Effects of Administration of Estrogens upon Enzymes of Rat Pituitary

II. Aconitase, Succinoxidase, and Transaminase*

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SUMMARY

Succinoxidase, aconitase, and glutamic-oxalacetic transaminase have been studied in the pituitary and liver of male rats during the induction of pituitary tumors by treatment with diethylstilbestrol.

In the pituitary the two representatives of the tricarboxylic acid cycle were decreased by approximately the same amount. The changes in these enzymes occurred soon after the beginning of the treatment, and thereafter their activities remained constant, whereas the pituitary slowly underwent marked gross alterations.

Pituitary transaminase first increased and then decreased in both diethylstilbestrol- and placebo-treated animals; thus any effects of the diethylstilbestrol are masked by the placebo effect.

In the preceding publication (12) it was shown that significant changes occur in certain enzymes of the pituitary as the result of treatment with diethylstilbestrol (DES). For example, \( \beta \)-glucuronidase was increased by 40 per cent soon after initiation of treatment; prolonging the treatment for up to 2 months resulted in no further increase in the activity of this enzyme. However, in this work no overt tumors, such as have been described by other workers (3, 4, 6, 15, 16, 22, 32), were found, and the changes were considered to reflect the situation associated with the limited amount of pituitary hyperplasia which occurs in the initial stages.

It was thus of interest to study the enzymology of pituitaries which had been treated until definite tumors were produced. In the present study, animals were treated for up to 420 days, resulting in grossly abnormal pituitaries, some as large as 20 times the normal size. Animals were sacrificed at intervals, and succinoxidase, aconitase, and glutamic-oxalacetic transaminase were studied in the pituitary and liver.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, grown in the Loyola colony, were used in this work. They were housed in an air-conditioned room and offered fox chow pellets and water ad libitum to the time of sacrifice, which was effected by sharp blows on the back of the neck.

DES was administered by subcutaneous implantation of a tablet containing 25 mg. every 30 days. The implantation was carried out without anesthetic; the small incision was closed with a wound clip. Placebo tablets were of the same size and contained only inert ingredients (lactose, starch, and magnesium stearate).1 Immediately after the sacrifice of an animal, the pituitary gland and a sample of liver were removed and weighed on a torsion balance. The tissues were then homogenized in chilled homogenizing medium, diluted, and used immediately. All enzyme meas-

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urements were made in duplicate at two homogenate levels. In each case, the conditions used showed the procedure to be linear with enzyme concentration.

Succinoxidase was determined by the method of Schneider and Potter (20) as described by Umbreit et al. (26), in the microflasks described by Melchior and Hilker (11). The fluid volume in each flask was 0.3 ml. and contained 10 μmoles phosphate, 15 μmoles succinate, 4 μmoles cytochrome c, 12 μmoles calcium chloride, and 0.12 μmoles aluminum chloride at pH 7.4. Tissue concentration was about 2 mg. of liver or 4 mg. pituitary per flask; from three to seven pituitaries were pooled for each determination. One unit of succinoxidase was defined as the μliters of oxygen consumed per hour per mg. nitrogen. The succinoxidase experiments gave results linear with time. Nitrogen was determined by the Ziegler (30) modification of Thompson and Morrison (25). Since the cytochrome c is added in this method, the assay may be assumed to be a valid measure of succinic dehydrogenase activity, which has been shown to be the rate-determining component of the succinoxidase system in most tissues under the conditions used (21).

Aconitase was assayed by the optical method devised by Racker (18). The reaction catalyzed is

\[
\text{Citrate (or D-isocitrate) } \rightarrow \text{Cis-aconitate } + \text{H}_2\text{O},
\]

and the rate is followed by measuring the absorption of the cis-aconitate at 2400 A.

Absorption was measured in a Beckman D.U. spectrophotometer; temperature was controlled in the cell compartment at 25° C. The enzyme was unstable when homogenates were prepared in water or buffer, but was found to be stable for several hours when homogenized in 0.05 m phosphate, pH 7.4, containing 5.4 mm sodium citrate. The cuvettes contained 2.0 ml. of 0.0435 m sodium citrate in 0.05 m phosphate, pH 7.4, and 1.0 ml. tissue homogenate. Optimal concentrations were about 1 mg. liver and 1.6 mg. pituitary; homogenates were centrifuged in a clinical centrifuge for 5 minutes immediately before use. The reaction was zero order for over 40 minutes with the amount described; optical density changes in the control tubes were negligible. A unit of aconitase was defined as that amount of enzyme which causes a rate of increase in the optical density of 0.001 per minute at 2400 A under the described conditions.

Glutaric-oxalacetic transaminase was determined by the procedure developed by the Sigma Chemical Company (23), which is basically similar to that described by Henley and Pollard (5). The reaction mixture contained 100 μmoles L-aspartate, 165 μmoles phosphate, 0.2 mg. reduced DPN, 200 units malic dehydrogenase (23), 25 μmoles a-ketoglutarate in a final volume of 3 ml.; disappearance of the reduced form of the DPN is followed in the spectrophotometer at 3400 A. All reagents except the homogenate and the a-ketoglutarate were mixed just before the determination, and 2.0 ml. of this was added to the cuvettes. The homogenate was then prepared and 0.5 ml. of a suitable dilution added to the cuvette. Tissue concentrations were of the order of 0.6 mg. pituitary and 0.15 mg. liver; no centrifugation was necessary. After exactly 10 minutes, the a-ketoglutarate was added to start the reaction. Control tubes contained distilled water in place of the a-ketoglutarate.

Homogenates prepared in distilled water were not stable; homogenizing in buffer produced a stable preparation which always yielded a lower initial value than the water homogenates. Thus, it was necessary to homogenize in distilled water and then dilute immediately with 0.1 m phosphate buffer, pH 7.5. The unit of transaminase was defined as that which would cause a decrease in the optical density of 0.001 per minute at 3400 A.

The significance of changes between several groups of measurements was determined by the analysis of variance method described by Snedecor (24).

RESULTS

Gross changes.—The treatment with DES has a marked effect on body weight and pituitary size of the animals, as shown previously (12) with somewhat shorter periods. As demonstrated by other workers (32), the effect on pituitary size was highly variable. For example, the range of pituitary weights in the group of thirteen rats treated from 300 to 399 days was from 12.1 to 153 mg.; thus, some pituitaries were of extraordinary size, and others were within the normal range of weights. It is further noted that there was no way of predicting before sacrifice which animals would have markedly abnormal glands; no difference was apparent in the general appearance, size, or activity of the animals. However, as can be seen from Chart 1, there was a definite increase in the average pituitary weight of the treated animals, and the difference in average size became more pronounced as the treatment was continued. As the glands became enlarged they became a deeper red; the largest glands had lost all gross resemblance to normal pituitary.

Succinoxidase.—As is apparent in Table 1, the rate of oxidation of succinic acid by pituitary
homogenates was significantly depressed by the treatment with DES, the mean value for all treated animals showing 72 per cent of the activity found in control animals. Activity in liver was not significantly altered.

Aconitase was decreased by treatment with DES to 76 per cent of the value found in control animals (Table 1). It was further noted that placebo treatment had no significant effect on aconitase activity. In the liver, aconitase activity showed an average increase of about 20 per cent. This was the result of a marked increase soon after the start of the treatment; thus, the value increased from 3.67 units in the controls to 5.07 units in animals treated for 20-40 days. As treatment was continued, the activity of liver aconitase gradually returned to normal.

It was of interest to determine whether the continued treatment led to progressive decrease in the activity of these enzymes in the pituitary. The values were subdivided into several groups according to the length of treatment, but in no case was there any tendency for the effects to become more pronounced as exposure to the agent was prolonged. For example, succinoxidase in the pituitaries of seven animals treated for less than 200 days was 408 ± 38, whereas the five animals treated for more than 200 days showed an average value of 428 ± 3, whereas the six treated for longer periods averaged 45 ± 2 units. Both changes were significant at the 0.99 confidence level. It seems evident that the changes in transaminase reflect some nonspecific effect of the treatment, rather than an effect of the hormone per se. The most striking effect of treatment was the marked decrease of the activity of liver transaminase.

### Table 1

**Effects of Diethylstilbestrol Administration on Enzymes of Pituitary and Liver**

The units used are described in the text; the values are expressed as mean ± the standard error. Numbers in parentheses represent the number of separate values; each value was obtained in quadruplicate.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Aconitase (units/mg)</th>
<th>Succinoxidase QO(N)</th>
<th>Glutamic-oxalacetic transaminase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.51 ±0.05</td>
<td>122 ± 8</td>
<td>50 ± 1</td>
</tr>
<tr>
<td>(16)</td>
<td></td>
<td>(15)</td>
<td>(16)</td>
</tr>
<tr>
<td>Placebo-treated</td>
<td>1.55 ±0.07</td>
<td>125 ± 6</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>(11)</td>
<td></td>
<td>(13)</td>
<td>(8)</td>
</tr>
<tr>
<td>DES-treated</td>
<td>1.18 ±0.06*</td>
<td>90 ± 6</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>(19)</td>
<td></td>
<td>(15)</td>
<td>(20)</td>
</tr>
<tr>
<td>Liver:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>3.67 ±0.10</td>
<td>451 ±12</td>
<td>233 ±11</td>
</tr>
<tr>
<td>(21)</td>
<td></td>
<td>(28)</td>
<td>(16)</td>
</tr>
<tr>
<td>Placebo-treated</td>
<td>3.83 ±0.16</td>
<td>405 ±12</td>
<td>231 ±14</td>
</tr>
<tr>
<td>(12)</td>
<td></td>
<td>(28)</td>
<td>(9)</td>
</tr>
<tr>
<td>DES-treated</td>
<td>4.47 ±0.16</td>
<td>416 ±22</td>
<td>187 ± 6</td>
</tr>
<tr>
<td>(18)</td>
<td></td>
<td>(14)</td>
<td>(16)</td>
</tr>
</tbody>
</table>

* Significance level (compared with untreated plus placebo-treated), 0.1 per cent.
† Significance level, 1 per cent.

### DISCUSSION

The treatment with DES results in an enlarged gland in which secretory cells may be presumed to be significantly diluted with nonsecretory cells (29, 32, 33). The present study shows that two representative enzymes of the tricarboxylic acid cycle, aconitase and succinoxidase, decrease significantly as a result of this treatment.

Among other changes in pituitary enzymology
which are known to occur upon treatment with DES is a marked increase in the amino acid-incorporating system (31); thus, estrogenic substances cause an increase in the activity of this system in both pituitary and target organs (14). This is not unexpected in the target organs, where the immediate result is a rapid growth of new tissue; it is less obvious how this results in an inhibition of hormone secretion by the pituitary. Possibly the synthesis of proteins other than follicle-stimulating hormone is speeded up, causing a decreased secretion of the hormone by competitive inhibition or by draining the supply of protein precursors.

It is apparent that the response of pituitary to high DES levels results in a shift of the enzymology toward the general pattern characteristic of rapid growth. Specifically, aconitase has been shown to be lowered in mouse and rat tumor tissues (27), whereas succinoxidase activity has been observed to be diminished in many tumors, including rat hepatoma (17, 20), mouse hepatoma (19), and a variety of human neoplasms (13). On the other hand, a rapid rate of amino acid incorporation has been found in a variety of tumors (10, 28).

Thus, the present data are consistent with the postulate that the shift in enzymology, which presumably reflects the physiological mechanism for inhibiting the secretions of the pituitary, is characterized by an increasing growth rate. In the normal cyclic interplay of the target organ and the pituitary, this would have no noticeable effect on the size; but, under the abnormal constant stimulus used in studies such as this, the gland continues to grow at an increased rate.

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