Chemical Reactivity of Carcinogenic Aminofluorenes
Color Reactions with Montmorillonite

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The hypothesis presented in this article serves a twofold function: (a) a means of predicting whether an aminofluorene is carcinogenic or not and (b) an indication of a possible site in the aminofluorene molecule which is capable of combining with some cell substance. This study deals with the chemical reactivity of an extensive number of aminofluorenes and aminofluorenone and involves their complex formation with montmorillonite, a clay mineral. In a number of cases the above reaction produced marked color changes. There are, however, certain types of montmorillonite clay minerals which are free from ferric iron and do not produce color reactions with aromatic amines. A discussion of the chemical and physical properties of montmorillonite would be out of place in this journal. The reader is, therefore, referred to two books dealing with that subject. It will be shown that those aminofluorenes which produce color changes undergo tautomerism, yielding an activated form. Furthermore, it will be postulated that this activated tautomer, a dipole, is involved in carcinogenesis. Color reactions due to complex formation of montmorillonite with aromatic amines are not new. However, a new mechanism for the color reactions of certain aminofluorenes will be introduced. A method by which carcinogenic activity or inactivity of aminofluorenes can be predicted will be indicated.

MATERIALS AND METHODS

The amines used are listed in Table 1. They are isomers of aminofluorene and aminofluorenone. Some of the aminofluorenes contain nuclear substitutes. Two amino-9,9-spirobifluorenes are also included. The montmorillonite is a bentonite obtained from Wyoming.

To an intimate mixture of 5-10 mg. of amine and 0.5 gm. of montmorillonite, obtained by being ground in an agate mortar, was added dropwise 2 or more ml. of water until a paste or slurry was formed, depending upon the amount of water added. Striking color reactions appeared in ten out of 27 amino compounds tested. The color-reacting compounds, without exception, had an amino group in the 2-position. Three other 9-aminofluorenes containing a hydroxy group in the 1-, 3-, or 5-position produced indefinite color reactions, which changed upon standing. The characteristic colors of five aminofluorenes were imparted to their complexes upon interaction with montmorillonite, whereas 2,7-diamino-9-benzylfluorene did not impart its purple color to its complex. It is not clear why this compound behaved differently from the other fluorenes. The same behavior was observed with 2,5- and 2,7-diamino-9-benzylfluorene.

The data are shown in Table 1. Information on reported and predicted carcinogenic activities is also included in the table. The predicted activities are presented as either active or inactive, whereas the found activities are shown in greater detail. Carcinogenic activity, as reported in the literature, was determined with the acetyl derivatives. Only 9-amino-9-benzylfluorene was tested as the free amine and its acetyl derivative. Since the acetyl group is removed by hydrolysis in vivo, no distinction is made between an aminofluorene and its acetyl derivative.

DISCUSSION

A significant finding of the tests, shown in Table 1, is the sharp contrast in the activity of certain aminofluorenes as compared with the corresponding aminofluorenone. 2-Aminofluorene, 2,5- and 2,7-diamino-9-benzylfluorene interact with montmorillonite, forming dark-colored complexes, whereas no color changes occur in complexes of montmorillonite with the corresponding aminofluorenone. It is noteworthy that 9-amino- and 2,9-diamino-9,9-spirobifluorene also do not form colored complexes with montmorillonite. The difference between the color-reacting aminofluorenes and the noncolor-reacting aminofluorenone and amino-spirobifluorines is that the aminofluorenes have hydrogen on the 9-carbon, whereas the two latter types of compounds do not have any hydrogen on the 9-carbon. It is, therefore, concluded that the color-reacting amino-
flourenes undergo tautomerism, forming a dipolar structure:

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

Additional evidence for this conclusion is as follows: Dipole structures of fluorene containing certain substituents in the 9-position have been reported in the literature. When the substituents are trimethylamino, triethylamino (11), and pyridine (24), the compounds designated by the following dipole structures,

\[
\begin{align*}
\text{N} + \\
\text{R}_3 & \\
\text{C} - & \\
\text{S} + (\text{CH}_3)
\end{align*}
\]

are unstable and colored. When the substituent in the 9-position of fluorene is dimethyl sulfide, the compound

\[
\text{S}^+ (\text{CH}_3)_2 \\
\text{C} -
\]

is also colored and unstable (12, 23). It was shown in a number of cases that the presence of a substituent in the 2-position of fluorene enhances the lability of hydrogen attached to the 9-carbon (22). As an example, 2-acetylfluorene is readily oxidized to fluorenone-2-carboxylic acid by the action of potassium hypochlorite at room temperature, whereas fluorene under the same conditions does not yield a trace of fluorenone (26). Furthermore, the unstable dipoles 9-fluorylidene-2-amino fluoridinium and fluorylidene dimethyl sulfide are stabilized by the introduction of nitro groups in the 2- and 2,7-positions of the fluorylidene (10, 21). On the basis of these facts and the fact that 2-amino fluoridinum-montmorillonite complex is colored and is exceedingly stable (the dark green color of an aqueous suspension of this complex remained unchanged for more than 5 years), it is deduced that under such mild treatment as is involved in the complex formation a proton from the 9-carbon in 2-amino fluoridine is transferred to the nitrogen in the 2-position. The same applies to the dark blue complexes of 2,5- and 2,7-diamino fluoridines.

Very little is known about the chemical properties of 1-, 3-, and 4-amino fluoridines. These three isomers and 9-amino fluoridine do not form colored complexes with montmorillonite. Just as there are differences in the chemical reactivities of ortho, meta, and para positions of toluene and alpha and beta positions of naphthalene, it is probable that there are also differences in the chemical reactivity between the 2-position as compared with the 1-, 3-, and 4-positions of fluorene. It is possible that 1-, 3-, and 4-amino fluoridines undergo tautomerism, forming dipoles which are not colored in contrast to the intensely colored dipole of 2-amino fluoridine. 7-Hydroxy-2-amino fluoridine forms a permanganate-colored complex with montmorillonite. Here the hydroxyl group is phenolic, and it is expected that the phenolic hydrogen would be much more labile than the hydrogen attached to the 9 carbon. The dipole would, therefore, have the following structure,
the difference being that the negative charge is on the oxygen instead of the 9-carbon. When the phenolic group is in the 1-, 3-, or 5-positions in 2-aminofluorene, the observed color reactions indicate a decrease in the effect of the 2-amino group upon the degree of ionization and tautomerism. When the hydroxyl group is attached to the 9-carbon in 2-aminofluorene, the group is alcoholic, and its hydrogen is less labile than the one attached to the 9-carbon (25). This compound forms a green complex with montmorillonite (a lighter shade than that obtained with 2-aminofluorene). Complete immobilization of the hydrogen on the 9-carbon is obtained by the introduction of a benzyl group on the same carbon.

There appears to be a correlation between color reaction and carcinogenicity. Both reactivities occur in an aminofluorene when the amino group is in the 2-position. Furthermore, both depend upon the presence of hydrogen on the 9-carbon. The activities responsible for color reaction and carcinogenicity are attributed to dipole structure formed by the transfer of a proton from the 9-carbon to the amino group in the 2-position. In predicting an aminofluorene to be carcinogenic, it is assumed that the dipole is intensely colored (green, dark green, or dark blue). If the dipole is light-colored (light green or lavender), it occurs in a concentration too low to be active. Although the dipole of 7-hydroxy-2-aminofluorene is highly colored (purple), the author hesitates to make a prediction at this time on the carcinogenic activity of that compound, because its dipole structure is different from that of 2-aminofluorene. The brown color formed by 3-hydroxy-2-aminofluorene is due to air oxidation, for a moist sample without montmorillonite also turns brown upon standing. A tinge of purple, if formed, would be masked by the brown. 9,9-Dimethyl-2-aminofluorene does not have any hydrogen attached to the 9-carbon.

### Table 1: Color Reactions of Aminofluorenes with Montmorillonite, and Carcinogenic Activity

| Compound No. | Substituent | Color of Aminofluorene | Color of Complex | Carcinogenic Activity
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Isomers of aminofluorene</td>
<td>Predicted</td>
</tr>
<tr>
<td>1</td>
<td>1-Amino</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Inactive</td>
</tr>
<tr>
<td>2</td>
<td>2-Amino</td>
<td>Red</td>
<td>Red</td>
<td>Active</td>
</tr>
<tr>
<td>3</td>
<td>3-Amino</td>
<td>Peach</td>
<td>Peach</td>
<td>Inactive</td>
</tr>
<tr>
<td>4</td>
<td>4-Amino</td>
<td>Orange</td>
<td>Orange</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>9-Amino</td>
<td>Brownish red</td>
<td>Brownish red</td>
<td>*</td>
</tr>
<tr>
<td>6</td>
<td>2,5-Diamino</td>
<td>Purple</td>
<td>Charcoal gray</td>
<td>Active</td>
</tr>
<tr>
<td>7</td>
<td>2,7-Diamino</td>
<td>Dark green</td>
<td>Dark blue</td>
<td>Active</td>
</tr>
</tbody>
</table>

8-Aminofluorene containing a nuclear substituent

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Substituent</th>
<th>Color of Aminofluorene</th>
<th>Color of Complex</th>
<th>Carcinogenic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1-Hydroxy</td>
<td>Dark gray acquired tinge of purple</td>
<td>Inactive</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3-Hydroxy</td>
<td>Dark color changed to brown</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5-Hydroxy</td>
<td>Grayish blue changed to lavender</td>
<td>Inactive</td>
<td>Weak or inactive (3, 9)</td>
</tr>
<tr>
<td>17</td>
<td>7-Hydroxy</td>
<td>Purple</td>
<td>Active</td>
<td>Weak (18)</td>
</tr>
<tr>
<td>18</td>
<td>9-Hydroxy</td>
<td>Dark blue</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>7-Methoxy</td>
<td>Green</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>7-Benzyl</td>
<td>Orange</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>9,9-Dimethyl</td>
<td>Light green</td>
<td>Inactive</td>
<td>Inactive (27)</td>
</tr>
<tr>
<td>22</td>
<td>7-Iodo</td>
<td>Dark green</td>
<td>Active</td>
<td>Weak (10)</td>
</tr>
<tr>
<td>23</td>
<td>7-Chloro</td>
<td>Dark green</td>
<td>*</td>
<td>Very potent (18)</td>
</tr>
<tr>
<td>24</td>
<td>7-Fluoro</td>
<td>Dark green</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

9-Benzylfluorene with nuclear substituents

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Substituent</th>
<th>Color of Aminofluorene</th>
<th>Color of Complex</th>
<th>Carcinogenic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>2,5-Diamino</td>
<td>Yellow</td>
<td>Inactive</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>2,7-Diamino</td>
<td>Light brown</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

9,9-Spirobifluorene with nuclear substituents

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Substituent</th>
<th>Color of Aminofluorene</th>
<th>Color of Complex</th>
<th>Carcinogenic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>2-Amino</td>
<td></td>
<td>Inactive</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>2,2'-Diamino</td>
<td></td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

* The colorless amines and the complexes having various gradations of gray are designated by a dash.
† This observation was first reported by Hendricks and Alexander (8) and confirmed by the author.
‡ The crystals were orange, but when finely ground they yielded a yellow powder.
and thereby precludes the possibility of this compound’s existing as a dipole; consequently, it should be inactive. Likewise, all fluorenones should be inactive because of the absence of hydrogen on the 9-carbon. Miller et al. (18) reported 2-aminofluorenone to be weakly active and suggested the possibility that the activity of the fluorenone derivative as well as that of 9-hydroxy-2-aminofluorenone might be owing to 2-aminofluorenone formed from it in vivo. According to the hypothesis, 9-hydroxy-2-aminofluorenone should be carcinogenic. The activity of 2-aminofluorenone might be accounted for by a one-step enzymatic reduction to the corresponding fluorenol, rather than a two-step reduction to 2-aminofluorenone. Similar metabolic transformations might occur with 2,5- and 2,7-diaminofluorenones. Although all fluorenones are predicted to be inactive, the three mentioned above, however, might yield carcinogenic 9-hydroxy derivatives. The seeming difference between the predicted and found activities of 2-aminofluorenone is, therefore, not real. The only discrepancies that occur in the predicted and found activities involve the 1-, 3-, and possibly the 4-aminofluorenes.

Following the discovery by Miller and Miller (14, 16, 17) that certain aminoazo dyes are chemically bound to liver protein, it was found that 2-aminofluorenone is also chemically bound to liver protein (8, 15, 30). The present study suggests a possible site in aminofluorenone capable of combining with protein. The constituents of an aminofluorene-montmorillonite complex are rigidly held together by electrostatic attraction of the positively charged nitrogen of aminofluorene and a negatively charged group, e.g., carboxyl in protein.

**SUMMARY**

The interaction of isomers of aminofluorene and aminofluorenone with montmorillonite was studied. Most of the fluorene derivatives having an amino group in the 2-position produced a color change. Evidence is presented showing that the color reaction involves tautomerism forming an activated dipole. It is postulated that the activated tautomers are carcinogenic. The nitrogen in an aminofluorenone is considered to be the most active site in the molecule capable of interacting with a protein.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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