Succinic Dehydrogenase and Cytochrome Oxidase in Epidermal Carcinogenesis Induced by Methylcholanthrene in Mice*

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The role of the alkalies, alkaline earths, and of iron, copper, and zinc in epidermal carcinogenesis in mice has been described (4) and the integration of the chemical, physical, and histological changes in mouse epidermis undergoing carcinogenesis has been reviewed by Cowdry (7). The investigations on the minerals revealed that the calcium, iron, zinc, and copper contents of hyperplastic (methylcholanthrene-treated) epidermis were significantly less than that of normal epidermis, and furthermore the content of these metals in the squamous cell carcinoma was reduced to even lower values. Since calcium may be associated with the succinic dehydrogenase system (15, 21, 27), and copper and iron with cytochrome oxidase (20), experiments were undertaken to study these enzymes in the epidermis of mice under the carcinogenic influence of methylcholanthrene.

EXPERIMENTAL

Swiss mice of both sexes were painted with methylcholanthrene (0.6 per cent weight per volume in reagent grade benzene) as in our previous work (4). The mice were sacrificed 5 days after the last application of either benzene or carcinogen. The epidermis of normal controls, benzene-treated controls, and of methylcholanthrene-treated mice was removed from the dermis at 41° to 42° C. on a constant temperature hot plate, or it was scraped off at room temperature. For the final stage in our carcinogenic series, we used the transplantable squamous cell carcinoma of Cooper, Firminger, and Reller (6). This tumor has now passed through 40 generations and has remained practically the same morphologically.

After removal of the various specimens of treated epidermises, they were immediately dropped into a test tube (submerged in an ice bath) containing 1 cc. of redistilled water, and after weighing, the tissue was homogenized by the technic of Potter and Elvehjem (16). The carcinomas were first ground in an unglazed mortar and then homogenized. Succinic dehydrogenase and cytochrome oxidase were determined by the method of Schneider and Potter (21) upon the tissue homogenate at a concentration of 10 per cent. As recommended by Potter, succinic dehydrogenase was tested at two different tissue concentrations, and cytochrome oxidase at three, from which the auto-oxidation of ascorbic acid was determined.

In our previous investigations on the role of the minerals in epidermal carcinogenesis, the basis of reference was nucleoprotein phosphorus (4). Since the nucleoprotein phosphorus content is nearly the same in normal and hyperplastic epidermis and in the transplantable squamous cell carcinoma used in our investigations (23), the conventional method used by practically all workers in enzymology of expressing the results on a dry weight basis was employed. Moreover, the extent of the decrease in the calcium, copper, and zinc contents in hyperplastic epidermis is the same whether nucleoprotein phosphorus or the wet weight of the tissue is selected as a basis of reference (23-25). Also, the decrease in the total lipid is practically the same when lipid protein or the dry weight of the fat-free tissue is employed as a basis of reference (26). It would, therefore, appear that the diminution of some of the chemical constituents in hyperplastic mouse epidermis was independent of the basis of reference. Although the same premise might not hold for the activity of the enzymes, the dry weight appeared to be as good a basis of reference as any. The increase in the activity of succinic dehydrogenase in the carcinoma and of cytochrome oxidase in late hyperplastic epidermis and in the carcinoma may be partially explained on a morphological basis since

1 Unpublished experiments.

2 The authors are indebted to Dr. Van R. Potter of McArdle Memorial Laboratory, Madison 6, Wisconsin, for the assay of two samples of cytochrome c.
Cowdry and Paletta (8) have shown that the cell volume of both spinous and basal cells shows a progressive increase as the hyperplasia progresses, and decreases in the carcinoma. The latter were induced by methylcholanthrene and are not strictly comparable to the transplantable tumor employed by us. The same authors have also demonstrated that the nucleocytoplasmic ratio of both spinous and basal cells decreases in hyperplastic epidermis and increases in the carcinoma, both being less than that of normal epidermis.

**RESULTS**

The results on normal untreated, benzene-treated, and methylcholanthrene-treated epidermises are shown in Table I and are expressed in the usual way, that is, micro-liters of oxygen consumed per mgm. dry weight per hour. The Q_{O_2} values for cytochrome oxidase activity were corrected for the degree of homogenization of the tissue according to the method of Schneider and Potter (21). In all of our previous investigations the epidermis was removed at 50° to 51° C. (2), but this temperature was found to have a destructive effect upon succinic dehydrogenase and cytochrome oxidase activities in the epidermis of young mice whose dermis is quite thin and permits too much heat to pass to the epidermis. However the epidermis removed at 41° to 42° C. gave Q_{S} values of 3.9 and 25.0 respectively which agreed very well with Q_{O_2} values of 3.6 and 24.8 for epidermis removed at room temperature. The epidermis was either scraped off at room temperature or removed at 41° to 42° C. for the determinations reported here. Two groups of mice about 1 year of age gave Q_{S} values of 2.8 and Q_{O_2} of 22.2 which values are somewhat lower than those for the normal young mice.

TABLE I: SUCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE IN EPIDERMAL CARCINOGENESIS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Epidermis removed at room temperature by</th>
<th>Suceinic dehydrogenase</th>
<th>Cytochrome oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>scraping</td>
<td>Q_{S}</td>
<td>3.1</td>
<td>24.7</td>
</tr>
<tr>
<td>&quot;</td>
<td>4.8</td>
<td>23.8</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>3.0</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>10 Average</td>
<td></td>
<td>3.6</td>
<td>24.8</td>
</tr>
<tr>
<td>Epidermis removed at 41-42°</td>
<td>Q_{S}</td>
<td>4.4</td>
<td>25.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.0</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>4.9</td>
<td>24.9</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>2.8</td>
<td>29.5</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>4.6</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td>12 Average</td>
<td></td>
<td>3.8</td>
<td>21.1</td>
</tr>
<tr>
<td>67 Average</td>
<td></td>
<td>3.9</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Since benzene is used as a solvent for the carcinogen, its effect on the enzymes was studied. Mice which received 3 applications of benzene on alternate days were sacrificed 5 days after the last treatment (all benzene and methylcholanthrene-treated mice were killed 5 days after the last application of either benzene or of the carcinogen) had a Q_{S} value of 2.7 and Q_{O_2} value of 21.9 while the epidermis treated with 6 paintings of benzene on alternate days showed a Q_{S} value of 2.5 and a Q_{O_2} value of 23.8. Therefore benzene alone has a slight inhibitory effect upon the activity of the enzymes.
The results on succinic dehydrogenase and cytochrome oxidase activity of the transplantable squamous cell carcinoma are shown in Table II. The results are expressed in the usual way under "dry weight" and also under "total solids." Since we have recently been able to remove the epidermis at room temperature, it has been possible to determine the water content of normal, and hyperplastic epidermis to compensate for the increased amount of water. The late hyperplastic epidermises, therefore, contained nearly twice as much cytochrome oxidase activity as did the normal.

The succinic dehydrogenase and cytochrome oxidase activities of the transplantable squamous cell carcinoma are shown in Table II. The results are expressed in the usual way under "dry weight" and also under "total solids." Since we have recently been able to remove the epidermis at room temperature, it has been possible to determine the water content of normal, and hyperplastic epidermis to compare the water content of the latter with the transplantable carcinoma. These studies showed that the water content of the carcinoma was 22 per cent greater than that of normal. Under the caption "total solids" the dry weight of the tumors in each sample has been increased by 22 per cent to compensate for the increased amount of water. The average $Q_{o2}$ value for the tumors was 16.1 and under "total solids" 13.1, which values are respectively about 4.5 and 3.5 times greater than that of normal. The average $Q_{o2}$ value for the tumors was 41.6 and under "total solids" 34.1, which values are respectively about 4.5 and 3.5 times greater than that of normal.

### Table II: Succinic Dehydrogenase and Cytochrome Oxidase in Epidermal Carcinogenesis

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Succinic dehydrogenase</th>
<th>Cytochrome oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry weight</td>
<td>Total solids</td>
</tr>
<tr>
<td>Tumors</td>
<td>$Q_{o2}^*$</td>
<td>$Q_{ox}^*$</td>
</tr>
<tr>
<td>&quot;</td>
<td>12.3</td>
<td>10.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>17.6</td>
<td>14.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>13.2</td>
<td>10.8</td>
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<tr>
<td>&quot;</td>
<td>11.7</td>
<td>9.2</td>
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<tr>
<td>&quot;</td>
<td>15.7</td>
<td>12.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>18.7</td>
<td>15.3</td>
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<tr>
<td>&quot;</td>
<td>21.3</td>
<td>17.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>18.3</td>
<td>15.0</td>
</tr>
<tr>
<td>Average</td>
<td>16.1</td>
<td>13.1</td>
</tr>
</tbody>
</table>

### DISCUSSION

The results on succinic dehydrogenase and cytochrome oxidase activity of the metals, calcium, copper, iron, and zinc are shown graphically in Fig. 1 where the percentage of change in the metal nucleoprotein phosphorus ratio is plotted against the time and the number of applications of the carcinogen and where the $Q_{o2}$ is likewise plotted against the latter two variables. It can be seen that there is no change in the activity of succinic dehydrogenase until the epithelial cells become carcinomatous when it is increased significantly. The investigations of Swingle, Axelrod, and Elvehjem (27) showed that calcium stimulates the succinic dehydrogenase system in homogenates. Potter and Schneider (15) have furthermore demonstrated that the activating effect of added calcium and aluminum to the homogenates is probably due to a multiple dilution effect, caused by the dissociation of cytochrome $c$, aluminum ions, and calcium ions away from the succinic dehydrogenase and cytochrome oxidase. Later Schneider and Potter (21) postulated that the necessity of calcium and aluminum for maximum activity of the succinic dehydrogenase system, and of aluminum for the cytochrome oxidase system was probably indirect.

In this study (Fig. 1) a decrease of nearly 60 per cent in the calcium nucleoprotein phosphorus ratio in the hyperplastic epidermis had no effect upon the activity of succinic dehydrogenase. However, in the tumors which are very low in calcium, the succinic dehydrogenase activity is high. It would, therefore, appear that either the effect of the tissue calcium was indirect or that sufficient calcium was added to the medium to compensate for the loss induced by the carcinogen. Since Potter and DuBois have shown that zinc has an inhibitory effect upon succinic dehydrogenase activity (17), perhaps the decrease in the zinc content of hyperplastic epidermis and in the tumors may account for an increased activity of this enzyme, or zinc may play an important role in its regulation.

An inspection of Fig. 1 reveals that the epidermis which had received 6 and 12 applications of the carcinogen has a greater cytochrome oxidase activity than normal. Moreover, this increase is pronounced in the carcinoma (18 and 24 paintings) at which time the papillomas and carcinomas begin to appear. (Our samples of late hyperplastic epidermis did not contain any papillomas or carcinomas.) In the carcinoma the activity of cytochrome oxidase is less than in late hyperplasia, but it is greater than that of the normal. Since cytochrome oxidase contains iron and since copper is essential for the formation and maintenance of cytochrome oxidase activity of heart muscle in rats and probably other tissues (20), an explanation for the increase in the activity of this enzyme where the copper and iron contents are decreased is not easy. However, the regulation of the cytochrome oxidase activity may be conditioned by the quantity of iron and copper in the
Fig. 1.—Succinic dehydrogenase, cytochrome oxidase, iron, calcium, copper, and zinc in epidermal carcinogenesis induced by methylcholanthrene. CA represents carcinoma.

cells. Fig. 1 also reveals that up to 30 days the contents of the metals are decreasing with time, and are in a state of some flux, and during this interval there is no appreciable change in the enzymes. However, from 30 to 60 days there is no further decrease in the metal contents, and it is during this time that the cytochrome oxidase activity begins to rise which would indicate a definite change in the cells prior to malignancy, in which state the activity of this enzyme decreases somewhat.

Care was taken to avoid necrotic tissue in the transplantable carcinoma since Elliott and Greig (11), the first to show that tumors were low in succinic dehydrogenase, later demonstrated that necrotic tumor tissue had an inhibitory effect upon this enzyme (12). How-
ever, Albaum and Potter showed that fresh tumor tissue free of necrotic material was amply adequate for the assay of this enzyme, but that autolysis produced inhibitory substances (1).

It is impossible here to relate our studies with the investigations of others on many types of tumors, so only those investigations pertaining to skin will be considered. In general, the activity of succinic dehydrogenase and cytochrome oxidase is less in tumors than in their normal prototypes (13, 22). Dickens and Weil-Malherbe (10) found that the respiration and glycolysis of carcinoma of the vulva were typical of those of malignant growths which have a high aerobic and anaerobic glycolysis. Leukoplakia showed only a slight increase in the latter when compared with normal skin. However, these investigators used whole skin for the normal, a large part of which is dermis, so that the analyses do not relate, as ours do, specifically to epidermis.

Salter and his co-workers (9) have found that the QO₂ of tumor tissues in general increases less in response to succinate and p-phenylenediamine than does the QO₂ of normal tissue. These investigators found that virus-induced papillomas removed at 6 and 7 weeks gave a 2 to 3 fold increase in QO₂ response to succinate and p-phenylenediamine whereas the virus-induced papillomas removed at 10 and 14 weeks and described as benign did not show an increase. This observation indicated to these workers that the papilloma had begun to behave like a malignant growth. In a subsequent paper, Salter and his colleagues removed tissues at 46, 55, and 79 weeks, the first specimens being benign papillomas, and the latter carcinomatous (19). All showed a loss of cytochrome system response to the substrates succinate and p-phenylenediamine. Kidd, Winzler, and Burk have made similar observations on virus-induced tumors of the rabbit, and have found that normal rabbit skin gave a greater QO₂ response to succinate and p-phenylenediamine than did V-2 carcinoma (14). The virus-induced Shope papilloma gave the same low QO₂ response to substrates as did the V-2 carcinoma. These investigators also demonstrated that the metabolism of the V-2 carcinoma is characteristic of malignant cells generally.

On the other hand, Berenblum, Chain, and Heatley (3), using rabbit epidermis, observed no differences in oxygen uptake, aerobic and anaerobic glycolysis, and RQ of normal epidermis and a Shope virus papilloma. The values they obtained for normal epidermis were nearly identical with those of the papilloma, and very similar to the values cited in the literature for squamous carcinoma.

Rosenthal and Drabkin, in a study of the response of normal and malignant tissues to succinate and p-phenylenediamine, reported (18) that normal epidermal tissues fall into two groups as follows: (a) tissues with a high oxidative response towards the substrates (kidney cortex, liver, brain cortex, and probably smooth and striated muscles); (b) tissues with low response towards the substrates (gastrointestinal mucosa, lung, and possibly skin, mammary gland, and lymphatic tissue). Benign and malignant rat tumors and human carcinomas exhibited low oxidative responses similar to group (b). These investigators claim that the oxidative behavior incident to malignancy can be expected to occur only in group (a), where spontaneous tumors rarely occur.

In contrast to the observations cited above, we have found that the activity of cytochrome oxidase in late hyperplastic mouse epidermis is greater than normal, but less than the squamous cell carcinoma, and that the activity of succinic dehydrogenase is unchanged from the normal in hyperplastic epidermis, but increases significantly in the carcinoma.

In a comparison of the chemical changes in the early stages of carcinogenesis with tissue found in a carcinomatous tumor, it must be remembered that the cellular material analyzed in a carcinoma, which consists mainly of cancer cells together with some stroma, is more homogeneous than the cellular material presented by the several samples of epidermis obtained from the skin of 8 to 12 different mice during the intermediate stages of carcinogenesis. In this latter material, the skins of individual mice show considerable variation in the degree of chemical hyperplasia. Moreover, since cancer arises always in a sharply circumscribed area of a treated epidermis, there are considerable differences of the degree of chemically precancerous hyperplasia in the same skin, and the increase in cytochrome oxidase in this late hyperplasia may be due to the increase in the number of scattered foci where cancer is about to originate.

**SUMMARY**

Succinic dehydrogenase and cytochrome oxidase activity of mouse epidermis undergoing carcinogenesis by methylcholanthrene has been investigated. The activity of succinic dehydrogenase in the normal and hyperplastic epidermis is the same, but almost a fourfold increase occurs when the cells become carcinomatous. Cytochrome oxidase activity in early hyperplastic epidermis (6 and 12 paintings of methylcholanthrene) was slightly greater than normal, but the activity of late hyperplastic epidermis (18 and 24 applications of the carcinogen) was nearly twice that of normal. In the carcinoma the activity of this enzyme was less than that of late hyperplastic epidermis, but greater than that of the normal. The relationship of the enzymes to the metals, calcium, iron, copper, and zinc, is briefly discussed.
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