THE CYTOCHROME C OXIDASE OF THE HOUSE FLY, MUSCA DOMESTICA L.

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Since it is now possible to effect a partial purification of some components of the cytochrome system by solubilization, this method has been used here to investigate the properties of the cytochrome *c* oxidase of the house fly, *Musca domestica* L.

Although it has frequently been assumed that the vital pathways in cellular oxidation are similarly constituted in most organisms, some recent evidence has suggested the existence among the insects of specific variations on the general theme. The original observations of Keilin (1925) and of Warburg and Negelein (1931) on the flight muscles of insects indicated the presence of three hemochromogens, designated as *a*, *b*, and *c*, which have since been found in the tissues of the majority of animals and plants. These cytochromes have been shown to function reciprocally in such a manner as to transfer electrons from substrate to oxygen during biological oxidation. For example, if succinate is the substrate, electrons from succinate are passed, in the presence of succinic dehydrogenase (and other factors: Slater (1949 *c*)) to cytochromes *b*, *c*, *a* and a_3, in order, and thence to oxygen. The latter two organic components, *a* and a_3, constitute the cytochrome *c* oxidase.

In addition to the cytochromes mentioned above, Keilin and Hartree (1949) have recently demonstrated the presence of another cytochrome, *e*, in the thoracic muscles of the blow fly, *Phormia regina*, as well as in tissues of other organisms. The peak absorption of *e* was at 554 mμ. It has been suggested further by Sanborn and Williams (1950) that some insects may have still other protein-hemochromogens that differ qualitatively from the classical components. They have described from the midgut of *Platysamia cecropia* a new constituent of the cytochrome system, called cytochrome *x*, which has a reduced band at 551 to 560 mμ. At the temperature of liquid air this broad band underwent contraction to yield a single band at 554 mμ. The weak band at 547 mμ (due to cytochrome *c*) which appears in mammalian tissues was not found at this temperature. From these observations the authors have suggested that cytochrome *x* and *e* are identical. Cytochrome *x* was thought to have replaced both *b* and *c*, which they consider absent despite the presence of succinic dehydrogenase and cytochrome oxidase activities. There are some difficulties in this interpre-

tation. For one thing, it appears unlikely that such activities would be present in the absence of *b* and *c* in view of the possible identity between cytochrome *b* and succinic dehydrogenase (Pappenheimer and Hendee (1949); Slater (1949 *b*)); and the occurrence of $a + a_3$. Further, in a recent paper by Slater (1949 *b*) on the cytochrome system of kidney, *b* and *c* were found to be present, but with their absorption bands fused. For these reasons, additional investigations of the "new" component would be desirable.

Despite such complications, most of the evidence still indicates that insect cytochromes are very similar to those found in mammalian tissues. There occur, however, during the development of holometabolous insects (Williams (1948); Sacktor (1951 *a* and 1951 *b*)) quantitative changes in the activity of the cytochrome system of a sort which are not observed (Boell (1945); Albaum, Novikoff, and Ogur (1946)) in the development of vertebrates. The opportunity thus presented by insects for correlating enzymatic function with developmental events accentuates the need for more precise information than has been available concerning the characteristics of their cytochrome system.

Methods

The house fly, *Musca domestica* L. was used in all experiments described.

Insoluble cytochrome *c* oxidase preparations were made by a modification of the procedures of Keilin and Hartree (1947) and Slater (1949 *b*). Crushed adult flies, approximately 15 gm., were collected on muslin, thoroughly washed with 5 liters of tap water, and squeezed hard to remove water. This process was repeated until the wash water was colorless. To the washed pulp was added, per gram wet weight of flies, 2 ml. of 0.2 M phosphate buffer, pH 7.4. This mixture was then homogenized for 2 minutes in a Waring blendor. In order to remove pieces of cuticle the suspension was filtered through several layers of cheese-cloth and was then centrifuged for 20 minutes at approximately 1,000 *g*. The supernatant was cooled to 0–4°C. and brought to pH 5.7 with 1.0 N acetic acid. The precipitate was collected immediately by centrifuging at 0–4°C. at 1,000 *g*. for 15 minutes, and was suspended in a volume of 0.1 M phosphate buffer, pH 7.4, equal to that of the precipitate.

The weight of the fat-free dried enzyme preparation was determined by the method of Slater (1949 *b*), as follows: 1.0 ml. of enzyme preparation was diluted with 5.0 ml. water, and 1.0 ml. of 20 per cent trichloracetic acid was added. The precipitate was collected by centrifugation, and the supernatant siphoned off. The precipitate was subsequently washed and centrifuged first with 5.0 ml. of 50 per cent EtOH and later with 5.0 ml. of 96 per cent EtOH. The residue was dried to constant weight at 100°C.

"Soluble" cytochrome *c* oxidase preparations were made by following the procedure of Smith and Stotz (1950) and Stotz (personal communication). House fly extracts (the supernatant obtained after centrifugation for 45 minutes at 1,000 *g*) containing sodium cholate and trypsin, which dissolved the oxidase, were subjected to fractionation with ammonium sulfate.

The activity of the cytochrome *c* oxidase preparations was determined with a Beck-

man model DU spectrophotometer according to the technique previously described (Sacktor (1951 *a*)). To 3.0 ml. of the buffered reduced cytochrome *c* mixture[1] was added the desired amount of enzyme preparation. The initial reading was made as rapidly as possible and subsequent readings were taken at 20 or 30 second intervals for approximately 3 minutes.

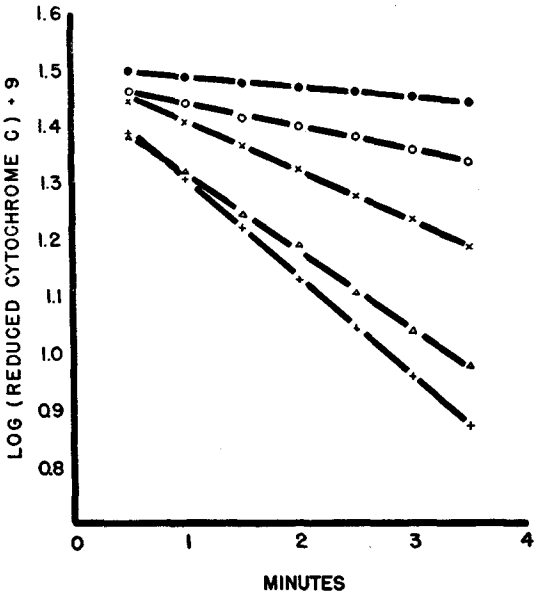

FIG. 1. The oxidation of reduced cytochrome *c* as a function of time for different concentrations of an insoluble oxidase preparation. The following symbols, ●, ○, ×, △, and +, designate the oxidation in the presence of, respectively, 0.01 ml., 0.025 ml., 0.05 ml., 0.075 ml., and 0.10 ml. of the oxidase preparation.

RESULTS

The heart muscle preparations of Keilin and Hartree have been the standard used for measurements of cytochrome *c* oxidase activity. Similar preparations were made from house flies. Examination of these with a low dispersion visual spectroscope showed that they displayed bands corresponding to those of cytochromes *c*, *b*, and $a + a_3$.

The activity of such a preparation is shown in Fig. 1, in which log (reduced cytochrome *c*) is plotted against time for five concentrations of the insoluble oxidase. The activity of the enzyme was determined by the slopes of the lines in such plots. In Fig. 2 the values of d log (reduced cytochrome *c*)$/dt$ obtained for different enzyme concentrations appear as a function of the enzyme con-

[1] Cytochrome *c* was obtained from the Sigma Chemical Co.

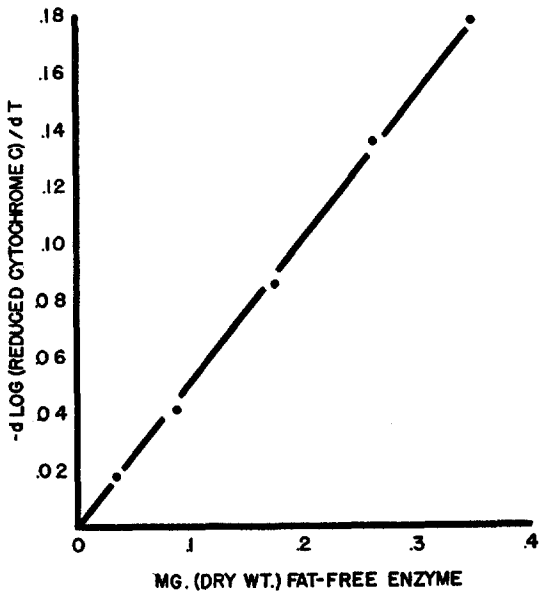

FIG. 2. The activity of an insoluble cytochrome *c* oxidase preparation as a function of the enzyme concentration.

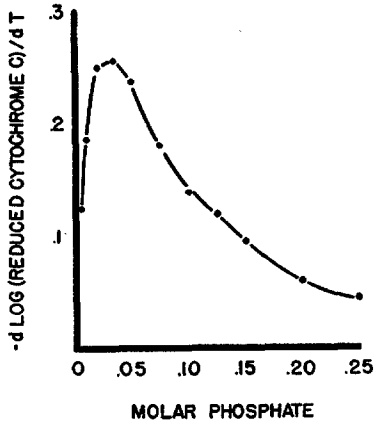

FIG. 3. The effect of the phosphate buffer concentration on the activity of the insoluble cytochrome *c* oxidase.

centrations. These results, in conjunction with those of previous reports (Sacktor (1951 *a* and 1951 *b*)), prove that insect cytochrome *c* oxidase is capable of oxidizing mammalian cytochrome *c*.

The importance of phosphate buffer concentration in manometric measurements of the enzyme activity was demonstrated by Quinlan-Watson and Dewey

(1948) and by Slater (1949 *a*) with heart muscle preparations. Fig. 3 shows the effect of varying buffer concentration on the cytochrome *c* oxidase activity of

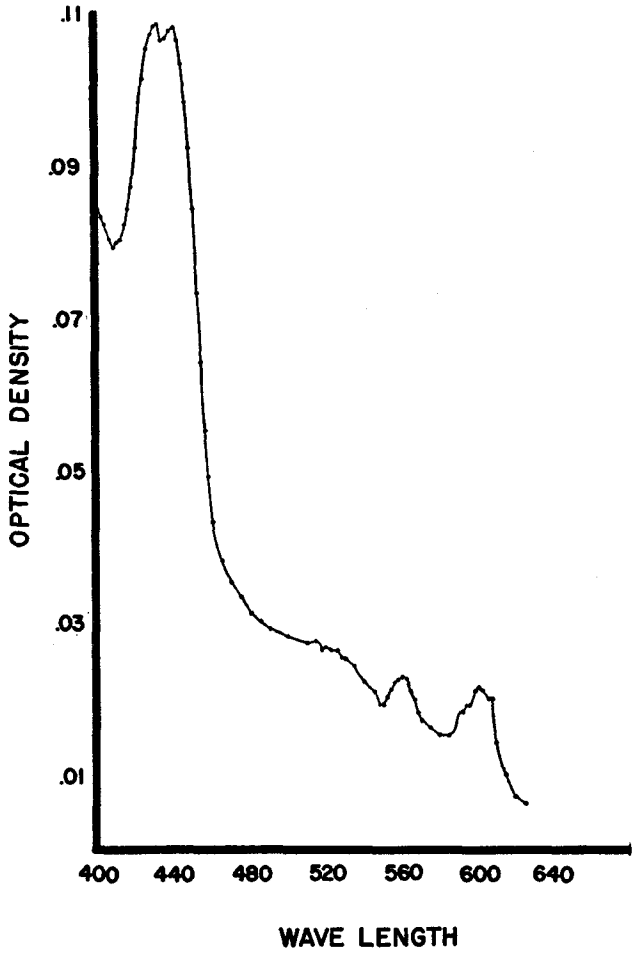

FIG. 4. A typical absorption spectrum of a dithionite-reduced "soluble" cytochrome *c* oxidase preparation. Preparation diluted to 0.9 mg. dry weight/ml. Slit width varied from 0.02 to 0.06 mm. depending on the wave length. Sensitivity maintained constant.

house flies, as measured spectrophotometrically with an insoluble preparation. The data demonstrate that the optimum phosphate concentration is approximately 0.02 to 0.05 M. These values are in agreement with those found by the investigators mentioned above.

The experiments with insoluble preparations showed that the house fly possesses considerable cytochrome *c* oxidase activity. By means of the recently

developed technique of Smith and Stotz (1950), "soluble" preparations also were made with house flies. A typical absorption spectrum of such a preparation of cytochrome *c* oxidase, reduced with dithionite, is shown in Fig. 4. Cytochrome $a + a_3$ can be identified by the alpha peak at 598 to 602 mμ and the Soret peak at 440 mμ. In addition to the oxidase peaks, there are cytochrome *b* peaks at 428 to 432 mμ and 556 to 562 mμ. Cytochrome *c* (peak at 550 to 552

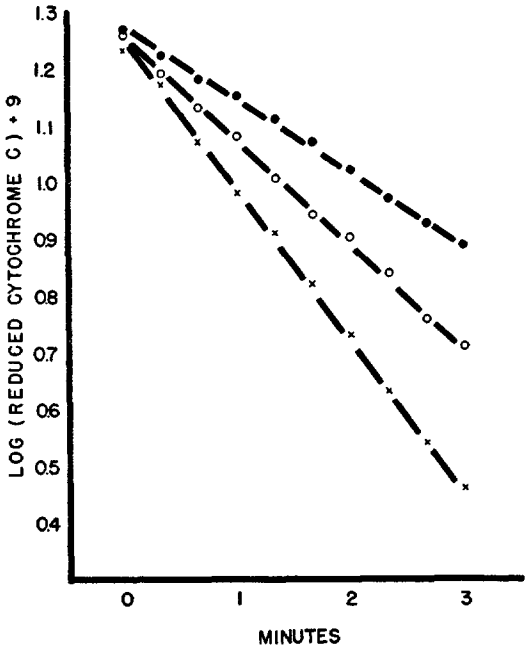

FIG. 5. The oxidation of reduced cytochrome *c* as a function of time for different concentrations of a "soluble" oxidase preparation. The following symbols, ●, ○, ×, designate the oxidation in the presence of, respectively, 0.02 ml., 0.03 ml., and 0.04 ml.

mμ) is absent in this preparation. With mammalian preparations, Smith and Stotz by diluting in order to decrease the concentration of cholate and ammonium sulfate, and Eichel *et al.* (1950) by changing the concentration of desoxycholate, have separated the oxidase from cytochrome *b*. These methods of separation have not been successful with preparations made with house flies.

Further data on the absorption spectrum of the insect oxidase aid in its characterization. An oxidized "soluble" preparation exhibited only a Soret peak at 414 to 418 mμ. Addition of cyanide to a dithionite-reduced oxidase caused a decrease in the magnitude of the Soret peak but had no effect on the alpha peak. This suggests that, as in mammalian tissue, the Soret peak of the

oxidase is due primarily to cytochrome a_3 whereas the alpha peak is due primarily to a. The addition of oxidized cytochrome c plus the reducing agent, hydroquinone, to an oxidized "soluble" preparation resulted in the appearance

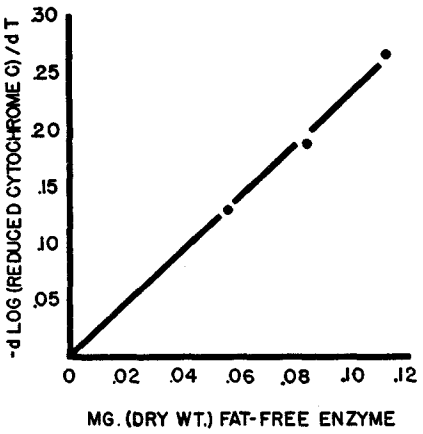

FIG. 6. The activity of a "soluble" cytochrome c oxidase preparation as a function of the enzyme concentration.

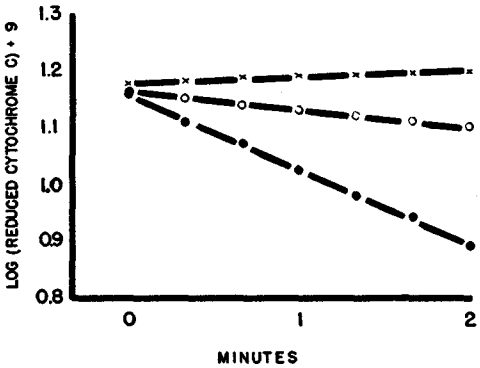

FIG. 7. The effect of cyanide on the oxidation of reduced cytochrome c. The symbol, ●, designates the oxidation in the absence of cyanide. The symbols, ○ and ×, designate the oxidation in the presence of 6.6×10^{-7} M and 3.3×10^{-4} M cyanide, respectively.

of reduced cytochrome c and the reduced band of cytochrome $a + a_3$. The peak at 556 to 562 mμ did not appear, which suggests that this peak is due to cytochrome b, since b is not involved in the oxidation of hydroquinone.

The activity of the "soluble" oxidase preparation is demonstrated in Fig. 5, which shows plots of log (reduced cytochrome c) against time for three concentrations of enzyme. In Fig. 6 the slopes are plotted as a function of enzyme concentration.

Since cyanide is known to inhibit cytochrome *c* oxidase (85 per cent inhibition for rat brain oxidase by 3.25×10^{-6} M cyanide, according to Albaum, Tepperman, and Bodansky (1946)), the effect of this inhibitor on a "soluble" oxidase preparation from the fly was investigated. As shown in Fig. 7, cyanide concentrations of 3.3×10^{-4} and 6.6×10^{-7} M caused complete and 76 per cent inhibition, respectively.

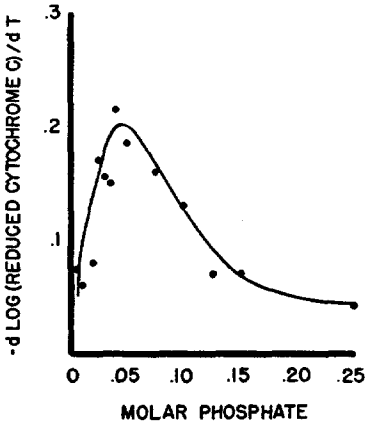

FIG. 8. The effect of the phosphate buffer concentration on the activity of the "soluble" cytochrome *c* oxidase.

TABLE I

Partial Purification of Cytochrome C Oxidase

Preparation	*W*	Purification
Water brei	0.12	—
Insoluble	0.54	4.5 ×
"Soluble"	2.40	20.0 ×

In Fig. 8 is shown the effect of phosphate concentration on the oxidase activity of a "soluble" preparation. As with the insoluble preparation, the optimum phosphate concentration is approximately 0.03 to 0.06 M.

DISCUSSION

A comparison of the activity of the insoluble and "soluble" cytochrome *c* oxidase preparations here studied with that previously obtained with a crude water brei of house flies (Sacktor 1951 *a*)) demonstrates that a partial purification of this enzyme has been achieved. Utilizing the following relationship,

$$W \text{ (specific enzyme activity)} = \frac{d \log \text{ (reduced cytochrome } c)}{dt \times \text{milligrams (dry weight) fat-free enzyme}},$$

the degree of purification can be calculated. These results are shown in Table I.

These degrees of purification of house fly cytochrome *c* oxidase are comparable with those obtained with mammalian tissue. Smith and Stotz (1950) reported a sevenfold increase in activity of their "soluble" preparation as compared to a phosphate extract. Eichel *et al.* (1950) obtained a sixfold increase of a similar "soluble" enzyme over their enzyme in insoluble form.

Fig. 4 shows that the "soluble" preparation exhibits the peaks, at 600 and 440 mμ, of reduced cytochrome *c* oxidase. The alpha peak, 600, is in agreement with the results of early investigations of insect cytochromes, and also with those of mammalian tissues. The Soret peak, however, seems to be shifted towards the blue end of the spectrum. Warburg and Negelein (1931) reported that this peak was at 448 in flight muscles of the bee. This is identical with Keilin and Hartree's results in 1939 with mammalian heart tissue. Stotz (personal communication) found, however, that the peak for his "soluble" preparation was at 445 mμ, and Eichel *et al.* (1950) showed that the Soret peak for their "soluble" preparation was at 440 mμ. The similarity between the present results and those of Eichel *et al.* does not necessarily indicate that the Soret peak of the "soluble" oxidase is at 440 mμ rather than at 445, since both preparations contained some cytochrome *b*. It is possible (Slater (1949 *b*)) that a strong absorption by *b* at 432 mμ caused a slight shift in the oxidase peak.

The peak at 432 mμ indicates the presence of reduced cytochrome *b*. This is identical with the results of other investigations. The apparent peak here at 560 as compared to 564, found by other investigators of both insects and mammals, suggests some denaturation of this component.

The recent results of Cooperstein and Lazarow (1951) enable one to compare the cytochrome *c* oxidase activity of various mammalian tissues with that of whole house flies. Utilizing their units for standard enzyme activity, *d* log (ferrocytochrome *c*) per minute for a 1:100 tissue dilution, the oxidase activity for whole flies (Sacktor (1951 *a*)) was found to be approximately 3.4. Thus the whole fly, which includes the probably inert cuticle and wings, has the same activity as do the rat rectus abdominus muscles, and about one-tenth that of rat heart tissue. It is likely then that insect flight muscles have even larger oxidase activity than skeletal muscles of vertebrates.

The effect of various concentrations of buffer on the activity of house fly oxidase is in agreement with the results of Quinlan-Watson and Dewey (1948) and Slater (1949 *a*), as measured manometrically with an insoluble mammalian tissue preparation. The work of the former authors showed an optimum concentration of 0.072 to 0.088 M when hydroquinone was the reducing agent and a slightly lower optimal concentration with ascorbic acid. Slater found optima of approximately 0.03 to 0.07 M when ascorbic acid and *p*-phenylenediamine, respectively, were used as the reducing agents. Cooperstein and Lazarow (1951) reported that the optimal buffer concentration for rat brain homogenates was 0.02 to 0.04 M when measured spectrophotometrically. With insoluble and "soluble" preparations from house flies the optimal buffer concentrations were

found to be, respectively, 0.02 to 0.05 M and 0.03 to 0.06 M, in spectrophotometric measurements.

The present study indicates that the cytochrome *c* oxidase of adult house flies is comparable, in all respects so far examined, with that of mammalian tissue, and that the methods described for preparing the oxidase of the latter are of sufficiently general applicability to give satisfactory results with house flies. In certain cases, because of the absence of hemoglobin and myoglobin, the use of insects may have particular advantages.

The correlation of enzymatic properties with developmental events has been facilitated by the characterization of adult cytochrome *c* oxidase. It will now be of interest to investigate the properties of the oxidase from the various developmental stages. Further, since it has been shown that the whole house fly has as much oxidase activity as does rat skeletal muscle, it may be inferred that some insect tissues contain considerable amounts of this enzyme. An investigation of the cytochrome oxidase activity of various insect tissues is now under way.

SUMMARY

1. Insoluble and "soluble" forms of cytochrome *c* oxidase were prepared from house flies by methods described for mammalian tissue. A 4.5-fold purification was accomplished by preparing the enzyme in insoluble form (as compared with a crude water brei). The oxidase in "soluble" form was purified 20.0-fold.

2. The absorption spectrum of the "soluble" preparation from flies was similar to that from mammalian tissue, and indicated the presence of cytochromes $a + a_3$ and *b*. In contrast with the corresponding mammalian enzymes these components were not separable by the existing techniques.

3. The Soret peak seen at 440 mμ and the alpha peak at 600 mμ were due. primarily to cytochromes a_3 and *a*, respectively.

4. The oxidase activity of whole house flies was comparable with that of rat abdominal muscle. In both organisms, this activity was inhibited by cyanide to approximately the same extent.

5. The optimum buffer concentration for oxidase activity, as measured spectrophotometrically, was approximately 0.02 to 0.06 M. This is in agreement with results obtained for mammalian enzyme, as measured both manometrically and spectrophotometrically.

6. The present study has demonstrated the following components of the cytochrome system in house flies: *b*, *c*, *a*, and a_3.

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