STUDIES ON CYTOCHROME b-555 FROM LARVAE OF THE HOUSEFLY, *MUSCA DOMESTICA* L.

I. PURIFICATION AND PROPERTIES OF CYTOCHROME b-555

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Running title: Housefly Cytochrome b-555

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I. PURIFICATION AND PROPERTIES OF CYTOCHROME b-555

1. Cytochrome b-555 was highly purified from larvae of the housefly, Musca domestica L. The absorption spectra of the purified cytochrome b-555 showed peaks at 358-360, 414, and 530 mμ in the oxidized form, and at 424, 528 and 555 mμ in the reduced. The α-band of the reduced form was asymmetric at room temperature and at neutral pH, but split into two distinct peaks at 552 and 556 mμ at liquid nitrogen temperature. The purified cytochrome contained protoheme as the prosthetic group and combined neither with carbon monoxide nor with cyanide.

2. In the ultracentrifugation, the cytochrome preparation showed a symmetrical and monodispersed pattern and its sedimentation constant, $s_{20,w}$, was 1.43 S. The molecular weight was determined to be 13,700 by the gel filtration method.

3. Cytochrome b-555 was reduced readily by sodium dithionite, slowly by cysteine, and anaerobically by ascorbate, but was not reduced non-enzymatically by NADH. The reduced

form was oxidized by potassium ferricyanide, beef heart ferricytochrome *c*, and very slowly by air (the apparent first-order velocity constant was 0.0046 sec^{-1}). The midpoint redox potential (E_h) of cytochrome *b*-555 was + 0.006 volt at pH 7.0 and at 12°C.

4. Purified cytochrome *b*-555 was reduced by NADH in the presence of the larval microsomal fraction or NADH-cytochrome b_5 reductase (EC 1.6.2.2) purified from rat liver microsomes. The reaction was inhibited neither by antimycin A nor by rotenone as in the case of microsomal NADH-cytochrome b_5 reductase systems of housefly larvae and mammalian liver.

5. Purified cytochrome *b*-555 seemed to be a solubilized form of cytochrome b_5 which existed both in the mitochondrial and microsomal fractions prepared from the larvae. Solubilization of larval cytochrome b_5 from the cytoplasmic membranes was discussed.

It has been found that cytochrome components change qualitatively and quantitatively during the metamorphosis of cecropia moth (1) and honey bee (2). Much attempts have been made to elucidate the differences in properties among larval, pupal,

and adult cytochromes of insects for clarifying their physiological functions. Yamanaka et al. (3) have reported that three cytochrome components, cytochromes c, b-563 and b-555, are readily extracted with a salt solution from the larval and pupal homogenates of the housefly, Musca domestica L., while cytochromes b-563 and b-555 are not isolated from adult flies by the same procedures as used for the larvae and pupae. Furtheremore, they have observed that although the cytochrome c content varies greatly during the metamorphotic stages there is no difference among larval, pupal and adult cytochromes c, judged from the reactivities of the cytochrome c preparations with cytochrome oxidases (EC 1.9.3.1 and 1.9.3.2). Ohnishi (4) has crystallized larval cytochrome b-563 and investigated its chemical properties. Cytochrome b-555 among the three cytochromes described above has not been highly purified and hence its properties have been obscure.

The present communication deals with the purification and some properties of larval cytochrome b-555 of the housefly. The results obtained here suggest that the purified cytochrome may be a solubilized form of cytochrome b_5 which exists in the larval particulate fraction but not in the soluble. Solubilization of membrane-bound cytochrome b_5 of the larvae is also discussed.

MATERIALS AND METHODS

Materials —— Third instar larvae of the housefly, Musca domestica L., were stored at -15°C until sufficient amounts of the materials were collected. Phenylthiourea, one of tyrosinase (EC 1.10.3.1) inhibitors (5), was synthesized according to the method of Kurzer (6). NADH was prepared by reduction of NAD^+ with yeast alcohol dehydrogenase (EC 1.1.1.1) and ethanol according to the method of Rafter and Colowick (7). Beef heart cytochrome c was prepared by the method of Hagihara et al. (8). NAD^+, rotenone and crystalline bovine pancreatic ribonuclease (EC 2.7.7.16) were purchased from Sigma Chemical Company, U.S.A. Antimycin A was purchased from Kyowa Fermentation Industry Co. Ltd., Tokyo. Crystalline trypsin (EC 3.4.4.4) was a product of Nutritional Biochemicals Corporation, Ohio, U.S.A. Purified rat liver microsomal NADH-cytochrome b_5 reductase and sperm whale myoglobin were supplied through the courtesy of Mrs. Y. Takesue (Institute for Protein Research, Osaka University, Osaka) and Mr. G. Miyazaki (Laboratory of Biophysics, Faculty of Engineering Science, Osaka University, Osaka), respectively.

Physical Measurements —— The concentrations of NADH, NADPH, antimycin A, larval cytochrome b-555, and beef heart cytochrome c were determined spectrophotometrically (9-13).

The measurements of low temperature absorption spectra

were performed according to the method of Kawai (14).

Spectrophotometric measurements were carried out with a Cary spectrophotometer, model 15 and 14, a Shimadzu spectrophotometer, type ZB-50, a Hitachi Perkin-Elmer spectrophotometer, model 139, and a split-beam recording spectrophotometer, using cuvettes of 1 cm light path. In measuring low temperature absorption spectra optical path of cuvettes was 1 or 2 mm.

Ultracentrifugal analyses were performed in a Hitachi UCA-1 analytical ultracentrifuge.

Molecular weight of cytochrome b-555 was determined by the methods of Archibald (15) and Andrews (16) with slight modifications.

The midpoint redox potential (E_h) of the cytochrome was determined by the method of Shichi and Hackett (17) with slight modifications. The reaction mixture in the main compartment of a Thunberg-type cuvette contained 250 mM potassium oxalate, 66 mM phosphate buffer of pH 7.0, 7.15 μM purified larval cytochrome b-555 and 1.0 mM iron (III) ammonium sulfate in a total volume of 0.97 ml, and 0.03 ml of various concentrations of freshly prepared iron (II) ammonium sulfate solution was placed in the side arm of the cuvette. The reference cuvette contained the same constituents as the sample cuvette except that iron (II) ammonium sulfate was omitted. After the reaction mixture was made anaerobic by the evacuation of

the atmosphere in the cuvette and the subsequent filling the cuvette with argon, iron (II) ammonium sulfate in the side arm was tipped in the solution in the main compartment. The difference absorption spectra (the sample reduced by iron (II) ammonium sulfate minus reference) were recorded in the wavelength region between 500 and 600 mμ after 5 min equilibrium period at 12°C. The fully reduced level of cytochrome b-555 was determined by adding a trace amount of sodium dithionite to the sample cuvette. The ratio of reduced (b^{2+}) to oxidized (b^{3+}) cytochrome b-555 was calculated from the following formula.

$$\frac{b^{2+}}{b^{3+}} = \frac{(\Delta A^{*}_{555\ m\mu} \text{ with iron (II)})}{(\Delta A_{555\ m\mu} \text{ with dithionite}) - (\Delta A_{555\ m\mu} \text{ with iron (II)})}$$

E'_0 of the pigment was determined from a plot of log (b^{2+}/b^{3+}) versus log (Fe^{2+}/Fe^{3+}) assuming that E'_0 of ferri-ferro oxalate system was 0.0 volt (18, 19).

Determination of Protein —— Concentration of protein was determined by the method of Lowry et al. (20), using bovine serum albumin as standard.

Oxidation of Larval Cytochrome b-555 by Beef Heart Ferricytochrome c —— Oxidation of the reduced larval cytochrome b-555 by beef heart ferricytochrome c was measured spectrophotometrically by the increase in the absorbance at 550 mμ at 19°C. The assay mixture contained purified rat liver

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* A: absorbance.

microsomal NADH-cytochrome b_5 reductase (final 0.8 μg protein), 100 μM NADH, 50 μM-ferricytochrome c, and 50 mM phosphate buffer (pH 7.4) in a total volume of 2.0 ml. The reaction was started by addition of 1 μl of 107 μM purified larval ferricytochrome b-555.

Fractionation of Larval Homogenate —— The differential centrifugal fractionations of the larval homogenates were performed as described in the following: Batches of 30 g of the fresh larvae were roughly minced with a razor and thoroughly homogenized using a Pottor-Elvehjem glass homogenizer in 270 ml of ice-cold 0.25 M sucrose-1 mM EDTA (pH 7.4) solution. The following procedures were outlined in Fig. 1.

Fig. 1

The operations were always carried out at 0-4°C. Both "mitochondrial" and "microsomal" fractions were washed once more with 1/2 of the initial volume of the sucrose-EDTA solution and suspended in the same medium at 0-2°C.

Determination of the Content of the Cytochrome b_5 in Larval Subcellular Fractions —— The content of cytochrome b_5 in the fractions prepared from larval homogenate was determined based on the difference absorption spectrum (NADH-

or NADPH-reduced minus oxidized) in the presence of rotenone with a split-beam recording spectrophotometer. The assay mixture contained 50 mM phosphate buffer (pH 7.4), 1 mM potassium cyanide, 25.4 μM rotenone, an appropriate amount of each fraction, and 100 μM NADH or NADPH, in a total volume of 3.0 ml. The reference cuvette contained the same constituents as the sample cuvette except that the reducing agent was omitted. The particulate fraction was allowed to stand overnight at 0°C in order to make endogeneous substrates exhausted. Before adding the reductant, oxygen was bubbled through a reaction mixture for 2 or 3 min to oxidize the cytochrome components in the fraction. The spectra were recorded approximately 1 min after the reaction was initiated by the addition of the electron donor to the sample cuvette.

RESULTS

I. Purification of Cytochrome b-555

About 500 g of larvae was homogenized with a Waring blendor in 1 liter of 50 mM phosphate buffer (pH 7.0) containing 2 mM EDTA and 5-10 mM phenylthiourea at 0-4°C. The latter reagent is an inhibitor of tyrosinase (5). The following operations were carried out at 0-4°C unless otherwise indi-

cated. To the homogenate was added solid ammonium sulfate to 40 % saturation. After being allowed to stand overnight, the mixture was filtered through a layer of Celite in a Büchner funnel. The filtrate was fractionated by ammonium sulfate, the precipitate which appeared between 50 and 90 % saturation of the salt was dissolved in an appropriate volume of distilled water and the resulting solution was dialyzed overnight against running tap water. When the dialyzed solution was put on an Amberlite CG-50 column which had been equilibrated with 0.02 M phosphate buffer (pH 7.0), cytochrome c and other basic proteins were adsorbed on the resin.

1. First Chromatography on DEAE-Cellulose Column

DEAE-cellulose was washed in turn with N HCl, N NaOH, a large volume of distilled water, and 0.05 M phosphate buffer (pH 7.0). When the effluent from the Amberlite CG-50 resin described above was put on a column of the DEAE-cellulose, cytochrome b-555 and the most of the dark brown pigments were adsorbed at the top of the column to make a dark brown band, but cytochrome b-563 passed through the cellulose column. The adsorbed cytochrome was eluted with 0.2 M phosphate buffer (pH 7.0).

2. Ammonium Sulfate Fractionation

The dark brown eluate obtained above was fractionated with ammonium sulfate. The precipitate obtained between 70

and 100 % saturation of the salt was collected by centrifugation and dissolved in a minimal volume of distilled water. The resulting solution was dialyzed overnight against 0.1 M phosphate buffer (pH 7.0).

3. Duolite A-2 Resin Treatment

Duolite A-2 resin of 100-200 mesh was washed with N NaOH and then with a large volume of water. The resin was suspended in 0.1 M phosphate buffer (pH 7.0) and fine resin particles were discarded by repeating decantation. Finally, the resin was equilibrated with 0.1 M phosphate buffer (pH 7.0). The dialyzed solution mentioned above was charged on a column of the Duolite A-2. The most of the brown pigments was adsorbed on the column, while cytochrome b-555 unadsorbed.

4. Second Chromatography on DEAE-Cellulose Column

The effluent from the Duolite A-2 column was again charged on a DEAE-cellulose column which had been buffered with 0.1 M phosphate buffer (pH 7.0). In this case, cytochrome b-555 was weakly adsorbed making a broad red band, which migrated down gradually on the column by developing with 0.1 M phosphate buffer (pH 7.0). Yellowish brown band of melanin pigments moved down faster than the red one of cytochrome b-555 under the conditions.

5. Concentration and Ammonium Sulfate Fractionation

After the reddish yellow eluate from the DEAE-cellulose column obtained above was dialyzed against 0.02 M phosphate

buffer (pH 7.0) overnight, the solution was again charged on a DEAE-cellulose column equilibrated with 0.05 M phosphate buffer (pH 7.0). Cytochrome b-555 adsorbed on the column was eluted with 0.2 M phosphate buffer (pH 7.0). Finely powdered ammonium sulfate was gradually added to the above red eluate with a gentle stirring until a slight turbidity appeared. After the mixture was allowed to stand overnight, the precipitate was collected by centrifugation. With the supernatant obtained above, the same procedures were repeated several times. The precipitates obtained above were combined and dissolved in 0.1 M phosphate buffer (pH 7.0). The solution thus obtained was used as the purified cytochrome b-555 preparation. The purification procedures are summarized in Table I.

Table I

II. Properties of Purified Cytochrome b-555

1. Absorption Spectra

The absorption spectra of the purified cytochrome b-555 are shown in Fig. 2. The absorption spectrum of the oxidized form showed a sharp peak at 414 mμ, and two smaller bands at

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Fig. 2

358-360 and 530 mμ with a shoulder at 558 mμ, besides the peak at 278 mμ due to protein absorption. The reduced form had major absorption peaks at 424, 528, and 555 mμ, and the α-band at 555 mμ was asymmetric with a pronounced shoulder at 560 mμ. The ratios in the absorbance among the peaks are as follows: $A_{424\ m\mu}(red.)/A_{555\ m\mu}(red.) = 6.8$, $A_{555\ m\mu}(red.)/A_{528\ m\mu}(red.) = 1.8$, $A_{424\ m\mu}(red.)/A_{414\ m\mu}(ox.) = 1.5$, and $A_{555\ m\mu}(red.)/A_{278\ m\mu}(ox.) = 1.2$.

At liquid nitrogen temperature (-196°C), the asymmetric α-band of the reduced form split into two distinct peaks at 556 and 552 mμ (Fig. 3). Although the β-band showed a more complex structure than the α-band, the Soret band did not split and only shifted from 424 to 422 mμ at the temperature.

Fig. 3

In neither the reduced nor the oxidized form the absorption spectra of purified cytochrome b-555 were altered in

the presence of carbon monoxide and potassium cyanide at neutral pH. This would suggest that the cytochrome did not combine with these reagents. Since the absorption spectrum of the oxidized form was obtained when air was bubbled through the dithionite-reduced preparation, cytochrome b-555 was autoxidizable. This autoxidation reaction was not influenced by carbon monoxide and cyanide.

2. Prosthetic Group

The absorption spectrum of the pyridine hemochrome derived from the purified cytochrome b-555 preparation (final concentrations of pyridine and NaOH were 30 % and 0.2 N, respectively) had maxima at 557, 525, and 417-420 mμ. From this result it is concluded that the cytochrome contained protoheme as the prosthetic group, as previously reported (3).

3. Molecular Weight

The most purified cytochrome b-555 preparation (the ratio of $A_{555\ m\mu}(red.)/A_{278\ m\mu}(ox.) = 1.2$) showed a symmetrical and monodispersed pattern in an ultracentrifugal field as shown in Fig. 4. The sedimentation constant, $s_{20,\ w}$, was

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Fig. 4

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calculated to be 1.43 S. The molecular weight of the larval

cytochrome was estimated by gel filtration on a column of Sephadex G-100, by a slight modification of the method of Andrews (16), as shown in Fig. 5. From the position of the

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Fig. 5

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elution volume of the pigment on the standard curve, the molecular weight was calculated to be 13,700. By the method of Archibald (15), the molecular weight of cytochrome b-555 was determined to be 18,000, assuming the partial specific volume to be 0.720 (Fig. 6). The apparent molecular weight became

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Fig. 6

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lower when the centrifugal run proceeded. This may indicate that the preparation was contaminated with a trace of impure protein(s) of higher molecular weight than the cytochrome. Therefore the value of 13,700 obtained by the gel filtration method seems to be more accurate for the molecular weight of the cytochrome than that estimated from the Archibald run.

4. Reduction and Oxidation

The purified cytochrome b-555 was reduced readily by sodium dithionite and slowly by cysteine (reduced to 21 % 5 hr after the addition of the reducing agent under aerobic conditions). It was anaerobically reduced very slowly by ascorbate (approximately 60 % reduced 12 hr after the reductant was added), but not reduced nonenzymatically by NADH even under anaerobic conditions. The reduced pigment was reoxidized by potassium ferricyanide, beef heart ferricytochrome c, and more slowly by air. The above facts suggest that the midpoint redox potential (E_0') of the purified cytochrome is approximately 0 volt. This interpretation was confirmed by the following experiment. In ferric-ferrous oxalate redox systems, the extent in the reduction of the cytochrome varies with the ratio of Fe^{2+}/Fe^{3+} (Fig. 7).

Fig. 7

From the results shown in Fig. 7, the E_0' value of cytochrome b-555 was calculated to be + 0.006 volt at pH 7.0 and at 12°C, assuming the E_0' value of ferric-ferrous oxalate system to be 0.0 volt (18, 19).

5. Intracellular Localization

The fact that larval cytochrome b-555 is extractable with a salt solution suggests that the cytochrome b-555 exists in the cells as a soluble protein. In order to elucidate the mode of existence of cytochrome b-555 in the cells, intracellular localization of the cytochrome was studied on the basis of the difference absorption spectra of the subcellular fractions obtained by differential centrifugation. Table II shows

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Table II

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the various enzymic activities contained in larval mitochondrial, microsomal, and soluble fractions (for preparation procedures of these fractions, see "METHODS AND MATERIALS", and Fig. 1). The exclusive localization of the cytochrome oxidase and succinate-cytochrome c reductase activities in the mitochondrial fraction isolated from larval homogenate indicates that the microsomal fraction was scarcely contaminated with the mitochordria. Whether the larval mitochondrial fraction was contaminated with the microsomes was not investigated in the present study. NADPH- and NADH-cytochrome c reductase activities of the larval microsomal fraction were about five

to three (sometimes two) times as high as the respective activities of the mitochondrial one. In the larval particulate fraction NADPH-cytochrome c reductase activity was about one-tenth as high as NADH-cytochrome c reductase activity. On the other hand, neither the cytochrome oxidase nor succinate-cytochrome c reductase activity was detected in the soluble fraction. However, the NADH- and NADPH-cytochrome c reductase activities found in the soluble fraction were 1 and 10 % of the respective activities in the microsomal one. These relationships among the various kinds of enzymic activities observed with the three larval fractions were quite similar to those found in mammalian liver.

It has been well known that antimycin A and rotenone inhibit the electron transfer in the respiratory chain specifically from cytochrome b to cytochrome c_1 and from NADH to NADH dehydrogenase (21), respectively. Thus, both the reagents inhibit the respiratory chain-linked pyridine nucleotide oxidizing system of liver mitochondria but not the NADH- or NADPH-cytochrome c reductase system mediated by the NADH- or NADPH-cytochrome b_5 reductase system in the microsomes and outer membranes of the mitochondria (22). In the larvae of housefly, as shown in Table III, NADH-cytochrome c reductase activity in the mitochondrial fraction was 39 and 33 % inhibited by antimycin A and rotenone, respectively. With

Table III

the microsomal fraction, either of the poisons scarcely inhibited NADH-cytochrome c reductase activity. Succinate-cytochrome c reductase activity was completely inhibited by both the reagents. It is uncertain at prsent why succinate-cytochrome c reductase activity was inhibited by rotenone. Both cyanide and azide inhibited completely the cytochrome oxidase activity. These effects of the inhibitors indicate that both mitochondrial and microsomal fractions isolated from larval homogenate correspond to the respective fractions from mammalian livers.

Both antimycin A and rotenone stopped the electron flow from succinate to cytochrome c in the larval mitochondria, while the NADH-cytochrome c reductase systems of liver particulate fractions via the NADH-cytochrome b_5 reductase system, and the NADH-cytochrome c reductase of larval microsomes were insensitive to the above reagents. Therefore, the cytochrome b_5 content in a larval fraction could be determined in the presence of the above inhibitors without being affected by the respiratory pigments in the inner membranes of the mitochondria. The NADH- or NADPH-reduced minus oxidized difference

absorption spectrum obtained both with the larval mitochondrial and microsomal fractions in the presence of rotenone were the same as that obtained with liver microsomal cytochrome b_5 (23, 24); i.e. absorption maxima were at 556, 527 and 424 mμ and a shoulder at 560 mμ (Fig. 8). As expected, the same difference absorption spectra as those exhibited in Fig. 8 were ob-

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Fig. 8

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tained when antimycin A was added to the reaction mixture in place of rotenone. The cytochrome b_5 contents estimated on the basis of the difference absorption spectra shown in Fig. 8 were 0.20-0.25 and 0.04-0.06 mμmoles/mg protein in the larval microsomal and mitochondrial fractions, respectively. The ratio of cytochrome b_5 content in the larval mitochondrial fraction to that in the microsomal one was four to five. This was in good agreement with that in rat liver as reported by Sottacasa et al. (22).

However, the difference absorption spectra obtained with the larval soluble fraction in the same way as with the particulate fraction showed no peaks even in the presence of a trace of the larval microsomal fraction, while on addition

of sodium dithionite, two absorption maxima appeared at 560 and 425 mμ. At low temperature (-196°C), the microsomal and soluble fractions prepared from larval homogenate exhibited the difference absorption spectra shown in Fig. 9. The

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Fig. 9

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spectrum of the larval microsomal fraction showed two distinct peaks at 552 and 556 mμ, and Soret band at 422 mμ, which was identical in position and pattern with that of the purified larval cytochrome b-555 (Fig. 3). On the other hand, that of the soluble fraction showed a single peak at 556-558 mμ and γ-band at 425 mμ. It is uncertain at present to what a component or components this spectrum is attributable except the cytochrome b-555.

7. Reduction of Purified Cytochrome b-555 in the Presence of Microsomal NADH-Cytochrome b_5 Reductase System

Curve A of Fig. 10 shows a typical "cyclic process" consisting in the reduction of purified larval cytochrome b-555 by NADH in the presence of the microsomal fraction prepared from larval homogenate and in its subsequent reoxidation by air. This proved that purified cytochrome b-555 preparation

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Fig. 10

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was enzymically active. The reduction of the purified cytochrome was not inhibited by antimycin A and rotenone as in the case of liver and housefly larval microsomal NADH-cytochrome b_5 reductase systems. The reduction of the purified cytochrome by NADH was also catalyzed by microsomal NADH-cytochrome b_5 reductase purified from rat liver (Fig. 10, curve B), which has been known to react specifically with cytochrome b_5 (25). The larval cytochrome b-555 was almost completely reduced (95 % reduced) by the enzyme under anaerobic conditions.

The apparent first-order velocity constant for the autoxidation of purified cytochrome b-555 was calculated to be 0.0046 sec^{-1} directly from the curves such as shown in Fig. 10 (curve A). The value obtained here was in good agreement with those of rat liver and rabbit brain microsomal cytochromes b_5 which have been reported to be 0.00466 and 0.0056 sec^{-1}, respectively (Hirota, Omura, Nishibayashi, and Sato, personal communication, 26).

Chance and Williams (27), and Chance and Pappenheimer (28) have studied the NADH-cytochrome b_5 reductase systems of rat liver and cecropia midgut, and derived a formula to calculate turnover number of an enzyme-substrate complex. They

have assumed that the reaction proceeds as shown in eq. (1),

$$E + S \rightleftharpoons E \cdot S \xrightarrow{k_3} E + P \qquad (1)$$

where, E represents oxidized cytochrome b_5, S NADH, and ES reduced cytochrome. The velocity constant, k_3 is expressed by the following equation (29),

$$k_3 = \frac{x_o}{p_{max} \times t_{\frac{1}{2}}\ off} \qquad (2)$$

where, x_o represents initial concentration of NADH, p_{max} the maximum steady state concentration of reduced cytochrome, and $t_{\frac{1}{2}}\ off$ the period between half-formation and half-decay of p. As the values of k_3 for the cyclic reduction and re-oxidation reaction of cytochrome b_5, Mordirzadeh and Kamin (30) have obtained by application of eq. (2) 0.03 sec^{-1} and 0.008 sec^{-1} with rat liver microsomes and with "solubilized preparation" prepared from lipase (EC 3.1.1.3) treated calf liver microsomes, respectively. With cecropia midgut homogenate Chance and Pappenheimer (28) have obtained 0.09-0.13 sec^{-1} (in the absence of cyanide) and 0.02 sec^{-1} (in the presence of cyanide) as the k_3 values, and Chance and Williams (27) 0.06 sec^{-1} and 0.02 sec^{-1} with rat liver microsomes in the absence and in the presence of the inhibitor, respectively.

However, these k_3 values cited above are obtained only with the membrane-bound cytochrome b_5. It seems important

for studies on physiological role and mechanism of the oxidation reaction of microsomal cytochrome b_5 to determine the rate constant with the solubilized cytochrome b_5 system. By applying eq. (2) to the experiments such as shown in Fig. 10 (curve A), a straight line was obtained when x_o was plotted against $v_{max}\ t_{\frac{1}{2}\ off}$, indicating the applicability of this equation to the NADH-cytochrome b_5 reductase system studied in the present investigation. Thus, k_3 was determined to be 0.0045 sec^{-1} with the system of larval microsomal fraction and soluble cytochrome b-555. This value is consistent with that of 0.0046 sec^{-1} calculated directly from the curve A in Fig. 10. The discrepancy in the k_3 values between the other authors' and the present studies may be due to various causes; use of external or internal cytochrome, difference in sources of microsomes, presence or absence of an inhibitor such as cyanide, use of intact or "solubilized" microsomes, or presence of any other oxidizing component in microsomes than air.

DISCUSSION

Homogeneity of Purified Cytochrome b-555 ——— The fact

that the purified cytochrome b-555 preparation obtained in the present investigation has shown an asymmetric α-band in the reduced form may suggest that the preparation has contained more than one cytochrome components. However, no cytochrome components other than cytochrome b-555 have been detected in the preparation by the electrophoretic and ultracentrifugal analyses, and chromatographies of the crude cytochrome preparation on a DEAE-cellulose column and on a DEAE-Sephadex A-50 column under various conditions (to be published). Highly purified preparations of mammalian microsomal ferrocytochrome b_5 also show an asymmetric α-band (31-33) which resembles that of the reduced larval cytochrome b-555. Further, the asymmetric α-band in the reduced form of the larval cytochrome b-555 splits into two distinct peaks at 552 and 556 mμ at low temperature (Fig. 3) just as has been found with liver microsomal ferrocytochrome b_5 which exhibited two split peaks at 552 and 558 mμ in the low temperature absorption spectrum (33-38). Furthermore, Estabrook (35) has reported that at low temperature two distinct peaks of the split α-band of rat liver ferrocytochrome b_5 bound to cytoplasmic membranes appear simultaneously when various amounts of NADH are added. The similar spectrum has been obtained by Shichi and Hackett (39) with purified and particle bound reduced cytochromes b-555 of mung bean. Therefore, the asym-

metric α-band is one of characteristics of the larval cytochrome b-555. It is uncertain what gives rise to difference among mammalian, plant, and insect cytochromes with respect to the positions of the two peaks formed by splitting of the α-band at low temperature (556 and 558 mμ).

Relationship between Purified Cytochrome b-555 and Larval Membrane-bound Cytochrome b_5 —— The following lines of evidence indicate that the purified larval cytochrome b-555 is similar to, or identical with cytochrome b_5 which is detected spectrophotometrically in both the mitochondrial and microsomal fractions isolated from larval homogenate (Fig. 8).

(1) Both the cytochromes showed difference absorption spectra similar to each other at room temperature (Figs. 2 and 8).

(2) Even at low temperature (-196°C) absorption spectra of both the reduced hemoproteins were virtually identical with each other (Figs. 3 and 9).

(3) With the soluble fraction prepared from larval homogenate, any peak was not detected in the difference absorption spectra (NADH- or NADPH-reduced minus oxidized) in the presence of a trace of the larval microsomal fraction (under aerobic conditions) or of purified rat liver microsomal NADH-cytochrome b_5 reductase (under anaerobic conditions) (unpublished observations). The low temperature difference absorption spectrum of the soluble fraction (reduced by dithionite) showed a single peak at the α-band (Fig. 9).

(4) When the soluble fraction of larvae was subjected to pass through a DEAE-cellulose column equilibrated with 20 mM phosphate buffer (pH 7.0), no cytochrome component was adsorbed on the cellulose column (unpublished observations).
(5) Purified larval cytochrome b-555 was reduced by NADH under the catalytic action of larval microsomal fraction or of purified rat liver microsomal NADH-cytochrome b_5 reductase. This enzyme has been known to react specifically with cytochrome b_5 (Fig. 10).

The studies on intracellular localization of larval cytochromes have made it sure that cytochrome b_5 exists in the particulate fraction of the larval cells but not in the soluble, in contrast with mung bean cytochrome b-555 which is known to be present both in the soluble and particulate fractions (40). Several causes will be considered that have made the larval membrane-bound cytochrome b_5 solubilized:
(1) Freezing and thawing treatment of larvae has released the cytochrome from the membranes.
(2) During the extraction procedures of cytochrome hydrolytic enzyme(s) such as proteinase(s) and lipase(s) present in the larvae have solubilized the membrane-bound hemoprotein.
(3) The ionic linkage by which the cytochrome is bound to the membranes has been cleaved by the effect of such high ionic strength as 40 % saturated ammonium sulfate.
(4) When the membranes in which the cytochrome is buried has

been destroyed by vigorous homogenization in the hypotonic medium (50 mM phosphate buffer solution), release of the pigment has occurred.

No proteinase activity has been detected in the extract of the larval homogenate with 40 % saturated ammonium sulfate (unpublished observations). Thus, it is unlikely that larval cytochrome b_5 has been solubilized by the action of proteolytic enzymes.

Relationship between Purified Larval Cytochrome b-555 and Other Cytochrome b_5-like Hemoproteins from Various Sources —— In Table IV, b-type cytochromes isolated from various sources are compared with one another. They are very similar to one another with respect to their chemical and physiological properties. It has been reported that a crude preparation of calf liver microsomal cytochrome b_5 solubilized by lipase treatment is separated into two parts by DEAE-Sephadex A-25 column chromatography or starch gel electrophoresis (41), and a cytochrome b_5 preparation solubilized with the aid of Nagarse (EC 3.4.4.16) or trypsin from rabbit liver microsomes is also divided into two bands by a DEAE-cellulose column chromatography (42). Kajihara and Hagihara (41) have thought two possibilities for the solubilization of particle-bound cytochrome b_5 by the proteolytic enzymes and that any preparations of cytochrome b_5 liberated by proteases may not

consist of the native cytochrome molecules but heme-peptide fragments. Recently, without the aid of proteolytic enzyme Ito and Sato (43) has succeeded in solubilizing cytochrome b_5 from rabbit liver microsomes by deoxycholate and triton X-100 treatments. The preparation obtained by them is found to be homogeneous ultracentrifugally and electrophoretically, and its molecular weight is determined to be about 25,000 on the basis of heme content and of behavior on dextran gel. Further, by trypsin digestion they have converted cytochrome b_5 solubilized by the above detergents to the cytochrome b_5 of lower molecular weight which is found to be identical with the cytochrome b_5 obtained directly by trypsin digestion of rabbit liver microsomes.

On the other hand, throughout the purification steps the crude preparation of larval cytochrome b-555 has not been separated into two or more cytochrome components. Further the purified cytochrome b-555 preparation has been found to be homogeneous ultracentrifugally, electrophoretically, and chromatographically. It has been also reported that the crude mung bean cytochrome b-555 preparation is not separated into two or more cytochrome components and highly purified preparation is pure on the basis of ultracentrifugal patterns and chemical analysis of N-terminal amino acid (17, 44). These facts would suggest that both the larval and mung bean cytochrome preparations consist of intact hemoprotein molecules.

Raw et al. (45) have found that treatment of pig liver mitochondria with 10 % ethanol releases cytochrome b_5-like hemoprotein from the membranes, whereas the same treatment of the microsomes does not release any cytochrome b_5. Sottocasa et al. (22) have reported the similar observation; namely, hypotonic treatment solubilizes most part of the cytochrome b_5-like hemoprotein from rat liver mitochondria, but not cytochrome b_5 from the microsomes. Furthermore, Raw et al. (45) have observed that a mitochondrial cytochrome b_5-like hemoprotein purified from pig liver is not reduced by cysteine, while Strittmatter (24) has found that microsomal cytochrome b_5 purified from rabbit liver is reduced by the reagent. In our experiments, when the microsomal fraction isolated from larval homogenate was incubated for 15 hr in 50 mM phosphate buffer (pH 7.0) containing ammonium sulfate (40 % saturated) and the mixture was centrifuged, cytochrome b-555 was not detected spectrophotometrically in the resulting supernatant (unpublished observations). In the case of larval cytochrome b-555 the purified pigment has been slowly reduced by cysteine (21 % reduced 5 hr after the addition of the reducing agent under aerobic conditions). In comparison with the mitochondrial cytochrome b_5-like hemoprotein and the microsomal cytochrome b_5 described by other authors in respect to their properties, further investigations are

necessary to investigate whether purified larval cytochrome b-555 originates from mitochondrial cytochrome b_5-like hemoprotein or microsomal cytochrome b_5, or both.

The Mitochondrial and Microsomal Electron Transfer Systems of Larvae and Adult of the Housefly —— By our preliminary experiments, the larval mitochondria have not possessed any appreciable α-glycerophosphate-cytochrome c reductase activity which is found with adults flies by other workers (46), and cytochrome P-450 has been detected in the larval microsomes (0.13 mμmoles/mg protein) but not in the mitochondria. In microsomes of adult housefly (Musca vicina L.) Ray (47) has reported the existence of microsomal electron transfer hemoproteins, cytochromes b_5 and P-450. Further experiments are necessary to clarify the aspects of changes in cytochrome b_5 content, in the mode of existence of the cytochromes in membraneous structure, and in activities of the microsomal and mitochondrial electron transfer systems during the metamorphosis of the housefly.

Acknowledgement

The author wishes to thank Dr. T. Hiroyoshi (Department of Genetics, Medical School, Osaka University, Osaka) for cultivation of houseflies, Prof. B. Hagihara (Department of Biochemistry, Medical School, Osaka University, Osaka) for the split-beam recording spectrophotometric analyses, Dr. T. Omura and Mrs. Y. Takesue (Institute for Protein Research, Osaka University, Osaka) for their generosity in supplying purified rat liver microsomal cytochrome b_5 and NADH-cytochrome b_5 reductase, Mr. G. Miyazaki (Laboratory of Biophysics, Faculty of Engineering Science, Osaka University, Osaka) for his generosity in supplying sperm whale myoglobin, and Dr. K. Kawai (Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka) for the measurements of low temperature absorption spectra.

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Table I

Summary of purification procedure of cytochrome b-555 of housefly larvae[1)]

Fraction	Total protein[2)] (mg)	Total cytochrome b-555[3)] (μmoles)	Specific content (μmoles cytochrome /g protein)
1. Extract	105,000	----[4)]	----
2. $(NH_4)_2SO_4$ 50-90 % sat.	93,600	----	----
3. CG-50 treated	73,200	----	----
4. 1st DEAE-cellulose eluate	3,020	1.66	0.550
5. $(NH_4)_2SO_4$ 70-100 % sat.	408	1.42	3.48
6. Duolite A-2 treated	316	1.34	4.24
7. 2nd DEAE-cellulose eluate	23.8	0.697	29.3

1) About 3.0 kg of larvae was used as the starting material.

2) Concentration of protein was determined by the method of Lowry et al. (20).

3) Cytochrome b-555 content was estimated spectrophotometrically (12).

4) With these fractions, the presence of cytochromes c and b-563 prevented the determination of cytochrome b-555 contents.

Table II

Activities of cytochrome c reductases and oxidase in various fractions of larval homogenate[1)]

Fraction	Reductase activity[2)] (μmoles cyt. c red./mg protein/min)			Cyt. oxidase[3)] (sec^{-1}/mg protein/2 ml cuvette)	Total protein[4)] (mg)
	NADH-cyt. c	NADPH-cyt. c	succ.-cyt. c		
Mitochondrial fraction"	0.154	0.0109	0.121	0.534	108
Microsomal fraction"	0.543	0.0533	0.000	0.0767	71.4
Soluble fraction"	0.0048	0.0051	0.000	0.000	1200

1) Figures show a typical experimental results of seven experiments.

2) The activities were measured spectrophotometrically at 24°C by following the reduction rate of beef heart ferricytochrome c. The assay mixture consisted of an appropriate amount of each fraction (4.2 μg-1.0 mg protein), 50 mM phosphate buffer (pH 7.4), 50 μM ferricytochrome c, and 1 mM potassium cyanide. The total volume of the reaction mixture was 2.0 ml. The reaction was started by the addition of

100 μM NADH, NADPH, or 10 mM succinate. The activities were calculated from the initial reaction velocities and expressed as μmoles cytochrome c reduced/mg protein/min.

3) The activities were assayed at 24°C by following the decrease in the absorbance at 550 mμ of beef heart ferrocytochrome c. The reaction mixture contained 15 μM ferrocytochrome c in 2.0 ml of 50 mM phosphate buffer (pH 7.4). The reaction was started by the addition of each fraction (23 μg-1.0 mg protein). The activities were expressed as the first-order velocity constant (sec^{-1})/mg protein/2 ml cuvette according to the method of Smith and Conrad (48).

4) Concentration of protein was determined by the method of Lowry et al. (20).

Table III

Effect of inhibitors on the various enzymic activities of larval fractions

Figures indicate the inhibition in percentage. Experimental conditions were the same as for Table II, except that the inhibitor (25.4 μM rotenone, 5.87 μM antimycin A, 1 mM cyanide or azide, or an appropriate amount of ethanol) was further added and that the reaction was started after incubation with the inhibitor or ethanol for 5 min at 24°C. To calculate per cent of inhibition, the activities in the presence of ethanol were taken as 100 %.

Fraction	NADH-cyt. c		NADPH-cyt. c		Succ.-cyt. c		Cyt. oxidase	
	Anti-mycin A	Rotenone	Anti-mycin A	Rotenone	Anti-mycin A	Rotenone	KCN	NaN_3
"Mitochondrial fraction"	39	33	34	0	100	100	100	100
"Microsomal fraction"	6	4	20	3			100	100

Table IV

Comparison of properties of larval cytochrome b-555 with those of cytochrome b_5-like hemoproteins from various sources

α, β, γ, and γ_{ox} mean positions of α-, β-, and γ-bands of the reduced cytochrome and γ-band of the oxidized, respectively, M.W. molecular weight, E_o' midpoint redox potential of the hemoprotein, + positive, and - negative. Mt, Ms, and Sol. are mitochondrial, microsomal, and soluble fractions, respectively.

	Microsomal cytochrome b_5 [1]	Mitochondrial cytochrome b-556 [2]	Plant cytochrome b-555 [3]	Housefly cytochrome b-555
Source	Rabbit liver Ms	Pig liver Mt	Mung bean seedlings	Housefly larvae
Extraction	Lipase treatment	Freezing-thawing in 10 % ethanol	Water	Buffer + $(NH_4)_2SO_4$
α, β, γ (γ_{ox})	556, 526 423 (413)	556, 528 423 (412)	555, 527 423 (413)	555, 528 424 (414)
M.W.	16,900	15,000	13,500	13,700
E_o'	+ 0.02	+ 0.014 (pH 6.5)	- 0.03	+ 0.006
Reduction by cysteine	+	-	+	+ (slow)
Isoelectric point	Acidic	Acidic	Acidic	Acidic
Autoxidation	+	+	+	+
Reactivity with CO and cyanide	-	-	-	-
Intracellular localization	Ms	Mt	Mt, Ms Sol.	Mt, Ms
Reducibility by NADH oxidase system	+	+	+	+

1) references (11, 25, 49) 2) references (45, 50)
3) references (17, 39, 40, 44, 51).

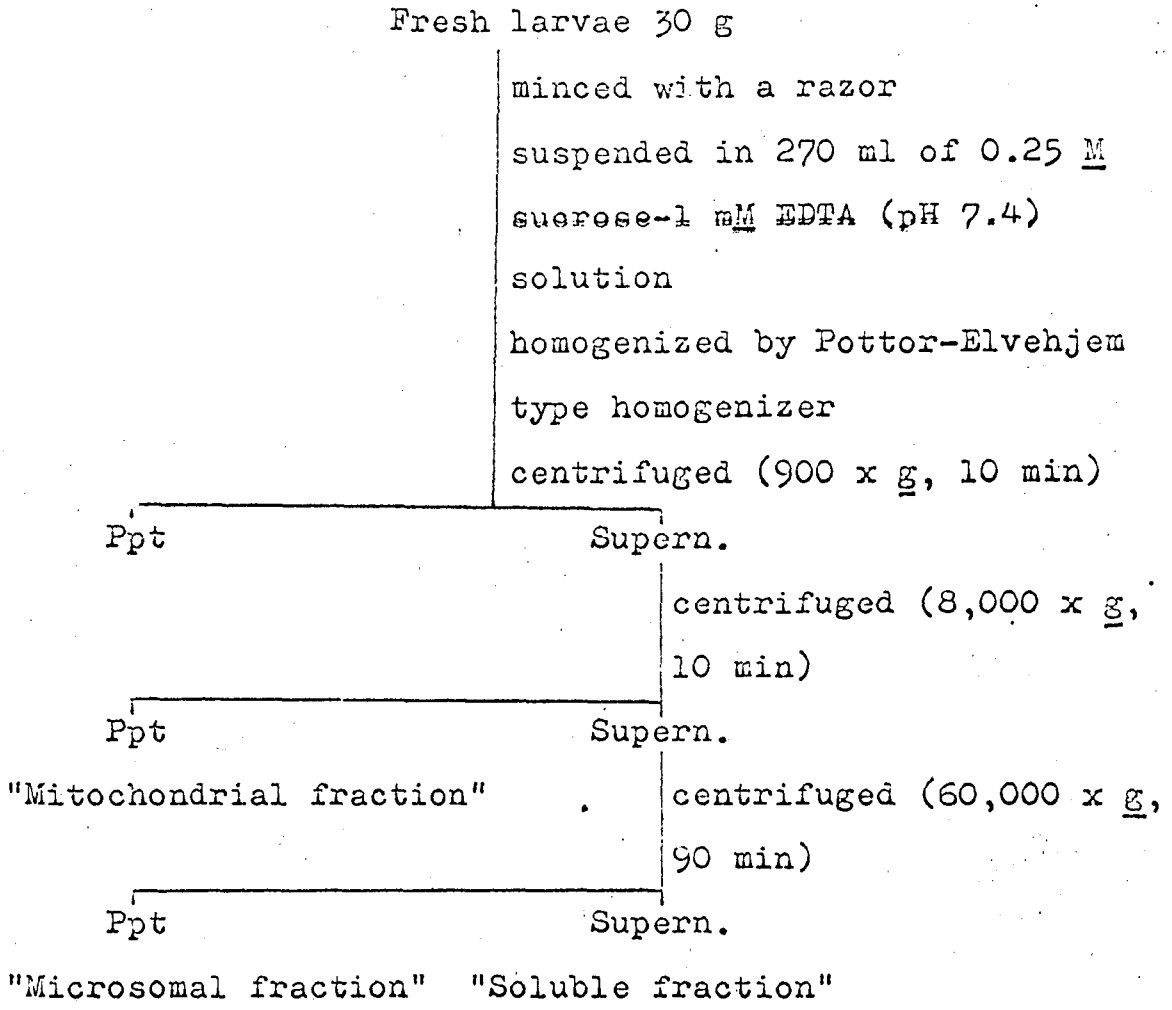

Fig. 1. Preparation of various fractions from larval homogenate.

Fig. 1. Preparation of various fractions from larval homogenate.

Both "Mitochondrial" and "Microsomal" fractions were washed once more with 1/2 of the initial volume of 0.25 M sucrose-1 mM EDTA (pH 7.4) solution and suspended in the same medium at 0-2°C.

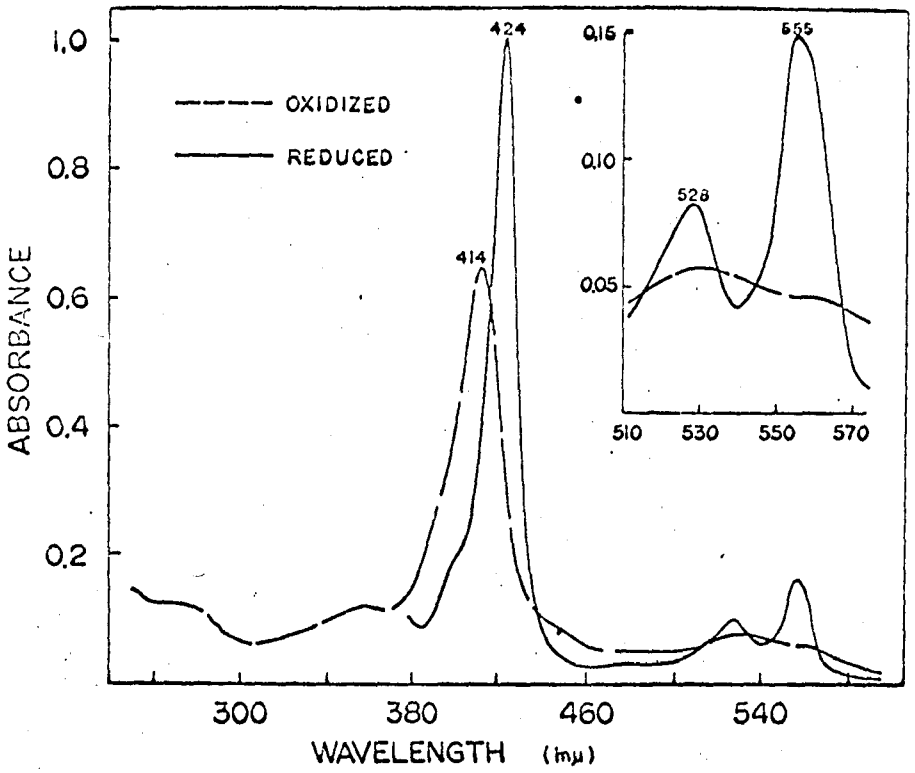

Fig. 2. Absorption spectra of purified larval cytochrome b-555.

Fig. 2. Absorption spectra of purified larval cytochrome b-555.

The spectra were measured in 0.1 M phosphate buffer (pH 7.0) at 24°C. ----, Oxidized form; ——, dithionite-reduced.

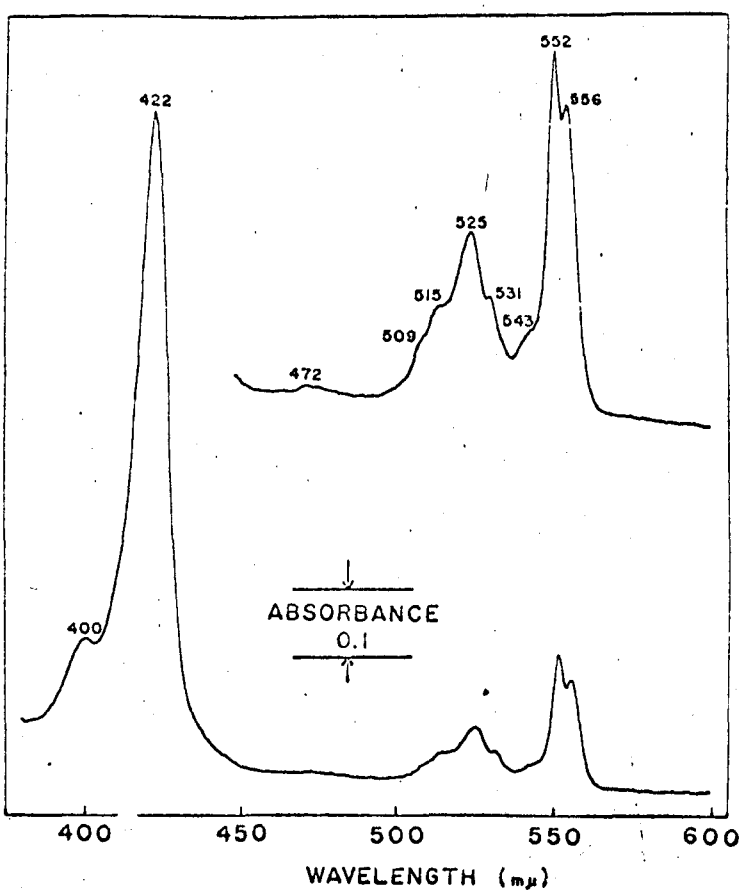

Fig. 3. Low temperature absorption spectra of purified larval cytochrome b-555.

Fig. 3. Low temperature absorption spectra of purified larval cytochrome b-555.

The purified cytochrome b-555 preparation (about 10 μM) which had been dialyzed against 0.1 M phosphate buffer (pH 7.4) was mixed with an equal volume of glycerol and reduced with sodium dithionite. The reference cuvette contained an equal volume mixture of glycerol and buffer. The sample and reference cuvettes were first cooled in liquid nitrogen, secondly warmed to induce devitrification of the glycerol mixtures, and finally recooled in liquid nitrogen. Absorption spectra were recorded with a Cary model 14 spectrophotometer. Optical path of cuvettes was 1 mm. The insert shows the spectrum when cuvettes of 2 mm. light path were used. The effective band widths were 0.5 mμ.

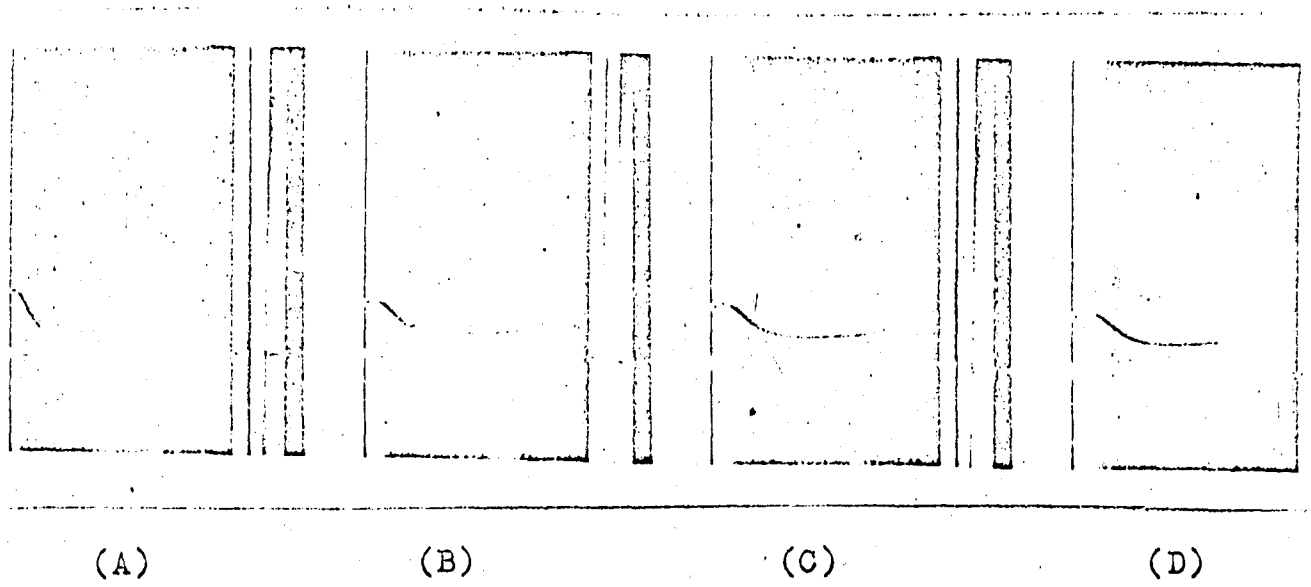

Fig. 4. Sedimentation patterns of purified larval cytochrome b-555 preparation at 54,700 rpm in 0.2 M phosphate buffer (pH 7.0) and at 16.5°C.

Fig. 4. Sedimentation patterns of purified larval cytochrome b-555 preparation at 54,700 rpm in 0.2 M phosphate buffer (pH 7.0) and at 16.5°C.

The protein concentration was approximately 0.3 %. The photographs were taken at 30 min (A), 60 min (B), 90 min (C), and 105 min (D) after the velocity had reached the maximum.

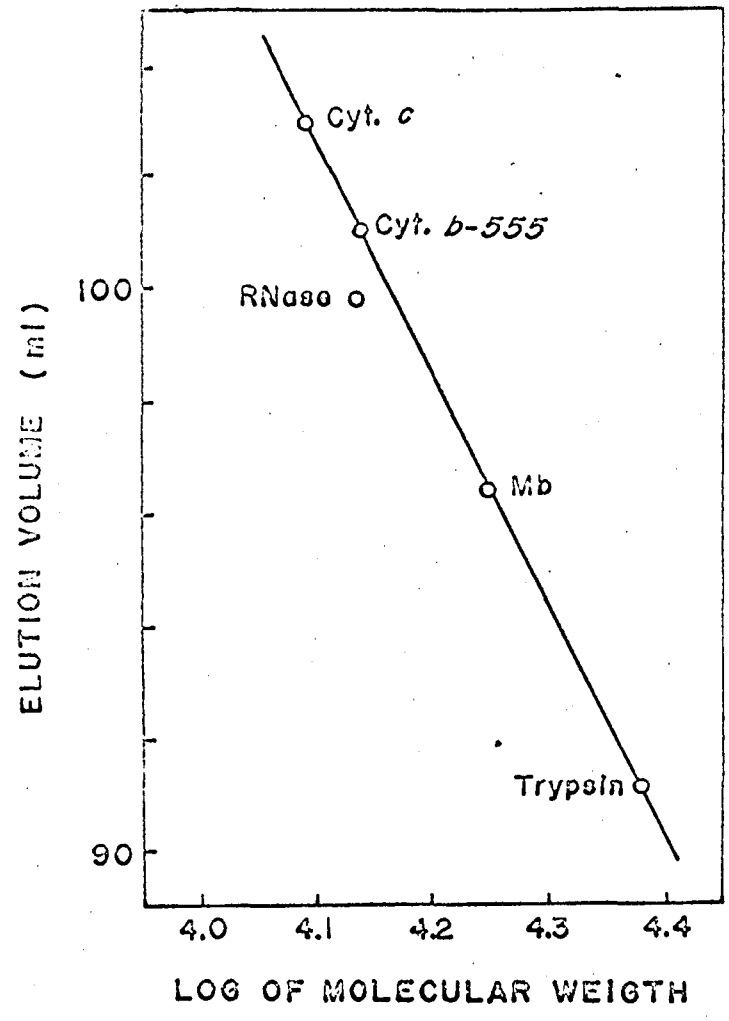

Fig. 5. Estimation of molecular weight of larval cytochrome b-555 by gel filtration.

Fig. 5. Estimation of molecular weight of larval cytochrome b-555 by gel filtration.

The following proteins were dissolved in 1.0 ml of the equilibration buffer and were applied to the Sephadex G-100 column (1.5 x 74 cm): Crystalline beef heart cytochrome c, 5 mg (mol wt, 12,400); crystalline bovine pancreatic ribonuclease 5 mg (mol wt 13,700); sperm whale myoglobin 5 mg (mol wt 17,800); trypsin 5 mg (mol wt 24,000). The gel column was equilibrated with 0.2 M phosphate buffer (pH 7.0), and used at 3-5°C. Elutions were carried out with the same buffer as used for equilibrating the gel at a flow rate of about 12 ml per hr, and 2.0 ml fraction was collected. The hold-up volume (V_o) of the column was determined with blue dextran 2000 (Pharmacia), which has an average molecular weight of 2,000,000. Ribonulease and trypsin in column effluents were estimated by absorbance at 280 mμ, cytochrome c at 530 mμ, myoglobin at 582 mμ, and cytochrome b-555 at 414 mμ. Cyt. c; cytochrome c, RNase; ribonuclease, Cyt. b-555; cytochrome b-555, Mb; myoglobin.

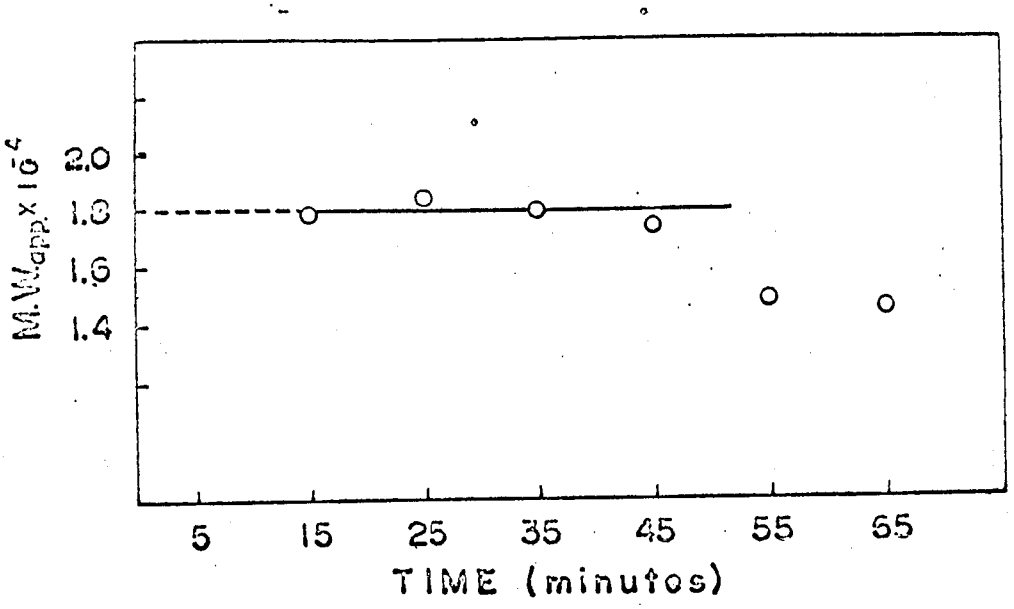

Fig. 6. Changes in apparent molecular weight of purified cytochrome b-555 preparation during an Archibald run.

Fig. 6. Changes in apparent molecular weight of purified cytochrome b-555 preparation during an Archibald run.

Apparent molecular weight was plotted against period of centrifugation. The initial protein concentration was 0.21 % in 0.1 M Tris-HCl buffer (pH 7.5). The run was performed at 20,600 rpm and at 17.1°C.

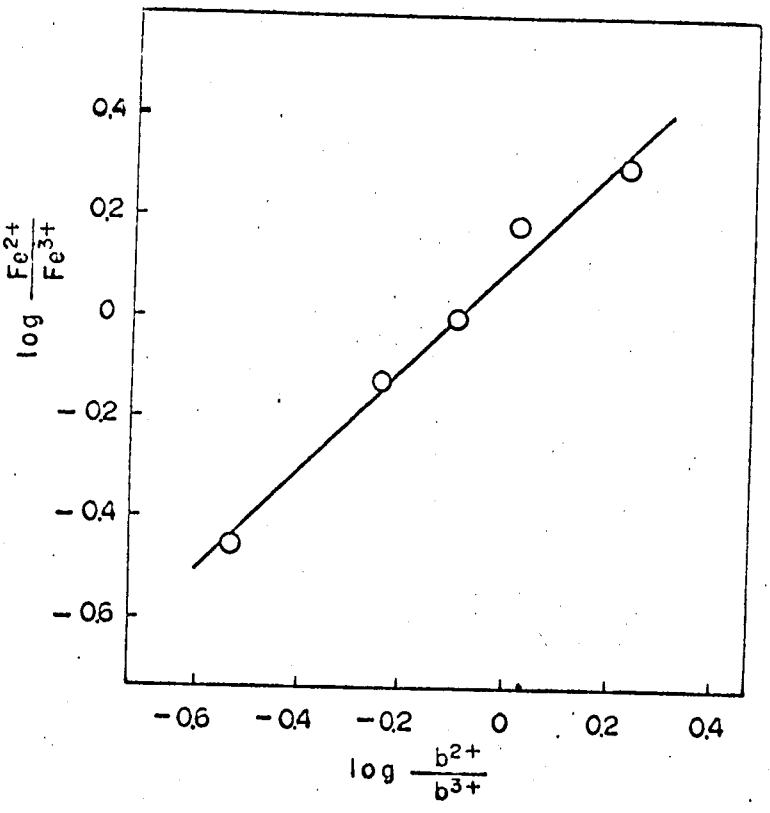

Fig. 7. Relationship between the ratio of the concentrations of ferrous to ferric ions (Fe^{2+}/Fe^{3+}) and the extent of reduction of larval cytochrome b-555 (b^{2+}/b^{3+}) at pH 7.0 and at 12°C.

Fig. 7. Relationship between the ratio of the concentrations of ferrous to ferric ions (Fe^{2+}/Fe^{3+}) and the extent of reduction of larval cytochrome b-555 (b^{2+}/b^{3+}) at pH 7.0 and at 12°C.

The reaction mixture consisted of 250 mM potassium oxalate, 66 mM phosphate buffer (pH 7.0), 7.15 μM purified cytochrome b-555 and 1.0 mM ferric ammonium sulfate in a total volume of 0.97 ml in the main compartment of a Thunberg-type cuvette. Freshly prepared ferrous ammonium sulfate solution (0.03 ml) with various concentrations was placed in the side arm. The reference cuvette contained the same components as the sample cuvette except for ferrous ammonium sulfate. Under anaerobic conditions the extent of reduction of the cytochrome was determined on the basis of the difference absorption spectrum after the ferrous ammonium sulfate solution had been tipped in the reaction mixture of the main compartment and the equilibrium in the reaction had been established. The fully reduced level of the cytochrome was determined by adding a trace amount of sodium dithionite to the sample cuvette.

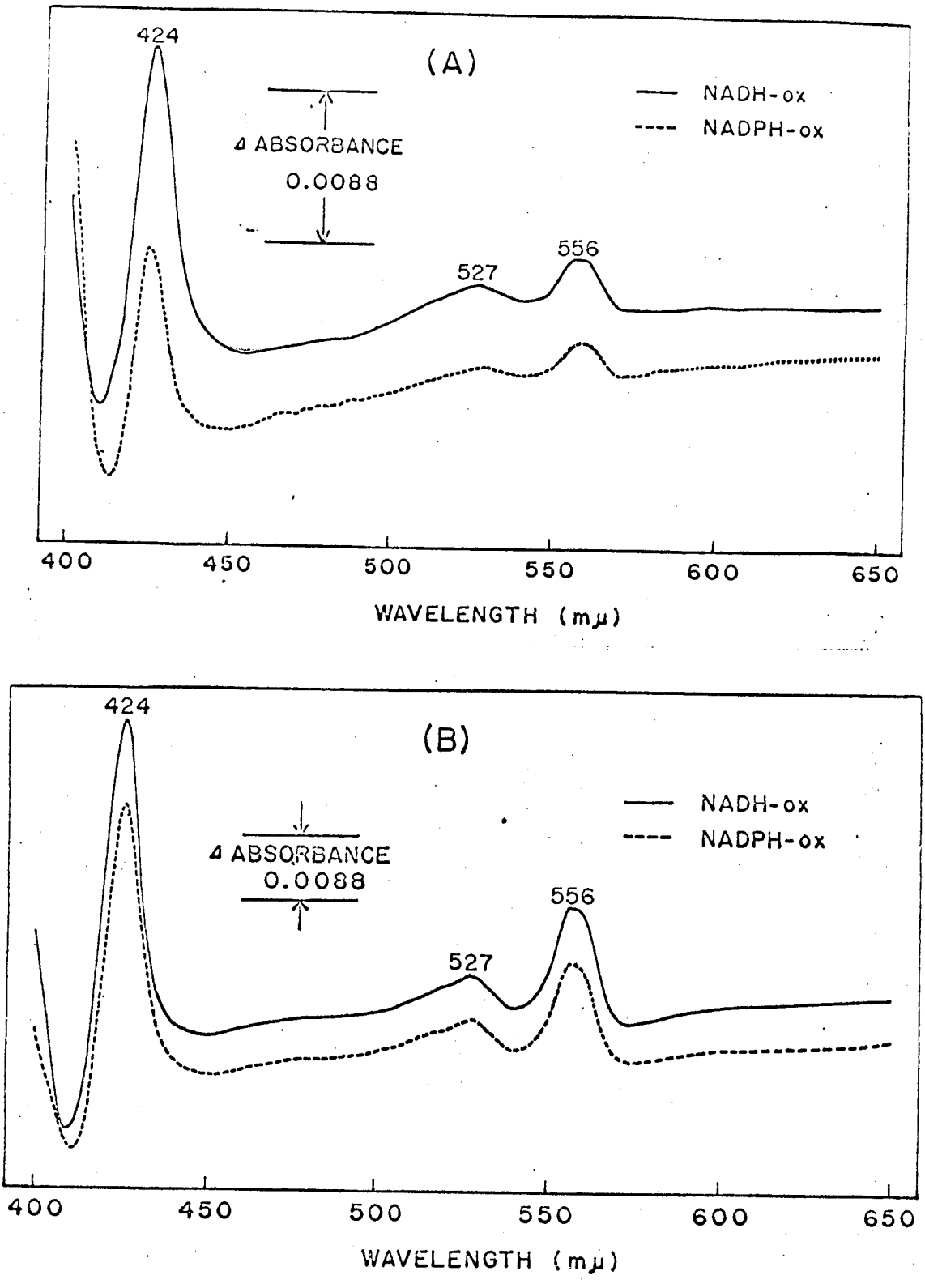

Fig. 8. NADH- or NADPH-reduced minus oxidized difference absorption spectra of larval mitochondrial and microsomal fractions at room temperature.

Fig. 8. NADH- or NADPH-reduced minus oxidized difference absorption spectra of larval mitochondrial and microsomal fractions at room temperature.

Assay mixture consisted of 50 mM phosphate buffer (pH 7.4), 1 mM potassium cyanide, 25.4 μM rotenone, an appropriate amount of each fraction, and 100 μM NADH or NADPH. The total volume of the reaction mixture was 3.0 ml. The reference cuvette contained the same constituents as the sample cuvette except that the reducing agent was omitted. The particulate fraction was allowed to stand overnight at 0°C in order to consume endogeneous substrates. Before adding the reductant oxygen was bubbled through the reaction mixture for 2 or 3 min to make cytochrome components oxidized in the fraction. Spectra were recorded with a split-beam recording spectrophotometer at room temperature approximately 1 min after the reaction was initiated by the addition of the electron donor to sample cuvette. (A) shows the difference absorption spectra of mitochondrial fraction and (B) those of microsomal one. ——, NADH-reduced minus oxidized; ----, NADPH-reduced minus oxidized.

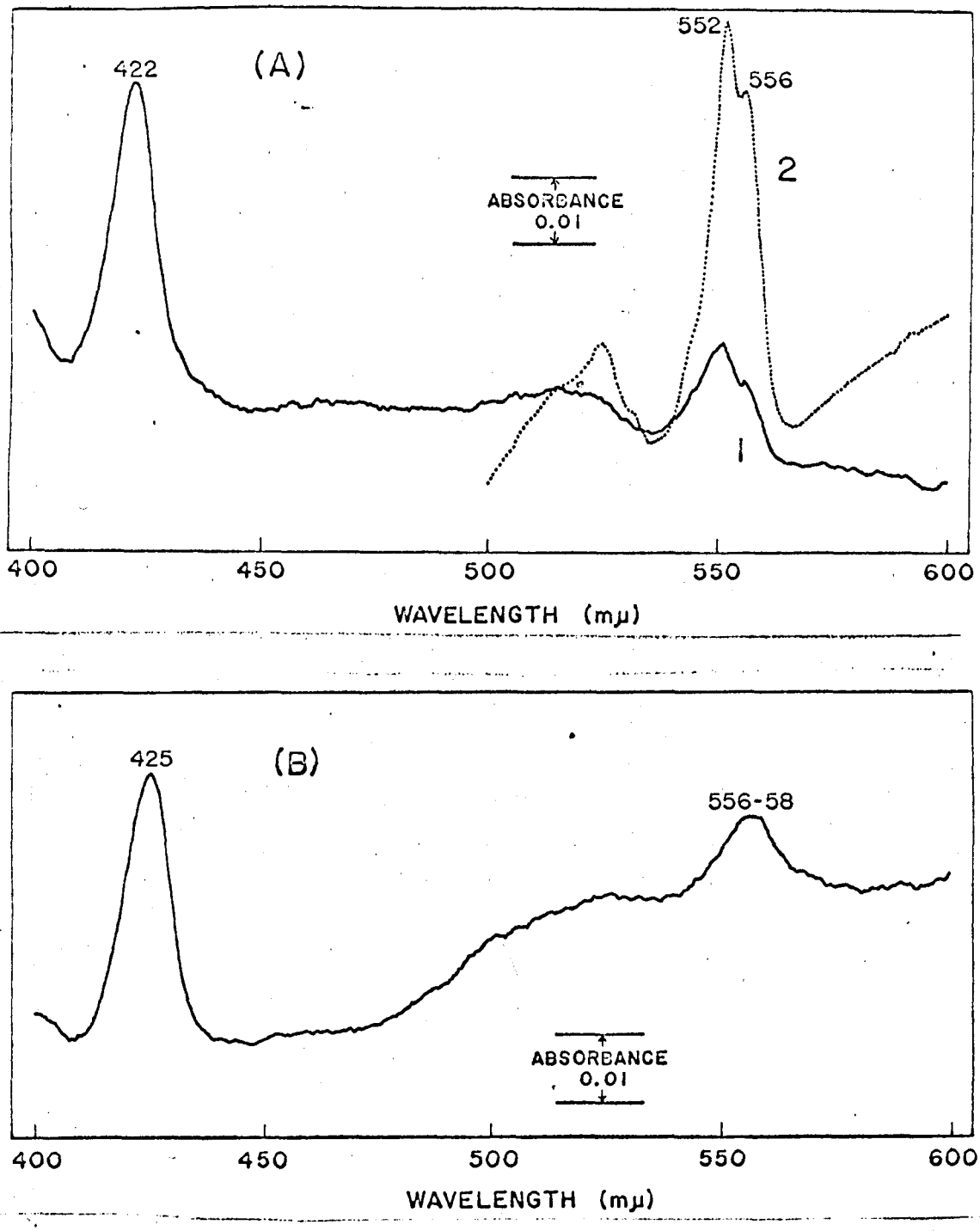

Fig. 9. Reduced minus oxidized difference absorption spectra at -196°C of larval microsomal and soluble fractions.

Fig. 9. Reduced minus oxidized difference absorption spectra at -196°C of larval microsomal and soluble fractions.

(A) represents NADH-reduced minus oxidized difference absorption spectra of the larval microsomal fraction.
Curve 1: The sample cuvette contained 0.5 ml of larval microsomal fraction (18 mg protein/ml), 1.5 ml of 0.25 M sucrose-0.05 M phosphate buffer (pH 7.4), 0.02 ml of 2.54 mM rotenone, and 0.01 ml of 30 mM NADH.
Curve 2: The sample cuvette contained 1.0 ml of the microsomal fraction (20 mg protein/ml, cytochrome b_5 content of this fraction was higher than that of microsomal fraction used for curve 1), 1.0 ml of the sucrose-phosphate buffer solution, 0.02 ml of 2.54 mM rotenone, and 0.01 ml of 30 mM NADH.

Both in curves 1 and 2 the reference cuvette contained a mixture of an equal volume of the microsomal fraction, an inhibitor and sucrose-phosphate buffer.

(B) represents dithionite-reduced minus oxidized difference absorption spectrum of larval soluble fraction. The sample and reference cuvettes contained 2.0 ml of the soluble fraction (final 6 mg protein).

The particulate fraction was allowed to stand overnight at 0°C in order to exhaust endogeneous substrates. Before adding the reducing agent, oxygen was bubbled through a

reaction mixture for 2 or 3 min to oxidize cytochrome components in the fraction. The sample cuvette was cooled in liquid nitrogen approximately 1 min after addition of the reductant, and absorption spectra were recorded with a Cary spectrophotometer, model 14. Optical path of cuvettes was 2.0 mm. The effective band widths were 0.5 mμ.

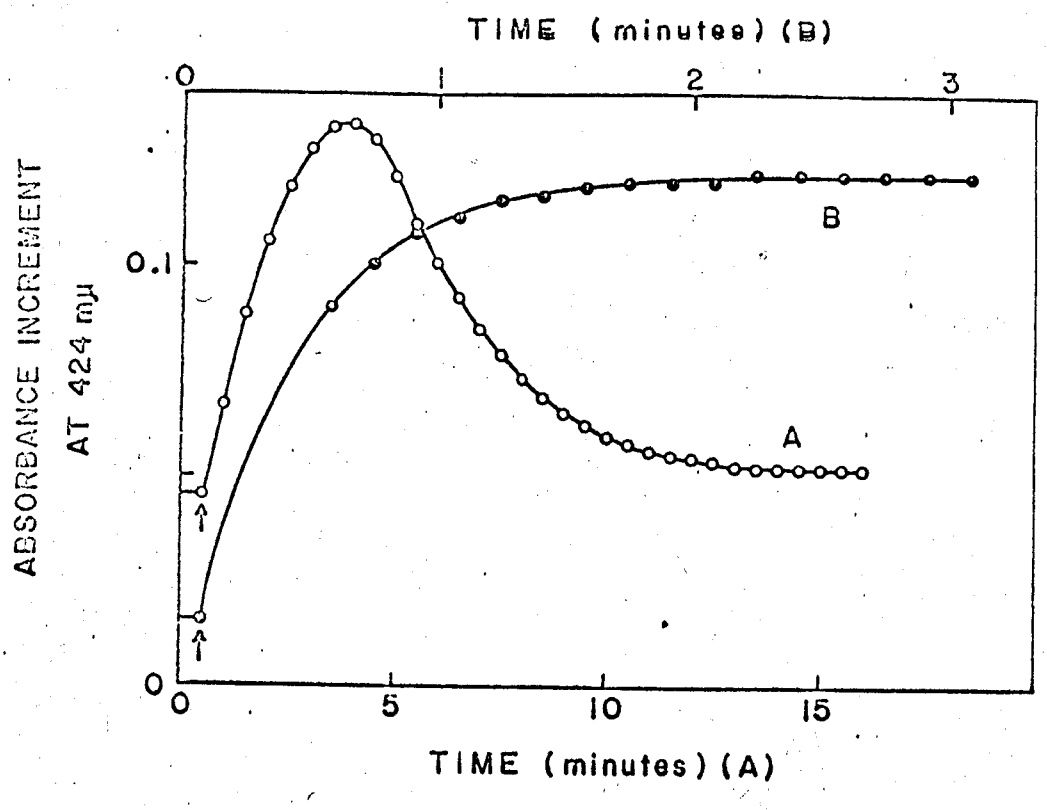

Fig. 10. Reduction of purified larval cytochrome b-555 by NADH in the presence of larval microsomal fraction and purified rat liver microsomal NADH-cytochrome b_5 reductase, and reoxidation of the reduced cytochrome by air.

Fig. 10. Reduction of purified larval cytochrome b-555 by NADH in the presence of larval microsomal fraction and purified rat liver microsomal NADH-cytochrome b_5 reductase, and reoxidation of the reduced cytochrome by air.
Curve A: The sample cuvette contained larval microsomes (final 0.30 mg protein; NADH-cytochrome c reductase activity, 0.115 µmoles cytochrome c reduced/mg protein/min), 4.28 µM purified ferricytochrome b-555, 1 mM potassium cyanide, and 50 mM phosphate buffer (pH 7.4) in a total volume of 2.0 ml. The reaction was started by adding 0.890 µM NADH (at the arrow on the figure). The change in the absorbance at 424 mµ was traced with time under aerobic conditions at 24°C.
Curve (B): The sample cuvette contained purified rat liver microsomal NADH-cytochrome b_5 reductase (final 0.8 µg protein), 1.07 µM purified ferricytochrome b-555, and 100 mM phosphate buffer (pH 7.4) in a total volume of 2.0 ml. The reaction was started by adding 100 µM NADH (at the arrow on the figure). The increase in the absorbance at 424 mµ was followed under anaerobic conditions at 24°C.

STUDIES ON CYTOCHROME b-555 FROM LARVAE OF THE HOUSEFLY, MUSCA DOMESTICA L.

II. ISOELECTRIC POINT, AMINO ACID COMPOSITION AND CIRCULAR DICHROIC SPECTRA

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Running title : Housefly Cytochrome b-555

STUDIES ON CYTOCHROME b-555 FROM LARVAE OF THE HOUSEFLY, MUSCA DOMESTICA L.*, **

II. ISOELECTRIC POINT, AMINO ACID COMPOSITION AND CIRCULAR DICHROIC SPECTRA

1. A crude larval cytochrome b-555 preparation was separated into two or three components by the method of isoelectric focusing. The two main components had much the same or the same absorption spectra, catalytic activity, molecular weight, amino acid composition, and circular dichroic spectra in the Soret region. For three components the pI values at 0-2°C were 4.24, 4.28, and 4.32, respectively. A number of explanations was suggested for the heterogeneity of larval cytochrome b-555.

2. The circular dichroic spectra of ferri- and ferro-cytochrome b-555 suggested that the protein moiety of the molecule provided an asymmetrical environment to all of the chromophores in the molecule. With respect to the sign of the CD peak in the Soret region of hemoproteins containing protoheme several hypotheses were presented.

3. The amino acid composition of larval cytochrome b-555 was as follows: Lys_5, His_2, Arg_2, Asp_{10}, Thr_3, Ser_4, Glu_{12}, Pro_1, Gly_4, Ala_6, Val_6, Met_1, Ile_2, Leu_2, Tyr_2, Phe_3. The

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* The previous paper was published in J. Biochem., 65, 581 (1969)

** This research was supported in part by a grant-in-aid from the U.S. National Institutes of Health (GM 05871-12).

contents of tryptophan, cysteine and amide were not determined. In comparison with cytochrome b_5 from various sources larval cytochrome b-555 contained the fewest amino acid residues (65 amino acid residues other than tryptophan and cysteine), lower contents of histidine, proline, and leucine, and higher contents of alanine and valine.

The previous paper (1) reported some of the properties of purified larval cytochrome b-555 and it was concluded that this hemoprotein is a solubilized form of cytochrome b_5 localized in the larval particulate fraction. The present investigation was undertaken to obtain isoelectric point, amino acid composition, and circular dichroic (CD*) spectra of this hemoprotein.

MATERIALS AND METHODS

Materials —— Alumina C_γ gel was prepared by the method of Wilstätter (2). All other materials used were as described in the previous paper (1).

Preparation of Larval Cytochrome b-555 —— Larval cytochrome b-555 was isolated from the housefly, Musca domestica L. essentially as described previously (1) but several modifi-

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* The abbreviations used are as follows: CD, circular dichroism; ORD, optical rotatory dispersion; A, absorbance.

cations were made in the purification procedure. The dark brown eluate from the first DEAE-cellulose column was fractionated with ammonium sulfate (see ref. (1) for preparation procedures). The precipitate obtained between 70 and 100 % saturation of the salt was dissolved in a minimal volume of water and the resulting solution was dialyzed against water. The dialyzate was used as the crude cytochrome b-555 preparation.

This crude preparation was charged on the second DEAE-cellulose column buffered with 0.1 M phosphate buffer (pH 7.0). When developed with 0.1 M phosphate buffer (pH 7.0) cytochrome b-555 migrated gradually down the column forming two separate red bands, and fractions of 8 ml were collected. After all the main fractions with reddish yellow color were combined and dialyzed overnight against 0.02 M phosphate buffer (pH 7.0) the dialyzate was again applied to a DEAE-cellulose column equilibrated with 0.05 M phosphate buffer (pH 7.0). Cytochrome b-555 adsorbed on the column was eluted with 0.2 M phosphate buffer (pH 7.0). The concentrated solution was dialyzed against 0.005 M phosphate buffer (pH 7.0), and then an appropriate amount of alumina C_γ gel was added to the dialyzed solution. The adsorbed cytochrome was eluted three times by washing the gel with 0.005 M phosphate buffer (pH 7.0). The eluates were combined and used as the purified larval cytochrome b-555 preparation, in which the ratio of $A_{555\ m\mu}$(reduced)/$A_{280\ m\mu}$(oxidized) was 1.3.

Molar Extinction Coefficients —— The molar extinction coefficients of larval cytochrome b-555 were calculated from

the extinction coefficient of its pyridine hemochrome using a value of $\varepsilon_{557\ m\mu} = 34.7 \times 10^3\ \underline{M}^{-1}cm^{-1}$ for pyridine ferroprotoporphyrin (3).

Pyridine was added to the pigment solution with a known absorbance followed by sodium hydroxide solution. Finally a trace of dithionite was added and the absorption spectrum was immediately recorded as rapidly as possible at room temperature. The final concentrations of pyridine and sodium hydroxide were 30 % (v/v) and 0.2 N, respectively.

Isoelectric Focusing —— For the determination of isoelectric points the method of Svensson (4) was employed at 0-2°C, using a 1 % (v/v) ampholyte solution (LKB-Produkter AB, Stockholm). The pH gradient was made to cover the range between pH 3 and 5. A maximum load of about 1 W was applied for 48 hours at 0-2°C to focus the hemoprotein. Fractions of 30 drops were collected. The pH value of each fraction was determined at exactly the same temperature as that of the column during focusing, using a Hitachi-Horiba pH-meter equipped with a scale expander, type F-5, which had been standardized at pH 4 and 7. Cytochrome b-555 in the fractions was estimated from the absorbance at 414 mμ.

Measurement of Catalytic Activity —— The catalytic activity of cytochrome b-555 was examined in both the following systems: (1) a sample cuvette contained purified rat liver microsomal NADH-cytochrome b_5 reductase (EC 1.6.2.2) (0.8 μg

protein), 100 μM NADH, 50 μM ferricytochrome c, and 50 mM phosphate buffer (pH 7.4) in a total volume of 2.0 ml. The reaction was started by addition of 48 mμM larval ferricytochrome b-555, and the reduction of beef heart ferricytochrome c was followed spectrophotometrically by the increase in the absorbance at 550 mμ. (2) An assay mixture contained larval microsomes (for preparation procedures, see ref. (1)) (42 μg protein), 3.98 μM ferricytochrome b-555, 1 mM potassium cyanide, and 50 mM phosphate buffer (pH 7.4) in a total volume of 2.0 ml. The reaction was started by adding 100 μM NADH. The change in the absorbance at 424 mμ was traced with time under aerobic conditions.

Amino Acid Analyses —— Approximately 400 μg of sample was hydrolyzed in 0.5 ml of twice distilled 5.7 N HCl in a sealed tube *in vacuo* at 110°C. Hydrolysis was conducted for 24 or 72 hours. Analyses were performed by the method of Spackman *et al.* (5), using a Beckman Amino Acid Analyzer 120B.

CD Spectra —— The CD spectra were measured on a JASCO/ORD/UV-5 spectropolarimeter equipped with a CD attachment at 22-24°C. The slit width of the instrument was programmed to yield constant light intensity throughout the spectral region. Cells with fused quartz windows and of path lengths from 1.0 to 10 mm were used. A sample dissolved in 0.1 M phosphate buffer (pH 7.0) in a cuvette was reduced by addition of solid sodium dithionite. Full reduc-

tion of the cytochrome throughout the CD measurement was monitored by recording the absorption spectra immediately before and after each scan. Base lines were recorded and subtracted from the dichroism curves. The ellipticities, $[\theta]$, were calculated from the expression,

$$[\theta] = 3300 \times (\varepsilon_L - \varepsilon_R),$$

and are expressed as derees$\cdot$cm$^2\cdot$decimole^{-1}.

Determination of Concentration —— The concentrations of NADH, larval cytochrome b-555 and beef heart cytochrome c were determined spectrophotometrically (6, 7, see Table II in "RESULTS"). The concentration of protein was determined by the method of Lowry et al. (8), using bovine serum albumin as the standard.

Spectrophotometric Measurements—— Spectrophotometric measurements were carried out with a Cary spectrophotometer, model 15 and a Hitachi Perkin-Elmer spectrophotometer, model 139. The optical path lengths of cuvettes were from 1.0 to 10 mm.

RESULTS

Separate Components —— At the steady state of isoelectric focusing a crude larval cytochrome b-555 preparation showed two

or three red bands on the column. On draining, however, only a single peak was obtained because of the close proximity of the bands (Fig. 1). Assignment of components to the fractions was

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Fig. 1

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confirmed by direct viewing during draining. The pH of the fractions were determined at 0-2°C to be 4.24, 4.28, and 4.32, which were supposed to correspond to the isoelectric points for the cytochrome components, respectively. The absorption spectra of the 4.24 and 4.28 components, which were recorded in the drains both in the oxidized and reduced forms at room temperature, were very similar to those of mammalian microsomal cytochrome $\underline{b}_5$. This indicates that the crude larval cytochrome b-555 preparation contains one kind of spectrally uniform cytochrome and not other types. The approximate molar ratio of the 4.24 component to the other components were estimated to be 1:1:trace assuming that the components had the same extinction coefficient in the visible region. There was only a trace of the 4.32 component so it was not studied further.

On the other hand, the same crude preparation as that used for isoelectric focusing gave two red bands (in approximately

equal amounts) on chromatography on a DEAE-cellulose column (Fig. 2). By separate isoelectric focusing the isoelectric

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Fig. 2

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point for the component in the first peak (marked "I" in Fig. 2) was determined to be 4.28, while that for the second component (marked "II" in Fig. 2) was 4.24. The two components revealed a single red band in either focusing. The 4.28 component is referred to as cytochrome b-555 (I) and the 4.24 component as cytochrome b-555 (II), hereafter. At this stage of purification (the second DEAE-cellulose chromatography), the two components were spectrally indistinguishable, and no difference was found between them in either their catalytic activities, or their CD spectra in the Soret region in either the oxidized or the reduced state.

Each component, cytochromes b-555 (I) and (II) was further purified separately by chromatography on a Sephadex G-100 column (1.1 x 133.5 cm), and by adsorption on and elution from alumina C_γ gel (see "MATERIALS AND METHODS"). The different behavior in adsorption and elution on the C_γ gel between the two components was not observed. The two components purified by the alumina gel

had much the same amino acid composition (Table I). The samples

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Table I

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used for determination of the molar extinction coefficient, measurements of CD spectra, and amino acid analyses were a mixture of all three components, cytochromes b-555 (I) and (II), and a minor component.

In the preparation described previously (1) there was no obvious separation of different components on a DEAE-cellulose column (Fig. 3). Fractions after fractions number 150 to 400 (not shown in Fig. 3) contained no cytochrome b-555. Melanin-like pigments were eluted in fractions around the peaks

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Fig. 3

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marked "X" in Fig. 3. From the Rf value on chromatography on a DEAE-cellulose column cytochrome b-555 (I) in this preparation (marked "I" in Fig. 2) seems to be identical with cytochrome b-555 preparation purified previously (marked "I" in Fig. 3).

Molar Extinction Coefficient —— The extinction coefficients and absorbance ratios of larval cytochrome b-555 are given in Table II.

Table II

Amino Acid Composition —— The results of amino acid analyses of the purified sample are summarized in Table III.

Table III

A large excess of acidic amino acids over basic ones and of hydrophilic amino acids over hydrophobic ones is noticeable. This feature is in good accordance with the low isoelectric points and solubilities in water and a salt solution (Table III and Fig. 1). From the composition of amino acids other than cysteine and tryptophan the formula weight was calculated to be 8,020. On the basis of protein and heme contents, the molecular weight of the cytochrome was 11,300.

The previous paper (1) has reported that purified larval cytochrome b-555 is a solubilized form of cytochrome b_5 localized

in the larval particulate fraction. In comparison with cytochromes b_5 from various sources reported by other workers (9-12) larval cytochrome b-555 contains the fewest amino acid residues (65 amino acid residues other than tryptophan and cysteine), lower contents of histidine, proline, and leucine, and higher contents of alanine and valine.

Circular Dichroic Spectra —— The circular dichroic spectra of larval ferri- and ferrocytochrome b-555 are shown in Fig. 4.

Fig. 4

In the Soret region a large negative trough at 416 mμ of ferricytochrome b-555 was prominent reflecting a simple optically active transition of the Soret absorption band. Positive peaks at 483, 391, and 337 mμ, a negative trough at 304 mμ, and three broad negative troughs in the region of metal-ligand transition, from 500 to 600 mμ, suggest that the protein moiety provides an asymmetrical environment to the heme in the molecule. A broad negative CD band around 300 mμ, a positive peak at 284 mμ and a negative trough at 270 mμ seem to be associated with transitions of the prosthetic group, protoheme, as well as of aromatic chromophores. In the far-ultraviolet region the dichroic spectrum showed negative troughs at 221 and 211 mμ. The spectrum charac-

teristic of α-helical proteins and polypeptides allows the conclusion of the presence of a right-handed α-helix form in the protein moiety of the cytochrome. When the height of the negative ellipticity band around 221 mμ was used to estimate the α-helix content, using the value $[\theta]_{222\ m\mu} = -4.0 \times 10^4$ for helical poly-L-glutamic acid as a standard (13), a value of 21 % for larval ferricytochrome b-555 was obtained assuming 66 peptide chromophores per heme (see Table III). This value for the α-helix content may be a maximum one because the contribution of completely denatured cytochrome to the ellipticity at 221 mμ was not taken into consideration in the calculation. It has been established that the negative trough at 221 mμ involves contributions not only from the α-helix but from β- and random coiled structures. Moreover, the presence of optically active transitions other than those associated with the amide transitions of the polypeptide chain, heme in the case of cytochrome, further complicates the quantitative interpretation of the data in this wavelength region. Therefore, any estimate of the helical content of cytochromes based on either CD or ORD measurement may be of limited use.

The dichroic spectrum of the reduced protein was significantly different from that of the oxidized protein. The major Soret negative trough was at about 427 mμ instead of 416 mμ as in ferricytochrome, the displacement being similar to that observed in the absorption spectra, and the CD spectra of both

oxidized and reduced cytochrome represent an approximate reflection of the absorption spectra with respect to the wavelength axis. This prominent ellipticity at about 427 mμ was accompanied by a positive peak at 413 mμ and a negative trough at 402 mμ. In the region between 500 and 570 mμ complicated features in the CD spectrum were observed. The α and β absorption bands at 555 and 528 mμ, respectively, of ferrocytochrome b-555 seemed to exhibit five distinct negative ellipticities at about 559, 552, 525, 508, and 490 mμ. The five negative dichroic troughs were at about the same wavelengths as the absorption maxima of the low temperature spectrum of ferrocytochrome (1). The present data suggest that the α and β bands of ferrocytochrome b-555 contain multiple transitions at room temperature and that circular dichroic spectroscopy is an extraordinarily sensitive method to resolve these transitions under ordinary conditions. In the near ultraviolet region there were a negative trough at 364 mμ, a curve at 340 mμ and a positive peak at 317 mμ. The negative and positive bands at 298 and 284 mμ seem to reflect complex optically active transitions of both aromatic amino acid groups and protoheme. The reduction of the cytochrome accompanied a slight decrease in the ellipticity at 222 mμ.

DISCUSSION

Separate Components —— A different behavior of larval cytochrome b-555 on a DEAE-cellulose column was noticed from preparation to preparation (Figs. 2 and 3). This difference was not caused by a difference in a column size because the separation of the two components was achieved on a shorter column (3.0 x 11.0 cm) than that used for the results in Fig. 2 (unpublished observations). At present there is no satisfactory interpretation on this difference because of a lack of enough evidence. Further work is required on this heterogeneity among preparations.

By isoelectric focusing the crude cytochrome b-555 preparation was separated into three components, of which components (I) and (II) had the same properties in several respects. The reason why the crude preparation contained three electrophoretically different components is not clear at present but several explanations are possible. Heterogeneity usually depends on differences in primary, secondary, tertiary, or quarternary structure of proteins. Even with a difference only in the primary structure there are several possible explanations taking into account of much the same amino acid compositions of the two components (Table I).
(1) The two components may have approximately the same amino acid sequence in the molecule: but (1a) a different distribution of the amide groups within the molecule may cause sufficient shift of the dissociation constants of some ionizable groups to account for a difference in electrophoretic mobility, as suggested in the

case of myoglobin (14) and cytochrome c (15); (1b) there may be several additional amino acid residues to the molecule of one of the components, as in the case of liver microsomal cytochrome b_5 (16); (1c) one or two neutral amino acids in one of the components may be replaced by acidic or basic ones in the other. (2) Occasionally, the two components may have the same amino acid composition and very close isoelectric points but have quite different amino acid sequences. Causes for differences in the secondary, tertiary, or quarternary structure of hemoproteins also remain to be elucidated.

Amino Acid Composition —— Cytochromes b-555 (I) and (II) (lower purity) had less proline, tyrosine, and phenylalanine than a mixture of both components (most purified) (Tables I and III). This is probably because of the technical difficulty in the quantitative analysis of a small amount of sample (approximately 100 µg protein) and due to destruction of these amino acids during acid hydrolysis.

Larval cytochrome b-555 contains the fewest amino acid residues among cytochromes b_5 from various sources reported by other workers (2-12). This fact may suggest that larval cytochrome is extracted as a heme-peptide, contrary to the previous conclusion (1).

From amino acid sequence determination and chemical modification studies Strittmatter has suggested (12) that two histidine residues are involved in the heme binding in cytochrome b_5. The fact that larval cytochrome b-555 has only two histidine

residues is, therefore, of great advantage in locating the liganding groups.

CD Spectra —— Urry has reported (17) that low spin complexes have large CD bands at 260 mμ and that the rotational strength and positions of these dichroic bands would be affected by the position of the iron with respect to the heme plane and by the nature and relative orientations of the ligands in the fifth and sixth coordination position. However, larval ferricytochrome b-555 of low-spin type had no significant ellipticity around 250 mμ, while rabbit liver microsomal ferricytochrome b_5 (low-spin type) solubilized by endogeneous cathepsin showed a positive peak at 250-1 mμ (unpublished data). Larval cytochrome b-563 (low-spin type) from the housefly, Musca domestica L. had no positive peak in the 250-260 mμ region in either the oxidized or the reduced CD spectrum (to be published). Although these data are inconsistent with Urry's suggestion, further data on the CD spectra of other hemoproteins must be accumulated before a definite conclusion can be safely drawn.

Studies on optical rotatory dispersion and circular dichroism of hemoproteins with protoheme as the prosthetic group, e.g. hemoglobin, myoglobin, catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), and cytochrome b_2 (EC 1.1.2.3), have been undertaken by many workers (18-23). These studies will throw light on elucidation of the different functions of hemoproteins with the same prosthetic group. Larval ferri- and ferrocytochrome b-555

showed negative dichroic peaks in the Soret region. ORD spectra of another b-type cytochrome, yeast ferri- and ferrocytochrome b_2, have been reported to exhibit negative Soret Cotton effects (23). Since a negative Cotton effect in an ORD spectrum is equivalent to a negative trough in a CD spectrum, the heme in both b-type cytochromes may be in the same steric environment provided by the protein molecule. On the other hand, many other workers have observed that in myoglobin and hemoglobin which are oxygen-carrying hemoproteins, and in peroxidase, a hydrogen peroxide-binding hemoprotein, the case is reverse both in the oxidized and reduced states (18, 19, 21). From these results it is suggested that in general both in the reduced and oxidized forms, a b-type cytochrome has a negative Cotton effect in the Soret region while non-b-type protohemoproteins (hemoglobin, myoglobin, peroxidase, etc.) have a positive one. On the basis of this hypothesis, the sign of a Soret Cotton effect may be correlated to the different functions of the hemoproteins containing protoheme: a negative Cotton effect to an electron-transferring function, a positive one to an oxygen- and hydrogen peroxide-carrying function. Moreover hemoproteins in the former group do not react with extrinsic ligands (carbon monoxide, cyanide, azide, etc.), while those in the latter do. Catalase and lamprey hemoglobin are, however, exceptional to this hypothesis (20, 22). Further studies are required to explain why this is so.

On the basis of the ORD and CD spectra both in the oxidized

and reduced forms of hemoglobin, myoglobin, cytochrome b_2, larval cytochromes b-555 and b-563, and rabbit liver microsomal cytochrome b_5 reported by other authors and in the present investigation (18, 19, 23, to be published), another hypothesis is proposed: protohemoproteins have the same sign in the Soret CD spectra both in the oxidized and reduced forms.

Furthermore, many other workers have reported that myoglobin, hemoglobin, catalase, and peroxidase coordinate with external ligands such as cyanide, azide, oxygen, or hydrogen peroxide to form hemoprotein-external ligand complexes which exhibit the same sign Cotton effects in the Soret region as the original hemoproteins (18-20, 24, 25). This fact suggests a third hypothesis that complexes of protohemoproteins with extrinsic ligands exhibit the same sign in the Soret Cotton effects as the original hemoproteins.

The CD spectra of ferro- and ferricytochrome oxidase (EC 1.9.3.1) exhibit a similar pattern to those of larval ferri- and ferrocytochrome b-555 in the Soret region, but their signs of ellipticity are opposite (26). Cytochrome oxidase contains heme a bound to the protein moiety by a non-covalent linkage just as in the case of protohemoproteins, and binds an oxygen molecule resulting in a stable oxygenated complex. Moreover it coordinates both with carbon monoxide and cyanide to form complexes which exhibit the same sign in the Soret Cotton effects as the original cytochrome (26). These facts support the above considerations.

X-Ray diffraction analysis on crystals of cytochrome b_5 and other studies will prove the above speculations on the Soret CD sign and clarify the structure of the heme environment.

The author wishes to thank Dr. T. Hiroyoshi (Department of Genetics, Medical School, Osaka University, Osaka) for cultivation of houseflies, Mr. Y. Yoshida (Department of Biochemistry, Medical School, Osaka University, Osaka) for his generosity in supplying purified rabbit liver microsomal cytochrome b_5, and Mr. M. Saita, Miss. C. Toyosaki, and Mr. S. Yoshikawa in the author's laboratory for their technical assistance in isoelectrophoretic focusing, amino acid analyses, and measurements of CD spectra, respectively.

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Table I

Amino acid compositions of larval cytochromes b-555 (I) and (II)

Hydrolysis of both cytochromes b-555 (I) and (II) was performed for 24 hours on a single sample. Cysteine, methionine, amide, and tryptophan were not analyzed.

The concentration of cytochrome b-555 was determined spectrophotometrically on the basis of the extinction coefficient of 120×10^3 $M^{-1}cm^{-1}$ for the oxidized form at 414 mμ (see Table II).

Amino acid	Cytochrome b-555 (I) $A_{555\ m\mu}(red.)/A_{280\ m\mu}(ox.) = 1.00$		Cytochrome b-555 (II) $A_{555\ m\mu}(red.)/A_{280\ m\mu}(ox.) = 1.05$	
	moles/heme	nearest integer	moles/heme	nearest integer
Lys	5.89	6	5.75	6
His	2.12	2	1.80	2
Arg	2.29	2	1.84	2
Asp[1)]	11.4	11	10.4	10
Thr	3.61	4	3.54	4
Ser	5.38	5	4.65	5
Glu[1)]	14.3	14	13.5	14
Pro	+	(1)	+	(1)
Gly	4.90	5	5.08	5
Ala	6.17	6	5.99	6
Cys	-[2)]	-	-	-
Val	7.35	7	7.09	7
Met	-	-	-	-
Ile	1.73	2	1.87	2
Leu	2.15	2	2.41	2
Tyr	1.01	1	1.40	1
Phe	2.36	2	2.45	2
Try	-	-	-	-

1) Glutamine was not differentiated from glutamic acid, as well as asparagine from aspartic acid.

2) Not measured.

Table II

Spectral properties of purified larval cytochrome b-555

The molar extinction coefficients of larval cytochrome b-555 were determined after its conversion to pyridine hemochrome using a value of $\varepsilon_{557\ m\mu} = 34.7 \times 10^3 \cdot M^{-1} cm^{-1}$ for the molar extinction coefficient of pyridine ferroprotoporphyrin (3).

Absorption maxima		mμ
	Oxidized form	278, 360, 414, 530
	Reduced form	424, 528, 555
Extinction ratios		
	A (red., 555 mμ)/A (ox., 278 mμ)	= 1.3
	A (ox., 278 mμ)/A (ox., 414 mμ)	= 0.18
	A (red., 424 mμ)/ A (red., 555 mμ)	= 6.8
	A (red., 528 mμ)/A (red., 555 mμ)	= 0.56
Molar extinction coefficients		$M^{-1} cm^{-1}$
	Absolute (ox., 414 mμ)	120 x 10^3
	(ox., 530 mμ)	11.6 x 10^3
	(red., 424 mμ)	179 x 10^3
	(red., 528 mμ)	14.6 x 10^3
	(red., 555 mμ)	26.3 x 10^3

Table III

Amino acid composition of larval cytochrome b-555

A single sample was hydrolyzed. Cysteine, amide, and tryptophan were not analyzed.

The concentration of cytochrome b-555 was determined spectrophotometrically on the basis of the extinction coefficient of 120×10^3 $M^{-1}cm^{-1}$ for the oxidized form at 414 mμ (see Table II).

Total cytochrome b-555 was a mixture of both cytochromes b-555 (I) and (II) (refer to "Separate Components" in "RESULTS").

(Table III)

Amino acid	Total cytochrome b-555					
	$A_{555\ m\mu}^{(red.)}/A_{280\ m\mu}^{(ox.)}$ = 0.96		$A_{555\ m\mu}^{(red.)}/A_{280\ m\mu}^{(ox.)}$ = 1.3			
	24 hr hydrolysis		24 hr hydrolysis		72 hr hydrolysis	
	moles/heme	nearest integer	moles/heme	nearest integer	moles/heme	nearest integer
Lys	6.45	6	4.56	5	4.81	5
His	3.07	3	2.01	2	1.84	2
Arg	2.67	3	2.08	2	1.73	2
Asp[1)]	11.6	12	9.18	9	9.57	10
Thr	3.99	4	2.94	3	2.94	3
Ser	6.34	6	3.46	4	3.47	4
Glu[1)]	15.3	15	11.6	12	11.8	12
Pro	2.56	3	1.23	1	0.865	1
Gly	5.97	6	4.34	4	4.32	4
Ala	6.70	7	5.85	6	5.77	6
Cys	-[2)]	-	-	-	-	-
Val	7.51	8	6.16	6	6.36	6
Met	1.12[3)]	1	1.19[4)]	1	1.23[4)]	1
Ile	2.06	2	1.75	2	2.27	2
Leu	2.74	3	2.37	2	2.26	2
Tyr	2.10	2	2.12	2	2.33	2
Phe	3.17	3	2.56	3	2.96	3
Try	-	-	-	-	-	-
Total residues		84		64		65

1) Glutamine was not differentiated from glutamic acid, as well as asparagine from aspartic acid.

2) Not measured.

4) Only free methionine was calculated. Derivatives of methionine were not included in the calculation because of a very small amount.

3) Only methionine sulfoxide was calculated. Methionine and methionine sulfone were not included in the calculation because of a trace.

Fig. 1. Elution diagram of crude larval cytochrome ^{a} $\underline{b}$-555 preparation after isoelectric focusing.

The pH gradient was made to cover the range between pH 3 and 5, using a 1 % (v/v) ampholyte solution (LKB-Produkter AB, Stockholm). The hemoprotein was focused at a maximum load of about 1 W for 48 hours at 0-2°C. Fractions of 30 drops were collected. The pH of each fraction was measured at 0-2°C. Cytochrome $\underline{b}$-555 in the fractions was estimated from the absorbance at 414 mμ.

—— • —— • —— pH at 0-2°C,

—— o —— o —— Absorbance at 414 mμ.

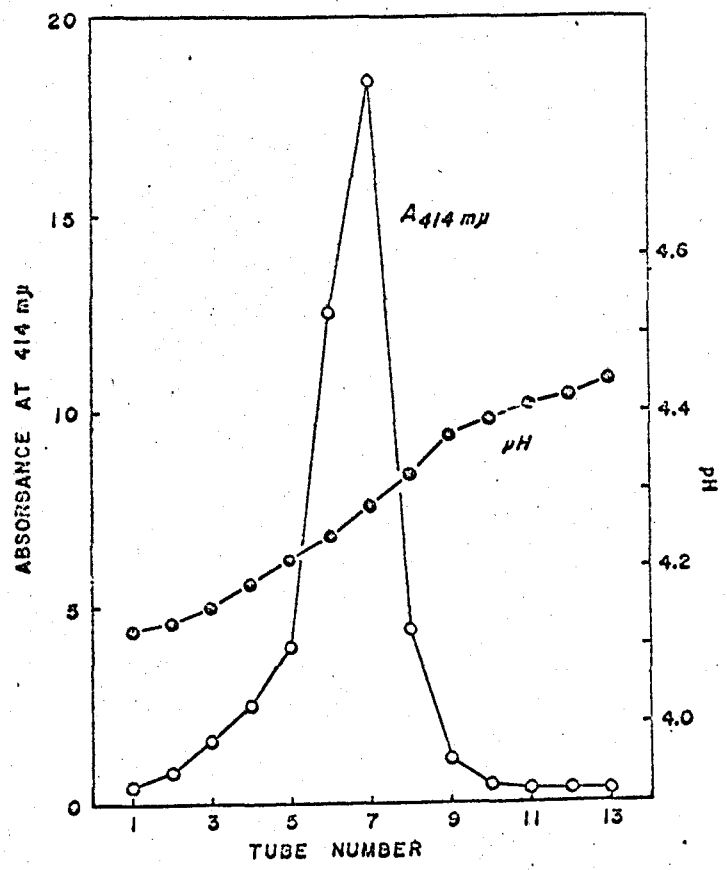

Fig. 1. Elution diagram of $\overset{a}{\wedge}$ crude larval cytochrome b-555 preparation after isoelectric focusing.

Fig. 2. Separation of a crude larval cytochrome *b*-555 preparation on a DEAE-cellulose column.

A crude larval cytochrome *b*-555 preparation was developed with 0.1 *M* phosphate buffer (pH 7.0) on a DEAE-cellulose column (4.2 x 33.5 cm) which had been equilibrated with the same buffer. Fractions of 8.0 ml were collected.

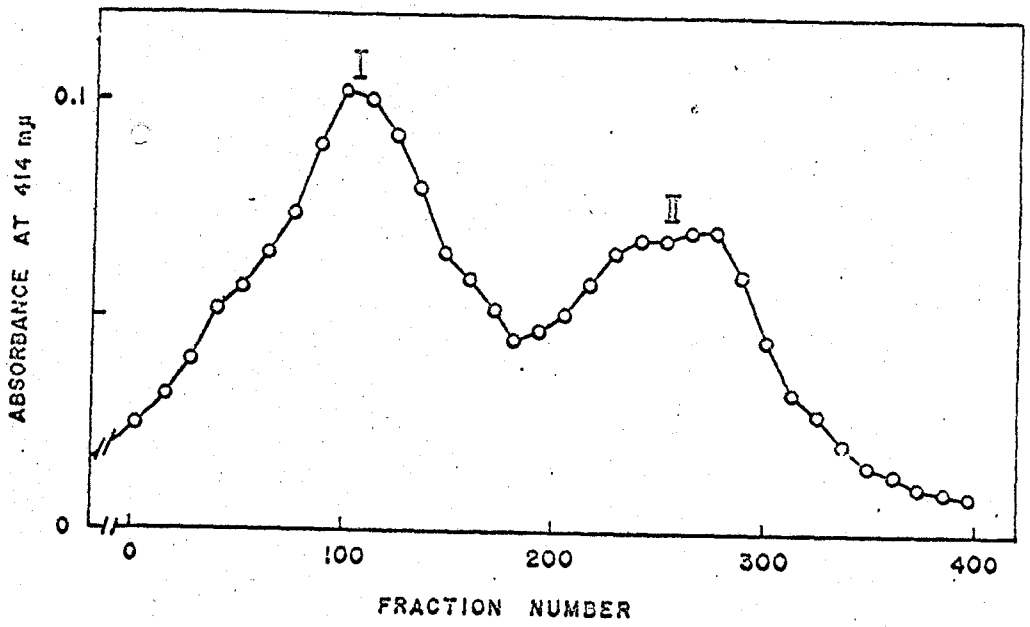

Fig. 2. Separation of a crude larval cytochrome b-555 preparation on a DEAE-cellulose column.

Fig. 3. Rechromatography of another crude larval cytochrome *b*-555 preparation on a DEAE-cellulose column.

A crude larval cytochrome *b*-555 preparation (for preparation, see ref. (1)) was charged on a DEAE-cellulose column (3.0 x 11.0 cm) buffered with 0.1 *M* phosphate buffer (pH 7.0) and developed with the same buffer. Fractions of approximately 4.2 ml were collected.

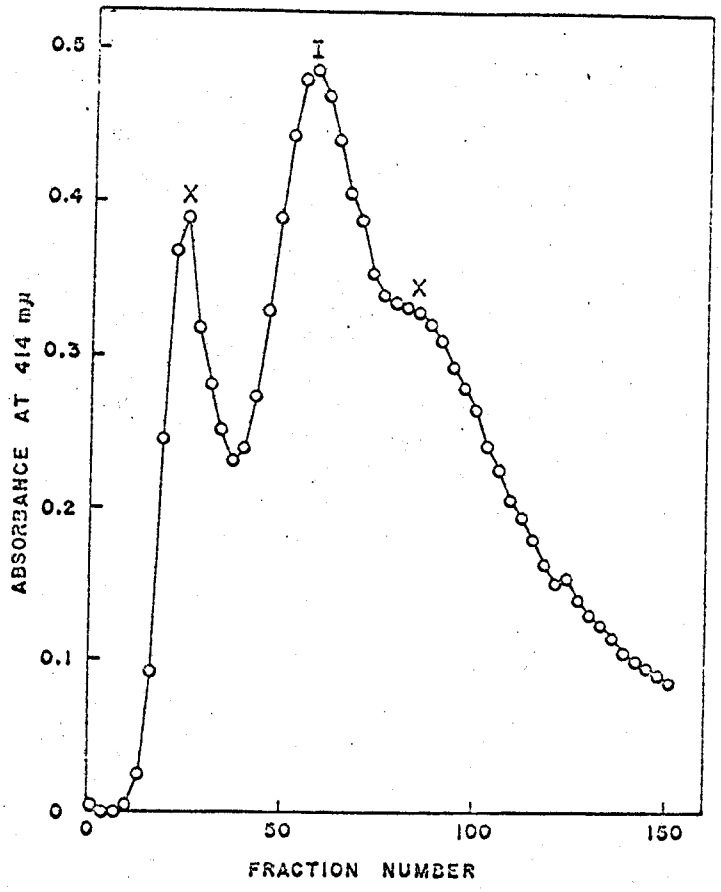

Fig. 3. Rechromatography of another crude larval cyto-. chrome b-555 preparation on a DEAE-cellulose column.

Fig. 4. Circular dichroic spectra of larval cytochrome b-555 in 0.1 M phosphate buffer (pH 7.0) at 22-24°C.

Molar ellipticity, $[\theta]$, in degrees·cm^2/decimole.

---- Oxidized,

—— Reduced with $Na_2S_2O_4$.

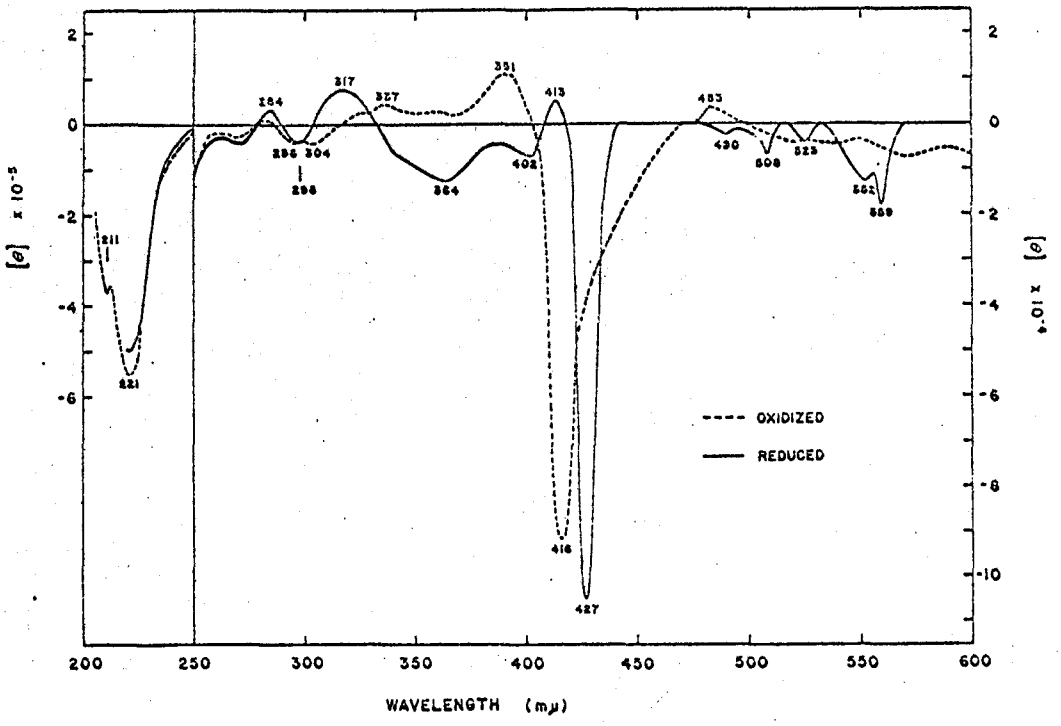

Fig. 4. Circular dichroic spectra of larval cytochrome b-555 in 0.1 M phosphate buffer (pH 7.0) at 22-24°C.