COLORIMETRIC MICRODETERMINATION OF CYTOCHROME c OXIDASE

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The Nadi reaction, discovered by Ehrlich (1885), has been used for many years as a test for indophenol oxidase. The reaction consists in the formation of indophenol blue from dimethyl-p-phenylenediamine and α-naphthol and is given by most animal and plant cells. It is now generally held that indophenol oxidase is identical with cytochrome c oxidase, and the Nadi reaction is often employed for the histochemical detection of cytochrome oxidase.

During an attempt to determine cytochrome oxidase in cellular fractions which could be prepared only in small amounts, tests were made in order to learn whether the Nadi reaction would permit the quantitative determination of micro amounts of cytochrome oxidase. Mitochondrial suspensions from the kidney of the rat were used as a source of the enzyme. Unexpectedly, the formation of indophenol blue by small aliquots of mitochondrial suspensions containing 10 to 60 μ of total N was found to be proportional to the concentration of enzyme and to the time of incubation during the first few minutes. These observations seemed to make it worth while to study the reaction in more detail and to establish optimal conditions for a quantitative test.

Reagents

Phosphate Buffer—Phosphate buffer of pH 7.8 was used to neutralize the acidity of dimethyl-p-phenylenediamine hydrochloride and to bring the pH into the range of 7.0 to 7.4.

α-Naphthol—The commercial preparation (Eastman Organic Chemicals) was recrystallized once from petroleum ether for use in the routine tests. It was recrystallized twice for determination of “indophenol blue units.” α-Naphthol is slightly soluble in water, but readily soluble in 40 per cent alcohol, and does not precipitate in 20 per cent alcoholic solutions. Solutions of α-naphthol of 0.05, 0.1, and 0.2 per cent concentration in 20 to 25 per cent ethyl alcohol were prepared by diluting a 1 per cent stock solution of recrystallized α-naphthol in 40 per cent ethyl alcohol 20, 10,

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and 5 times, respectively, with 20 per cent ethyl alcohol. These solutions were kept in the refrigerator and seemed to be stable. Although ethyl alcohol in higher concentrations strongly inhibits or destroys cytochrome oxidase, concentrations of less than 5 volumes per cent were found to cause no inhibition of the enzyme.

Dimethyl-p-phenylenediamine Hydrochloride—A commercial preparation (Eastman Organic Chemicals) which was almost colorless was employed without further purification. Although dimethyl-p-phenylenediamine is quite autoxidizable at neutral and alkaline pH, the autoxidation is slow at the acid pH (about 2.7) of the hydrochloride in water. The solution could be kept on ice for some hours, becoming only slightly colored by autoxidation during this time.

Since α-naphthol and dimethyl-p-phenylenediamine react in equimolar concentrations when forming indophenol blue, the same molar concentrations of both reagents are required. It was found necessary to use about 40 per cent more of the (impure) commercial dimethyl-p-phenylenediamine hydrochloride than of the recrystallized α-naphthol. This figure was arrived at by measuring the amounts of indophenol blue formed by different proportions of the two reagents after oxidation with K₂Cr₂O₇. It is not desirable to employ more of the dimethyl-p-phenylenediamine hydrochloride, because the additional buffer required for the neutralization of the hydrochloride inhibits the enzyme.

Cytochrome c—A commercial preparation (Delta Chemical Works) was used; this was stated by the supplying company to be more than 92 per cent pure. During the enzymatic tests, cytochrome c was kept in the reduced state by the excess of dimethyl-p-phenylenediamine hydrochloride.

Solvents—Chloroform-alcohol (9:1) was used in most cases for the extraction of the indophenol blue from the test solutions. When coarse emulsions formed during the extraction, it was not necessary to wait until the phases had completely separated. After standing for $\frac{1}{2}$ to 1 minute, the small layer in the separatory funnel consisting of a coarse chloroform-water emulsion was separated, together with the chloroform layer, into a graduated cylinder. The addition of 1 to 2 ml. of fresh chloroform-alcohol (9:1) immediately clarified coarse or fine emulsions, and the volume could be read. The small amount of water above the chloroform did not interfere with the colorimetric measurement. Two extractions with chloroform-alcohol (9:1) were sufficient in most cases to extract the indophenol blue completely if enough water had been added.

Method

In a 100 × 13 mm. test-tube, 0.20 ml. of 1/40 M phosphate buffer solution, pH 7.8, 0.25 ml. of 0.1 per cent α-naphthol solution in 22 per cent ethyl alcohol, 0.35 ml. of 0.1 per cent dimethyl-p-phenylenediamine hydro-
chloride solution, 0.15 ml. of 0.02 per cent cytochrome c solution, and distilled water to make a final volume of 1.5 ml. (the test solution included) were warmed for 2 minutes in a constant temperature water bath at 37°, and the test solution containing 10 to 60 γ of mitochondrial N was added. After 1 to 5 minutes incubation at 37°, and as soon as the test solution had become dark blue, the formation of indophenol blue was stopped by adding about 0.5 ml. of chloroform-alcohol mixture (9:1) and vigorous shaking. The tube was then cooled in ice. The solution was transferred to a 125 ml. separatory funnel by rinsing the test-tube several times with small portions of chloroform-alcohol (9:1). After addition of about 75 ml. of water, the indophenol blue was completely extracted by twice shaking with 5 to 10 ml. of chloroform-alcohol (9:1). The extracts were collected in a 10 ml. or 25 ml. graduated cylinder (with a ground-in stopper), and the volume was read or adjusted to the desired amount with chloroform-alcohol mixture. The color intensity of the indophenol blue in the chloroform extract was determined with the Evelyn photoelectric colorimeter, filter No. 540. A blank containing the reagents without test solution was run together with the sample.

Calculation of Results—The "photometric densities," corresponding to the colorimeter readings for the sample and blank, were read from the table. These values were multiplied by the volumes of the extracts in ml., divided by 10, and thus the photometric densities of the indophenol blue in 10 ml. of solvent were obtained. The photometric density of the blank was deducted from that of the sample. The result was expressed in indophenol blue units, as defined in the following paragraph, by dividing by 1.46 when chloroform was used as solvent and the Evelyn colorimeter with filter No. 540 for the measurement. The indophenol blue units of the sample (corrected for the blank) were divided by the number of minutes of incubation and by the mg. of total N of the sample, and the resulting indophenol blue units per minute per mg. of N were taken as a basis of comparison for different samples.

Indophenol Blue Units—In order to compare cytochrome oxidase activities when measured with different instruments, it is possible to refer the photometric densities of indophenol blue from unknown samples to the photometric density of a standard amount of indophenol blue, and this proportion will be independent of the type of colorimeter. As such a standard, the photometric density of indophenol blue in 10 ml. of solvent, formed from 0.1 mg. of pure (twice recrystallized) α-naphthol and an equimolar amount of dimethyl-p-phenylenediamine hydrochloride by oxidation with K2Cr2O7, was chosen arbitrarily as 1 indophenol blue unit. If this amount of indophenol blue is extracted with the same solvent as it is used for the enzyme test, and is measured with the same colorimeter and the same light filter, the photometric densities of indophenol blue
from unknown samples will represent the same proportions of this unit amount in all colorimeters.

Fig. 1 shows the photometric densities of indophenol blue formed from different concentrations of twice recrystallized \( \alpha \)-naphthol and equimolar amounts of dimethyl-\( p \)-phenylenediamine hydrochloride by oxidation with \( K_2Cr_2O_7 \). The dye was extracted with chloroform or carbon tetrachloride or ether and measured colorimetrically in 10 ml. of the solvents. It can be seen that the photometric densities are proportional to the concentration of indophenol blue. Fig. 1 also indicates that the photometric densities of indophenol blue, formed from 0.1 mg. of \( \alpha \)-naphthol and the equimolar amount of amine, measured with the Evelyn photoelectric colorimeter with filter No. 540 in 10 ml. of solvent, are 1.46 in chloroform, 1.52 in carbon tetrachloride, and 1.64 in ether. These factors permit the calculation of indophenol blue units as defined above.

**Test of Method**

The influence of time of incubation, concentration of enzyme and reagents (phosphate buffer, \( \alpha \)-naphthol, dimethyl-\( p \)-phenylenediamine hydro-
chloride, and added cytochrome c), and of the pH was tested. The possibility of interference by reducing or oxidizing agents which might be present in test solutions was examined.

During the earlier part of the experiments (and before the influence of the time of incubation was completely understood), constant times of incubation of 5 minutes were applied, and 20 per cent lower concentrations of α-naphthol and dimethyl-p-phenylenediamine hydrochloride were employed than those finally adopted. That is why the scale of the ordinates on Figs. 3 to 6 is 5 times higher than with the activities referred to 1 minute's incubation, and why the activities were smaller than those measured under the conditions finally adopted. However, the slopes of the curves are not affected by these changes, and the characteristic influence of the factors under discussion can be seen from the curves. In the experiments of Fig. 2 and in Tables I to III, activities of mitochondrial suspensions and total homogenates of the kidney and liver of the rat were measured for different concentrations of enzyme and different times of incubation. The conditions applied in these experiments were those finally adopted as optimal and indicated under "Method."

**Time of Incubation and Concentration of Enzyme**—As Fig. 2 shows, the formation of indophenol blue by a mitochondrial suspension from rat kidney increased proportionally with time of incubation during the first few minutes, and the rate then decreased rapidly. The duration of proportional increase depended on the concentration of mitochondrial suspension (enzyme). In the experiment of Fig. 2, the proportional increase lasted 5 minutes when the test solution contained 15 γ of total N, 2.5 minutes with 35 γ of N, and 2 minutes with 60 γ of N. Since the proportional increase of the formation of indophenol blue came to an end when about 0.6 indophenol blue unit was formed (blanks not deducted), while α-naphthol and amine were still present in excess, it is possible that the toxic effect of high concentrations of indophenol blue on the enzyme was responsible for the sudden decrease of the dye formation. This interpretation is supported by the observation that the range of proportional increase could be extended by diluting the samples with water while the concentration of the reagents was maintained constant. In the technique adopted here, the disturbing effect was avoided by adjusting the time of incubation to the concentration of enzyme. This was done simply by observing the samples and interrupting the incubation as soon as between 0.3 to 0.6 indophenol blue unit was formed. Such an amount of dye could be easily estimated from the aspect of the incubated samples.

**Concentration of α-Naphthol and Dimethyl-p-phenylenediamine Hydrochloride**—Fig. 3 indicates that maximal formation of indophenol blue required a certain excess of α-naphthol and dimethyl-p-phenylenediamine hydro-
chloride, and that further increase of the reagents had no influence. The slope of the curve may suggest that the sudden decrease of the dye formation described in the preceding paragraph was also due to a lack of excess of α-naphthol and amine. Although this possibility should not be excluded, the above interpretation seems more probable.

**Phosphate buffer; Added NaCl and KCl**—The experiment (Fig. 4) indicates that the formation of indophenol blue by a mitochondrial suspension from rat kidney was strongly depressed when the concentration of phosphate buffer exceeded 0.0044 M. This was probably a salt effect, since NaCl and KCl when added in addition to 0.0033 M phosphate buffer also inhibited the enzymatic formation of the dye, as may be seen from Fig. 4.

The strong influence of phosphate buffer on cytochrome oxidase was also noted by Slater (1) and of salts by Smith and Stotz (2).

**Added Cytochrome c**—Added cytochrome c (Fig. 5) had optimal effects in concentrations of 0.7 to 2.0 mg. per cent (about 0.5 to 1.5 $\times 10^{-6}$ M) when the mitochondrial sample contained 27 $\gamma$ of total N. It was found in other experiments that the optimal concentrations of added cytochrome c varied from about 1.0 to 2.0 $\times 10^{-6}$ M when the N content of the mitochondrial samples was increased from 10 to 60 $\gamma$. The effects were within the range of error of the method, but the accuracy may be improved by adjusting the concentration of added cytochrome c approximately to that of the enzyme.

**Influence of pH**—Fig. 6 shows that the cytochrome oxidase activity of a mitochondrial suspension from the kidney of the rat was optimal from pH
6.4 to 8.0 and fell off rather steeply below and above this pH. The pH was measured with the Beckman pH meter; phosphate buffer (0.0033 M) was employed from pH 5.7 to 6.9 and borate buffer (0.0033 M) from pH 7.1 to 8.8.

**Micro Range**—It may be important in certain experiments to determine extremely small amounts of cytochrome oxidase. No systematic studies were made, but the following experiments may facilitate such an application of the method.

When mitochondrial suspensions containing less that 10 μ of N were tested, the formation of indophenol blue was no longer proportional to the concentration of enzyme. It was too low when the concentrations of the reagents were those indicated under "Method." However, the proportionality could be approximately restored when the concentrations of the reagents (phosphate buffer, cytochrome c, α-naphthol, and dimethyl-p-phenylenediamine hydrochloride) were decreased about one-third. This effect is probably due to the inhibition of the dye formation by too high concentrations of buffer and cytochrome c, as shown in Figs. 4 and 5.

When very small amounts of enzyme are tested, the accuracy is further limited by the fact that the values of the samples approach those of the blanks. It was observed that the blanks remained very low if the temperature was held at 0° (ice cooling) and the time extended to 1 hour or longer. It may therefore be favorable to measure very small amounts of cytochrome oxidase by incubation at 0° for 1 hour or longer.
Test of Interference by Oxidizing or Reducing Agents—Unknown samples may contain oxidizing agents other than cytochrome oxidase which form indophenol blue or reducing agents which inhibit its formation. In order to estimate this interference, activities of mitochondrial suspensions of rat kidney were determined in the presence of NaN₃, a specific inhibitor of cytochrome oxidase. Rather high concentrations of NaN₃ (about $3 \times 10^{-3}$ M) were required to inhibit the activity of the suspension completely. This made it difficult to distinguish the salt effect from specific inhibition. Stannard and Horecker (3) have shown that only the free hydrazoic acid and not the ions inhibit cytochrome oxidase. The relatively high concentrations of NaN₃ necessary for complete inhibition may thus find an explanation, since at the optimal pH of 6.5 to 8.0 of enzyme activity NaN₃ solutions con-

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<th>Time of incubation</th>
<th>Indophenol blue units per min. per mg. N</th>
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<tbody>
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<td>Samples containing 22.8 γ N</td>
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<tr>
<td>min.</td>
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<tr>
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<td>3.98</td>
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<td>6</td>
<td>4.15</td>
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<tr>
<td>8</td>
<td>2.94</td>
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tain for the most part ions and relatively little free acid. When samples of mitochondrial suspensions or total homogenates from rat kidney or liver, all containing 35 γ of N, were treated with an excess of NaN₃ ($1.5 \times 10^{-2}$ M) and compared with the blanks containing the reagents alone, the NaN₃-inhibited mitochondrial suspensions formed only a little more, and the NaN₃-inhibited total homogenates a little less, indophenol blue than did the blanks with the reagents alone.

The possible interference by oxidizing and reducing agents was also tested by heating mitochondrial suspensions or total homogenates for 2 minutes at 70°, thus destroying cytochrome oxidase, and adding the heated samples to a mitochondrial suspension of known activity. It was concluded from these experiments that the concentration of reducing agents present in the small samples is in general too low to cause a marked inhibition of the indophenol blue formation by the enzyme. However, no definite conclusions should be made about this point before the same samples have been tested.
by the present method and by other methods, especially the manometric method, and the results compared.

*Testing of Mitochondrial Suspensions and Total Homogenates from Rat Kidney and Liver*—Mitochondria of the kidney (cortices only) and liver were prepared according to Hogeboom, Schneider, and Palade (4) in an 0.88 M sucrose solution and resedimented once for purification. The "fluffy layer" above the mitochondrial sediments was partially removed. When preparing kidney mitochondria, the dark brown sediment at the bottom of the centrifuge tube containing "droplets" was discarded. The final sediments were suspended in distilled water.

In the experiment illustrated by Fig. 2 a mitochondrial suspension from rat kidney was tested. It can be calculated from Fig. 2 and from the times

<table>
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<th>Time of incubation (min.)</th>
<th>Indophenol blue units per min. per mg. N</th>
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<td>Samples containing 20 γ N</td>
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of incubation and N content of the samples that the activity of this preparation amounted to 5.9 indophenol blue units per minute per mg. of N. Several other mitochondrial suspensions, tested under the same conditions, all showed activities between 5.5 and 6.5 units per minute per mg. of N.

Table I indicates the results of an experiment with a mitochondrial suspension from rat liver. The formation of indophenol blue increased proportionally with time of incubation during the first 6 minutes when the sample contained 22.8 γ of N, during the first 3 minutes when the sample contained 41.1 γ of N, and during the first 2 minutes with 68.5 γ of N. The activity of this preparation amounted to 4.0 indophenol blue units per minute per mg. of N. For two other mitochondrial suspensions from the liver, activities of 3.6 and 3.7 indophenol blue units per minute per mg. of N, respectively, were found.

Table II shows determinations with a total homogenate of rat kidney.
The formation of indophenol blue increased proportionally with the time of incubation during the first 9 minutes when the suspension contained 20 and 40 \( \gamma \) of N, and during 4 to 5 minutes when the suspension contained 70 \( \gamma \) of N. The activity of this suspension amounted to 1.5 indophenol blue units per minute per mg. of N. Two other total homogenates of rat kidney showed activities from 1.3 to 1.4 indophenol blue units per minute per mg. of N.

Table III summarizes the results of determinations with a total homogenate of rat liver. The formation of indophenol blue increased proportionally with the time of incubation during the first 14 minutes when the suspension contained 24.2 \( \gamma \) of N, during 10 minutes with 48.4 \( \gamma \) of N, and during 8 minutes with 72.6 \( \gamma \) of N. The activity of this preparation amounted to 0.6 to 0.7 indophenol blue units per minute per mg. of N and was rather low. Two other total homogenates from the liver showed activities of 0.82 and 0.96 indophenol blue units per minute per mg. of N, respectively.

**SUMMARY**

A calorimetric method for the determination of small amounts of cytochrome oxidase, based on the formation of indophenol blue from \( \alpha \)-naphthol and dimethyl-\( p \)-phenylenediamine hydrochloride (Nadi reaction), is described. The optimal concentrations of the reagents were determined on mitochondrial suspensions from rat kidney as the source of the enzyme. The formation of indophenol blue was proportional to the amount of enzyme (mitochondrial suspensions containing 10 to 60 \( \gamma \) of total N) and was also proportional to the time of incubation during the first few minutes. When cytochrome oxidase activity was expressed in indophenol blue
units per minute per mg. of N as defined in the text, mitochondrial suspensions from rat kidney contained 5.5 to 6.5 units, those from the liver 3.6 to 4.0 units, total homogenates from the kidney 1.3 to 1.5 units, and total homogenates from the liver 0.7 to 1.0 unit.

BIBLIOGRAPHY

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