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REDOX STATE OF RESPIRATORY CHAIN ENZYMES AND POTASSIUM TRANSPORT IN SILKWORM MID-GUT

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SUMMARY

The midgut of *Hyalophora cecropia* actively transports potassium from hemolymph to lumen and the energy for this process appears to be intimately linked to oxidative metabolism. In the present investigation, we monitored concurrently the rate of active transport and the redox levels of the components of the respiratory chain in the intact tissue under a variety of experimental conditions. Approximately equal concentrations of cytochromes a_3, a, c and b-557 were found. Other investigators (Pappenheimer, Jr, A. M. and Williams, C. M. (1954) J. Biol. Chem. 209, 915, Shappirio, D. G. and Williams, C. M. (1957) Proc. R. Soc. Lond. Ser. B 147, 233 and Chance, B. and Pappenheimer, Jr, A. M. (1954) J. Biol. Chem. 209, 931) have identified cytochrome b-557 with b_5 and found that it exists primarily in an extramitochondrial location.

Steady-state experiments demonstrated that all these cytochromes were approximately 50 % reduced while active transport proceeded at a high rate in regular cecropia Ringer containing 32 mM KCl. When the potassium concentration was reduced, the active transport decreased and all the cytochromes became more oxidized. Addition of 1 mM cyanide inhibited active transport by 90 % and caused a 100 % reduction of all cytochromes. Redox state and short circuit current (I_{sc}) kinetics measured as the tissue was made anoxic showed that all the respiratory enzymes, except cytochrome b-557, became fully reduced at a faster rate than the rate of inhibition of the I_{sc}. The rate of cytochrome b-557 reduction followed kinetically the I_{sc}.

These observations are interpreted in a scheme where cytochrome b-557 (possibly b_5) branches off cytochrome c from the conventional respiratory chain, utilizing cytochrome a_3 as the terminal oxidase for both branches. Cytochrome b-557 may be involved in providing a direct link between oxidative metabolism and active transport in the midgut of the silkworm.

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INTRODUCTION

The midgut of the silkworm (*Hyalophora cecropia*) actively transports potassium from blood (hemolymph) to lumen at a comparatively high rate during its last stages of larval development [1]. The energy for this process appears to be derived mainly from oxidative metabolism, since the transport process is extremely oxygen dependent [2]. The midgut epithelium consists of a single layer of two types of cells: goblet cells and columnar cells [3]. Potential profiles of the isolated gut [4] indicate that the transport site is located on the lumen side of the epithelium. Which cell type is responsible for the potassium transport has not been directly established, but the morphology of the apical border of the goblet cells is reminiscent of other "mitochondrial pumps" and has led Anderson and Harvey [3] as well as Harvey and Zerahn [5] to propose that the goblet cell alone is active in potassium transport. The apical cell membrane of the goblet cells is thrown into microvilli which project into the cavity. Each microvillus contains a mitochondrion. Of particular interest is the appearance of areas in which the mitochondrial outer membrane seems to fuse with the cell membrane of the microvillus [3]. This observation and the lack of demonstrable $(Na^+ + K^+)$-ATPase activity in mitochondria-free cell membrane preparations of the gut epithelium (Wood and Ellory, personal communication) suggest the possibility of a direct coupling between the mechanism of oxidative metabolism and the mechanism for active transport of potassium [6].

The present investigation seeks to study the coupling process between respiration and active transport by simultaneously monitoring the rate of active transport and the redox state of the respiratory chain enzymes under conditions which affect specifically either the respiratory chain or the transport process.

METHODS

Instrumentation

The spectrophotometric equipment used is of two types: (1) differential wavelength-scanning (the "split beam"), in which the light from a monochromator falls alternately on two samples and the differences in optical absorption are recorded as a "difference spectrum" (Yang and Legallais [7] and Yang [8]), and (2) dual-wavelength differential ("the double beam"), in which two beams of light at different wavelengths alternately fall on a single sample and the differences in optical absorption measure the redox state of a respiratory enzyme as a function of time (Chance [9, 10]).

The chamber utilized for these studies is shown in Fig. 1. It is essentially a flattened version of an Ussing double-chamber with clear windows to permit spectrophotometric measurements. Each pair of half-chambers is connected by a slit measuring 15×1.5 mm. Fastening the gut pieces over the slits separating the half-chambers was facilitated by a grooved ridge surrounding each slit; the gut pieces were tied down over the slits with thread loops which fit into the grooves making a tight seal with little edge damage. Each of the four half-chambers contained 15 ml of cecropia Ringer solution, which was stirred and oxygenated by vigorous bubbling with 100 % oxygen. The potential difference across each tissue was measured with calomel electrodes and current was passed through the midgut via Ag/AgCl electrodes. Both types

of electrodes were connected to the bathing solutions; these bridges were introduced through a plastic cap on top of the chamber. An automatic voltage clamp that compensated for the resistance of the solution between the potential difference bridges

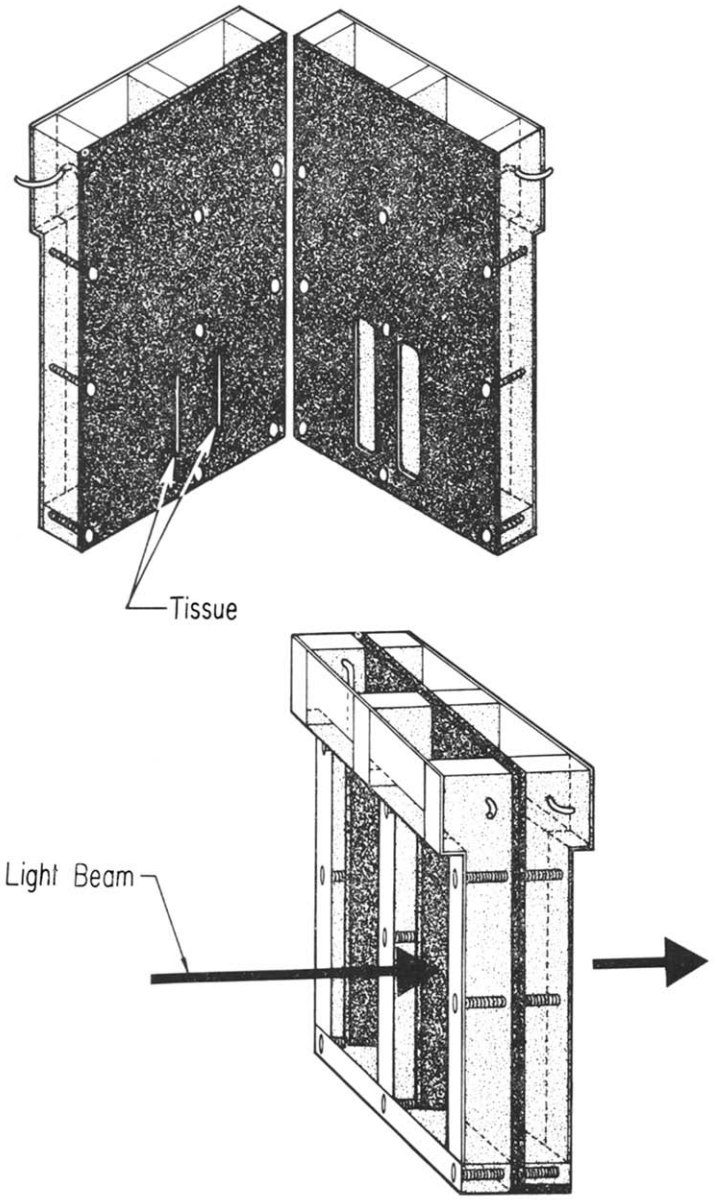

Fig. 1. Schematic diagram of the chamber utilized in the present studies. (a) Shows the slits on which the tissue was mounted. (b) Shows the two sides of the chamber screwed together. Four electrically isolated compartments are separated when the pieces of tissue are in place, allowing the measurement of the potential difference and I_{sc} for each tissue. Light was passed through the chamber in the direction indicated by the arrow.

was used to pass the appropriate current through the midgut to maintain short circuit conditions. The short circuit current (I_{sc}) obtained in this manner was utilized as a measure of net potassium transport as demonstrated by Harvey et al. [11]. Concurrently, the light beams were passed through the chamber for the spectrophotometric measurements, as shown in Fig. 1b.

Biological materials and solutions

Midguts were removed from mature fifth-instar larvae of *Hyalophora cecropia* (L) using techniques similar to those of Nedergaard and Harvey [12]. For the experiments with the split beam, the midgut was divided longitudinally to yield two pieces as nearly symmetrical as possible, which were mounted on the two slits shown in Fig. 1. In the double-beam experiments the whole excised midgut was mounted on one side and the light beams directed to that side. After the tissue was mounted it was allowed to equilibrate at the appropriate temperature for approx. 1 h in a temperature-controlled holder which could be attached to either spectrophotometer. The short circuit current of each tissue sample was monitored and the experiment started after the currents had equilibrated.

The composition of regular cecropia Ringer was 32 mM KCl, 166 mM sucrose, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM Tris · HCl. In some experiments low potassium Ringer was utilized; in this solution 28 mM choline was substituted for 28 mM KCl. Uncouplers and inhibitors of oxidative metabolism were added to these solutions at times and concentrations indicated in Results.

RESULTS

Composition of the cytochrome chain

A typical reduced minus oxidized spectrum of the silkworm midgut at 20 °C is shown in Fig. 2. This is a difference spectrum obtained with the split beam apparatus. The tissue in one pair of half-chambers is in an anoxic, reducing environment obtained by adding sodium dithionite and bubbling with 100 % N_2, and its respiratory chain enzymes are 100 % reduced. The tissue in the other half-chamber is in an environment with an excess of dissolved oxygen obtained by bubbling with 100 % O_2 and the addition of H_2O_2; its respiratory chain enzymes are 100 % oxidized. The difference spectrum under these circumstances reflects the absorbance changes caused by the reduction of each of the cytochromes and can be used to calculate their relative amounts. This spectrum shows the well-known absorption peaks for the reduced cytochrome $a(+a_3)$ at 605 nm, $c(+c_1)$ at 550 nm, and $a_3(+a)$ at 445 nm. The

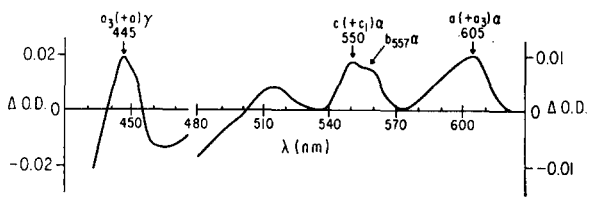

Fig. 2. Typical reduced minus oxidized difference spectrum of intact silkworm midgut. Notice the presence of cytochromes a_3, a, $c(+c_1)$, and b-557 with peaks of approximately equal heights.

TABLE I

AVERAGE RELATIVE CONCENTRATIONS OF CYTOCHROMES IN *H. CECROPIA* MIDGUT ($n = 7$)

λ_1, λ_2 = peak and reference wavelengths (nm), respectively. E = extinction coefficient ($mM^{-1}cm^{-1}$), as calculated by Chance and Williams [13] (the value for cytochrome *b*-557 was assumed to be equal to that of *b*-564).

Component	λ_1–λ_2	E	Average relative concentration
a	605–630	16	1.0
a_3	445–465	91	0.76
$c+c_1$	550–540	19	0.74
b-557	557–575	20	0.62

absorption peak at 564 nm characteristic of cytochrome *b* is not apparent in this spectrum, or it may be masked by the larger peak observed at 557 nm. By arbitrarily referring all the concentration values to cytochrome *a* the relative molecular composition of the chain can be calculated from the respective extinction coefficients at the wavelengths indicated in Table I. The average relative concentrations shown in that table indicate that all these cytochromes are present in approximately equimolar amounts, although the average cytochrome *b*-557 concentration is slightly lower than the others.

Cytochrome redox state and active transport inhibition

The relationship between the cytochrome redox level and the rate of active transport was tested in the split beam apparatus under a variety of experimental conditions known to affect active transport. Since a direct relationship exists between the K^+ concentration of the bathing medium and active transport rate in this tissue [11], one series of experiments was performed to test the effects of K^+ concentration on cytochrome redox level. The action of cyanide was tested thereafter in the same experimental series. The experimental protocol was as follows:

(1) Control: the two halves of the gut were initially bathed in regular cecropia Ringer (32 mM K^+) to obtain baseline values for the short circuit current (I_{sc}) and for the difference in optical spectra between the two halves.

(2) Low K^+: the solution bathing one gut half (experimental side) was changed to low K^+ Ringer (4 mM KCl+28 mM choline chloride). The solution bathing the reference gut half remained unchanged through all these steps.

(3) Recovery: returned to the same condition as under control.

(4) Cyanide: 1 mM NaCN (final concentration) added to the experimental side.

(5) 0 % Reduction: washed out cyanide until recovery of the I_{sc} was obtained in regular Ringer bubbled with 100 % O_2. At this time, H_2O_2 was added to the experimental side. The measured optical absorption in this condition provided the 0 % reduction level of the tissue in the experimental side.

(6) 100 % reduction: the regular cecropia Ringer was now bubbled with 100 % N_2 to which sodium dithionate was added. The optical absorption in this condition provided the 100 % reduction level of all cytochromes in the experimental side.

A typical difference spectrum obtained by subtracting the baseline spectrum

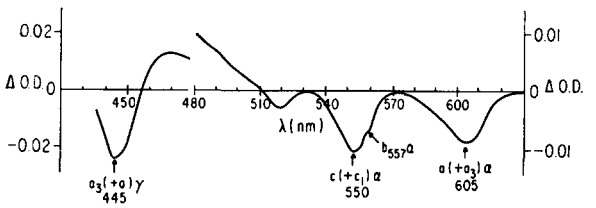

Fig. 3. Difference spectrum of the midgut obtained when the experimental side is placed in a low K^+ Ringer solution and the control side is in regular Ringer. All the cytochromes become oxidized under these conditions.

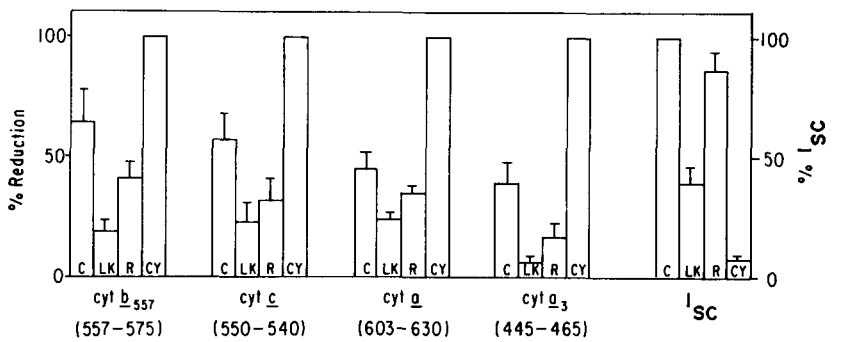

Fig. 4. Percentage reduction and short circuit current (I_{sc}) for tissue which followed the full protocol described in Results. C, control; LK, low K^+; R, recovery; CY, cyanide. Wavelengths in parentheses are in nm.

obtained in Step 1 from the low K^+ spectrum obtained in Step 2 (a measure of the change in redox state resulting from the low potassium treatment) is shown in Fig. 3. All the cytochromes are seen to become oxidized in the low K^+ solution.

The average results of seven experiments following the full protocol are shown in Fig. 4. The redox levels observed in Steps 1–4 are expressed as percentages on the optical absorption scale determined in Steps 5 and 6; the active transport rate is also shown for comparison purposes. One surprising result is that all the cytochromes, including *a* and a_3, display a substantial level of reduction under control conditions when the tissue is actively transporting K^+ at a high rate. When the K^+ concentration is reduced to 4 mM, the active transport rate falls to less than half while all the cyto-

TABLE II

PERCENTAGE CHANGES IN REDOX STATE AND POTASSIUM TRANSPORT REDUCTIONS ARE POSITIVE AND OXIDATIONS ARE NEGATIVE

Experimental condition	Cytochromes				I_{sc}
	a_3	*a*	*b*-557	*c*	
Cyanide ($5 \cdot 10^{-4}$ M)	36	36	27	24	−10
Dinitrophenol ($5 \cdot 10^{-5}$ M)		24	30	18	−53
Amytal ($5 \cdot 10^{-3}$ M)	−16	−22	−18	−41	−74
Oligomycin (20 μg/ml)	0	0	0	0	0

chromes become oxidized. Return of regular Ringer to the bathing solutions causes reductions in all the cytochromes but not a recovery to the initial redox level; in contrast, there is almost complete recovery of the active transport rate. Adding 1 mM NaCN to the experimental tissue causes complete reduction of the cytochromes and virtually complete inhibition of active transport in this tissue.

Blockers and uncouplers of the respiratory chain have diverse effects on the cytochrome redox state and the rate of active transport in cecropia midgut. Results of experiments utilizing cyanide, dinitrophenol, amytal, and oligomycin are summarized in Table II. Cyanide at a concentration of 0.4 mM produced a lesser reduction of the cytochromes and a smaller decrease in I_{sc} than 1 mM. Both amytal and dinitrophenol inhibited active transport, but caused opposite changes in the respiratory chain components: amytal oxidized the chain, whereas dinitrophenol reduced it. Oligomycin did not affect the I_{sc} nor did it produce a significant change in redox state; since it is not known whether oligomycin enters the cells of this tissue, no conclusions can be drawn from these results.

Kinetic studies

Concurrent changes in redox state and I_{sc} were monitored in this tissue as it was made partially anoxic by bubbling with 100 % N_2 for 4–5 min, and subsequently reoxygenated and returned to control conditions with 100 % O_2. The double-beam spectrophotometer was utilized to measure continuously the redox state of a single cytochrome, while the I_{sc} was measured simultaneously during the cycle to partial anoxia and reoxygenation. Since only one respiratory enzyme could be measured at a time with this apparatus, the cycle had to be repeated for each component of the cytochrome chain. This presented little problem because the cycle was fully reversible

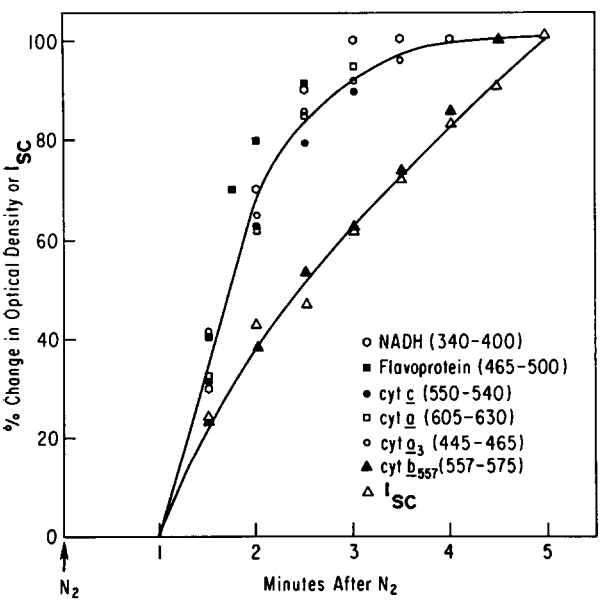

Fig. 5. Percentage changes in absorbance and I_{sc} as the tissue was made anoxic with N_2 added at time 0. Symbols are shown in the figure. Wavelengths in parentheses are in nm.

if the short circuit current was allowed to be inhibited by anoxia to no more than 80 % of its control value. This maneuver stopped the transient decay in I_{sc} before a new steady state was established, as shown in Fig. 5. During reoxygenation, on the other hand, the I_{sc} returned to the relative steady state which obtains under control conditions (Fig. 6). The experiments were performed at 13 °C to better resolve the kinetic responses of the respiratory enzymes; their redox changes were measured at the peak-and-reference wavelength pairs indicated in the figures. All values were normalized to the maximal change for each variable to allow direct comparisons of rates of change of each species. In the transition to anoxia (Fig. 4) it can be seen that NADH, flavoprotein, cytochromes *c*, *a*, and a_3 have a relatively rapid time course, reaching 100 % reduction while the I_{sc} is still decreasing (plotted upward for comparison purposes). The rate of change shown by cytochrome *b*-557 is clearly slower, and its transient reduction seems to closely match the rate of decrease of the I_{sc}.

A similar pattern is obtained for the kinetic changes due to reoxygenation of the tissue, shown in Fig. 6. NADH, flavoprotein, cytochromes *c*, *a* and a_3 became reoxidized at a rate at least twice as fast as that of the recovery of the I_{sc}. In this part of the cycle, cytochrome *b*-557 became reoxidized at a considerably slower rate than the recovery of the I_{sc} or the rate of reoxidation of any of the other components of the cytochrome chain.

The average results of the experiments in which midguts were subjected to the anoxia-reoxygenation cycle are shown in Table III. The results are expressed in terms of half-times calculated as the time to 50 % reduction or reoxidation of respiratory chain enzymes and the corresponding time to 50 % inhibition or recovery of the I_{sc}. No significant difference was found among the half-times of NADH, flavoprotein, cytochrome *a* and a_3 for either reduction or oxidation steps; therefore, these were all

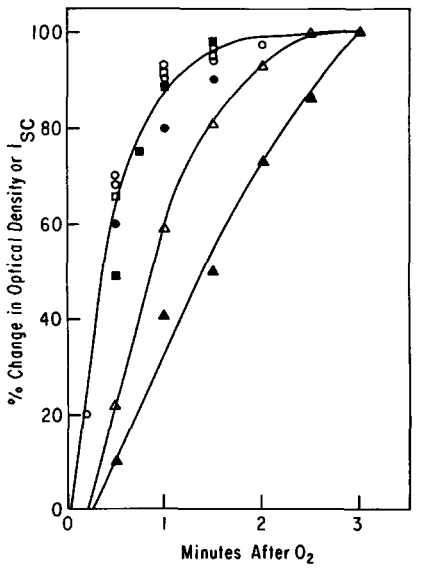

Fig. 6. Percentage changes in absorbance and I_{sc} as the tissue was reoxygenated following partial anoxia. All the symbols are as in Fig. 5.

TABLE III

AVERAGE HALF-TIMES FOR TRANSITIONS TO ANOXIA AND REOXYGENATION

Values are means ±S.E. at 13 °C. Number of experiments in parentheses.

Measured parameter	t_{50} $O_2 \rightarrow N_2$ (min)	t_{50} $N_2 \rightarrow O_2$ (min)
NADH, flavoproteins, cytochromes *c*, *a* and a_3	1.89±0.07 (11)	0.44±0.03 (11)
Cytochrome *b*-557	2.41±0.30 (4)	1.43±0.23 (3)
I_{sc}	2.43±0.06 (15)	0.85±0.02 (14)

averaged together in the table. No significant differences were shown between the half-times of *b*-557 reduction and I_{sc} inhibition during the transition to anoxia. On the other hand, there is a highly significant difference ($P < 0.001$) between the average half-times for the respiratory chain components and the half-times of both *b*-557 and the I_{sc}. Since the need for reproducibility of the cycle did not allow time for completion of the cytochrome *b*-557 reductions whereas the other components did reach their maximal effects, the differences in half-times actually underestimate the differences in kinetics.

The reoxygenation part of the cycle also showed no significant differences among the half-times of the various respiratory chain components but a significant difference between these and the half-times for *b*-557 and I_{sc}. In contrast to the situation in the hypoxic step, *b*-557 became reoxidized significantly more slowly than than the recovery of the I_{sc}.

DISCUSSION

Composition of the respiratory chain

The optical spectrum for the intact tissue (Fig. 2) shows the presence of large peaks for cytochromes *a*, a_3, *c* ($+c_1$) and *b*-557. The results shown in Table I indicate that similar concentrations of all these cytochromes are present in cecropia midgut. The present observations contrast with those of Pappenheimer and Williams [14] and Shappirio and Williams [15], who found a very low concentration of cytochrome *c*, as compared to cytochrome *a*, in tissue homogenates of midgut. Since cytochrome *c* is quite water-soluble [16], it is possible that upon homogenization it dissolved in the supernatant and therefore, only low concentrations of this cytochrome were found in the studies by the aforementioned investigators [14, 15].

Pappenheimer and Williams [14] and Shappirio and Williams [15], working with tissue homogenates, found that the most prevalent cytochromes in this tissue were $a+a_3$ and *b*-557. In these studies, the broad absorption band from 550 to 565 nm with a peak at 557–558 nm was intense enough to obscure the α peak of cytochrome *b*. When a mitochondrial fraction was separated from a microsomal fraction by these investigators, most of the *b*-557 band appeared in the microsomal fraction whereas a small amount of *b*-564 could be identified in the mitochondrial fraction. These results,

as well as kinetic studies by Chance and Pappenheimer [17] led these investigators to the conclusion that cytochrome *b*-557 was identical with b_5.

The present studies did not attempt to clarify the identity of *b*-557. Much more work is needed before the nature of this cytochrome is clearly established, especially, because of the more recent identification of multiple *b*-type cytochromes [18]. However, the weight of the aforementioned evidence favors its identity with b_5, and a probable distribution on the outer mitochondrial membrane and the microsomes.

Relationship between cytochrome b-557 and the conventional respiratory chain

Although cytochrome *b*-557 might be located outside the mitochondrion [15], its redox state was always found to respond in concert with that of the mitochondrial respiratory enzymes under a variety of experimental conditions (Fig. 3, Table II), suggesting that in intact tissue it is coupled to the conventional chain. The slower kinetics of *b*-557 indicate that it is not included as an extra member in the conventional chain, but acts as a branch from the chain as proposed by Pappenheimer and Williams [14]. Therefore it is proposed that the scheme shown in Fig. 7 would account for the experimental observations.

Communication between cytochrome *b*-557 and the respiratory chain is necessary to explain the observation that *b*-557 is reduced by cyanide in the intact preparation (Fig. 4 and Table II), in contrast to its cyanide insensitivity in tissue homogenates [15].

This result has two implications: firstly, the terminal oxidase for cytochrome *b*-557 in the intact tissue is probably the conventional cytochrome a_3 since this enzyme is specifically affected by cyanide; secondly, the failure of a_3 to act as a terminal oxidase for *b*-557 in tissue homogenates suggests that in the intact tissue there is electron transfer from *b*-557 to a_3, this transfer being broken when the cells are disrupted. According to the scheme presented in Fig. 7, communication between cytochromes *b*-557 and a_3 requires the presence of cytochrome *c*. As discussed earlier, cytochrome *c* may not be present in tissue homogenates, causing the break in electron flow between *b*-557 and a_3. In the intact tissue, a physical link may actually be present which corresponds to the "bridges" seen between goblet cell mitochondria and the microvillar membrane [3].

Amytal oxidizes both cytochrome *b*-557 and *c*. On the basis of the present experiments it is difficult to determine whether the oxidation of *b*-557 is due to a block of FP_1 or a block at FP_2 transmitted through cytochromes *b* and *c*. In the scheme presented above, $NADH_1$ and FP_1 have been added because their presence would be expected if *b*-557 had the properties of cytochrome b_5.

The similarity between the kinetics of *b*-557 and active transport suggest the presence of a link between them. At present, little is known about the nature of this

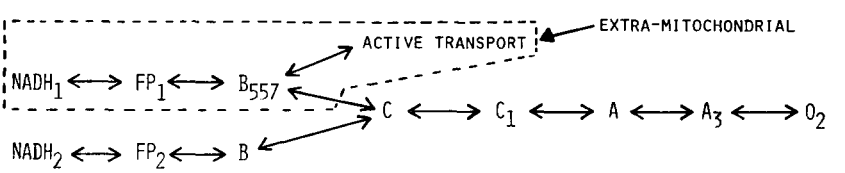

Fig. 7. Schematic representation of the cytochrome chain in the intact silkworm midgut.

link; however, this link could be the means of transferring energy from the cytochrome chain to the active transport site.

Midgut metabolism, potassium transport, and the respiratory chain

The oxidation of all cytochromes elicited by amytal and low K^+ indicates that these cytochromes are somewhat reduced in the control state. The partial reduction of cytochrome a_3 is particularly noteworthy since it does not correspond to any of the metabolic states postulated by Chance and Williams [13] for isolated mitochondria in the presence of oxygen. This effect probably is not due to a lack of oxygen because reducing the oxygen content of the bubbling gas to 50 % does not change either the I_{sc} or the oxygen consumption (Wood, J. and Harvey, W. R., personal communication). This partial reduction of cytochrome a_3 has also been found in intact gastric mucosa [19], heart muscle [20] and brain [21].

Harvey et al. [2] have calculated the theoretical minimum power output necessary to maintain the rate of potassium transport sustained by the midgut in vitro, and they concluded that the transport activity must consume a large part of the total oxidative energy budget of the tissue. Yet, when the cecropia midgut is placed in a low K^+ solution, the rate of active transport decreases but the rate of oxygen consumption remains unaltered [2]. This observation contrasts sharply with the behavior of many other transporting epithelia, such as gastric mucosa [22], frog skin [23], and toad bladder [24], in which the rate of oxygen consumption is closely linked to the rate of active transport. Since the potassium transport process in midgut is highly oxygen dependent [2] (see Figs 4 and 5), there appears to be an interesting one-way relationship between oxygen consumption and active transport.

The transition from a low K^+ solution to a normal K^+ solution causes a reduction of all cytochromes concomitant with a doubling of the active transport rate (Fig. 4). If this were to be compared to a mitochondrial State 4 to State 3 transition [13], oxidation of the cytochromes would be expected. Therefore, changes in K^+ concentration may not affect the cytochrome chain through changes in ADP concentration but, rather, act at a site closer to the substrate end of the chain. More extensive kinetic studies need to be performed to determine the exact site of action.

The results presented in this paper, as well as observations in other tissues [21–23] indicate that the interaction between cytochrome a_3 and oxygen in intact tissues may differ from that found in isolated mitochondria. The partial reduction of cytochrome a_3 discussed earlier and the lack of change of the oxygen consumption rate when all the cytochromes become oxidized in the low K^+ solution indicate that the affinity of cytochrome a_3 for oxygen may be lower in intact tissue. In isolated mitochondria, the oxygen consumption rate is directly proportional to the concentration of reduced cytochrome a_3 [25]. It might be possible that certain factors exist in intact tissue which regulate the oxygen affinity; these factors could be lost in the process of mitochondrial isolation.

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