THE CYTOCHROME SYSTEM IN THE CECROPIA SILKWORM, WITH SPECIAL REFERENCE TO THE PROPERTIES OF A NEW COMPONENT*

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INTRODUCTION

To a considerable degree, our knowledge of the cytochrome system in insects is based on the initial studies of Keilin (5, 6). Subsequently, in the absence of more detailed appraisal of this enzyme system in insects, two assumptions have seemed acceptable: (1) that the cytochrome system is of general distribution in the tissues of insects; and (2) that the system is organized to include the classical components, a, b, c, and cytochrome oxidase.

During the past 3 years we have found exceptions to both these assumptions. Studies performed on the *cecropia* silkworm and its near relatives have demonstrated that the cytochrome system undergoes marked alterations during the lifespan of the insect. Quantitative alterations, which will be considered elsewhere in greater detail, consist of a breakdown of the cytochrome system at the end of larval life and its resynthesis at the outset of adult development.

For our present purposes, we may state that the progress of metamorphosis is also accompanied by qualitative changes in the cytochrome system. Whereas the adult insect contains a system that includes the classical components a, b, and c, in the caterpillar this type of organization is encountered only in the heart and intersegmental muscles. Elsewhere in the larval insect the components b and c are replaced by an apparently undescribed component which may conveniently be termed cytochrome x (20).

In the reduced state this new component is characterized by a broad and indivisible absorption band extending between 551 and 562 mμ. Although present in all larval tissues except the heart and intersegmental muscles, it occurs in highest concentration in the walls of the midgut. In all these sites, it is invariably accompanied by a second cytochrome that is spectroscopically identical with the usual $a + a_3$—a complex which is generally presumed to include cytochrome oxidase (7).

In consequence of the breakdown of the cytochrome system late in larval life (at the beginning of the prepupal stage), x disappears abruptly and completely at that time and cannot be demonstrated in the pupa or in the adult

moth. x is, therefore, a cytochrome whose function is apparently confined to the larval stage of the insect. During this period, however, the presence of x and the apparent absence of b and c reflect an unusual organization of the cytochrome system.

In the present investigation we have attempted to gain some insight into the nature of this organization with special reference to the functional significance of cytochrome x.

Materials and Methods

1. Preparation of Tissues.—Midguts of full grown *cecropia* silkworms proved to be the most favorable tissue for studying the cytochrome system in the larval insect. Caterpillars were anesthetized with carbon dioxide (19), opened longitudinally, and the relatively enormous midgut removed intact. Each gut was cut open, washed in insect Ringer's solution, and used in the various studies outlined below.

In many experiments the midguts were converted to brei by homogenization in insect Ringer's solution, according to the method of Potter and Elvehjem (14). Such homogenates were freed from endogenous substrates by a series of three or four centrifugations (20 minutes at 11,000 × g) and resuspensions in Ringer's solution. Final suspensions were made with 0.1 M phosphate buffer at pH 7.4.

2. Reagents.—

(a) *Water.* Glass-redistilled water was used throughout.

(b) *Phosphate buffer.* 0.4 M phosphate, pH 7.4, was prepared by titrating 0.4 M KH_2PO_4 into 0.4 M $Na_2\ HPO_4$ until the proper pH, as measured by the glass electrode, was attained.

(c) *Insect Ringer's solution* was prepared according to the formula of Ephrussi and Beadle (3).

(d) *Sodium succinate* was recrystallized according to the method of Potter and Schneider (15), dissolved in water, and brought to pH 7.4 with dilute HCl.

(e) *Cytochrome c* was prepared by the method of Keilin and Hartree (9) and dialyzed against redistilled water. Material purchased from the Sigma Chemical Co., St. Louis, was equally satisfactory.

(f) *Malonic acid.* Merck "reagent grade" was dissolved in water, neutralized with NaOH, and diluted to 1.0 M.

(g) *Potassium cyanide.* Mallinckrodt "analytical reagent" was used without further purification. It was assumed to be 100 per cent KCN in calculations of molarities.

3. Spectroscopic Studies.—

(a) *Of intact midguts.* Large fragments were cut from midguts and folded into a plastic sleeve 5 mm. in height and 7 mm. in internal diameter. This sleeve rested on a glass slide which was placed on the stage of a compound microscope and viewed in transmitted light, using a low power objective and a Zeiss spectroscopic ocular. Reagents were added by draining and flooding the plastic chamber.

(b) *Of homogenates.* Homogenates were placed in Carrel flasks having a light path of approximately 10 mm. and viewed in transmitted light by means of a Hartridge reversion spectroscope.

4. Manometric Studies.—Warburg manometers having vessels of 15 ml. capacity

were used. Homogenate, buffer, substrates, inhibitors, and water were added, according to schedules outlined below, to yield total fluid volumes of 3 ml.; 0.2 ml. of 20 per cent KOH was placed in the center well along with the customary strip of filter paper. All experiments were performed at 25.2 ± 0.05°C. at a shaking rate of 110 to 120 cycles/min. and an amplitude of 2 cm.

Cytochrome oxidase and succinoxidase activities were assayed by the methods of Schneider and Potter (13, 17), as modified by Quinlan-Watson and Dewey (16). Inhibition studies using potassium cyanide were performed according to the advice of Laties (12).

<div align="center">RESULTS</div>

1. Spectroscopic Observations.—

(a) *On intact midguts.* When viewed with an ocular spectroscope intact segments of larval midgut show the absorption bands diagrammed in Fig. 1. The broad band between 551 and 562 mμ is a distinguishing feature of cytochrome *x*. Subdivision of the band is never observed even when the optical depth of
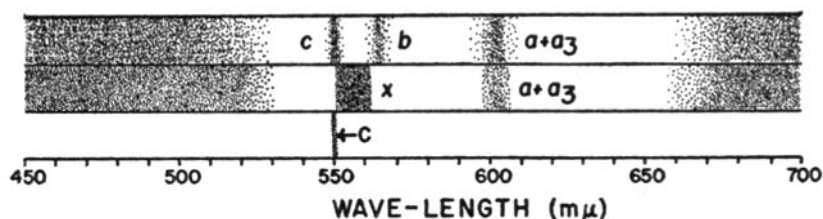

Fig. 1. Absorption spectra of reduced components. Top, thoracic muscles from adult *cecropia* moth. Middle, midgut of full grown larva. Bottom, solution of pure cytochrome *c*.

the preparation is greatly reduced. For the sake of comparison, the spectrum of the cytochromes in the thoracic muscles of the adult *cecropia* moth is also diagrammed in Fig. 1, along with that of an aqueous solution of pure, reduced cytochrome *c*. Though all the pertinent bands occur within a narrow zone of the spectrum, there can be little doubt that cytochrome *x* differs spectroscopically from *b* and *c*.

(b) *On homogenates.* The data from spectroscopic observation of homogenates are summarized in Table I.

The results show that, like cytochrome *b* in the presence of succinic dehydrogenase, cytochrome *x* is reduced by succinate. But, like cytochrome *c* and unlike *b*, it is also reduced by ascorbate (2, 18). Moreover, like cytochrome *c* and unlike *b*, its oxidation is blocked by cyanide. But, like cytochrome *b* and unlike *c*, its oxidation is also blocked by urethane (5). It is therefore evident that cytochrome *x* shows a combination of certain of the properties of cytochromes *b* and *c*.

Finally, we may note the curious fact that malonate blocks the reduction of x, not only by succinate, but also by ascorbate. This observation is of interest since, in the classical organization of the cytochrome system, a distinguishing feature of the action of ascorbate is its direct reduction of cytochrome c without the intervention of succinic dehydrogenase or of cytochrome b: for this reason malonate does not ordinarily interfere with the oxidation of ascorbate by cytochrome c. Yet, we observe in Table I that malonate does interfere with the oxidation of ascorbate by cytochrome x. Thus, this presumably specific

TABLE I

Time for Reappearance of Absorption Bands at 557 mμ and 603 mμ after Equilibration of Midgut Homogenates with Air

Components	Time for reappearance	Remarks
	min.	
1. Unwashed homogenate	15–20	
2. Washed homogenate	>240	Promptly reappears if ascorbate or succinate is added
3. Washed homogenate + succinate	2–3	
4. Washed homogenate + ascorbate	<1	
5. Washed homogenate + succinate + KCN (0.001 M)	0	Bands fail to disappear on aeration
6. Washed homogenate + ascorbate + KCN (0.001 M)	0	" "
7. Washed homogenate + succinate + malonate (0.01 M)	>90	Faintly visible after 4 hrs.
8. Washed homogenate + ascorbate + malonate (0.01 M)	>90	
9. Washed homogenate + succinate + urethane	557 band = 0 min. 603 band = 3 min.	Band at 557 mμ fails to disappear on aeration

inhibitor of succinic dehydrogenase must gain intimate access to cytochrome x —a result which suggests that succinic dehydrogenase, itself, is a part of cytochrome x.

2. Manometric Studies.—

(a) *Cytochrome oxidase in midguts.* As previously indicated, cytochrome x is invariably accompanied by an absorption band at 603 mμ. Since this component, $a + a_3$, is thought to include cytochrome oxidase, manometric studies were performed on homogenates of midguts, according to the schedule outlined in Fig. 2. The curves in this figure show that washed homogenates of midguts contain a high cytochrome oxidase activity, proportional to the concentration of brei, and amounting to 210 microliters of oxygen per hour per milligram of

nitrogen. Negligible activity is observed in the absence of ascorbate, or in the presence of cyanide, or after exposure to 100°C. for 10 minutes.

(b) *Succinoxidase in midguts.* By the use of succinate as substrate, the presence of an intact succinoxidase system was demonstrated. As indicated in Fig. 3,
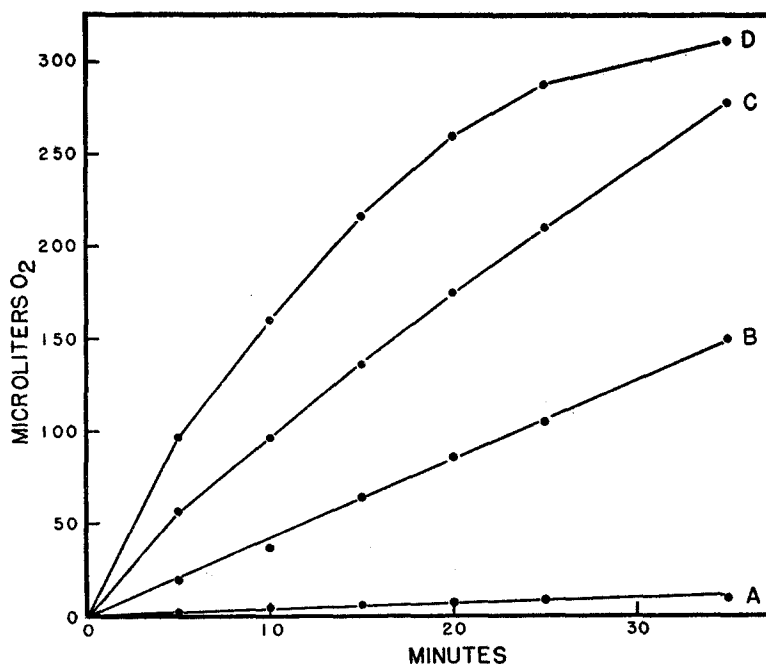

FIG. 2. Cytochrome oxidase assay on homogenates of midguts. Total volume 3.0 ml. Flasks contained the following materials (final concentrations specified). All flasks contained 0.04 M phosphate, pH 7.4.

A. Flasks with heated homogenate (1.3 per cent); or 0.01 M KCN; or without ascorbate. (All essentially the same.)

B. Ascorbate 0.014 M; homogenate 1.3 per cent.

C. Ascorbate 0.014 M; homogenate 2.6 per cent.

D. Ascorbate 0.014 M; homogenate 2.6 per cent; cytochrome c 1.6 $\times$ 10^{-4} M.

succinoxidase activity was about one-tenth as great as that of cytochrome oxidase.

(c) *Action of malonate on cytochrome oxidase and succinoxidase activities.* Experiments described in (a) and (b) above were repeated with the addition of malonate (0.01 M). Results are summarized in Table II. We observe that malonate inhibited the oxidation of succinate and ascorbate to the same degree. This result confirms the spectroscopic observations in Table I, and is further sup-

port for the proposition that cytochrome x combines the properties of succinic dehydrogenase and of cytochromes b and c.
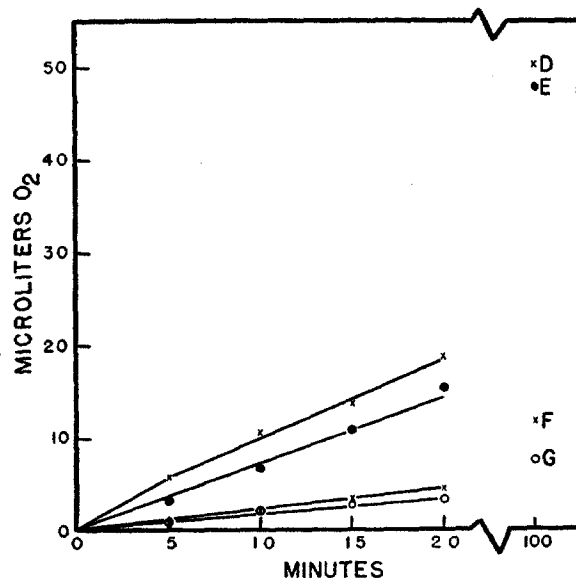

FIG. 3. Succinoxidase assay of midgut homogenates (final concentrations specified) All flasks contained 2 per cent homogenate and 0.04 M phosphate, pH 7.4. In addition they contained the following substances and distilled water to give a final volume of 3.0 ml.

D. 0.013 M succinate, pH 7.4.

E. 4.8 × 10⁻⁵ M cytochrome c, 0.013 M succinate, pH 7.4.

F. 0.01 M KCN, 0.013 M succinate, pH 7.4.

G. No succinate.

TABLE II

Effects of Malonate on Cytochrome Oxidase and Succinoxidase Activities of Washed Homogenates of Midguts

Test system	Q_{O_2} (N)	Inhibition
	μl. O₂/mg. N/hr.	*per cent*
Succinoxidase system + NaCl (0.01 M)	29	
Succinoxidase system + malonate (0.01 M)	20	31
Cytochrome oxidase system + NaCl (0.01 M)	424	
Cytochrome oxidase system + malonate (0.01 M)	306	28

3. Attempts to Extract Cytochrome x.—With the exception of cytochrome c, the components of the succinoxidase system are bound to subcellular particles

from which their extraction is difficult or impossible. Cytochrome x also be-
haves in this manner. Extraction was unsuccessful in phosphate buffer of vary-
ing molarities and pH values, trichloracetic acid, alkaline-isotonic-KCl, strong
KCl solution, distilled water, or alkali. Therefore, in this respect, cytochrome x
differs from cytochrome c and resembles b, a, and succinic dehydrogenase.

DISCUSSION

The experimental results indicate that cytochrome x is an enzyme which
combines certain of the properties of succinic dehydrogenase and of cytochromes
b and c. As far as we can discover, such a substance has not been described
previously. In terms of the position of its absorption band, cytochrome x re-
sembles a cytochrome designated as b_2 by Bach, Dixon, and Zerfas (1), and a
cytochrome designated as b_1 by Keilin (8). The relation of cytochrome x to
these two components will now be briefly considered:

1. *Relation to Cytochrome b_2 (Lactic Dehydrogenase of Yeast).*—Although the
cells of bakers' yeast present the classical spectrum of cytochromes b, c, and a,
Bach et al. report the presence of a further component which, after extraction
and purification, is characterized in the reduced state by an absorption band
at 556 mμ. Further studies served to identify this "cytochrome b_2" as the
lactic dehydrogenase of yeast.

In view of the spectroscopic similarity between cytochrome b_2 of yeast and
cytochrome x of insects, we have performed Thunberg studies on homogenates
of midguts, according to the method of Potter (13) and Bach et al. (1). The
time for reduction of 1:10,000 methylene blue in these experiments was: with
succinate, 4 minutes; with lactate, incomplete reduction in 90 minutes. In the
absence of any significant activity as lactic dehydrogenase, it seems certain
that cytochrome x is a different enzyme from b_2 of yeast.

2. *Relation to Cytochrome b_1.*—According to Keilin and Hartree (8), "In
higher organisms a typical and well developed cytochrome system with its
characteristic absorption spectrum is found mainly in skeletal muscles, in
heart muscles, and in the grey matter of the brain. In cells of most other tissues
such as kidney, liver, pancreas, etc., cytochrome is somewhat modified. Spectro-
scopically . . . the bands of b and c are replaced by a broad band, b_1." This type
of organization is also observed in certain microorganisms, such as *Bacillus
licheniformis* and *B. megatherium* (10).

These observations are of unusual interest since b_1 is, therefore, spectro-
scopically identical with the cytochrome x of insects. More recently, Keilin (11)
has demonstrated that b_1 is, in fact, a complex resulting from the fusion of the
absorption bands of cytochrome b and c with that of a new cytochrome, termed
cytochrome e. For, at the temperature of liquid air, the spectrum of b_1 splits
into the separate bands of these three components. Furthermore, even in the
thoracic muscles of bees and in many other organisms in which b_1 is absent and

in which the system is organized according to the classical scheme, the absorption band of the new cytochrome e becomes visible at the temperature of liquid air.

For our present purposes, the relation of cytochrome x to the complex of cytochromes, b_1, or to Keilin's new cytochrome e, is of obvious significance. On this account we have applied the method of low temperature spectroscopy to homogenates of midguts, according to the method of Keilin (10, 11).[1] Control observations were made on the thoracic muscles of the blowfly, *Phormia regina* (4). The results are recorded in Table III.

The flight muscle presented a spectrum identical to that reported for this

TABLE III

Spectrum of Components of Cytochrome System at Room Temperature and at Temperature of Liquid Air

Source	Component	Position of absorption	
		At 25°C.	At −196°C.
		$m\mu$	$m\mu$
Flight muscle	$a + a_3$	603	598
" "	b	565	562
" "	c	550	547, 546*
" "	e	Not visible	554
Midgut	$a + a_3$	603	598
"	x	551–562	554

* Satellite band.

tissue by Keilin. But, in preparations of midgut, the spectrum of cytochrome x behaved differently from that described by Keilin for b_1. Instead of splitting into components, the broad band of cytochrome x underwent contraction at the temperature of liquid air to yield a single, narrow band at 554 mμ. Thus, the attempt to establish a relation between cytochrome x and the complex b_1 had the unexpected result of demonstrating that cytochrome x was spectroscopically identical with Keilin's cytochrome e.

Cytochrome e is reported by Keilin to have as wide a distribution as the other components of the cytochrome system. However, attempts to explore its kinetics are handicapped by two serious difficulties: (1) on account of its low concentration, its spectrum is visible only at the temperature of liquid air, and, thus, under circumstances incompatible with kinetic studies; and (2) it

[1] We are indebted to Professor J. R. Loofbourow and Dr. J. F. Scott, of the Massachusetts Institute of Technology, for their assistance in these spectroscopic studies at low temperatures.

has been reported only in the company of succinic dehydrogenase and cyto-chromes b and c, so that kinetic studies are complicated by the functions of these other components.

The possibility that cytochromes x and e are merely quantitative variations of the same enzyme is therefore of technical interest, since the study of cyto-chrome x is not handicapped by the above-mentioned difficulties. Under these circumstances the properties of cytochrome e become accessible through the study of the cytochrome in the larval insect. Of greater theoretical interest are the observations that, (1) the cytochrome oxidase of the caterpillar is active in the apparent absence of cytochrome c, the substance which is nor-mally considered to be its only substrate, and (2) the succinic dehydrogenase activity in the larval tissue is inseparably associated with the new cytochrome.

SUMMARY

1. In the majority of tissues in the *cecropia* silkworm, cytochromes b and c are apparently absent, being replaced by a hitherto undescribed component which we have tentatively termed cytochrome x.

2. Spectroscopically, the new cytochrome is characterized in the reduced state by a broad and indivisible absorption band extending from 551 to 562 mμ.

3. The enzyme can be demonstrated only in the larval stage of the insect and undergoes breakdown prior to pupation. It occurs in highest concentra-tion in the walls of the larval midgut and resists extraction, being apparently bound to subcellular particles.

4. Evidence is presented, based on spectroscopic and manometric studies in the presence of various substrates and inhibitors, that cytochrome x is a single component which mimics certain of the properties of cytochromes b and c and of succinic dehydrogenase.

5. Detailed studies served to differentiate cytochrome x from the cyto-chromes b_1 and b_2 which it resembles spectroscopically.

6. On the basis of spectroscopic studies at the temperature of liquid air, it is concluded that cytochrome x closely resembles, and may be identical with, the component which Keilin has recently described as cytochrome e.

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