Effect of Various Synthetic Estrogens on the Respiration of Ascites Tumor Cells in Vitro*

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SUMMARY

The effects of the synthetic estrogens diethylstilbesterol, hexosterol, dienesterol, and phloretin were compared with their capacity to inhibit the endogenous respiration of intact Ehrlich ascites, Adenocarcinoma 755 ascites, and Ehrlich-Lettré ascites cells. The Ehrlich ascites was found to be far more susceptible to respiratory inhibition than either the Adenocarcinoma 755 or the Ehrlich-Lettré ascites. Diethylstilbesterol and hexosterol were found far more effective inhibitors of endogenous respiration than dienesterol or phloretin. Substrate-induced respiration of lysed ascites cells was inhibited by the hormonal agents listed to approximately the same degree as that observed in whole cells. The addition of the synthetic estrogens dissolved in ethanol markedly increased the capacity of these agents to inhibit the respiration of intact tumor cells. The relative activity of these compounds and the relative susceptibility of the tumor types to these synthetic estrogens were unaltered by the presence of high levels of ethanol.

Diethylstilbesterol, a widely used synthetic estrogen, has been employed in the chemotherapy of human prostatic and breast cancer (7) and has been demonstrated to inhibit the growth of an experimental mouse adenocarcinoma (12). In tissue homogenates, diethylstilbesterol, estradiol, and many other estrogenically active materials are capable of inhibiting dehydrogenase systems at levels of $10^{-5}$ and $10^{-4}$M (2, 3, 6, 8, 9). This ability to inhibit respiratory pathways in tissue homogenates is also a property of many compounds containing phenolic or quinone functional groups regardless of their estrogenic activity (2). The studies, reported in this communication, were carried out to determine the effect of compounds exhibiting varying degrees of estrogenic activity on the endogenous respiration of intact cells and the respiratory rate of lysed preparations of three ascites cell types.

MATERIALS AND METHODS

Ehrlich and Ehrlich-Lettré ascites tumor cells were grown in Swiss Albino mice, the Adenocarcinoma 755 ascites was grown in C57BL mice. All cells were harvested 10 days after inoculation and washed 2 times with Robinson’s solution (10). Only cells relatively free of erythrocytes were used. Respiration of 0.125-ml aliquots of packed cells suspended in Robinson’s solution was determined at 37°C by standard Warburg technic. The synthetic estrogens were dissolved in a minimal amount of dilute sodium hydroxide and added to the cell suspension prior to a 10-minute equilibration period. Control flasks received an equivalent amount of NaOH. The final pH of the reaction mixtures was in all cases 7.4. Respiration was measured by readings taken every 10 minutes for a period of 30 minutes.

Lysed ascites cells were prepared by suspending washed cells in distilled water and allowing them to stand at room temperature for 30 minutes. The lysed cell suspensions were then added to the reaction vessels at levels equivalent to that employed with the intact cells.

RESULTS

The effects of diethylstilbesterol, phloretin, hexosterol, and dienesterol on the endogenous respiration of the intact Ehrlich ascites, Adenocarcinoma 755 ascites, and the Ehrlich-Lettré ascites are shown in Table 1. At this concentration of cells and estrogenically active materials, it can
be observed that the compounds diethylstilbesterol and hexosterol were far more powerful inhibitors of endogenous respiration in the Ehrlich ascites and the Adenocarcinoma 755 ascites than either phloretin or dienesterol. Under these particular conditions (Table 1) no inhibition was observed in the Ehrlich-Lettre ascites. Unpublished data, however, demonstrated that higher concentrations of these estrogenically active materials produced respiratory inhibition in the Ehrlich-Lettre ascites. At these higher concentrations in the effectiveness of diethylstilbesterol and hexosterol as compared with the same concentration of these compounds in intact cell preparations. In contrast, phloretin and dienesterol produced no significant change in activity.

Experiments on inhibition of endogenous respiration of washed ascites cells by diethylstilbesterol, hexosterol, phloretin, and dienesterol, dissolved in absolute ethanol, are presented in Table 2. The concentration of ethanol in the final reaction mixture was approximately 7 per cent.

<table>
<thead>
<tr>
<th>COMPOUNDS*</th>
<th>Ehrlich ascites</th>
<th>Adenocarcinoma 755 ascites</th>
<th>Ehrlich-Lettre ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact†</td>
<td>Lysed‡</td>
<td>Intact†</td>
</tr>
<tr>
<td>None</td>
<td>520 ± 27</td>
<td>357 ± 52</td>
<td>632 ± 21</td>
</tr>
<tr>
<td>Diethylstilbesterol</td>
<td>270 ± 23</td>
<td>128 ± 47</td>
<td>435 ± 30</td>
</tr>
<tr>
<td>Phloretin</td>
<td>511 ± 65</td>
<td>232 ± 42</td>
<td>570 ± 19</td>
</tr>
<tr>
<td>Hexosterol</td>
<td>280 ± 43</td>
<td>101 ± 47</td>
<td>509 ± 13</td>
</tr>
<tr>
<td>Dienesterol</td>
<td>474 ± 36</td>
<td>270 ± 58</td>
<td>624 ± 35</td>
</tr>
</tbody>
</table>

* Final molarity was 4.0 × 10⁻⁴ M, dissolved in 6 × 10⁻⁴ M NaOH. Equivalent amount of alkali added to control flasks. Final pH 7.4.
† Cells washed 2 times in Robinson’s salts; 125 mg. of cells, suspended in Robinson’s salts, was added to each flask. Values are the average of three experiments run in duplicate. S.E. = standard error.
‡ Cells washed 2 times in Robinson’s salts; 125 mg. of cells, suspended in distilled water for 30 min. at room temperature (25°C), was added to each flask. Values are the average of at least four experiments run in duplicate. S.E. = standard error. Each reaction vessel contained: pyruvate, 0.011 M; ATP, 0.0007 M; MgCl₂, 0.008 M; nicotinamide, 0.04 M; potassium phosphate (pH 7.4), 0.01 M; fumarate, 0.011 M, and estrogenic materials as indicated.

(4.0 × 10⁻⁴ M) diethylstilbesterol and hexosterol were more powerful against the Ehrlich-Lettre ascites than was either phloretin or dienesterol. The effect of diethylstilbesterol, hexosterol, phloretin, and dienesterol on the oxygen uptake of lysed ascites cells is presented in Table 1. Lysis appeared to increase the capacity of phloretin to inhibit respiration of Ehrlich ascites cells, whereas diethylstilbesterol, hexosterol, and dienesterol had an effect similar to that of whole cell preparations.

Rupture of the cell membrane of 755 ascites caused no increase in effectiveness of the four estrogens studied. Studies with lysed Ehrlich-Lettre ascites, however, showed a slight increase in the effectiveness of diethylstilbesterol and hexosterol as compared with the same concentration of these compounds in intact cell preparations. In contrast, phloretin and dienesterol produced no significant change in activity.

Experiments on inhibition of endogenous respiration of washed ascites cells by diethylstilbesterol, hexosterol, phloretin, and dienesterol, dissolved in absolute ethanol, are presented in Table 2. The concentration of ethanol in the final reaction mixture was approximately 7 per cent.

<table>
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<tr>
<th>COMPOUNDS†</th>
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<th>Adenocarcinoma 755 ascites</th>
<th>Ehrlich-Lettre ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETOH</td>
<td>290 ± 23</td>
<td>290 ± 23</td>
<td>347 ± 33</td>
</tr>
<tr>
<td>ETOH+diethylstilbesterol</td>
<td>72 ± 22</td>
<td>119 ± 7</td>
<td>224 ± 27</td>
</tr>
<tr>
<td>ETOH+phloretin</td>
<td>160 ± 14</td>
<td>190 ± 33</td>
<td>330 ± 30</td>
</tr>
<tr>
<td>ETOH+hexosterol</td>
<td>90 ± 10</td>
<td>91 ± 36</td>
<td>160 ± 20</td>
</tr>
<tr>
<td>ETOH+dienesterol</td>
<td>130 ± 14</td>
<td>900 ± 10</td>
<td>330 ± 18</td>
</tr>
</tbody>
</table>

* Cells washed 2 times in Robinson’s salts; 125 mg. of cells, suspended in Robinson’s medium, was added to each flask. Values are the average of three experiments run in duplicate; 0.2 ml. ethanol with or without hormonal agents added per flask, in a final vol. of 3.0 ml. S.E. = standard error.
† Final molarity of estrogenic substances was 4.0 × 10⁻⁴ M.
As can be seen by comparing the respiration of control groups in Table 1, this concentration of ethanol by itself was sufficient to cause marked inhibition of endogenous respiration. In these studies, the Ehrlich ascites was most sensitive to the addition of the estrogenically active materials, and hexosterol and stilbesterol were more powerful inhibitors than phloretin and dienesterol. It is interesting to note that the respiration of the Ehrlich-Lettré ascites found to be insensitive to synthetic estrogens at $4 \times 10^{-8}$M final concentration, in aqueous suspension (Table 1), was inhibited by this level of estrogens when dissolved in ethanol (Table 2).

**DISCUSSION**

Diethylstilbesterol, hexosterol, dienesterol, and phloretin were selected for study in an attempt to compare compounds which vary markedly in estrogenic activity, but yet are strikingly similar in molecular weight and steric configuration. This approach to the problem was thought more logical than a comparison of potent synthetic estrogens such as diethylstilbesterol, also an effective inhibitor of respiratory pathways in ascites cells (11) and tissue extract (2, 3, 6, 8, 9), with that of hydroquinine (11), for example.

In all cases, the relative effects of these hormonal agents were the same, the compounds falling into two separate groups. Diethylstilbesterol and hexosterol were found to be equally effective in inhibiting the respiration of these cells, whereas the compounds dienesterol and phloretin were far less active in inhibiting respiration of these particular cells. The estrogenic activity of these compounds was in the following order of decreasing activity: hexosterol, diethylstilbesterol, dienesterol, and phloretin (4, 13). Phloretin is an extremely weak estrogen, far weaker than the other three compounds employed (4). As can be noted from the tables, phloretin possessed activity in inhibiting respiration to the same degree as the comparatively active estrogenic material dienesterol. Although there is correlation between estrogenic activity and respiratory inhibition of the three ascites cell types in the case of diethylstilbesterol and hexosterol, there exists little correlation in the case of the compounds dienesterol and phloretin.

On the basis of these data, it can be concluded that the inhibition observed in the endogenous respiration of ascites cells is not related directly to the estrogenic activity of these compounds. This is not surprising, since the concentration of these estrogenic materials needed to produce a minimal inhibition of respiration in the presence or absence of the cell membrane is approximately 500-fold that necessary to produce an estrogenic response.

The observation that various cell types are more resistant to respiratory inhibition by these compounds than other cell types is of interest. This is in correlation with the work of other investigators (3, 6, 8, 14), who have demonstrated that there was a marked variation in the capacity of compounds with estrogenic activity to inhibit respiration in tissue extracts from various tissues. This marked variation in cell type resistance cannot be attributed solely to the cell membrane as the data in Table 1 indicate.

The effect of ethanol in increasing the capacity of these compounds to inhibit respiration in intact ascites cells (Table 2) is not known. Such a high concentration of ethanol (7 per cent) is lethal to the intact cells, producing considerable respiratory inhibition in its own right as well as increasing tissue permeability (1). The effect of ethanol cannot be attributed solely to this phenomenon, since the data presented in Table 1 indicate that the presence or absence of the cell membrane has a minor effect on the capacity of these compounds to inhibit tissue respiration. The possibility does remain that the presence of the ethanol permits a somewhat greater solubility of these compounds so they are far more able to penetrate the areas of the cell high in lipide such as the mitochondria. This might allow more of these respiratory inhibitors to reach the intracellular sites where their inhibitory action would occur. In this case it is of interest to note that with diethylstilbesterol and ethanol it is possible to produce significant inhibition in the Ehrlich ascites tumor system with concentrations of less than $1 \times 10^{-5}$M (unpublished data). We have been unable to detect alcohol dehydrogenase in these ascites cells. Thus it is reasonable to assume that under these conditions these ascites tumors are not preferentially metabolizing ethanol. A clarification of the mechanisms whereby certain tumor types are more resistant than others to the action of these compounds, as well as an understanding of the mechanistic reasons why diethylstilbesterol and hexosterol are far more powerful in inhibiting respiration than phloretin and dienesterol, may give further insight into comparative intermediary metabolism and problems involved in selective tissue toxicity. An understanding of these basic problems is a prime prerequisite to the development of the field of rational cancer chemotherapy.

*The Ehrlich-Lettré ascites is far more resistant to proges-
terone, in vitro, than the Ehrlich ascites (C. Werner, private communication).*
ACKNOWLEDGMENTS

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REFERENCES

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