# THE INTERMEDIARY CARBOHYDRATE METABOLISM OF EPIDERMIS

## II. Assay for Succinic Dehydrogenase and Cytochrome Oxidase*

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Recent studies have shown that homogenates of rat epidermis can oxidize various substrates of the Krebs cycle: succinate, fumarate, oxalacetate, α-ketoglutarate, and malate (1).

The present report applies the homogenate method to the analysis of rat epidermis for the activity of its succinic dehydrogenase, the group of enzymes responsible for the oxidation of succinate. These enzymes were chosen for assay because they are one of the most active groups of respiratory enzymes in epidermis (1, 2). In general, the method of Schneider and Potter has been followed (3).

Enzyme assay may be used to compare the activities (or concentrations) of enzymes in tissues under various conditions: normal, under stress, during growth, in diseased states. Differences in enzyme activity may provide clues about disease mechanisms, or about the methods by which the organism adjusts to its environment.

In the assay of tissues for enzyme activity, not only must the many factors involved in a single enzyme reaction be considered, but also the fact that the enzyme in question is probably only one of a number of enzymes operating in the over-all reaction. Therefore, the measurement of succinic dehydrogenase activity requires that this enzyme actually be the limiting factor. That is, an excess of all other factors must be added, and enzymes which follow succinic dehydrogenase in the enzyme chain must be shown to have greater activities than succinic dehydrogenase. Then the rate of oxygen utilization will reflect the activity of the succinic dehydrogenase.

The conditions for succinic dehydrogenase assay are relatively simple to control. Only a few factors and only one other enzyme, cytochrome oxidase, need be considered. The chemistry of the over-all reaction is outlined in Fig. 1.[1] When succinate is oxidized to fumarate by succinic dehydrogenase, cytochrome *c* is reduced. Then cytochrome oxidase reoxidizes the reduced cytochrome *c* and simultaneously removes oxygen from the medium to yield water. In the assay of these two enzymes, the rate of disappearance of this oxygen is measured. It is clear

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[1] This reaction has been studied in great detail by biochemists. Since the present survey of respiratory enzymes of epidermis was restricted to broad reactions, those insoluble enzymes acting before cytochrome *c* were assayed as succinic dehydrogenase and those after as cytochrome oxidase. For an account of the details of this system read reference (4)

that for the succinic dehydrogenase assay to be valid, the capacity of the cytochrome oxidase, which functions at the end of the reaction chain, has to be greater than that of the enzymes preceding it. No animal tissue thus far studied has failed to demonstrate an excess of cytochrome oxidase activity.

## EXPERIMENTAL METHODS

The measurement of oxygen uptake by the Warburg apparatus was the assay tool. Homogenates of epidermis from white rats were prepared in distilled water as in the previous report (1). The homogenization time was always one minute. Manometric experiments were conducted in the same manner and the data plotted for the first 2½ hours. The rate of oxygen uptake during this period, $QO_2$ (microliters of oxygen utilized per hour per milligram dry homogenate), was considered to be characteristic of the conditions under study.

The factors investigated in the previous report (1) were reinvestigated to determine their optimum concentrations for succinic dehydrogenase activity and for cytochrome oxidase activity. To ascertain these optimum values, one

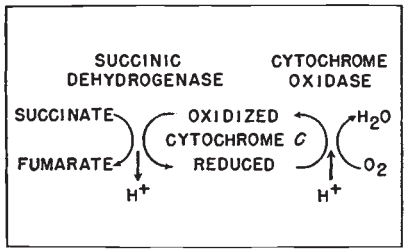


FIG. 1. Reaction pathways regulated by succinic dehydrogenase and cytochrome oxidase.

factor at a time was varied while the others were held constant at supposedly optimum levels.

## RESULTS

*Succinic dehydrogenase*—Table 1 shows the results of experiments to determine optimum concentrations of factors important for succinic dehydrogenase activity in epidermis homogenates. The asterisks indicate the concentrations used in the final assay method. By inspection, the reason for selection is obvious for cytochrome *c*, succinate, adenosine-5-phosphoric acid (AMP), and $MgCl_2$. For the other factors, it is not clear. The concentration of phosphate adopted is close to the isotonicity of the cell. While $Al^{+++}$ and $Ca^{++}$ ions did not stimulate oxygen uptake of epidermis homogenates, the concentrations found to be optimum for liver (3) were used for epidermis.

*Cytochrome oxidase*—For this enzyme assay, excess cytochrome *c* and ascorbic acid are needed, the latter to reduce cytochrome *c* as rapidly as it is oxidized by cytochrome oxidase. The ascorbic acid replaces the succinic dehydrogenase system and allows the investigator to test the capacity of the cytochrome oxidase.

In experiments with ascorbic acid, a correction must be made for the oxygen

TABLE 1

*Experiments to determine the optimum concentration of the factors for succinic dehydrogenase assay of epidermis homogenates*

One factor was varied while the others were held constant at the following concentrations: cytochrome *c* 3.3 × $10^{-5}$ M; AMP $10^{-3}$ M; $MgCl_2$ 3 × $10^{-3}$ M; phosphate 0.0125 M; $AlCl_3$ and $CaCl_2$, each $10^{-4}$ M; succinate 0.05 M. Asterisks mark concentrations selected for assay method.

| Cytochrome *c* | | Succinate | |
|---|---|---|---|
| Concentration | $QO_2$ | Concentration | $QO_2$ |
| 0 | 1.43 | 0.33 × $10^{-2}$ M | 1.39 |
| 3.3 × $10^{-8}$ M | 1.53 | 0.83 × $10^{-2}$ M | 1.66 |
| 3.3 × $10^{-7}$ M | 2.32 | 1.67 × $10^{-2}$ M | 2.08 |
| 3.3 × $10^{-6}$ M | 2.74 | 2.50 × $10^{-2}$ M | 2.29 |
| *3.3 × $10^{-5}$ M | 2.81 | 3.33 × $10^{-2}$ M | 2.27 |
| 6.6 × $10^{-5}$ M | 2.81 | *5.00 × $10^{-2}$ M | 2.36 |

| $AlCl_3$, $CaCl_2$ | | Phosphate | | Amp $MgCl_2$ | |
|---|---|---|---|---|---|
| Concentration of each | $QO_2$ | Concentration | $QO_2$ | Concentration | $QO_2$ |
| 0 | 2.77 | 0 | 2.73 | 0 | 1.86 |
| $10^{-6}$ M | 2.62 | 1.25 × $10^{-4}$ M | 2.48 | $10^{-4}$ M, 3 × $10^{-4}$ M | 2.10 |
| $10^{-5}$ M | 2.81 | 1.25 × $10^{-3}$ M | 2.53 | *$10^{-3}$ M, *3 × $10^{-3}$ M | 2.30 |
| *$10^{-4}$ M | 2.70 | *1.25 × $10^{-2}$ M | 2.50 | $10^{-2}$ M, 3 × $10^{-2}$ M | 2.25 |
| $10^{-3}$ M | 2.17 | 1.25 × $10^{-1}$ M | 2.66 | | |
| | | 1.25 M | 0.42 | | |

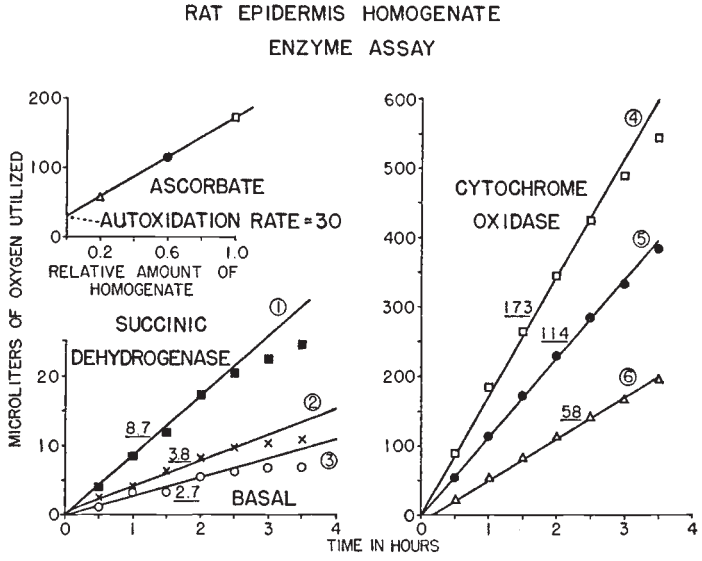


FIG. 2. Data for this assay experiment were obtained with the reaction mixtures shown. in Table 3. The encircled numbers refer to the vessels listed in Table 3. The underlined numbers are the slopes of the curves and represent the rates of oxygen uptake. Two levels of homogenate were used for the succinic dehydrogenase assay and three for the cytochrome oxidase assay. The ascorbate autoxidation rate was obtained by plotting the slopes of the three cytochrome oxidase curves against their respective homogenate concentrations and by extrapolating to zero homogenate concentration. The dry weight of one ml. of this homogenate was 9.5 mg.

TABLE 2

*Experiments to determine the optimum concentrations of the factors for cytochrome oxidase assay of epidermis homogenates*

Three concentrations of homogenates were used for each value. One factor was varied while the others were held constant at the following concentrations: phosphate, 0.0125 M; cytochrome *c*, $5 \times 10^{-5}$ M; ascorbate, 0.015 M. Asterisks mark concentration selected for assay method.

| Cytochrome *c* | | | Ascorbate | | | |
|---|---|---|---|---|---|---|
| Concentration | $QO_2$ | | Concentration | $QO_2$ | | |
| | Exp. 1 | Exp. 2 | | Exp. 1 | Exp. 2 | Exp. 3 |
| *$0.5 \times 10^{-4}$ M | | 13.7 | *$1.5 \times 10^{-2}$ M | 19.1 | | 8.6 |
| $1.25 \times 10^{-4}$ M | 11.0 | 14.6 | $3.8 \times 10^{-2}$ M | | 20.0 | 6.9 |
| $2.5 \times 10^{-4}$ M | 12.5 | | $5.3 \times 10^{-2}$ M | 23.2 | 24.4 | |

TABLE 3

*The reaction mixtures for succinic dehydrogenase and cytochrome oxidase assay of rat epidermis homogenates*

The homogenate was prepared by grinding epidermis scrapings in distilled water at 0°C. for 1 minute. The total volume of each reaction mixture was 3.0 ml. The gas was air, the bath temperature 37°C., the equilibration period 15 minutes.

| Vessel | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| | *ml.* | *ml.* | *ml.* | *ml.* | *ml.* | *ml.* |
| $10^{-3}$ M cytochrome *c* | 0.1 | 0.1 | 0.1 | 0.25 | 0.25 | 0.25 |
| 0.02 M K AMP | 0.2 | 0.2 | 0.2 | — | — | — |
| 0.10 M $MgCl_2$ | 0.1 | 0.1 | 0.1 | — | — | — |
| 0.125 M phosphate pH 7.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| $4 \times 10^{-3}$ M $AlCl_3$ | 0.1 | 0.1 | 0.1 | — | — | — |
| $4 \times 10^{-3}$ M $CaCl_2$ | 0.1 | 0.1 | 0.1 | — | — | — |
| 0.75 M K succinate | 0.2 | 0.2 | — | — | — | — |
| 0.114 M Na ascorbate | — | — | — | 0.4 | 0.4 | 0.4 |
| Homogenate | | | | | | |
| Undiluted | 1.0 | — | 1.0 | 1.0 | — | — |
| Diluted 1:1 | — | 1.0 | — | — | — | — |
| 3:2 | — | — | — | — | 1.0 | — |
| 1:4 | — | — | — | — | — | 1.0 |
| Water | 0.9 | 0.9 | 1.1 | 1.05 | 1.05 | 1.05 |

uptake due to autoxidation of ascorbic acid. Experience has shown that this autoxidation rate cannot be determined accurately by measuring the respiration of the various factors in the absence of the homogenate. A better method is to measure the oxygen uptake of three different amounts of homogenate plus constant amounts of factors, plot the oxygen uptake, and extrapolate to zero concentration of homogenate (3). This extrapolation value is a more accurate value for ascorbate autoxidation (Fig. 2). Therefore, for each concentration of ascorbate studied, three levels of homogenate were used.

Table 2 shows the results of experiments to determine the optimum concentrations of cytochrome *c* and ascorbic acid for cytochrome oxidase activity. The lowest values are optimum for other tissues (3). Since the slight stimulation of epidermis homogenates by higher concentrations is not significant, the optimum concentrations for liver were used for epidermis.

*The assay method*—Schneider and Potter devised a convenient procedure using six Warburg vessels for the simultaneous assay of a tissue homogenate for both succinic dehydrogenase and cytochrome oxidase (3). This procedure was feasible for assaying epidermis homogenates. Table 3 shows the reaction mixtures in the six vessels for the combined enzyme assay of epidermis.

In Fig. 2 are plotted the manometric data from an assay experiment. The enzyme assay values are calculated in the following manner:

Basal $QO_2$

$$\frac{\text{2.7 } \mu\text{l. } O_2 \text{ per hour per ml. homogenate}}{\text{9.5 mg. per ml. homogenate (dry weight)}} = \text{0.28 } \mu\text{l. } O_2 \text{ per hour per mg.}$$

| | | | |
|---|---|---|---|
| Succinic dehydrogenase | | | |
| Vessel | 1 | 2 | |
| Homogenate dry weight (mg.) | 9.5 | 4.75 | |
| Oxygen uptake ($\mu$l. $O_2$/hr.) | 8.7 | 3.8 | |
| Basal rate ($\mu$l. $O_2$/hr.) | 2.7 | 1.35 | |
| Corrected enzyme activity ($\mu$l. $O_2$/hr.) | 6.0 | 2.45 | |
| Divide by dry weight (mg.) | 9.5 | 4.75 | |
| $QO_2$ succinic dehydrogenase ($\mu$l. $O_2$/hr./mg.) | 0.63 | 0.52 | 0.58 (av.) |

| | | | | |
|---|---|---|---|---|
| Cytochrome oxidase | | | | |
| Vessel | 4 | 5 | 6 | |
| Oxygen uptake ($\mu$l. $O_2$/hr.) | 173 | 114 | 58 | |
| Ascorbate autoxidation rate ($\mu$l. $O_2$/hr.) | 30 | 30 | 30 | |
| Corrected enzyme activity ($\mu$l. $O_2$/hr.) | 143 | 84 | 28 | |
| Divide by dry weight (mg.) | 9.5 | 5.7 | 1.9 | |
| $QO_2$ cytochrome oxidase ($\mu$l. $O_2$/hr./mg.) | 15.1 | 14.7 | 14.7 | 14.8 (av.) |

Table 4 lists the results of assays on seven rat epidermis homogenates. The $QO_2$'s are corrected for basal rate and autoxidation.

*Application of the assay method*—The enzyme assay method reported in this paper is being used in the study of epidermis at different stages in the hair cycle in the rat. Among the many striking anatomical changes which occur in skin during the hair cycle is the increase in thickness of the epidermis during early anagen, the growth stage (5). Preliminary experiments show that both succinic

TABLE 4

*Assay of rat epidermis homogenates for succinic dehydrogenase and cytochrome oxidase*

All values were determined in duplicate. They are corrected for the basal rate and the autoxidation rate of ascorbate. $QO_2$ = microliters of oxygen per hour per milligram of dry homogenate.

| Rat | Succinic Dehydrogenase $QO_2$ | Cytochrome oxidase $QO_2$ | Basal Rate $QO_2$ |
|---|---|---|---|
| 1 | 1.74 | 13.9 | 0.90 |
| 2 | 1.38 | 9.1 | 0.85 |
| 3 | 2.27 | 14.5 | 0.80 |
| 4 | 1.84 | 12.1 | 0.75 |
| 5 | 2.11 | 13.5 | 0.81 |
| 6 | 2.19 | 13.1 | 0.62 |
| 7 | 1.70 | 11.4 | 0.63 |
| Mean | 1.89 | 12.5 | 0.76 |
| S.D. | ±0.37 | ±1.8 | |
| S.E.M. | ±0.14 | ±0.7 | |

dehydrogenase activity and cytochrome oxidase activity in epidermis homogenates increase during early anagen. This increase appears to be more evident when enzyme activity is referred to deoxyribonucleic acid content rather than dry weight of the homogenates. When complete, this study will be reported in detail. The nucleic acid content of the epidermis homogenates is being determined by a modification of the Scott procedure (6, 7).

## SUMMARY

1. A method for assaying epidermis homogenates for succinic dehydrogenase and cytochrome oxidase activities has been reported. The optimum concentrations of the factors involved in these enzyme reactions have been determined for homogenates of rat epidermis.

2. With these optimum conditions, rat epidermis homogenates have shown a $QO_2$ succinic dehydrogenase of 1.89 ± 0.37 and a $QO_2$ cytochrome oxidase of 12.5 ± 1.8.

3. The importance of assaying tissues for their enzyme activities lies in the extension of the method to the study of tissues under abnormal conditions with the hope that changes in enzyme activity may be correlated with functional and structural abnormalities. Such correlations might provide clues about the pathogenesis of disease.

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## DISCUSSION

Dr. Frederick Urbach (*Buffalo, N. Y.*): Dr. Griesemer's study is most impressive. Having been interested in respiration and oxygen tension of skin, I heartily agree that a better reference standard than dry weight of tissue must be found. This is particularly true in all studies performed on the skin of small animals, where the content of physiologically inert constituents is very large.

It is also of interest that one of Professor Otto Gans' associates reported to the 10th International Congress in 1952 that acanthosis of any cause was associated with a marked increase in cytochrome oxidase. Our own studies on tissue oxygen tension show a marked decrease in malignant tumors, and in diseases associated with acanthosis or cellular hyperplasia. This has been interpreted as being the result of excessive demand of oxygen by the tissues.

I would like to ask Dr. Griesemer whether a similar increase in enzyme content occurs in malignant tissue. Perhaps some sort of correlation between benign hyperplasia and malignant growth might be established by this method.

Dr. Robert D. Griesemer (In closing): Thank you, Dr. Urbach. I think your idea is very intriguing. Differentiation between benign and malignant tumors might be possible by means of enzyme assay. It would be made difficult by the fact that in malignant tissue the amount per cell of many substances including DNA is likely to change, with the result that DNA is no longer a reliable reference base. The problem could be handled in the manner in which Scott and Taft have done at the Huntington Memorial Laboratories at the Massachusetts General Hospital. In a study of butter yellow induced hepatomas in rats, they found a number of non-malignant pathological changes occurring before the tumors developed. They measured DNA, RNA, several phosphate compounds and a few other substances. In the normal and in all these non-malignant pathological changes, the concentrations of some of the above substances formed a characteristic pattern; but as soon as malignancy developed, this pattern was disorganized in random fashion. Thus one might detect early malignant changes in tissue by watching for distortion of the enzyme assay pattern of the normal cell.