PATHWAYS OF TERMINAL RESPIRATION IN MARINE INVERTEBRATES

II. The Cytochrome System of Aplysia*

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ABSTRACT

The classic spectrophotometric method for identification and characterization of respiratory enzymes has been used for the study of the cytochrome system of *Aplysia*. Particles have been prepared from the buccal mass and the gizzard muscles. Difference spectra taken on isolated particle suspensions show the presence of a complete cytochrome system composed of five components: cytochrome *a*, *b*, *c*, c_1, and a_3. As indicated by the peaks of the sharp absorption bands of their reduced forms, they are very similar to the cytochromes of mammals and yeast. Cytochrome a_3 has been identified as the terminal oxidase of *Aplysia* muscle by means of the spectrophotometric study of its carbon monoxide compound. Further evidence for the presence of a cytochrome system in *Aplysia* was obtained by assays of the catalytic activities of the isolated particles: succinic dehydrogenase, cytochrome oxidase, DPNH cytochrome *c* reductase. The cytochrome oxidase activity was strongly inhibited by carbon monoxide in the dark; the inhibition was totally relieved by light.

Cytochrome *c* has been extracted and purified from muscle tissue. Its spectrum is almost identical with that of the mammalian pigment both in the oxidized and reduced forms. From the hepatopancreas a new respiratory enzyme has been extracted which has many physical and chemical properties in common with cytochrome *h* from terrestrial snails.

INTRODUCTION

Evidence for the existence of a respiratory pigment between substrate and oxygen in all living cells was first given by Keilin (1925) who found cytochrome *c* in all organisms examined, irrespective of the type of oxygen carrier pigment in the blood.

As result of the past 30 years of research, the concept of the pathway to oxygen has increased greatly in complexity. The present view of the respiratory chain involves, beside pyridine-nucleotide enzymes and flavoproteins, a sequence of four or five cytochromes, intimately associated with the structure of mitochondria. Our knowledge of the nature of respiratory catalysts is based

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mainly upon studies made on yeast and mammalian liver mitochondria. However, research on the cytochrome system of bacteria (Smith, 1954) demonstrated that the components of the respiratory chain may vary from organism to organism. Because of the great differences among organisms, it is of interest to know whether the respiratory process is the same in all living forms, and if it is not, to determine the extent of their similarities and differences.

Previous studies on the respiratory system in marine invertebrates (Ghiretti-Magaldi *et al.*, 1957, 1958; Maggio and Ghiretti-Magaldi, 1958) indicated the presence in cephalopods and echinoderms (sea urchin eggs) of a cytochrome system very similar to that found in yeast and mammals. In cephalopods, which were taken as a representative group of invertebrates having hemocyanin in the blood, no participation of copper enzymes in tissue respiration could be demonstrated. However, the similarity of their cytochrome system to that of mammals, as indicated by the analysis of the difference spectra, is restricted to the system as a whole; differences can be found at the level of single components (Ghiretti-Magaldi and Ghiretti, 1958).

In this paper the results of a study on the cytochrome system of *Aplysia* (the sea hare) are reported. Marine gastropods are known to have hemocyanin in the blood, with the exception of a few groups containing erythrocruorins (Prosser, 1950). It is generally accepted that *Aplysia* too has hemocyanin (Eales, 1921). We have analyzed the hemolymph of *Aplysia* and found no oxygen carrier pigment (see Appendix). This fact, together with the presence of large amounts of myoglobin in muscles (Rossi-Fanelli and Antonini, 1957) makes this animal of interest from the point of view of the comparative biochemistry of respiration.

Two species of *Aplysia* were used: *A. depilans* and *A. limacina*. The respiration of tissue slices has been studied under different conditions. The presence of the cytochrome system in particles prepared from muscles has been investigated and the enzymatic activities of the components determined. Cytochrome *c* was extracted from muscle and its chemical and physical properties were compared with those of mammalian cytochrome *c*. Finally a new respiratory pigment has been found in *Aplysia* hepatopancreas which showed many properties in common with cytochrome *h* of the terrestrial snail *Helix pomatia*. A method of preparation and purification of the pigment is reported, as well as the study of its chemical, physical, and biological properties.

Material and Methods

Hemolymph was obtained from living animals by puncture of the heart or from the body cavity. For gas analysis the blood was collected under oil; 100 to 150 ml. of liquid were obtained from each animal (400 to 500 gm. body weight). The animal was opened and the organs were rapidly removed and frozen. The buccal mass, gizzard, and hepatopancreas were used. The buccal mass (1 to 2 gm.), called also the pharyngeal bulb, is a pear-shaped organ, deep red in color, which possesses several groups

of intrinsic muscles. The tissue is not homogeneous and slices are not recommended for manometric experiments. The gizzard or triturating stomach (0.5 to 1 gm.) is an annular, thick walled organ, colored red-orange, which has a dense coat of circular muscle fibers. The cavity is filled with semitransparent horny teeth inserted in the internal wall. These are easily detached when the organ is opened and from the small band of homogeneous muscle, slices can be easily prepared with a razor. The organ called hepatopancreas or liver or digestive gland is a compact, green or brown, voluminous gland which makes up the greater part of the visceral mass. It weighs from 50 to 100 gm. The hepatopancreas was removed from the body, put under sea water, and the intestine was carefully detached from its surface. All the organs were stored at −20°C. until used.

For preparation of the particles, the frozen muscles were ground in a meat grinder and suspended in 20 volumes of cold tap water for 30 minutes with continous stirring. The suspension was squeezed through gauze and the same treatment was repeated 5 to 8 times until the myoglobin and the viscous soluble proteins were almost completely extracted. The material was then homogenized in a Waring blendor for 7 minutes with 10 volumes of cold 0.1 M phosphate buffer, pH 7.3, and centrifuged at 700 *g* for 45 minutes. The sediment was resuspended in 5 volumes of the same buffer, homogenized again, and centrifuged as before. The supernatants were combined, diluted with an equal volume of buffer, and centrifuged at 1000 *g* for 1 hour. The clear opalescent liquid was filtered through glass wool and then centrifuged, first at 30,000 *g* and then at 107,000 *g* for 1 hour in the Spinco preparative centrifuge. The small sediments obtained were washed twice with phosphate buffer and finally suspended in 15 to 20 ml. of the same buffer.

The respiratory enzymes present in the particle suspensions prepared as above were identified by means of the classical method based on the appearance of the sharp absorption bands of their reduced forms. Difference spectra were taken with the technique described elsewhere (Ghiretti-Magaldi *et al.*, 1957). According to this technique, two samples of the particle suspension are used. One of them contains the respiratory chain in the oxidized state, in the other the pigments have been reduced by a chemical agent in the absence of air. The difference of absorbency between the two samples is then recorded over a convenient span of wavelengths, usually from 380 to 620 mμ. Cuvettes specially designed for work in anaerobiosis were used; readings of the optical density were taken at intervals of 4 or 2 mμ in a DU Beckman spectrophotometer with a photomultiplier attachment. The instrument was previously calibrated with a mercury lamp.

The enzymatic activities (succinic dehydrogenase, cytochrome oxidase, DPNH-cytochrome *c* reductase) were determined spectrophotometrically (Cooperstein *et al.*, 1950) or manometrically (Slater, 1949 *a*, 1949 *b*), either in the presence of ferricyanide (Quastel and Wheatley, 1938) or in air with and without KCN and methylene blue.

Respiration of the tissue slices was studied by the conventional method of Warburg at 25°C. and at different oxygen tensions. Inhibition by cyanide and by carbon monoxide was determined according to the methods recommended by Krebs (1935) and by Warburg (1926) respectively. Carbon monoxide was produced in the laboratory by dropping formic acid on hot sulfuric acid, and the absence of oxygen from it was checked before use.

Oxygen in hemolymph was analyzed manometrically by the method of Van Slyke

(1924). Free amino acids were detected by paper chromatography; proteins were estimated colorimetrically (Lowry *et al.*, 1951) or spectrophotometrically (Kalckar, 1947); iron and copper colorimetrically (Sandell, 1950). A beef heart preparation containing cytochrome oxidase and succinic dehydrogenase was prepared according to Keilin and Hartree (1937); cytochrome b_5 was prepared from beef liver by means of a modification of the methods described by Strittmatter and Velick (1956) and by Garfinkel (1956). Cytochrome *c* and DPNH were purchased from Fluka Inc., Switzerland; sodium succinate, sodium pyruvate, and α-ketoglutarate were commercial products recrystallized in the laboratory.

RESULTS

The Respiration of Muscle.—The Q_{O_2} of the gizzard muscle, as determined in air at 25°C., was found to have values from 0.35 to 0.70. Reduction of the

TABLE I

Effect of Some Intermediates of the Citric Acid Cycle on the Respiration of Aplysia Gizzard Muscle

Experiments on tissue slices. Final concentration of the substrates 0.01 M. Gas phase: air. Temperature = 25°C.

Substrate	Q_{O_2}	Relative rate
None	0.33	100
Succinate	1.80	550
Succinate + malonate	0.96	300
Succinate + malonate + fumarate*	0.90	280
Malonate	0.33	100
Fumarate	0.30	99
Citrate	0.64	194
Malate	0.56	170

* Final concentration of fumarate 0.003 M.

oxygen pressure from 21 to 5 per cent did not affect the rate of respiration, contrary to results in organs of other invertebrates (Barron and Tahmisian, 1948; Ghiretti-Magaldi *et al.*, 1958). No correlation was found between Q_{O_2} and the myoglobin content of muscles.

The action of several intermediates of the tricarboxylic acid cycle upon muscle respiration was studied. Whereas malate and fumarate had no effect, succinate increased the Q_{O_2} of gizzard muscle five times. Malonate inhibited the oxidation of succinate; the inhibition was not relieved by addition of catalytic amounts of fumarate (Table I).

Respiration of muscle was strongly inhibited by KCN: 84 per cent inhibition with 10^{-3} M cyanide (Table II). This is an indication that in *Aplysia* muscle, terminal respiration is catalyzed by metalloproteins and not by flavoproteins (Commoner, 1940).

The nature of the metal involved, that is the participation of iron or copper

in terminal respiration, was studied by using a mixture of CO and oxygen (95:5) as the gas phase. Carbon monoxide is known to be the most effective reagent for the identification of terminal oxidases, since it combines specifically with the iron atom of cytochrome oxidase (Warburg, 1926; Keilin and Hartree, 1939; Chance, 1953; Castor and Chance, 1955). If the carbon monoxide inhibition of respiration is found to be light-sensitive, then iron is the heavy metal (Warburg, 1946).

These inhibition studies present several difficulties when organs of marine invertebrates are used. The low endogenous respiration of the tissues, together with the presence of other pigments, makes questionable the response to carbon monoxide in the light and dark. In most of the experiments made with slices of *Aplysia* gizzard muscle, no inhibition of the endogenous respiration by carbon monoxide in the dark was observed. Light had no effect on the oxygen uptake. The same results were obtained when respiration was measured in the presence

TABLE II

Effect of Cyanide on the Endogenous Respiration of Aplysia Gizzard Muscle

Gas phase: air. Temperature = −25°C.

KCN	Q_{O_2}	Inhibition
M		*per cent*
0	0.39	
10^{-5}	0.40	0
10^{-4}	0.21	45
10^{-3}	0.06	84

of succinate. In several cases, however, carbon monoxide caused a small but definite *increase* in respiration (from 10 to 15 per cent). Since the early experiments of Fenn and Cobb (1932) on frog muscle, increase of respiration in the presence of carbon monoxide has been observed in various materials by several authors who postulated the existence of an enzymatic mechanism for the oxidation of CO to CO_2. (See Rothschild and Tyler, 1958 for bibliography.) Whether *Aplysia* muscle is a further example of this effect, we do not know. However, the results obtained cannot exclude the participation of iron porphyrin enzymes in terminal respiration, indicated by studies with particles isolated from muscle (see below). The only possible conclusion is that manometric methods are not adequate to clear up the problem.

The Cytochrome System of Muscle

Difference Spectra.—The spectrum representing the difference in optical density between the reduced and the steady-state oxidized forms of the pigments present in a particle suspension prepared from *Aplysia* muscle is shown in Fig. 1 (reduced-oxidized). Reduction was obtained anaerobically with

dithionite. Beginning with the long wave end of the spectrum, the α-band of cytochrome *a* is observed at 605 mμ. The fairly sharp peak at 562 mμ belongs to the α-band of cytochrome *b*, whereas in the region of 532 to 520 mμ is seen the β-band of cytochrome *b*. The trough in the region of 460 to 480

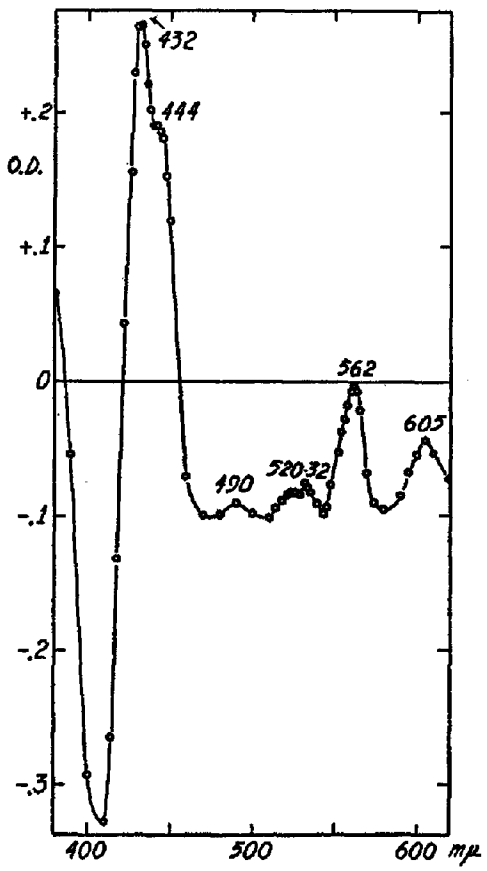

FIG. 1. *Aplysia limacina*. Buccal muscle particle preparation No. 9. Difference spectrum after reduction with dithionite.

mμ is caused by the disappearance of the absorption band of oxidized flavoproteins. In this as in all such spectra, a small band or shoulder is visible at 490 mμ which is very similar to that attributed by Keilin and Hartree (1939) to an oxidized flavoprotein. In the Soret region the distinctive band at 432 mμ is due to the *y* band of cytochrome *b* whereas the shoulder at 444 mμ is due to the *y* band of cytochrome a_3. The bands of cytochrome *c* are not visible in this as in other spectra. This soluble component was probably washed out

during the preparation of the particles. However, the presence of cytochrome *c* in *Aplysia* muscle was demonstrated by direct extraction of the pigment (see below). Further evidence for the presence at least in catalytic amounts of the pigment in the particles is given by the reduction of cytochromes *a* and a_3 observed when succinate was used instead of dithionite.

Fig. 2 illustrates the difference spectrum after reduction with DPNH. In this case, in addition to the absorption bands of cytochromes *a*, *b*, and a_3, in

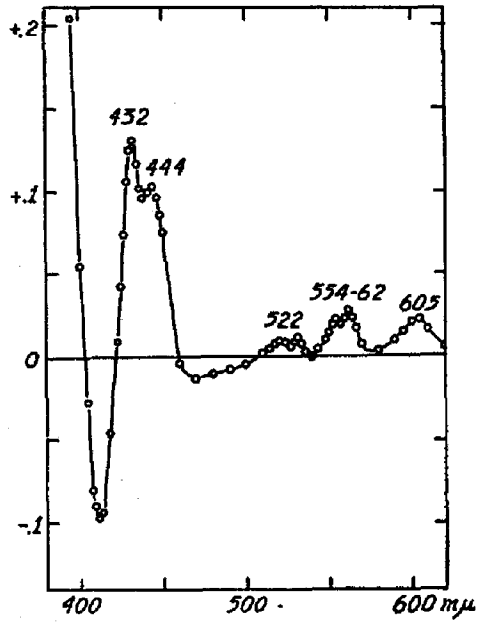

FIG. 2. *Aplysia limacina*. Buccal muscle particle preparation No. 10. Difference spectrum after reduction with DPNH.

the visible and the Soret regions, a shoulder at 554 mμ indicates the presence of cytochrome c_1.

Similar results were obtained in difference spectra taken from buccal mass muscle and from gizzard muscle. DPNH, succinate, and ascorbate were used as reducing agents (Table III). In all cases absorption bands of cytochromes *a*, *b*, a_3, and c_1, together with appreciable absorption due to flavoproteins, were observed.

Special attention was given to the further identification of the terminal oxidase. Carbon monoxide is known to react with mammalian cytochrome a_3 giving a compound with distinctive absorption bands in the Soret region. The method recommended by Chance (1953) was applied. In this method

two samples of material, both containing the respiratory chain in the reduced state, were used and the absorbancy between them was measured from 380

TABLE III

Peaks in Difference Spectra of Aplysia Muscle Cytochromes

Preparation No.	Reducing agent	α		β	γ			
		a	b + c_1	b	a_s	b	CO	
							Max.	Min.
Buccal muscle								
4	Ascorbate	605	560, 555	520	442	430	—	—
	Dithionite	605	561, 554	530	442	430	—	—
5	Dithionite	605	560, 554	530–522	442	432	—	—
6	Ascorbate	605	561	522	442	—	424	442
	Dithionite	605	562	532–522	440	432	—	—
8	Ascorbate	605	560	534–516	438	—	424	443
	Dithionite	605	561	532–520	442	431	—	—
9	Ascorbate	605	562, 554	532–522	444	430	426	446
	Dithionite	605	562	532–520	442	432	—	—
10	Succinate*	605	562, 552	528–520	444	430	428	446
	Dithionite	605	562	532–522	442	432	—	—
	DPNH	605	562, 554	532–522	444	432	—	—
Gizzard muscle								
5	Dithionite	605	562, 554	532–522	442	432	—	—
6	Ascorbate	605	562	532–520	442	432	423	442
	Dithionite	605	562, 554	532–520	436	—	—	—
8	Ascorbate	603	562	530–522	440	430	423	444
	Dithionite	600	562	524	444	430	—	—
11	Dithionite	605	562, 552	530	442	430	—	—

* The spectrum of the carbon monoxide compound was taken after treatment with ferricyanide.

to 480 mμ or more. Since the concentration of the pigments in the samples is the same, no absorption band was observed. Then carbon monoxide was bubbled through one of the cuvettes for 1 to 2 minutes and the spectrum was taken representing the difference of absorbancy between the reduced sample and

that reduced plus CO. The presence of cytochrome a_3 is demonstrated by the appearance of a peak at about 430 mμ and a trough at about 445 mμ.

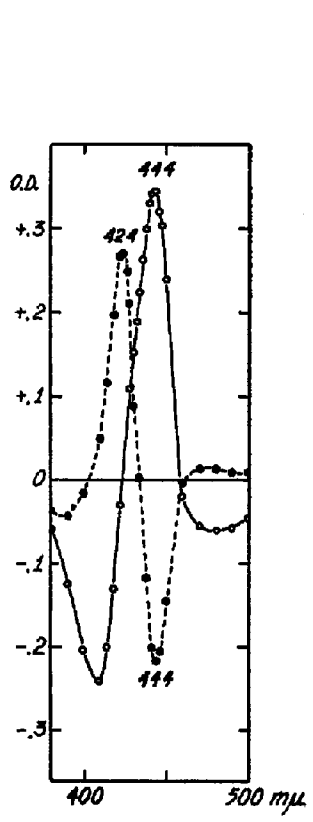

FIG. 3

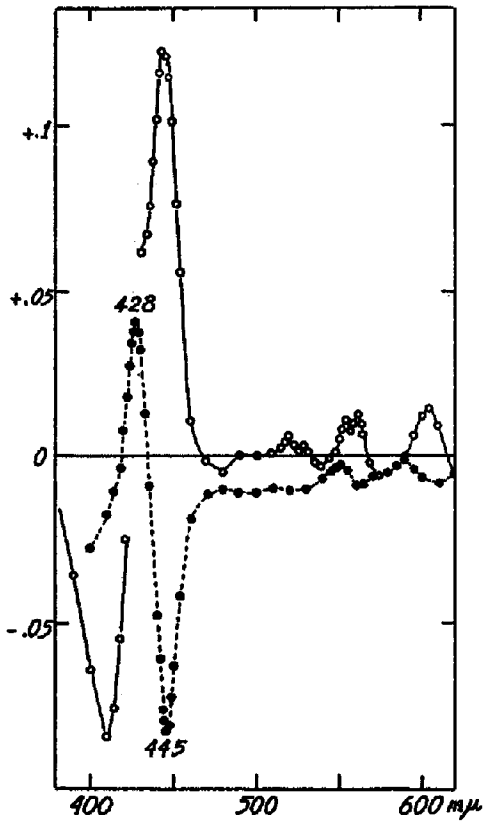

FIG. 4

FIG. 3. *Aplysia limacina*. Buccal muscle particle preparation No. 9. Difference spectra of the carbon monoxide compound. Solid line, succinate-treated minus oxidized. Broken line, succinate plus CO-treated minus succinate-treated.

FIG. 4. *Aplysia limacina*. Buccal muscle particle preparation No. 10. Difference spectra of the carbon monoxide compound after treatment with ferricyanide. Solid line, succinate-treated minus oxidized. Broken line, succinate plus CO-treated minus succinate-treated.

The difference spectrum of the carbon monoxide compound of *Aplysia* muscle is shown in Fig. 3. The peak of the absorption band is shifted towards lower wavelengths and lies at 424 mμ, whereas the corresponding trough is at 444 mμ. It is known that in mammalian tissues, such pigments as hemoglobin interfere with the reaction with CO: in presence of small concentrations

of hemoglobin the peak at 430 mμ of the CO compound of cytochrome a_1 appears at shorter wavelengths whereas the trough at 444 mμ is very little affected (Chance and Castor, 1952; Chance, 1952).

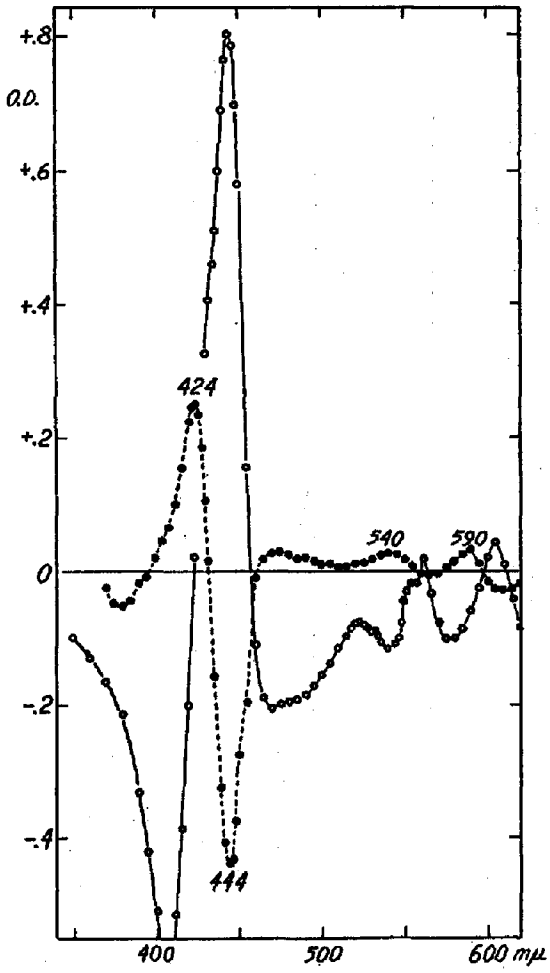

FIG. 5. Beef heart muscle particle preparation. Difference spectra of the carbon monoxide compound. Solid line, succinate-treated minus oxidized. Broken line, succinate plus CO-treated minus succinate-treated.

To remove this possibility, the oxidation of hemoglobin or myoglobin to ferric forms has been suggested; after oxidation they are not reduced to ferrous by succinate and hence do not contribute to the carbon monoxide spectrum. The ferrous form of *Aplysia* myoglobin combines with carbon monoxide and gives rise to an absorption band at 424 mμ. The particle suspension of *Aplysia*

muscle was therefore treated with ferricyanide for 2 to 4 hours, then dialyzed against phosphate buffer or washed by centrifugation, and the difference spectrum of the carbon monoxide compound taken as before using succinate as reducing agent. The new spectrum is illustrated in Fig. 4. As expected, the former peak at 424 mμ now appeared at about 428 to 430 mμ, indicating that cytochrome a_3 is the terminal oxidase as in mammalian tissues.

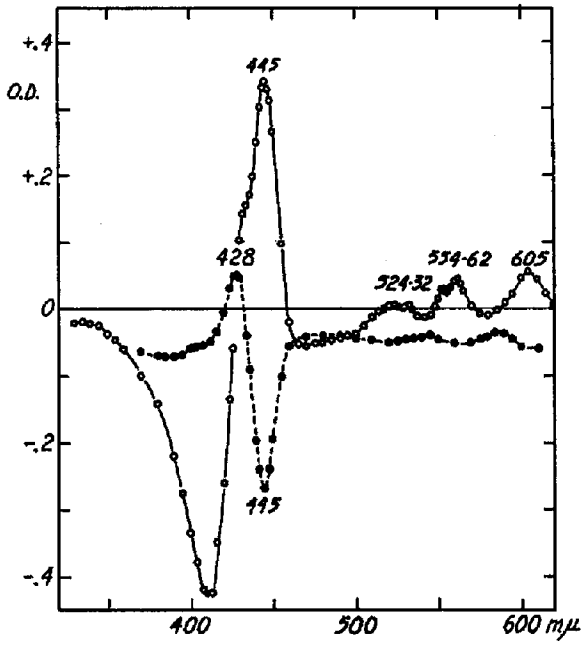

FIG. 6. Beef heart muscle particle preparation. Difference spectra of the carbon monoxide compound after treatment with ferricyanide. Solid line, succinate-treated minus oxidized. Broken line, succinate plus CO-treated minus succinate-treated.

The action of ferricyanide was also tested on a particle suspension prepared from beef heart. The preparation was made according to the same method used for *Aplysia* and the difference spectra taken before and after treatment with ferricyanide were used for comparison (Figs. 5 and 6). It is clear that much the same results was obtained.

Extraction and Purification of Cytochrome c.—Cytochrome *c* was prepared from *Aplysia* muscle by a slight modification of the method described by Hagihara *et al.* (1958). The tissue was minced in a meat grinder, suspended in 10 volumes of ammonium sulfate (85 per cent saturation) containing 0.5 M acetic acid, homogenized for a few minutes in a Waring blendor, and left at 4°C. for 48 hours. The suspension was then squeezed through cheesecloth, the solid material reextracted with ammonium sulfate (55 per cent saturation),

and the pH of the combined filtrates adjusted to 7.0. Solid ammonium sulfate was added to reach a specific gravity of 1.22 and 20 per cent trichloracetic acid was added to pH 4.5. The very light precipitate, which cannot be collected by centrifugation, was filtered through Celite hiflo and dissolved with alkaline water. The red-brown solution was dialyzed with stirring for 1 hour against distilled water. Prolonged dialysis resulted in a large loss of the cytochrome.

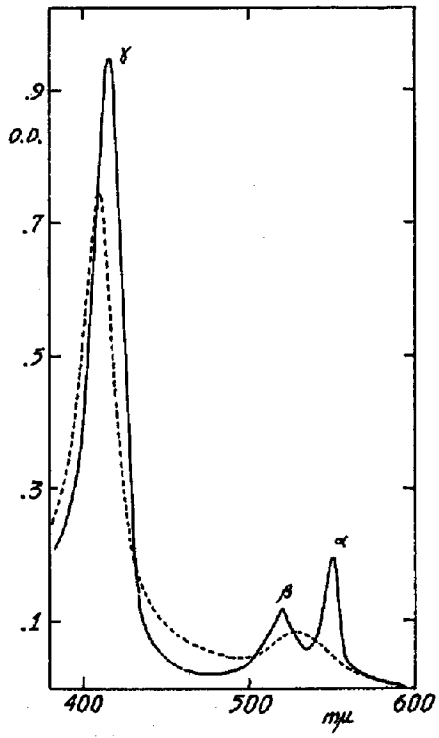

FIG. 7. Absorption spectrum of cytochrome *c* extracted from *Aplysia* muscle.

The ratio 550 Red/280 Ox (R_{280}^{550}) of the solution was 0.015–0.020. Adsorption on amberlite XE 64, mesh 120–230, was used for further purification. The resin had been equilibrated with 0.1 N (in NH_4^+ ions) ammonium phosphate, pH 7.0. The pigment was eluted with 10 per cent saturated ammonium sulfate, pH 9. The R_{280}^{550} was 1.10.

Aplysia cytochrome *c* has a spectrum almost identical with that of mammalian cytochrome *c* (Fig. 7). Its maxima are at 270, 410, and 530 mμ in the oxidized form and at 315, 414, 520, and 550 mμ in the reduced forms.

Enzymatic Activities of the Particle Suspension.—The activity of the complete succinic oxidase system in the particle suspension of *Aplysia* muscle was studied

manometrically in a gas phase of air with and without addition of mammalian cytochrome *c*. The oxygen uptake in the presence of succinate was found to be doubled when cytochrome *c* had been added (Table IV).

Succinic dehydrogenase activity was determined in the presence of ferricyanide as electron acceptor, with bicarbonate buffer and a gas phase of nitrogen and carbon dioxide. The activities of succinic dehydrogenase and of cytochrome oxidase in many preparations of buccal mass and gizzard muscle were determined also spectrophotometrically. The results are reported in Table

TABLE IV

Oxidation of Succinate and of Reduced Cytochrome c by a Particle Suspension (Preparation 2) from Buccal Mass Muscles of Aplysia limacina

Enzyme preparation 0.2 ml. (mg. 4.5 dry weight); 0.1 M sodium succinate 0.3 ml.; 0.1 M ascorbate or hydroquinone or *p*-phenylenediamine 0.3 ml.; 0.1 M phosphate buffer, pH 7.3, 0.5 ml.; cytochrome *c* 0.6×10^{-6} M (final concentration). Final volume 3.1 ml. Gas phase: air. Temperature = 25°C.

Substrate	O_2 uptake
	µl./30 min.
None	0
Succinate	10.5
Succinate + cytochrome *c*	20.1
Ascorbic acid	0
Ascorbic acid + cytochrome *c*	103.6
Hydroquinone	8.0
Hydroquinone + cytochrome *c*	80.6
p-phenylenediamine	22.9
p-phenylenediamine + cytochrome *c*	68.4

V. When cytochrome oxidase activity was studied manometrically, cytochrome *c* was reduced with ascorbate, hydroquinone, or *p*-phenylenediamine. The values obtained for the enzymatic activities are low if compared with the activities of mammalian oxidase, but comparable with those found in other marine invertebrates (Ghiretti-Magaldi *et al.*, 1958; Maggio and Ghiretti-Magaldi, 1958).

The effect of carbon monoxide on the cytochrome oxidase activity and the effect of light on the dissociation of the CO–cytochrome oxidase compound were also followed manometrically in the presence of a mixture of CO and oxygen (95:5). As temperature barometers, vessels containing only the gas mixture, and KOH in the central well, were prepared for each experiment. Controls were run in a mixture of nitrogen and oxygen (95:5). Fig. 8 shows

the results of a typical experiment using a muscle particle suspension from buccal mass. The inhibition by CO, which reached 90 per cent, was totally reversed by light.

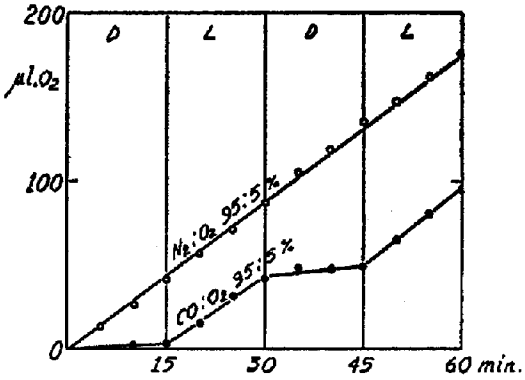

FIG. 8. *Aplysia depilans*. Buccal muscle particle preparation No. 12. Action of carbon monoxide in the light and dark on the cytochrome oxidase activity. The system contained: enzyme preparation 0.3 ml. (2.01 mg. protein); 0.1 M ascorbate 0.3 ml.; 0.1 M phosphate buffer 0.5 ml.; cytochrome *c* 3.2 × 10^{-5} M (final concentration). Temperature = 25°C. Blank values subtracted.

TABLE V

Succinic Dehydrogenase and Cytochrome Oxidase Activities of Particle Preparation from Aplysia Muscle

For succinic dehydrogenase determination the system was: KCN 10^{-3} M; cytochrome *c* 1.8 × 10^{-3} M; sodium succinate 1.7 × 10^{-3} M; 0.01 M phosphate buffer, pH 7.5.

For cytochrome oxidase determination the system was: reduced cytochrome *c* 2.5 × 10^{-5} M; 0.01 M phosphate buffer, pH 7.4.

Values represent the change in O.D. per minute per milligram of protein.

Organ	Preparation No.	Succinic dehydrogenase	Cytochrome oxidase
Buccal mass	5	0.077	
	9	0.019	0.457
Gizzard	5	0.044	0.447

DPNH cytochrome *c* reductase was studied spectrophotometrically in a number of preparations from *Aplysia* muscle. The values obtained are reported in Table VI together with the activity of some preparations of hepatopancreas.

Cytochrome h from Aplysia Hepatopancreas.—Recently J. Keilin (1956, 1957) extracted from the digestive gland of a terrestrial snail (*Helix pomatia*) a water-soluble pigment which in the reduced state shows bands at 422, 526.5, and 556 mμ. The pigment was called cytochrome *h*.

From the hepatopancreas of *Aplysia* we have obtained a hemoprotein which

has a spectrum very similar to that of cytochrome *h* both in the oxidized and in the reduced state. The pigment is therefore indicated here as *Aplysia* cytochrome *h*.

An acetone powder of the fresh organ was prepared by homogenization in a Waring blendor for 1 to 2 minutes with 20 volumes of cold (−20°C.) acetone. The suspension was rapidly filtered under vacuum and washed with cold acetone until the effluent fluid was colorless. The brown powder was then dried and kept in a desiccator until used. Water made alkaline (pH 8) with ammonia or dilute NaOH was used for extraction with continuous stirring in the cold. After centrifugation at 800 *g* for 45 minutes the supernatant was treated with 25 per cent basic lead acetate to reach a final concentration of

TABLE VI

DPNH Cytochrome c Reductase Activity of Particles Prepared from Aplysia Muscle and Hepatopancreas

The system was: DPNH 2×10^{-4} M; cytochrome *c* 1.8×10^{-5} M; KCN 10^{-3} M; phosphate buffer 0.01 M pH 7.5.

Organs	Preparation	Δ O.D./min./mg. protein
Buccal mass	30,000 × *g*	0.301
	107,000 × *g*	0.113
Gizzard muscle	30,000 × *g*	0.262
	107,000 × *g*	0.076
Hepatopancreas	Crude homogenate	0.106
	30,000 × *g*	0.106
	107,000 × *g*	0.039

2 per cent. The precipitate was centrifuged at 1000 *g* for 45 minutes and washed with alkaline water. The clear supernatants were brought to pH 6 with dilute acetic acid and the excess of lead acetate was precipitated with saturated sodium sulfate and centrifuged off. To the neutralized supernatant ammonium sulfate was added to 65 per cent saturation. The pH was adjusted to 7.5. After one night in the cold the precipitate was filtered on hiflo cell bed and washed with ammonium sulfate until the effluent liquid was colorless. The material was suspended in water, Celite was separated by filtration, and the fluid was centrifuged at 18,000 R.P.M. for 15 minutes in the cold. The brownish red solution was treated again with ammonium sulfate (final concentration 65 per cent). After a few hours the precipitate was filtered or centrifuged, washed, dissolved in water, dialyzed against water containing 0.001 M versene and finally against distilled water. The solution was concentrated under reduced pressure.

The preparation of cytochrome *h* from the hepatopancreas of *Aplysia* is

time-consuming, and the amount of pigment obtained was very small. In its oxidized form the pigment extracted from *Aplysia* hepatopancreas shows in the visible region of the spectrum a wide band with a maximum at about

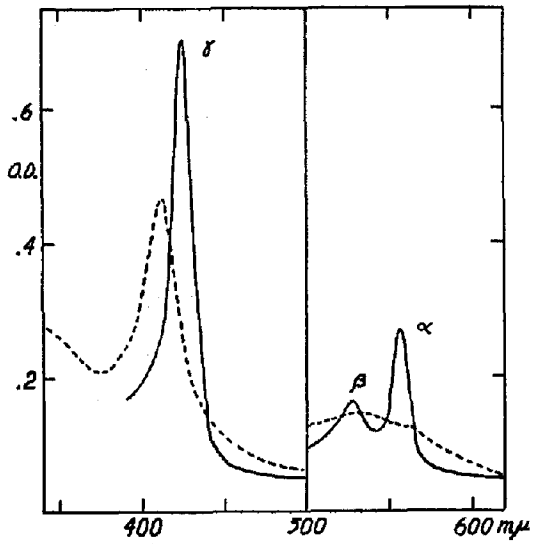

FIG. 9. Absorption spectrum of cytochrome *h* extracted from the hepatopancreas of *Aplysia depilans*. Broken line, oxidized form. Solid line, reduced form.

TABLE VII

Peaks in Spectra of the Respiratory Pigment (Cytochrome h) Extracted from Aplysia and Snail Hepatopancreas

		α	β	γ
Aplysia	Oxidized	—	536–540	410–412
	Reduced	556	526–528	423–424
*Helix**	Oxidized	562	536	408
	Reduced	556	526.5	422

* From J. Keilin (1956).

536–540 mμ, and a sharp Soret band with a peak at 410–412 mμ. The δ band is at 350 mμ. In the ultraviolet region the maximum is at 276 to 278 mμ and the ratio of extinctions at 276/260 is 1.5.

After reduction the pigment shows three distinct bands with peaks at 556, 526–528, and 424 mμ corresponding to the α-, β-, and γ-bands respectively (Fig. 9). These values are very similar to those obtained for *Helix* cytochrome *h*

(Keilin, 1956). In *Aplysia* the γ-band is slightly shifted toward higher wavelengths both in the oxidized and in the reduced forms (Table VII).

Aplysia cytochrome *h* is reduced by dithionite, lithium hydride, ferrous oxalate, cysteine, and glutathione. It is oxidized by ferricyanide and hydrogen peroxide. The compound cytochrome *h*-cyanide in its reduced form gives a spectrum with peaks at 424–426, 530–532, and 558–560 mμ. After treatment with 2 N NaOH and pyridine, followed by reduction with dithionite, the spectrum shows peaks at 415, 520–522, and 550–552 mμ. After reduction with ascorbate, the pigment is oxidized by beef heart cytochrome oxidase; the oxidation is inhibited by cyanide. The rate of oxidation is very slow if compared with the oxidation of mammalian cytochrome *c* and is complete only when large amounts of the oxidase are used. The pigment is reduced by DPNH and by sodium succinate in the presence of mammalian oxidase. Here again the rate of the reaction is much slower than that observed for mammalian cytochrome *c* under the same conditions.

DISCUSSION AND CONCLUSIONS

The cytochrome system present in the muscles of *Aplysia* is composed of five components: cytochrome *a*, *b*, *c*, c_1, and a_3. Cytochrome a_3 is the terminal oxidase. This conclusion results from the enzymatic and spectrophotometric analysis of tissue slices and particles prepared from the buccal mass and gizzard muscles. We have not established whether the properties of the separate components of the cytochrome system are the same as those of mammalian and yeast cytochromes. Work is in progress to isolate the components. From the point of view of comparative biochemistry it is interesting to find the same respiratory system in living forms so different structurally and taxonomically as molluscs and mammals. However in other marine invertebrates (annelids) the cytochrome system differs greatly from that of yeast and mammals (unpublished experiments). It will be interesting therefore to look for any correlation which might exist between the respiratory enzymes of the organism and the special physiological conditions of its natural environment.

Moreover, whereas in vertebrates there exists a chemical relationship between the respiratory enzymes of the cell (the cytochromes) and the blood oxygen carrier (the hemoglobins), in invertebrates the presence or absence of an oxygen-carrying pigment in the blood or, when present, its chemical nature, has nothing to do with the respiratory enzymes of the tissues. The haphazard distribution of blood pigments, not only in different groups of invertebrates, but even among species of the same family, is a puzzling problem of comparative biochemistry (Wald and Allen, 1957).

The chemical nature and the function of the pigment extracted from *Aplysia* hepatopancreas are not understood. Cytochrome *h* from *Aplysia* and from the terrestrial snail have the same spectrophotometric characteristics; they have

also many chemical properties in common. The hypothesis that *Helix* cytochrome *h* may be genetically related to helicorubin received support from the chemical similarities found between the two pigments. *Aplysia*, however, has no helicorubin in the intestine nor in any other part of the body (Phear, 1955). Only gastropods seem to possess cytochrome *h*; attempts to extract the pigment from the hepatopancreas of other molluscs (cephalopods and lamellibranchs) have failed.

TABLE VIII

Iron and Copper Content of Aplysia Blood

Experiment No.	Fe	Cu	Protein
	γ/ml.	*γ/ml.*	*mg./ml.*
1	0.5	—	1.0
2	1.3	—	1.2
3	0.5	3.5	1.1
4	0	2.6	1.1
5	0.5	2.7	1.5
6	0.3	2.8	1.3
7	0.2	4.0	2.0

TABLE IX

Oxygen Content and Oxygen Capacity of Aplysia Blood

	O_2	O_2 capacity	O_2 dissolved
	vol. per cent	*vol. per cent*	*vol. per cent*
Aplysia depilans	0.1–0.3	0.52	0.52
Aplysia limacina	0.1–0.3	0.61	0.51
Sea water		0.45	0.51

From its spectrum, cytochrome *h* could be classified among the *b* group (the peaks correspond exactly to those of cytochrome b_5 of mammalian mycrosomes), whereas its chemical properties are very close to those of cytochrome *c*. In *Aplysia*, cytochrome *h* can be extracted only from hepatopancreas: attempts to apply the same method to muscles were unsuccessful. On the other hand no cytochrome *c* could be extracted from hepatopancreas, either by the classical methods or by the method successfully applied to *Aplysia* muscle; moreover all attempts to prepare cytochrome b_5 from the fresh organ according to the method used for mammalian liver, gave negative results.

Finally, several experiments were made in order to elucidate the origin of cytochrome *h*; that is, whether the pigment is or is not a normal constituent of the hepatopancreas. The digestive organ of *Aplysia*, like that of all molluscs, has peculiar properties which have been carefully investigated by several

authors (Enriques, 1902; Bidder, 1950, 1957). In this organ, absorption of food precedes digestion, and therefore the cells appear full of granules of food. The algae present in the stomach and the intestine of *Aplysia* were identified, and *Ulva lactuca* was found to be the main component. It is also known that if marine molluscs are fed with marine bacteria these accumulate selectively in the hepatopancreas of the animal (Zobell and Feltham, 1937). We have observed that the digestive organ of *Aplysia* normally contains large numbers of marine bacteria. Several strains of these were separated from the organ and cultivated. All attempts to extract cytochrome *h*, either from *Ulva lactuca* or from large cultures of bacteria, gave negative results. It seems therefore that cytochrome *h* is a cellular component of *Aplysia* hepatopancreas.

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APPENDIX

In *Aplysia* the blood fills the body or hemocoele, from which it is collected into the venous sinuses. It is colorless and contains ameboid corpuscles (Cuénot, 1890). As soon as the blood is extracted from the body cavity or from the heart, even in the absence of air, a slight precipitation of the proteins is observed which increases within a few minutes. It has been claimed that in *Aplysia* "the oxygen carrier is hemocyanin, dissolved in the plasma" (Eales, 1921).

Iron and copper have been determined in the blood of both *A. depilans* and *A. limacina*. The results obtained show that the blood of *Aplysia* does not contain significant amounts of copper (Table VIII). Moreover the absence of any oxygen carrier was confirmed by manometric experiments: the oxygen content and the oxygen capacity of *Aplysia* blood were found to be equal to those of sea water (Table IX).

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