The Coenzyme I Oxidase System in Normal and Tumor Tissues

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To date, no detailed comparative study of the series of respiratory enzymes which constitute a system in the oxidation of reduced diphenolphosphoryl oxidase, (coenzyme I or DPN) in normal and tumor tissues has been published. The importance of this system, however, has found expression in the literature, which contains ample evidence that a considerable portion of oxidative cellular metabolism is carried out through the coenzyme I oxidase components.

Studies of certain parts of this oxidative system, with particular reference to the metabolism of tumors, have been limited. Euler and Hellström (7) found diaphorase and cytochrome oxidase present in Jensen sarcoma. Lockhart and Potter (31) observed that in three types of neoplasms there was no oxidation of DPNH in the absence of added cytochrome c but found that the reaction took place in the presence of additional cytochrome c. While numerous investigators have studied the terminal portion of this respiratory system, no comprehensive investigation has correlated these data with the initial part of the system; and, consequently, conclusions concerning the relative abilities of various tissues, normal and neoplastic, to catalyze the over-all aerobic oxidation of DPNH have not been established. This is due, in part, to the fact that the constitutive elements and mode of operation of certain parts of the chain have not been clarified definitively. Also, the study has been rendered difficult because of the failure to obtain some of the intermediates in soluble and stable form.

The system designated as coenzyme I oxidase can be resolved into the following known enzyme components: (a) diaphorase, a flavoprotein catalyzing the direct oxidation of DPNH, (b) cytochrome c reductase, which mediates the oxidation between DPNH and cytochrome c, and (c) cytochrome oxidase, which facilitates the oxidation of reduced cytochrome c. There is no general agreement as to what entities constitute the second portion of this chain, but it has been established that factors operating in this unit link the enzymatic removal of hydrogen from DPNH with the transfer of electrons to cytochrome c. Thus, in keeping with the general system as defined by Slater (35), cytochrome c reductase, as considered in this treatise, includes the activity of diaphorase, together with the essential elements which accomplish the oxidation of reduced diaphorase and the reduction of cytochrome c.

MATERIALS AND METHODS

Enzyme preparation.—The tissue extracts were prepared by a modified method of Keilin and Hartree (16). This consisted essentially of the extraction of the tissue with phosphate buffer and the precipitation of the enzymes with acetate buffer. A major difference in the two preparations, however, was the ability of the extracts prepared by the modified method to reduce cytochrome c enzymatically. This was absolutely essential in a system intended to measure total coenzyme I oxidase activity. Retention of the cytochrome c reductase activity was probably achieved by eliminating the copious washings prescribed by the method of Keilin and Hartree. While the enzyme preparation was not completely free from hemoglobin, its presence did not interfere in the spectrophotometric studies, since the enzyme preparation was also introduced into the "blank" cell.

As already noted, the comparative study undertaken in the present investigation was aimed to analyze total coenzyme I oxidase activity of cells. Schneider and Hogeboom (36) observed that by far the majority of cytochrome oxidase activity in mouse liver was found in the mitochondrial cell fraction. These authors (18, 15) also have shown that the presence of DPNH-cytochrome c reductase is not restricted to any definite cell structure. The greatest amount of this enzyme is found in the mitochondria and submicroscopic particles but was also present in the nucleus. Since the individual enzyme components of the coenzyme I oxidase system were found in various sedimentable and nonsedimentable fractions, only a general tissue preparation was studied, and no attempts were made to analyze cell fractions obtained by the methods of differential centrifugation.

The method of preparing the enzyme was as follows: Mice maintained on a standard Rockland mouse pellet diet were killed by exsanguination. The tissues were removed immediately, weighed, and extracted with cold 1/10 phosphate buffer, pH 7.5, 0.5 cc. being used for every 1 gm. of tissue. The extraction was accomplished by grinding the tissue in a mortar with a small amount of purified sand. Care was taken to rinse the mortar thoroughly with a small amount of the buffer, but the combined volumes used for extracting and rinsing did not

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The experimental reaction mixture for measurements of the total coenzyme oxidase system was made up of the following: (a) 1.5 cc. diluted enzyme, (b) 1.0 cc. DPNH solution prepared by nonenzymatic reduction or 0.8 cc. DPNH solution prepared by reduction with the glutamate system, and (c) glass-distilled water to give a final volume of 3.0 cc. In systems containing nonenzymatically reduced substrate, the "blank" Beckman cell contained enzyme and oxidized DPN. In experiments involving the use of enzymatically reduced DPNH, the "blank" contained enzyme and all the constituents used in the preparation of the substrate, except DPNH. Among these were nicotinamide, which was added to suppress the activity of the pyrophosphatases described by Kornberg and Lindberg (17).

Diaphorase determinations which required the presence of KCN and methylene blue were made with the same concentration of enzyme as used in the total system; 0.5 cc. of \(2 \times 10^{-4} M\) KCN and 0.1 cc. 5.0 \( \times 10^{-4} M\) methylene blue were added to both experimental and "blank" cells. The final volume of the reaction mixtures was 3.0 cc.

In the cytochrome c reductase studies, the concentration of KCN was as used in the diaphorase system. This, together with cytochrome c, was added to the "blank" also. Cytochrome c was either a commercial preparation or a product prepared by the method of Keilin and Hartree (16). The experimental results obtained for any given tissue preparation were independent of the cytochrome preparation. In all spectrophotometric determinations, DPNH was the last constituent added to the absorption cells. Optical densities were read within 15 seconds of the mixing time.

RESULTS AND DISCUSSION

Coenzyme I oxidase activity.—Preliminary results obtained by a colorimetric procedure designed to test the activity of the system under investigation revealed quantitative differences in reaction between two transplantable sarcomas and one transplantable adenocarcinoma, as compared to five normal tissues of mice. The method of assay was based on the ability of DPNH to decolorize 2,6-dichlorophenol indophenol, as reported by Haas (10). Though this reaction was nonspecific, the results were considered of sufficient significance to warrant a more detailed investigation of the reaction.

With the more definitive spectrophotometric method, the coenzyme I oxidase activity of the normal and tumor tissues was re-examined. The results are summarized in Chart 1, which represents the total system fortified by additional amounts of cytochrome c. The rates of activity for the respective tissues were found to be in general agreement with the data obtained in the aerobic colorimetric analysis. It is noteworthy that the results obtained in the study of normal tissues were not peculiar to any one animal strain, inasmuch as comparable results were obtained in the study of CSH, Rockland, and A mice. The low oxidative response of the transplantable sarcomas and adenocarcinomas was also substantiated. These values, however, fell essentially within the range of normal muscle, an observation which is in keeping
with the rather general finding that many enzyme systems in tumor tissue approximate the lower values for normal tissues. Of greater significance was the observation that hepatoma 98/15 in C3H mice showed a greatly diminished oxidative capacity when compared to normal liver.

Before investigating the specific causes for the failure of the coenzyme I oxidase system in tumor tissues, it was necessary to establish the enzymatic nature of the oxidation in question, since Singer and Kearney (32) have reported the nonenzymatic reduction of cytochrome c by pyridine nucleotide, as catalyzed by various flavins. The possibility of a nonenzymatic reduction was disproved, however, since heated enzyme preparations (90° C, for 5 minutes) of any one of the normal or tumor tissues failed to decrease the optical density at 340 mμ over a period of 15 minutes.

At this point, the possibility was entertained that the lowered oxidative response of tumors was peculiar to a system containing enzymatically reduced substrate. It was conceivable that similar studies, employing a substrate prepared by reduction with sodium dithionite, would yield quantitatively different responses. Pertinent to this idea was Slater's notation (34) that DPNH₃ prepared by a nonenzymatic modified method of Ohlmeyer (23) was unsatisfactory for certain parts of his oxidative studies. As illustrated in Table 1, the activity of certain tissues was not dependent on the type of substrate preparation. This was true of the less active tissues, viz., tumors, muscle, and brain. With the more active heart, liver, and kidney, oxidative values were considerably lower when the nonenzymatically reduced substrate was used. Presumably this was due to the interference of the products of oxidation of the reducing agent. The undesirability of the nonenzymatically reduced substrate was experimentally demonstrated in systems containing methylene blue and, more especially, in systems containing both methylene blue and cyanide. In such instances the inherent rate of autooxidation of the substrate was accelerated. This effect was also noted by Slater (34), who stated that the reason for this reaction is not known. Thus, though the nonenzymatically prepared substrate would have simplified the system under consideration, its disadvantages were such as to require the use of enzymatically reduced DPNH₃ in most experiments. Unless otherwise indicated, therefore, the data presented in this report were obtained with the latter kind of substrate.

**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzymatically reduced DPNH₃</th>
<th>Enzymatically reduced DPNH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>230</td>
<td>155</td>
</tr>
<tr>
<td>Kidney</td>
<td>112</td>
<td>80</td>
</tr>
<tr>
<td>Liver</td>
<td>60</td>
<td>33</td>
</tr>
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<td>Brain</td>
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<td>34</td>
</tr>
<tr>
<td>Muscle</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Adenocarcinoma in CSH mice</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>dbbB adenocarcinoma in DBA mice</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>S-180</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>S-37</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

**Diaphorase and cytochrome oxidase studies.**—In investigating the coenzyme I oxidase system for the factors essentially associated with the failure of the system in tumors, quantitative determinations of the diaphorase activity of the various normal and tumor tissues were first undertaken. Experimental systems designed to measure this activity contained cyanide to inhibit the cytochrome oxidase portion of the respiratory chain and methylene blue to by-pass the cytochrome system. It has been established that methylene blue is capable of oxidizing reduced diaphorase. Cyanide was employed at a final concentration of 2 × 10⁻⁴ M, which concentration was shown by
Lockhart and Potter (21) to be sufficient to block completely the activity of cytochrome oxidase. Was this the sole site of cyanide activity? The above-mentioned workers showed that, at a concentration of $10^{-2} \text{M}$ cyanide, the reduction of cytochrome c itself was inhibited, and they concluded that it is not unlikely that even $10^{-3} \text{M}$ cyanide has some depressant action on the reduction of cytochrome c. That $10^{-3} \text{M}$ cyanide does not inhibit diaphorase was reported by Adler et al. (1) and was later verified by Lockhart and Potter (21). Meyerhof and co-workers in 1938 reported that DPN itself reacts with cyanide (22). The investigations of Colowick and associates (4) not only confirmed the conclusions that the oxidized form of DPN is capable of forming a complex with cyanide but also presented evidence that reduced DPN is unaffected by cyanide. Hence, the locus of action of cyanide in the coenzyme I oxidase system seems primarily to be the terminal part of the oxidative chain rather than essential interaction with the components of the initial diaphorase system.

In the diaphorase determinations (Chart 2) the sarcoma and adenocarcinoma showed the lowest activity, though this again approached closely the activity of normal muscle. The low activity of these tumors was not shared by hepatoma 98/15, which was superior in activity even to the brain, though significantly lower than the analogous normal liver. These data suggested that, in the case of the hepatoma, the low coenzyme oxidase response was not associated primarily with limited diaphorase activity but rather pointed to a failure of the cytochrome components. In the case of the other tumors, however, low diaphorase activity, together with a reduced ability to complete the normal oxidative pathway through the cytochromes, may be essentially contributory to the inefficiency of the total system.

Direct measurements of cytochrome oxidase activity by the manometric method of Schneider and Potter (20) confirmed the supposition of a deficiency in the cytochrome system for both hepatoma and the other tumors analyzed (Chart 3). These results agree with the recent observations of Schneider and Hogeboom (28), who found a decrease in cytochrome oxidase in homogenates of hepatoma 98/15 as compared to that in normal liver. The earlier reports of Greenstein (8) and other investigators (30, 31, 36) likewise lend supporting evidence to the conclusion that cytochrome oxidase activity is lowered in neoplastic tissues.

Additional information on the failure of the cytochrome system in the tumor extracts used in the present study was afforded by spectrophotometric experiments in which the normal coenzyme I oxidase system was supplemented with either limited or excess amounts of cytochrome c. Chart 4 shows the increase in optical density at 550 mp ($+\Delta E_{540}$) in systems where the cytochrome oxidase is not inhibited. Cytochrome c, in these experiments, was used at a final concentration of $4.5 \times 10^{-4}$. The broad maximum clearly discernible in the curve for hepatoma indicates not only a somewhat delayed reduction of cytochrome c but
also a decreased cytochrome oxidase activity. The slope of the descending curve is due to cytochrome oxidase, the activity of which is not manifest until the oxidation of DPNH is near completion or has actually terminated. Unlike the heart, kidney, and liver where the oxidation of DPNH is completed very rapidly (Chart 5), hepatoma exhibited a falling off of the curve of absorption at 550 mμ only after approximately 3–5 minutes (Chart 4). The maximum of the curve also suggests the relative inefficiency of cytochrome oxidase for hepatoma. This can be deduced from the consideration that the maximum attained would be of a lower magnitude were the cytochrome oxidase more efficient than the reductase system. Such a phenomenon is illustrated by the heart extract. Further interpretation of the activity of hepatoma as represented in Chart 4 should be made in light of the relatively good diaphorase activity (Chart 2) and markedly superior cytochrome c reductase activity (Chart 7) exhibited under other experimental conditions.

With regard to the other tumors, Chart 4 suggests a low oxidase activity for sarcoma and adenocarcinoma. Here, the cytochrome c reductase activity is decidedly inferior to that of the hepatoma (Chart 7). Before concluding from the data in Chart 4 that the sarcoma and adenocarcinoma have a lower cytochrome oxidase activity than hepatoma, it should be remembered that the oxidation of DPNH was still in progress (Chart 5) when the extinction at 550 mμ began to decrease. However, a somewhat greater cytochrome oxidase activity for the hepatoma is indicated directly by the manometric results (Chart 3). (It should be noted here that the enzyme preparations used in the manometric studies were 30 times as concentrated as in the spectrophotometric experiments where enzyme solutions could be used at very high dilutions with reproducible results.)

That the metabolism of DPNH normally requires cytochrome oxidase is now generally accepted. Recently, Slater (34) has demonstrated that $5 \times 10^{-4}$ M KCN produced at 96.9 per cent inhibition of the cytochrome I oxidase system.

In addition, Slater observed that such inhibitors as azide, hydroxylamine, sulfide, and fluoride also depressed the cytochrome I oxidase system of heart muscle preparation. The present study confirms the inhibition of this system by several kinds of respiratory depressants. The effect of the following depressants on the heart, kidney, and liver preparations was examined: azide and hydroxylamine in final concentrations of $1.6 \times 10^{-4}$, p-chloromercuribenzoate, $1.6 \times 10^{-4}$, and phenyl mercuric nitrate, $3 \times 10^{-4}$ M. An appreciable inhibition of cytochrome I oxidase activity was observed in all experiments.

In determining the site of action of these depressants, the nature of the inhibitors should be borne in mind. p-Chloromercuribenzoate is known to react readily with sulphydryl groups. However, Barron and Singer (S) reported that heart flavoprotein does not contain essential -SH groups. Nor, according to these authors, is cytochrome oxidase -SH dependent. It is probable that this mercuiral as well as phenyl mercuric nitrate depresses not by formation of reversible mercaptides.
with essential -SH groups but by virtue of the general property of mercurials to act with proteins, possibly by combination with the peptide groups of proteins or with the N of the imidazole group. This was demonstrated by Haarman (9) in the reaction between inorganic Hg and proteins. With reference to cytochrome oxidase, Kreke and co-workers (18) have obtained depression of activity through the use of either of the above-mentioned mercurials. Slater (83) also found that the oxidation of cytochrome c was inhibited by p-chloromercuribenzoate. It seems altogether likely, therefore, that the coenzyme I oxidase system is inhibited because of inactivation of cytochrome oxidase and, possibly, the factors mediating the reductase portion of the chain. The latter supposition is included, since the existence of heme or other metallic components in this part of the system has been postulated. These, accordingly, would be susceptible to inhibition by certain of the depressants tested. Conclusions concerning diaphorase inhibition cannot be made with any degree of certainty.

The formation of hydrogen peroxide in the oxidation of reduced yellow enzyme has been reported (25). It was of interest, therefore, to examine the effect produced by the addition of catalase to the coenzyme I oxidase system. An acceleration of activity was anticipated in view of the fact that peroxide is toxic to a number of sensitive enzymes. Contrary to expectations, however, a decrease in oxidative activity was observed (Table 2). A related phenomenon reported by Altschul et al. (2) may afford a likely explanation for this reaction. These workers found that catalase at a concentration of 1 µg/cc was sufficient to inhibit completely the activity of soluble cytochrome oxidase. Inactivation of this enzyme in the coenzyme I oxidase system is, therefore, highly probable.

Cytochrome c.—Concerning cytochrome c content of tumors, values lower than normal have been reported by a number of investigators (6, 8, 36). Rosenthal and Drabkin (97) reported a lowered cytochrome c content in rat hepatoma compared to normal liver. Indirect evidence obtained in the present study supports the conclusion that cytochrome c is diminished in malignant tissues. This evidence was obtained by supplementing the coenzyme I oxidase system of normal and tumor tissue extracts with cytochrome c and determining the degree of stimulation of oxidase activity thus elicited. In the presence of cytochrome c (Chart 5), a tremendous acceleration of coenzyme I oxidase was exhibited by the tumors. Hepatoma 9B/15 was stimulated 50-fold; the other tumors, approximately 15-fold. The normal tissues, on the other hand, gave a much lower response. The oxidative rates for kidney and brain were accelerated 6 times; that for liver, 10 times. A relatively adequate amount of endogenous cytochrome c must be assumed in the case of the heart, since added cytochrome c increased the rate of oxidation of DPNH₂ only 3-fold. However, a high cytochrome c concentration in the tissue extracts prepared as described in this study is not to be expected even for heart, since the fractionation procedures employed were designed to remove certain soluble tissue metabolites. The catalytic effect of cytochrome c on the coenzyme I oxidase activity was reported also by Slater (84) who obtained an 11-fold increase in activity when cytochrome c was added to kidney preparation. A similar stimulation by cytochrome c of enzymes requiring coenzyme had previously been observed by other investigators (11, 21, 24).

Attempts to obtain a general notion of the actual cytochrome c content in the tissue extracts,

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Control + Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Heart</td>
<td>134</td>
<td>90</td>
</tr>
<tr>
<td>Kidney</td>
<td>120</td>
<td>70</td>
</tr>
<tr>
<td>Brain</td>
<td>55</td>
<td>35</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

* DPNH₂ was prepared by nonenzymatic reduction.

normal and tumor, were made by measuring the increase in absorption at 550 mµ before and after the addition of small quantities of sodium dithionite. A low cytochrome c value was indicated, notably in the case of tumors, all of which had +ΔE₅₅₀ values of 0.003–0.004. Heart, liver, and kidney gave values of 0.010–0.015. Obviously, such measurement yielded information on the concentrations of all tissues constituents, cytochrome c included, which show an increase in absorption at this wave length when treated with dithionite. Though not definitive, these data can be used in a negative manner, and it was found that, as such, they lent confirmatory evidence to the conclusions drawn from decisive experiments.

It should be emphasized here that, in analyzing the systems supplemented with cytochrome c, an absolute correlation between the degree of acceleration obtained and the original cytochrome c content cannot be made with certitude for all the tissues, since a number of factors directly influence the activity of the whole respiratory system. However, collectively the data strongly indicate a diminished cytochrome c concentration in the case of the hepatoma. This is not the sole factor responsible for negligible coenzyme I oxidase activity, since the addition of considerable amounts of cytochrome c, 1.6 × 10⁻⁴ M, did not give oxidative
rates equal to those of the analogous liver or of the heart and kidney. Lehninger states (19) that 1.5 \( \times 10^{-4} \) M cytochrome c is a saturating concentration for optimum activity of the succinoxidase system of rat liver.

Additional information on the specific activities of the components of the coenzyme I oxidase system was obtained by analyzing systems containing excess methylene blue (without cyanide) to facilitate the oxidation of reduced diaphorase. The acceleration of oxidase activity produced by added methylene blue is seen in Chart 6. The stimulation obtained is less than that observed with cytochrome c, but the molar concentration of the latter was somewhat higher. The results of Chart 6 again suggest a decreased diaphorase activity for hepatoma, as compared to liver, and would seem to provide some degree of support to the conclusion already deduced from more direct data, that the cytochrome system is not exclusively limiting in the coenzyme I oxidase activity of hepatoma.

**Cytochrome c reductase.**—Cytochrome c reductase determinations (Chart 7) revealed greatest and almost identical activities for liver and hepatoma. Hogeboom and Schneider (19), in their studies of particulate and nonparticulate fractions of liver and hepatoma 98/15, observed an actual increase in the activity of this enzyme in hepatoma mitochondria. The finding that cytochrome c reductase is not lowered in hepatoma is especially striking in view of the fact that the diaphorase activity (Chart 2) is lower than that of normal liver. It seems likely, therefore, that, while there is a decreased efficiency of the diaphorase system, a failure of the one or several components constituting the factor which mediates the system between diaphorase and cytochrome c does not occur. Adequate concentrations of these factors can be surmised also for heart, liver, and kidney. Unfortunately, knowledge of the nature and mechanism of action of the intermediates in this link is meager. A number of studies have implicated cytochrome b as an intermediate (5, 14, 24), but the recent work of Slater (34) has demonstrated its nonparticipation. Slater believes his BAL-sensitive factor, which operates between cytochrome b and cytochrome c in the succinoxidase system, also functions in the coenzyme I oxidase system in the position between diaphorase and cytochrome c. In support of this belief, Slater found that 0.01 M BAL inactivated the coenzyme oxidase system 94.6 per cent (35). Lehninger's finding (19) that phosphorylation is coupled to the oxidation of DPNH may help considerably in elucidating the nature of the participants and their mode of action as intermediates in this respiratory system.

It is perhaps worthy of mention that an alternative explanation for high cytochrome c reductase and low diaphorase activity for hepatoma might be associated with the catalytic acceleration of coenzyme I oxidase activity by cytochrome c. In the former experiments the systems were supplemented with cytochrome c, while, in the latter, the

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**Chart 6.**—The effect of 1.7 \( \times 10^{-4} \) M methylene blue on the coenzyme I oxidase activity of normal and tumor tissues of mice. \(-\Delta E_{540}\) values represent the decrease in optical densities \( \times 1,000 \).

**Chart 7.**—The cytochrome c reductase activity of normal and tumor tissues of mice. \(+\Delta E_{50}\) values represent the increase in optical densities \( \times 1,000 \).
determinations were made without added quantities of this carrier. Whatever the explanation, it seems fairly reasonable to assume from the data available that, in the case of hepatoma, the unit linking the removal of hydrogen from diaphorase with the electron transfer to cytochrome c is not critically diminished.

In evaluating the cytochrome c reductase activity of the other tumors analyzed in this study, a moderately good degree of activity is indicated. This is suggested not only by Chart 7 but also by the slopes of the respective curves in Chart 4. It is to be recalled, however, that diaphorase values were low (as was also cytochrome oxidase). It would not be improbable, therefore, that the deficiency in the operation of the cytochrome c reductase unit was due more to the failure of the first step in the oxidation of DPNH$_2$ than to a diminution.

It is of interest that, in the study of cytochrome c reductase systems containing cyanide, the authors were unable to obtain the maximum values for reduced cytochrome c when solid dithionite was added to the absorption cells after the experimental determinations were completed. In systems lacking cyanide, such was not the case. This suggested the formation of a cytochrome-cyanide complex which prevents reduction by dithionite. Indeed, Potter (26) observed that cytochrome c combines with cyanide in such a way that it cannot be reduced enzymatically. In the present study reduction was attempted by nonenzymatic methods. The formation of a cytochrome-cyanide complex has been further described by Horecker and Kornberg (14).

In reviewing the enzyme pattern for the normal tissues and in correlating this information with the data afforded by Chart 4, the following can be noted:

1. Liver manifests a marked ability to reduce cytochrome c, as evidenced in Chart 4 by the sharp rise of the curve to its maximum. The value of the maximum indicates that this system operates more efficiently than the cytochrome oxidase portion of the chain. This confirms the conclusions drawn from actual cytochrome c reductase determinations (Chart 7). A lower cytochrome c content for liver and kidney than for heart is suggested, since, given additional cytochrome c, the total coenzyme I oxidase activity of the first two tissues compares favorably with that of the heart. This is especially evident when it is recalled that the diaphorase and cytochrome c reductase systems are optimum for liver and closely approximated by kidney. The methylene blue experiments of Chart 6 supply additional information substantiating the same point.

2. With regard to the kidney and heart, the high cytochrome oxidase activity (Chart 3) explains the low maxima of the curves for these tissues. The appreciable cytochrome c reductase activity determines the time interval at which the system attains this maximum. For these two tissues, the very rapid oxidation of DPNH$_2$ is clearly shown by complete expenditure of the substrate in less than a minute (Chart 5).

3. A moderate diaphorase, cytochrome c reductase, and cytochrome oxidase activity is displayed by brain extract. The intermediate activity of cytochrome oxidase is also suggested to some degree by the slope of the descending curve in Chart 4, though, as was true of the tumors, the oxidation of DPNH$_2$ was still appreciable at this time and hence, too, the reduction of cytochrome c.

4. The low enzymatic activity of muscle tissue manifested by specific diaphorase and cytochrome c reductase determinations is further evidenced in Charts 4 and 5. Thus, cytochrome oxidase excepted, muscle shows greatly diminished concentrations of all of the components of the coenzyme I oxidase system.

The results of Charts 4 and 5 are of further significance in illustrating the direct linking of DPNH$_2$ oxidation with cytochrome c reduction. By making simultaneous determinations of de-
crease in optical density at 340 μg with increase in optical density at 550 μg, the close interdependence of these two systems was clearly established. In systems lacking cyanide but containing added cytochrome c, the over-all reactions were, in general, limited by the first part of the respiratory chain, while systems containing cyanide illustrated the importance of cytochrome c content.

In co-ordinating the results of this study, the enzyme patterns for the individual tissue extracts can perhaps best be summarized by representing a generalized picture of the degree of functioning of the known components of the coenzyme F oxidase system (Table 3). Conclusions as to the relative efficiency of each component are drawn from both direct and indirect information provided by the several lines of experimental approach.

SUMMARY

A comparative quantitative study of the ability of an acid-treated extract of various normal and tumor tissues of mice to oxidize DPNH₂ is presented. This oxidative system was analyzed quantitatively for the activity of its known constitutive enzymes: viz., diaphorase, cytochrome c reductase, and cytochrome oxidase. The responses of these units, together with those of cytochrome c and unknown factors mediating the system between reduced diaphorase and cytochrome c, are correlated to explain the over-all oxidative reactions obtained in the analysis of five normal tissues and several kinds of tumors. The impaired functioning of the complete coenzyme I oxidase system as displayed by hepatoma 98/15, when compared to normal mouse liver, is to be imputed chiefly to a failure of the cytochrome components of the respiratory chain, cytochrome c and cytochrome oxidase, though a somewhat diminished diaphorase activity has been demonstrated. The cytochrome c reductase activity was not lowered in hepatoma. A limiting activity of the factors mediating the oxidation of reduced diaphorase and the reduction of cytochrome c was likewise not indicated. The very low total oxidative reaction exhibited by the transplanted sarcomas and adenocarcinoma was shown to be due to a deficiency of a number of components, notably cytochrome c, cytochrome oxidase, and diaphorase. The cytochrome c reductase values were superior only to the muscle. The effect of various heavy metal and sulfhydryl depressants on the coenzyme I oxidase system was noted. A depression of the oxidative capacity was observed upon the addition of catalase. Evidence confirming the reaction between cytochrome c and cyanide is also presented by these studies.

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