previously. The pigment granules were separated by centrifuging and were washed once with distilled water. Each batch of granules was then suspended in 0.1M NaOH in 95 per cent alcohol and each tube was allowed to stand with periodic mixing by inversion for 15 minutes. At the end of this time the pigment was separated by centrifuging. The decanted supernatants were neutralized with...
suspended in 7 ml. of an aqueous solution of its content of dye and of radioactivity determined. The granules were re-
supernatant was separated by centrifuging, and after 15 minutes of intermittent agitation the supernatant was separated by centrifuging, and its content of dye and of radioactivity determined.

This wash was performed for a total of five times (three times in the first experiment). Because of the deep color of the acridine orange washes, the solutions were unsuitable for direct determination of radioactivity in the liquid scintillation spectrometer. For this reason all samples were wet ashed with perchloric acid and the S-35 precipitated as BaSO₄, was counted in the scintillation counter in thixotropic gel suspension as described previously.

b. Adsorption of chlorpromazine on pigment previously treated with acridine orange. Ten milligram portions of choroid pigments were treated with 7 ml. of solution containing 30 μmole acridine orange. The supernatant was removed after centrifuging and the pigment granules were washed with water by suspension and centrifuging. The granules were then resuspended in a solution of 2.5 μmole S-35-labeled chlorpromazine in 7 ml. solution at pH 6. A set of control tubes containing equal amounts of pigment not exposed to acridine orange was run in parallel. Because the radioactive supernatants were colorless, small aliquots of these could be counted directly in the liquid scintillation spectrometer in a dioxane-scintillant mixture. Comparison was made to a standard solution counted similarly.

4. Competition between acridine orange and chlorpromazine in vivo. In each experiment of this series two pigmented rabbits of identical color were used. One animal was given 5 mg. per kilogram of a solution of acridine orange injected intravenously once a day for 3 days. The control animal was given no such injection. On the fourth day each rabbit was given 5 mg. per kilogram of a solution of S-35-labeled chlorpromazine, administered intravenously. On the fifth day both rabbits were killed, their eyes dissected, and each of the eye samples was wet ashed so that the S-35, now sulfate, could be counted as a suspension of BaSO₄.

Results

1. Recovery of chlorpromazine and chloroquine from bovine choroidal pigment. The appearance of the developed chromatogram from a typical experiment on recovery of chlorpromazine and chloroquine from choroidal pigment granules is diagrammed in Fig. 1. It is evident from the diagram that the substance recovered from the pigment in the chlorpromazine part of the experiment has the same rate of migration on paper as chlorpromazine. It is equally evident that a different rate of migration is characteristic for chloroquine and that the material recovered from the pigment in this part of the experiment mi-
Accumulation of polycyclic compounds on uveal melanin

It was observed that both chlorpromazine and chloroquine are recoverable from the in vitro experiment essentially unchanged. There is no evidence on the paper for the existence of any additional substances.

2. Recovery of chlorpromazine from the uvea of pigmented rabbits. The results of a typical in vivo experiment with chlorpromazine are shown in Fig. 2. The diagram of the developed paper chromatogram is paralleled by a histogram of the radioactivity recovered from each half-inch strip of paper. It is evident that in addition to the chlorpromazine itself, a second area, corresponding closely in Rf to chlorpromazine sulfoxide, appears in the material recovered from rabbit uvea. There is a difference between the duplicate unknowns in that the two spots overlap more in one strip than in the other. Inspection of the spots for known chlorpromazine and the sulfoxide show that these, too, overlap. The difference in amount of overlap for the two runs is within tolerable limits. The histogram for radioactivity corresponds closely to the spots shown by color development. In each histogram there is an incisura at inch 11 corresponding to the junction point between known chlorpromazine and its

![Fig. 1. Recovery of chlorpromazine and chloroquine from uveal pigment in vitro.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932895/)

![Fig. 2. Recovery of chlorpromazine from uvea of pigmented rabbit.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932895/)
Table I. Displacement of chlorpromazine from pigment by subsequent treatment with acridine orange

<table>
<thead>
<tr>
<th>Tube number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</thead>
<tbody>
<tr>
<td>Per cent chlorpromazine adsorbed</td>
<td>70</td>
<td>78</td>
<td>79</td>
<td>69</td>
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<tr>
<td>pmole chlorpromazine adsorbed</td>
<td>1.75</td>
<td>1.90</td>
<td>1.97</td>
<td>1.73</td>
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<tr>
<td>Per cent adsorbed chlorpromazine removed by water:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash No. 1</td>
<td>2.48</td>
<td>2.56</td>
<td>2.22</td>
<td>4.30</td>
</tr>
<tr>
<td>Wash No. 2</td>
<td>0.28</td>
<td>0.38</td>
<td>3.48</td>
<td>1.12</td>
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<tr>
<td>Wash No. 3</td>
<td>0.41</td>
<td>0.13</td>
<td>2.86</td>
<td>1.30</td>
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<tr>
<td>Total per cent removed by water</td>
<td>3.17</td>
<td>3.07</td>
<td>8.56</td>
<td>6.72</td>
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<tr>
<td>Per cent removed by acridine orange:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash No. 1</td>
<td>8.05</td>
<td>7.94</td>
<td>9.00</td>
<td>7.67</td>
</tr>
<tr>
<td>Wash No. 2</td>
<td>5.05</td>
<td>2.18</td>
<td>4.91</td>
<td>1.31</td>
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<tr>
<td>Wash No. 3</td>
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<td>2.46</td>
<td>6.02</td>
<td>3.00</td>
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<td>Wash No. 4</td>
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<td></td>
<td>3.36</td>
<td></td>
</tr>
<tr>
<td>Wash No. 5</td>
<td></td>
<td>2.56</td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>Total per cent removed by acridine orange</td>
<td>15.12</td>
<td>12.58</td>
<td>26.04</td>
<td>15.90</td>
</tr>
<tr>
<td>pmole removed by acridine orange</td>
<td>0.26</td>
<td>0.25</td>
<td>0.50</td>
<td>0.27</td>
</tr>
<tr>
<td>pmole acridine orange adsorbed in one wash</td>
<td>2.50</td>
<td>1.85</td>
<td>3.50</td>
<td>3.25</td>
</tr>
<tr>
<td>pmole acridine orange adsorbed in three washes</td>
<td>4.40</td>
<td></td>
<td>3.40</td>
<td></td>
</tr>
</tbody>
</table>

Chlorpromazine, 2.5 pmole adsorbed on 10 mg. choroid pigment granules; three water washes, 7 ml. each, followed by three or five washes with 7 ml. containing 5 pmole acridine orange each time.

Sulfoxide. It is reasonable to conclude that in the living animal at least half of the administered chlorpromazine (as measured by radioactivity) is oxidized to the sulfoxide and that the compounds are held equally well by the uveal pigment.

3. Competition between chlorpromazine and acridine orange for uveal pigment granules in vitro.

a. Displacement of chlorpromazine from pigment by subsequent treatment with acridine orange. The results of four typical trials are given in Table I. It is evident that even five washes with acridine orange remove only 15 to 25 per cent of previously adsorbed chlorpromazine; this is not for lack of uptake of dye by the pigment. Indeed, on the first application of acridine orange, an average of ten times as many molecules of dye go on the pigment as the total number of molecules of chlorpromazine removed.

b. Adsorption of chlorpromazine on pigment previously treated with acridine orange. A typical experiment is shown in Table II. It appears from the results that the prophylactic adsorption of a pre-emp-
indicate fairly clearly that there is no chemical change inherent in the process of attachment of drug to pigment. Only chloroquine and chlorpromazine are recovered after adsorption in vitro. In vivo, although a significant portion of the chlorpromazine is changed to the sulfoxide, this substance, too, is held by pigment and no significant portion of the label appears in small molecules.

It may well be that this may explain the differences of reaction of different species to certain compounds, e.g., the difficulty of producing NP-207 choroidopathy in experimental animals. It may also explain differences in the reaction of the same species to different related compounds. For example, the differences in human choroidotoxicity between NP-207 and chlorpromazine may depend on differences in degree of in vivo oxidation of the two substances and the uveal accumulation of predominant amounts of original substance or oxidation product, as the case may be. This is a subject which requires further investigation.

The second series of experiments bears on the question of whether one might be able to utilize a harmless compound avidly taken up by pigment either before or after the potentially toxic therapeutic agent to allow the therapeutic agent to be used. For these trial experiments, chloroquine was used as an example of a less avid stand-in for the potentially toxic phenothiazines simply because radioactive-labeled chloroquine was available and labeled samples of the other compounds were not. Acridine orange was used as the avid harmless compound because radioactive-labeled chloroquine was available and labeled samples of the other compounds were not. Acridine orange was used as the avid harmless compound, partly because it was the substance with greatest affinity for pigment of those tested and partly because its color allowed colorimetric determination in the presence of colorless chlorpromazine. In actual practice it is anticipated that chlorpromazine will be used as the relatively harmless substance to protect against chloroquine or other phenothiazines. We have just prepared tritium-labeled chloroquine, and the chlorpromazine-chloroquine competition is now being investigated.

Table II. Adsorption of chlorpromazine on pigment previously treated with acridine orange

<table>
<thead>
<tr>
<th>Per cent chlorpromazine adsorbed on pigment</th>
<th>Control untreated</th>
<th>Pretreated with acridine orange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>μmole chlorpromazine adsorbed on pigment</td>
<td>76.7</td>
<td>72.4</td>
</tr>
<tr>
<td>μmole acridine orange on pigment</td>
<td>1.92</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Acridine orange, 30 μmole in 7 ml., offered to 10 mg. choroid pigment granules; 2.5 μmole labeled chlorpromazine then offered.

Table III. Uptake of labeled chlorpromazine by uveas of animals with and without pretreatment with acridine orange

<table>
<thead>
<tr>
<th>Micrograms chlorpromazine per gram tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreated animal</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Retina</td>
</tr>
<tr>
<td>Choroid</td>
</tr>
<tr>
<td>Iris</td>
</tr>
<tr>
<td>Ciliary body</td>
</tr>
</tbody>
</table>

Acridine orange dose, 5 mg. per kilogram on 3 successive days before chlorpromazine; chlorpromazine dose, 5 mg. per kilogram.

It would appear from the results of Section 3 that so many adsorption sites are left after treatment with 2.5 μmole chlorpromazine that there is still room for acridine orange to go on the pigment without displacing much chlorpromazine. Also there may be some advantage in adhesion to the compound first adsorbed, i.e., all sites may not be subjected to the identical binding forces even when one is on the steep portion of the adsorption isotherm. This hypothesis seems to be borne out by Section 3b, where a profound inhibition of attachment of chlorpromazine is shown to be caused by preadsorbed acridine orange. This cannot be due to the greater concen-
tration of acridine orange used, because even in preliminary experiments, where the pigment was pretreated with 5 μmole acridine orange, a similar inhibition of chlorpromazine adsorption was observed. Thus prophylactic treatment is more effective than an attempt to displace a compound already on the pigment. With this in mind the experiments reported in Section 4 were performed, and the results appear to bear out the idea that, at least for the choroid, prior treatment with acridine orange does reduce uptake of chlorpromazine given subsequently. One reservation must be made here; namely, that the choroid of the animal with acridine orange appeared less densely pigmented than the control on dissection. To date, histologic examination has not confirmed the idea that acridine orange causes choroid changes, and the results are presented upon the assumption that there is as much pigment in the control choroid as in that of the experimental animal.

The next step, trial of the principle in man, is beset by many uncertainties. The incidence of chloroquine retinopathy is quite low, so that an extremely large series of patients on chloroquine will be required to give any definitive answer to the question of how effective a prophylactic agent might be. The use of a more toxic compound such as NP-207 would be even a less attractive alternative. The final answer will have to wait in all probability until these types of choroidopathy or retinopathy can be reproduced in experimental animals. Then, trials in man can be done with some confidence. Until then any therapeutic use of this principle must necessarily be inconclusive.

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REFERENCES