A Study of the Cytochromes of *Octopus vulgaris* Lam.

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The presence of haematin compounds in those animals which have a copper pigment that serves as an oxygen carrier was first observed in 1886 by MacMunn. In 1925, Keilin confirmed MacMunn's observations and found cytochrome in several groups of invertebrates devoid of haemoglobin.

In 1940, Ball & Meyerhof, by direct spectroscopic observations, indicated the presence of iron–porphyrin compounds in some marine animals whose blood pigment is haemocyanin; they found cytochromes *a*, *b*, *c* and cytochrome oxidase in the heart and some body muscle of molluscs and crustaceans. In 1946, however, Warburg suggested that all those organisms might have also enzymes which are capable of transporting oxygen by means of copper. No further information can be found in the literature, and it is generally accepted that the process of oxygen utilization in molluscs is similar to that in mammals except for the substitution of haemocyanin for haemoglobin as oxygen carrier.

This paper brings direct evidence, based upon spectrophotometric and enzymic investigations, for the presence in particles isolated from the body muscle of *Octopus vulgaris* of a cytochrome system composed of cytochromes a, a_3, b and c. This evidence, however, does not exclude the possibility that other systems are operating in the terminal respiration of *Octopus*.

MATERIAL AND METHODS

Muscle preparation. The animal was bled and perfused with sea water according to the method commonly used in this laboratory (Bacq & Ghiretti, 1953). After half an hour no trace of haemocyanin was present in the perfusion liquid. The skinned mantle and tentacle muscles were collected and frozen at −20° before grinding in a meat grinder. All further manipulations were performed at about 3°. The ground material was weighed and suspended in about 5 vol. of cold distilled water for 30 min. The suspension was passed through cheese cloth and the solid material resuspended in the same volume of water. This treatment was repeated five times. At the end of this procedure the water was squeezed through muslin and the material was homogenized in cold phosphate buffer (0·1 M, pH 7·3) in a Waring Blendor for 7 min. About 2·5 l. of buffer was used/kg. of frozen muscle. The thick suspension so obtained was centrifuged at 2000 *g* for 30 min.; the sediment was collected, resuspended in an equal volume of buffer and again centrifuged. The combined supernatants were centrifuged at 30000 *g* for 1 hr. in the Spinco preparative centrifuge. The small sediment was washed in phosphate buffer and then suspended in an amount of the same buffer to give approx. 50 mg. dry wt./ml. This preparation can be stored at −20° for several days.

Difference spectra. All spectrophotometric measurements were made with a DU Beckman spectrophotometer with a photomultiplier attachment, 1 cm. light path cuvettes specially designed for work in anaerobiosis being used. The difference spectra (the difference between the reduced and the steady-state oxidized forms of the pigments present) were taken on the original thick particle suspension (about 50 mg. dry wt./ml.).

Enzyme activities. Oxygen uptake was determined manometrically at 24°. The activity of the complete succinic oxidase system was measured in the presence of excess of cytochrome *c*. Final concentrations in the Warburg flasks were: phosphate or aminotrishydroxymethylmethane (tris) buffer, 0·15 M; succinate, 0·05 M; cytochrome *c*, $2{\cdot}5 \times 10^{-5}$ M. Final volume, 3·0 ml.; 0·5 ml. of the original particle suspension was used in all the experiments. Succinic dehydrogenase activity was determined in the presence of KCN and of methylene blue (Slater, 1949*a*). Final concentrations were: phosphate or tris buffer, 0·15 M; succinate, 0·05 M; methylene blue, 0·001 M; cyanide, 0·01 M. Neutralized KCN and succinate were added from the side arm.

The activity of cytochrome oxidase was determined by the method recommended by Slater (1949*b*). Carbon monoxide inhibition and its light reversibility were measured according to Warburg (1927).

Chemicals. Eastman Kodak sodium succinate was crystallized twice by ethanol precipitation; cytochrome *c* was prepared from ox heart according to Keilin & Hartree (1937); diphosphopyridine nucleotide (DPN) 60% purity was purchased from Pabst; reduced DPN (DPNH) was obtained by reduction with alcohol dehydrogenase and was isolated as the tris buffer salt according to Loewus, Westheimer & Vennesland (1953).

RESULTS

Difference spectra

Fig. 1 shows the difference spectra (difference between the reduced and the oxidized forms) of the cytochromes in a suspension of particles from muscle. Reduction was obtained by addition, in the absence of air (gas phase, hydrogen), of solid sodium succinate (curve *A*) or dithionite (curve *B*).

The α band of cytochrome *a* is present at 605 mμ; the combined α bands of cytochromes *b* and *c* are at 560 mμ. The band at 522–525 mμ corresponds approximately to the β bands of cytochromes *c* and *b*.

A small shoulder at 485 mμ is very similar to the band attributed by Keilin & Hartree (1939) to an oxidized flavoprotein (it disappears on further reduction with dithionite). The presence of flavoproteins is demonstrated by the trough at about 470 mμ. In the Soret region only one band is present at 445 mμ which corresponds to the γ band of cytochrome a_3.

Further reduction with dithionite (curve *B*) results in an increase of the optical density of the peaks. Moreover a shoulder appears at 435 mμ corresponding approximately to the γ band of cytochrome *b*. The increase of the optical density might be an indication of the partial inactivation of the cytochromes present, or of the presence of particles devoid of succinic dehydrogenase and therefore not reducible by succinate.

The γ band of cytochrome *c* is not visible in these spectra. However, it is difficult to detect the γ band of cytochrome *c* in difference spectra. Difference spectra from bacteria (Smith, 1954) and from heart muscle and *Acetobacter pasteurianum* (Chance, 1952), all of which contain cytochrome *c*, do not show the γ band of this pigment. Even the difference spectra of rat-liver mitochondria taken with a very sensitive instrument show only a very faint γ band of cytochrome *c*, whereas its α band is clearly detectable (see fig. 8, Chance & Williams, 1956). This difficulty is probably related to the fact that the band of reduced cytochrome *c* is very near to the Soret band of oxidized cytochrome *b* and can therefore be cancelled by it in the difference spectra. Better signs of the presence of cytochrome *c* will be seen in Fig. 3. However, cytochrome *c* is certainly less abundant than cytochromes a_3 and *b*. This might well be due to losses of this water-soluble component

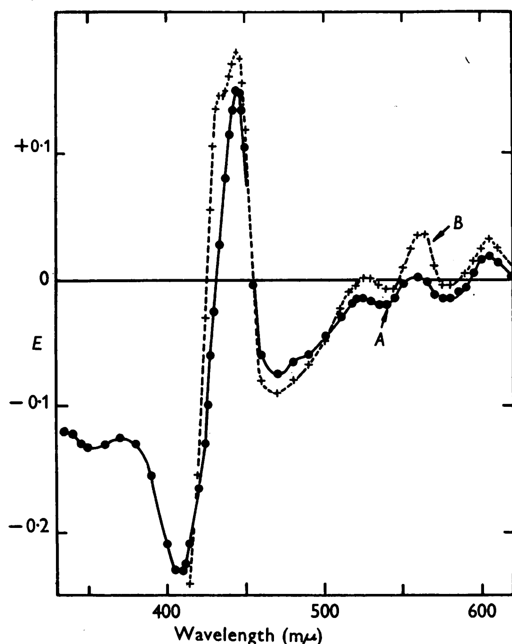

Fig. 1. Difference spectra of the cytochromes in a suspension of particles from muscle (50 mg. dry wt./ml.). Preparation N. 3. Curve *A* (●—●), reduction with sodium succinate; curve *B* (+ −− +), reduction with dithionite.

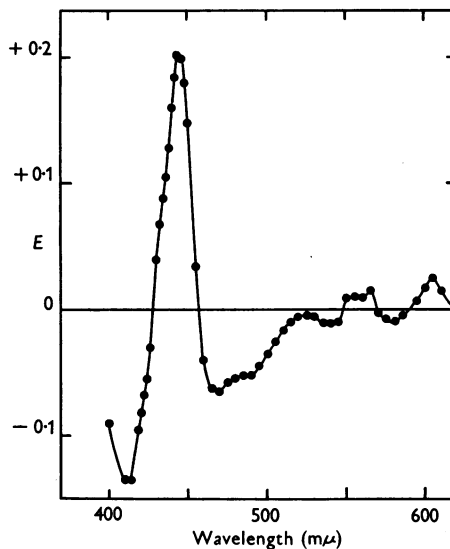

Fig. 2. Difference spectrum obtained by reduction with DPNH. Preparation N. 4.

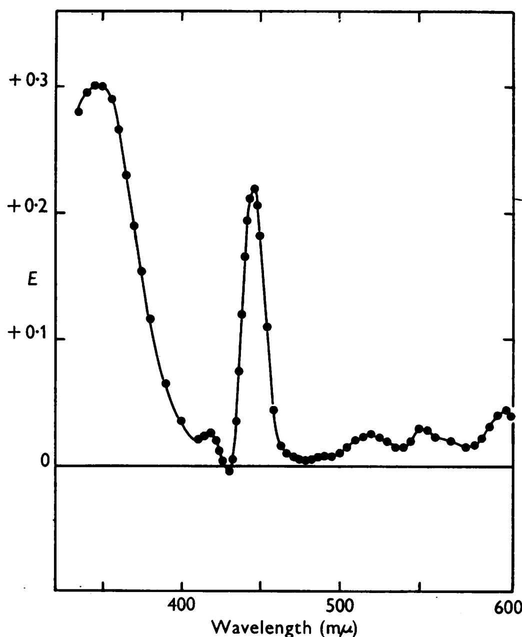

Fig. 3. Difference spectrum obtained by reduction with ascorbic acid. Preparation N. 5.

during the preparation, as will be seen in Fig. 5, which shows the effect of added cytochrome *c* on succinate oxidation. Since the preparation of muscle particles requires several prolonged washings with water, the results obtained with different preparations are not identical.

The effect of reduction by addition of DPNH is shown in Fig. 2. The α bands of cytochromes *a*, *b* and *c* are observed as well as a small β band of *b* and *c*. The shoulder at 485 mμ and the sharp peak in the Soret region at 445 mμ are also present. The γ band of cytochrome *b* is not visible.

In Fig. 3 the spectrum obtained by reduction with ascorbic acid is reported. In addition to the bands already observed after reduction with succinate and DPNH, it shows a small peak at 415 mμ corresponding to the γ band of cytochrome *c* and a large one at 345 mμ probably due to the presence of reduced pyridine nucleotides.

Cytochrome a_3 was identified also by the spectral study of its carbon monoxide compound. For this purpose the particle suspension was treated with ascorbic acid and then saturated with carbon monoxide. (The ascorbic acid plus carbon monoxide compound was read against the ascorbic acid-treated suspension.) Fig. 4 shows the difference spectra, a trough at 445 mμ and a peak at 430 mμ, between carbon monoxide-treated and reduced pigment. The corresponding values for heart-muscle particles from pig heart and for yeast according to Chance (1953) are at the very same position; older measurements from action spectra by Melnick (1942) do not

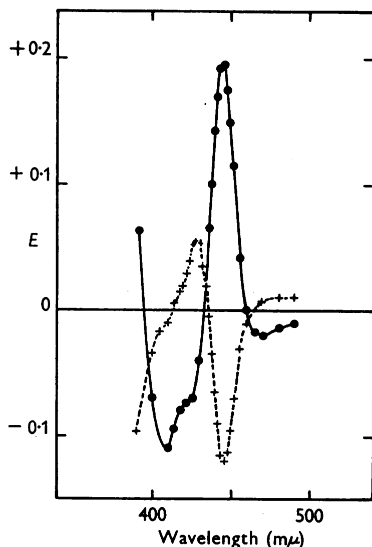

Fig. 4. Difference spectra of a particle suspension treated with carbon monoxide. Preparation N. 7. Solid line: ascorbic acid-treated, minus oxidized; broken line: ascorbic acid + carbon monoxide-treated, minus ascorbic acid-treated.

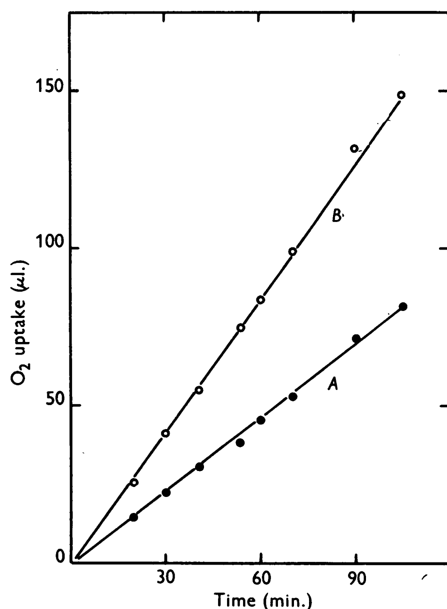

Fig. 5. Oxidation of succinic acid by a suspension of muscle particles alone (curve *A*) and in the presence of added cytochrome *c* ($2 \cdot 5 \times 10^{-5}$ M; curve *B*).

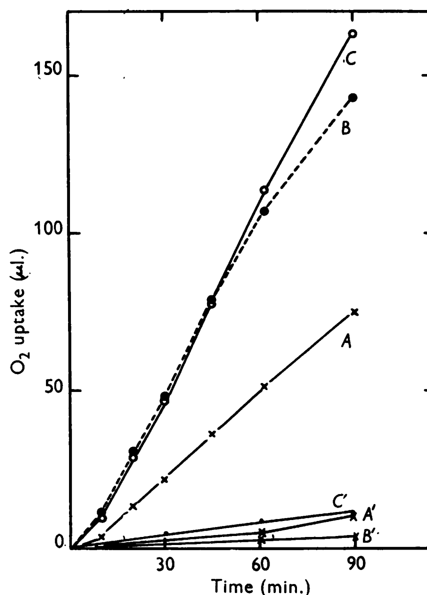

Fig. 6. Oxidation of cytochrome *c* by a suspension of muscle particles. Cytochrome was reduced with *p*-phenylenediamine (curve *A*), quinol (curve *B*) and ascorbic acid (curve *C*). Curves *A′*, *B′* and *C′*: O_2 uptake in the absence of cytochrome *c*.

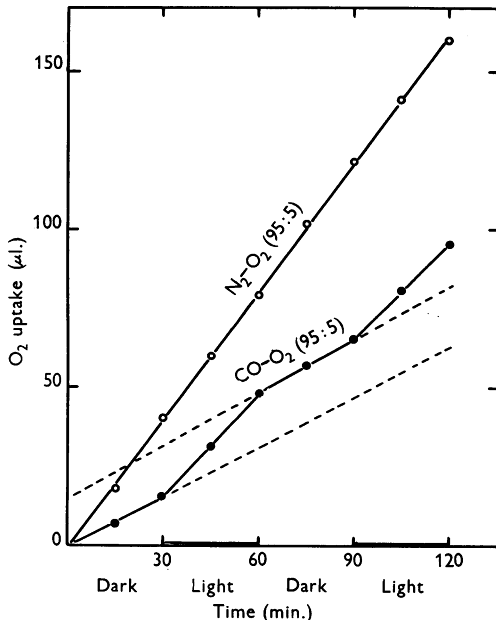

Fig. 7. Effect of alternate darkness and illumination on the oxidation of cytochrome *c* by a suspension of muscle particles in the presence of carbon monoxide.

agree with these values, but the Chance's evidence obtained from action spectra and from difference spectra seems decisive.

Oxidation of succinate

Sodium succinate was readily oxidized by the muscle preparation. The activity of the complete succinic oxidase system was measured in air with and without addition of cytochrome *c*. The effect of substrate concentration and of pH were also studied.

Addition of cytochrome *c* increased the oxidation of succinate (Fig. 5). High concentrations of substrate (up to 0·25 M) caused a definite inhibition. Addition of malonate (8×10^{-4} M) inhibited the oxidation of succinate. The optimum for both the activities (succinic oxidase system and succinic dehydrogenase) was found at pH 8·2. The pH optimum of mammal succinic dehydrogenase is 7·3; that of sea urchin sperm is 7·0 (Ghiretti & Libonati, 1957).

Cytochrome oxidase activity

The cytochrome oxidase activity of muscle-particle suspensions was determined by measuring the rate of oxidation of reduced cytochrome *c*. The cytochrome *c* was reduced by ascorbic acid, quinol or *p*-phenylenediamine (Fig. 6). In Fig. 7 a typical experiment of carbon monoxide inhibition of cytochrome oxidase and its reversibility by light is reported. The inhibition corresponded to 64% of the initial rate.

DISCUSSION

The existence of a cytochrome system composed of cytochromes a, a_3, b and c which can operate in the terminal respiration of *Octopus vulgaris* is demonstrated in these experiments. All the cytochromes found are identical with those of mammals and yeast as shown spectrophotometrically and enzymically.

The question whether this is the only operating system in the terminal respiration of *Octopus vulgaris* is still open. Beside iron the muscle contains copper and flavins. The figures found (μg./g. dry wt.) are: 47 Fe; 28 Cu; 2·5 flavin mononucleotide and 4·2 flavin-adenine dinucleotide (Ghiretti-Magaldi, Giuditta & Ghiretti, 1957). It was not possible to carry out inhibition experiments with cyanide and carbon monoxide on the respiration of whole muscle; this organ under the usual experimental conditions (slices or crude homogenate) has an extremely low Q_{O_2}. Moreover, a direct spectroscopic observation in whole muscle fails to show absorption bands corresponding to any of the cytochromes. In our experiments we had to use a quantity of particle suspension corresponding to 10–15 g. of fresh tissue, an amount which obviously cannot be used when the respiration of fresh muscle is measured.

SUMMARY

1. A method of preparing isolated particles from the muscle of *Octopus vulgaris* is described.

2. Difference spectra of the particles show the presence of a cytochrome system composed of cytochromes a, a_3, b and c.

3. Enzymic oxidation of succinate and cytochrome oxidase activity have also been demonstrated in these particles.

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Isolation and Properties of Malic Dehydrogenase from Ox-Heart Mitochondria

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Malic dehydrogenase, discovered independently by Batelli & Stern (1910) and Thunberg (1911), has been purified by Straub (1942) and recently has been isolated as a pure enzyme from acetone-dried powder of whole pig heart by Wolfe & Neilands (1956).

The oxidation of *meso*- and D(−)-tartrate by mitochondria of animal tissues has been reported (Kun & Hernandez, 1956; Kun, 1956). However, these studies did not solve the problem of the dehydrogenases involved in tartrate oxidation. Kun & Hernandez (1956) reported that various preparations of malic dehydrogenase oxidize *meso*- and D(−)-tartrate 'to a small and varying degree'. Upon re-investigation, we have found that all preparations of malic dehydrogenase consistently demonstrate activity toward these two isomers of tartaric acid and that the relative activities remain constant. These observations have led us to investigate the specificity of malic dehydrogenase.

The specificity of malic dehydrogenase has not previously been studied in detail, though Green (1936) reported that the enzyme did not oxidize D-malate or dihydroxyfumarate. Evidence will be presented for the view that malic dehydrogenase is an α-hydroxydicarboxylic acid dehydrogenase, catalysing the following reactions between substrate and diphosphopyridine nucleotide (DPN).

(1) L-Malate + DPN^+
$\rightleftharpoons$ oxaloacetate + reduced DPN (DPNH) + H^+.

(2) D(−)- or *meso*-Tartrate + DPN^+
$\rightleftharpoons$ oxaloglycollate + DPNH + H^+.

(3) Oxaloglycollate + DPN^+
$\rightleftharpoons$ dioxosuccinate + DPNH + H^+.

(4) Tartronate + DPN^+
$\rightleftharpoons$ mesoxalate (oxomalonate) + DPNH + H^+.

(5) α-Hydroxyglutarate + DPN^+
$\rightleftharpoons$ α-oxoglutarate + DPNH + H^+.

EXPERIMENTAL

Materials

The following substances were commercial preparations: diphosphopyridine nucleotide (DPN) 90% pure, reduced diphosphopyridine nucleotide (DPNH) 80% pure, aminotrishydroxymethylmethane (tris) and disodium ethylenediaminetetra-acetic acid (EDTA) from the Sigma Chemical Co., dihydroxyfumaric acid, dihydroxytartaric acid (the hydrate of dioxosuccinic acid), sodium mesoxalate (sodium oxomalonate) and sodium tartronate from the Aldrich Chemical Co., α-oxoglutaric acid, D(−)- and *meso*-tartaric acids from the California Foundation for Biochemical Research, and L-malic acid from Eastman Kodak Co. Oxaloacetic acid was prepared according to Heidelberger (1953).

Analytical methods

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer equipped with a photomultiplier attachment, and with the Cary recording spectrophotometer. Silica cells of 1 cm. light path and 1 ml. capacity were used for routine assay of enzyme activity, and cells of 10 and 5 cm. light path were used to obtain more accurate kinetic data. DPNH was determined by measurement of light absorption at 340 mμ, the extinction coefficient $6{\cdot}22 \times 10^6$ $cm.^2$ $mole^{-1}$ being used (Horecker & Kornberg, 1948). Protein was determined by the method of Warburg & Christian (1942) and standardized by total nitrogen determination of the most active and pure enzyme preparation. Assuming a nitrogen content of 16%, 1 mg. of enzyme protein/ml. gave an optical density at 280 mμ of 0·85 and a 280:260 mμ ratio of 1·35.

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