ENZYME ACTIVITY OF THE CYTOCHROME SYSTEM OF THE EGG AND LARVA OF THE CATTLE TICK (*BOOPHILUS MICROPLUS*)

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Abstract

The enzymatic activities of the succinate–cytochrome *c* reductase system, the NADH–cytochrome *c* reductase system, the NADH oxidase system, and cytochrome *c* oxidase were determined spectrophotometrically in particulate preparations of eggs and larvae of *B. microplus*.

All enzyme systems were detectable in the eggs of the tick and the activities remained relatively constant. At day 0, the day of hatching, an 80% increase in NADH–cytochrome *c* reductase was recorded. No other appreciable change occurred until about day 5 when there was a sharp increase in cytochrome *c* oxidase activity. This was followed by an increase in succinate–cytochrome *c* reductase on day 7. NADH oxidase did not increase until about day 9.

The reductases accounted for about 70% of the total oxidoreductase activity during the early larval phase but from day 9 the reductases declined, concomitant with an increase in oxidase activity. The oxidases reached a maximum of 66% of the total oxidoreductase activity about day 36.

These changes in the cytochrome system are indicative of the increasing metabolic activity of the larvae with age. The increased respiratory activity also reflects the increasing demand for energy at a time when the larvae are seeking a host.

Preliminary studies of the respiratory system in day 8 larvae of four strains (Yeerongpilly, Biarra, Ridgelands, and Mackay) of varying acaricide susceptibilities indicated some interstrain difference. The Mackay strain exhibited enzyme activities 2·5–3 times greater than the Biarra strain.

I. INTRODUCTION

The egg of the cattle tick *Boophilus microplus* contains a considerable quantity of a haemoprotein derived ultimately from the maternal blood meal and named "haemixodovin" by Bremner (1959). He extended work on the haem pigments of ticks first studied by Wigglesworth (1942). Tatchell (1971) found by acrylamide-gel electrophoresis that haemixodovin consisted of two components. O'Hagan (unpublished data) found that virtually all the haem from the haemixodovin can be found in the newly hatched larvae and the question naturally arose—does the tick use the haem derived from haemixodovin for synthesis of cytochromes or does it build its own cytochromes from iron, porphyrin precursors, and amino acids? In seeking the

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answer to this question the first stage undertaken, and described here, was to determine by enzyme activity studies which components of the cytochrome system were present. With this information now available the second stage, the isolation and spectral characterization of the individual enzymes of the system, is under way.

In carrying out the enzyme activity studies the previous work of Shappirio and Williams (1957) on the cytochrome system of the cecropia silkworm (*Platysamia cecropia*) was found to be of great value. They found considerable hour-to-hour and day-to-day variation in activity in the chilled and unchilled pupae. After initiation of adult development, day-to-day variation still occurred, but the overall increase in activity of the enzymes was progressive with age. Chino (1963) studied the respiratory enzyme system of the *Bombyx mori* silkworm egg and described the elements of the cytochrome system of the egg in both the early and later embryonic stages. In the later stage, the system is similar to that found for both mammalian and insect tissues, but in the early stage there are some outstanding differences. The most notable of these are that cytochrome *c* and succinate–cytochrome *c* reductase are absent (or present at extremely low activities), that cytochrome b_5 and cytochrome *c* oxidase (cytochrome $a+a_3$) predominate, and are mainly outside the mitochondria in the "lipid-rich particle fraction," and there is no NADH oxidase in the particle fractions nor NADH–cytochrome *c* reductase in the microsomal fraction.

The present investigations describe changes in enzyme activities associated with the cytochrome system in eggs and in larvae of *B. microplus*. Determinations of the succinate–cytochrome *c* reductase system, the NADH–cytochrome *c* reductase system, the NADH oxidase system, and cytochrome *c* oxidase (E.C.1.9.3.1) at successive stages from eggs, 14 days before hatching, to aged larvae (36 days) have indicated their growth and decline with age. Apart from its importance as a preliminary to studying the utilization of haem by the cattle tick, the present study has possible significance in aspects of the biochemistry of acaricide resistance, since Estabrook and Cohen (1969) have shown that cytochrome b_5 and the terminal oxidase P_{450} are most probably involved in hydroxylation reactions, which could account for the detoxification of some acaricides.

II. Materials and Methods

(*a*) *Eggs and Larvae*

Glass tubes each containing eggs (1·5 g) were incubated and maintained after hatching at 27±1°C and 85±5% relative humidity. Day 0 was the nominal day of hatching. The acaricide-susceptible Yeerongpilly reference strain was used for egg and larval enzyme activity assays. The enzyme activities of the larvae of acaricide-resistant Biarra, Ridgelands, and Mackay strains (Roulston *et al.* 1969) were determined on day 8 of their life cycle.

(*b*) *Preparation of Mitochondrial Pellet*

Whole eggs in one tube at the same stage of development were homogenized together. Larvae were separated from the egg-shell residue prior to homogenization, except on day 0 when both eggs and newly hatched larvae were present. The eggs or larvae were suspended in 5 ml of 0·32M sucrose and ground for 3 min at or near 0°C using an all-glass, motor-driven homogenizer of Potter–Elvehjem type. The preparation of the mitochondrial pellet followed exactly the method of Shappirio and Williams (1957) except that a Sorvall RC2B automatic refrigerated centrifuge was used. As with their preparation, ours would have contained some microsomal particles associated with the mitochondria. For assay, the final pellet was suspended in 10 ml of 0·32M sucrose.

(*c*) *Reagents*

Purified horse-heart cytochrome *c* and reduced nicotinamide adenine dinucleotide were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The oxidized and reduced forms were estimated spectrophotometrically. Solutions of these and other reagents used were prepared according to Shappirio and Williams (1957).

(*d*) *Spectrophotometric Data*

A Cary model 16 spectrophotometer coupled through a Cary model 1626 recorder interface to a Philips PM8100 chart recorder and to a Solartron digital voltmeter LM1440·3 was used to record enzyme activities and protein estimations.

(*e*) *Protein Determinations*

Enzymatic activities were expressed on a protein basis using the methods of Groves *et al.* (1968) which were described as being free from nucleic acid interference. Protein estimation by microKjeldahl determinations were made on some samples and compared with the results obtained with the Groves *et al.* procedure using bovine serum albumin (Sigma Chemical Co.) as standard, with good agreement.

(*f*) *Enzymatic Determinations*

Estimations of the four enzymatic activities were made using exactly the same concentrations and volumes of reagents and volumes of enzyme preparations as employed by Shappirio and Williams (1957). However, changes in absorbance were measured with the Cary model 16 spectrophotometer, its recorder interface, and the Philips chart recorder. Changes in absorbance shown on the continuous recording were linear within 20–30 s after mixing in the substrates and for 3–5 min afterwards. Rates were read from this linear portion of the traces.

III. Results

(*a*) *Nucleic Acid Contamination*

The nucleic acid contamination of the mitochondrial pellet was estimated by the ratio of absorbance at 280 nm to that at 260 nm. Contamination was relatively low for all preparations (as indicated from a mean ratio of $0{\cdot}865 \pm 0{\cdot}024$) and was within the limits suggested by Groves *et al.* (1968) for spectrophotometric determinations of protein without nucleic acid interference.

(*b*) *NADH–Cytochrome c Reductase*

The enzymatic activities (Table 1) in this system represent the sum of two possible pathways, one through cytochrome b_5 and the other via cytochrome *b* (Shappirio and Williams 1957).

There was no significant change in activity during incubation of the eggs, but on the day of hatching, day 0, when some unhatched eggs were still present, the activity increased approximately 80%. The peak of activity occurred on day 12 and declined thereafter.

In the presence of antimycin which inhibits the oxidation of NADH via cytochrome *b* (Fig. 4), the residual activity via cytochrome b_5 was recorded. Inhibition of the NADH–cytochrome *c* reductase activity was about 25–30% during the egg and early larval phase. By day 7 the inhibition had fallen to 11% and although it fluctuated somewhat, the overall picture was that it remained at this low level.

TABLE 1

ENZYMATIC ACTIVITIES IN EGGS AND LARVAE OF YEERONGPILLY STRAIN OF *B. MICROPLUS*

Age (days)*	NADH–cytochrome *c* reductase†			Succinate–cytochrome *c* reductase†	NADH oxidase‡			Cytochrome *c* oxidase§
		Antimycin A added to a final concn. of 1·2 μg/ml	Percentage uninhibited			Cytochrome *c* added to a final concn. of $7·5 \times 10^{-5}$M	Stimulation by cytochrome *c*	
−14	9·1	5·8	64	2·5	2·5	6·4	2·6	1·4
−10	10·1	8·0	79	1·6	3·0	5·5	1·8	1·7
− 7	8·3	6·0	72	1·3	2·8	6·5	2·2	0·8
− 3	7·7	5·6	73	1·8	3·2	6·2	1·9	1·4
− 1	7·0	6·1	88	1·7	2·2	5·0	2·2	1·1
0	13·1	9·3	71	2·2	1·9	6·3	3·3	1·9
1	11·1	8·2	74	2·4	2·5	6·0	2·3	1·2
3	10·0	7·8	78	1·7	1·5	5·5	3·6	1·5
5	11·8	8·7	74	2·8	2·7	6·8	2·5	3·4
7	12·6	11·3	89	4·0	2·9	5·8	2·0	4·6
8	14·6	12·5	85	4·5	2·0	5·6	2·8	5·0
9	15·4	11·5	75	5·2	5·9	6·0	1·0	3·7
10	14·6	13·7	94	5·7	3·0	6·0	2·0	6·6
11	14·5	12·7	88	4·4	4·3	5·7	1·3	6·7
12	16·9	13·7	81	5·3	6·0	9·5	1·6	5·8
14	12·2	9·7	79	4·5	7·3	8·1	1·1	5·3
17	11·6	10·4	90	4·9	4·7	5·2	1·1	5·4
21	8·8	7·7	87	6·0	4·3	4·5	1·0	5·0
24	13·4	11·9	89	5·6	4·1	5·6	1·4	5·0
28	11·8	10·6	90	4·2	3·4	4·6	1·3	5·0
36	8·5	5·1	61	4·3	13·3	16·4	1·2	11·3

* Day 0 = day of hatching.

† μmoles cytochrome *c* reduced per milligram protein per minute.

‡ μmoles NADH oxidized per milligram protein per minute.

§ μmoles cytochrome *c* oxidized per milligram protein per minute.

(*c*) *Succinate–Cytochrome c Reductase*

During the egg and larval stages (to day 5) there was little change in the enzyme activity (mean = $2{\cdot}008 \pm 0{\cdot}453$ μmoles cytochrome *c* reduced per milligram protein per minute). On subsequent days the activity more than doubled (mean = $4{\cdot}881 \pm 0{\cdot}636$), suggesting greater utilization of this pathway for electron movement (Table 1).

(*d*) *Total Reductase Activity*

The sum of the NADH–cytochrome *c* reductase and the succinate–cytochrome *c* reductase was considered to represent the total reductase activity. Succinate–cytochrome *c* reductase accounted for about 17% of the total reductase activity to day 5 (Fig. 1). Subsequently, electron transfer via this pathway rose to 40% at day 21

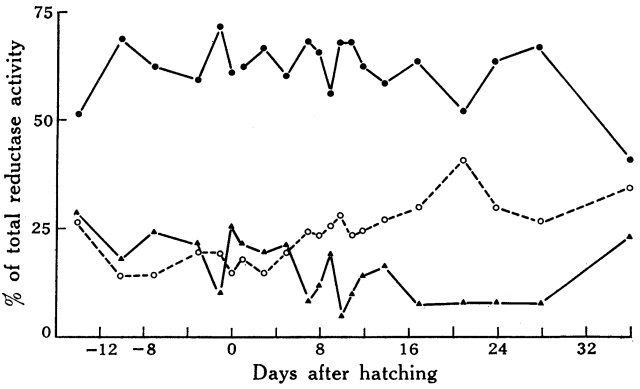

Fig. 1.—NADH–cytochrome *c* reductase activity via cytochrome b_5 (●) and via cytochrome *b* (▲), and succinate–cytochrome *c* reductase activity via cytochrome *b* (○). All values expressed as a percentage of the total reductase activity. NADH–cytochrome *c* reductase via cytochrome *b* obtained by difference of the reductase activity with and without the addition of 1·2 μg/ml of the inhibitor, antimycin A. Day 0 was the day of hatching of the larvae.

from which point it began to decline, although still contributing about 26% of the total reductase activity by day 28. In addition Figure 1 indicated that the NADH–cytochrome *c* reductase via cytochrome b_5 contributed between 50 and 70% of the total reductase activity.

The main physiological role in the respiratory system of the antimycin-sensitive NADH pathway via cytochrome *b* appeared to be associated with the embryonic development in the egg. At this time, 20–25% of the total reductase activity passed through cytochrome *b* to cytochrome *c*. After hatching, the decline in activity in the larvae compensated for the rise in activity of the succinate–cytochrome *c* reductase.

(*e*) *NADH Oxidase*

NADH oxidase followed a similar trend to the reductases during the egg and early larval stages. However, on day 9 a very rapid increase (2·9 times) occurred and the activity continued to rise until day 14 after which it declined. Table 1 and Figure 2

show that the addition of cytochrome *c* had a strong stimulatory effect on the oxidase system to day 8. Addition of cytochrome *c* after this time had less effect indicating that sufficient endogenous cytochrome *c* was present in the crude mitochondrial preparation and that it was no longer a limiting factor of NADH oxidase activity.

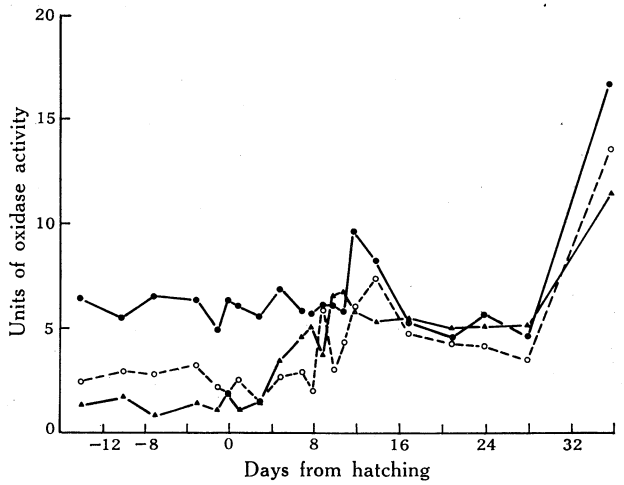

Fig. 2.—Activities of cytochrome *c* oxidase (▲) (μmoles cytochrome *c* oxidized per milligram protein per minute) and NADH oxidase (○) and NADH oxidase plus cytochrome *c* (●) (μmoles NADH oxidized per milligram protein per minute) of the Yeerongpilly strain. Day 0 was the day of hatching of the larvae.

(*f*) *Cytochrome c Oxidase*

Oxidation of reduced cytochrome *c* was detectable at low levels during the egg and early larval period. About 5 days after hatching the oxidase activity increased rapidly to reach a plateau which continued until day 28.

(*g*) *Total Oxidoreductase Activity*

The sum of the four enzyme system activities, i.e. NADH–cytochrome *c* reductase, succinate–cytochrome *c* reductase, NADH oxidase, and cytochrome *c* oxidase was regarded as comprising the total oxidoreductase activity. In each of the four systems activity increased at different times. The increase in NADH–cytochrome *c* reductase occurred on day 0, the day of hatching; cytochrome *c* oxidase on day 5; succinate–cytochrome *c* reductase on day 7; and NADH oxidase on day 9. An abrupt change in the percentage of NADH–cytochrome *c* reductase that was uninhibited by antimycin was also recorded on day 7 (Table 1). The change in the stimulatory effect of cytochrome *c* when added to NADH oxidase occurred on day 9.

The summary of the total oxidoreductase activity (Fig. 3) of developing larvae demonstrated the change in the relative proportions of each of the four enzyme systems with respect to age. The change from the embryo to the larva was accom-

panied by a relative increase in NADH–cytochrome *c* reductase and a decrease in NADH oxidase. The activities of cytochrome *c* oxidase and succinate–cytochrome *c* reductase remained relatively constant. It would appear that oxidoreductases in larvae are most active about day 12.

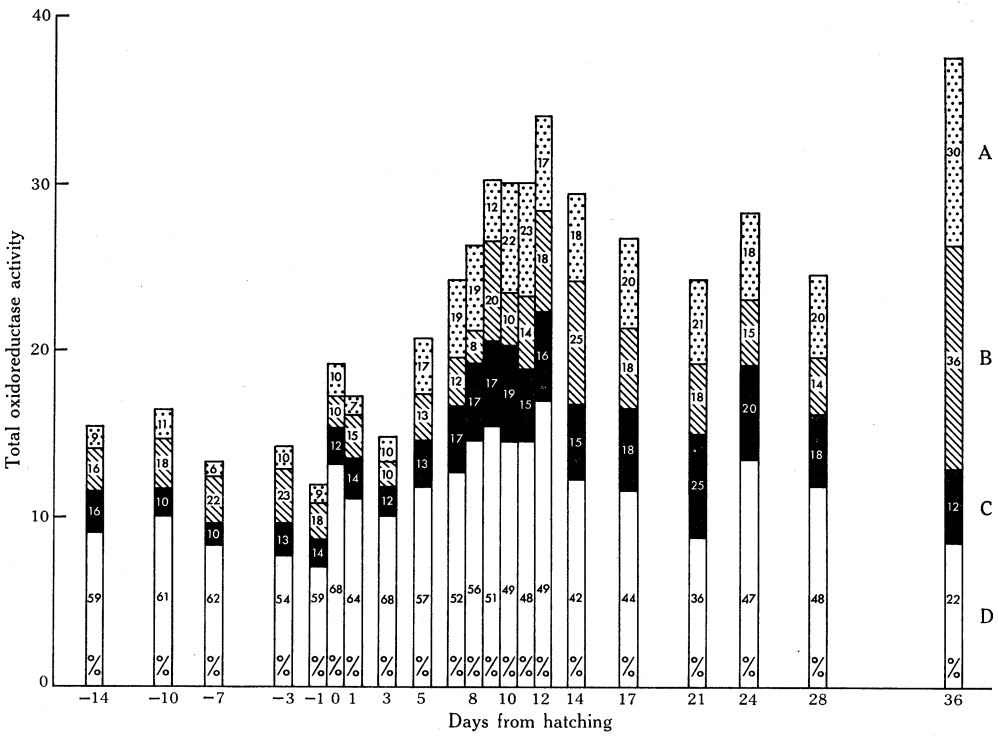

Fig. 3.—Summation of the oxidoreductase activity in the crude mitochondrial pellet of the eggs and larvae of Yeerongpilly strain to show relative activities. Day 0 was the day of hatching. Values on columns are percentages of the summed oxidoreductase activity for each of the four enzyme systems on a particular day. Activities are compared and summed on the basis of μmoles of substrates oxidized or reduced per milligram protein per minute. *A*, cytochrome *c* oxidase; *B*, NADH oxidase; *C*, succinate–cytochrome *c* reductase; *D*, NADH–cytochrome *c* reductase.

(*h*) *Comparison of Strains*

Day 8 was about midpoint of the rapid development phase for larvae of the Yeerongpilly strain. Hence, this time was chosen to compare the enzyme activities (Table 2) of the larvae of Biarra, Ridgelands, and Mackay strains.

Preliminary studies of the respiratory system showed that about 76% of the NADH–cytochrome *c* reductase of Biarra larvae was uninhibited by antimycin. Other strains showed antimycin insensitivity of the order of 88%. The Biarra strain also showed lowered activity for succinate–cytochrome *c* reductase and cytochrome *c* oxidase. The Mackay strain exhibited the highest enzyme activity which was 2·5–3 times that of the Biarra strain.

IV. Discussion

The pathways of electron transport of the respiratory system of insects have been described (Pappenheimer and Williams 1954; Shappirio and Williams 1957). This has been modified in Figure 4 according to Dickens (1961) and incorporates the cytochrome P_{450}–cytochrome b_5 relationship as suggested by Estabrook and Cohen (1969).

Table 2

STRAINS OF *B. MICROPLUS* LARVAE COMPARED FOR ENZYME ACTIVITY OF THE RESPIRATORY SYSTEM ON DAY 8 OF THE LIFE CYCLE

Strain	NADH–cytochrome *c* reductase*			Succinate–cytochrome *c* reductase*
		Antimycin A added to a final concn. of 1·2 μg/ml	Percentage uninhibited	
Yeerongpilly	14·6	12·5	85	4·5
Biarra	9·0	6·8	76	3·6
Ridgelands	15·0	13·3	89	8·2
Mackay	21·2	19·1	90	11·5

Strain	NADH oxidase†			Cytochrome *c* oxidase‡
		Cytochrome *c* added to a final concn. of $7·5 \times 10^{-5}$M	Stimulation by cytochrome *c*	
Yeerongpilly	2·0	5·6	2·8	5·0
Biarra	2·1	5·2	2·5	2·7
Ridgelands	2·4	5·4	2·3	4·9
Mackay	5·2	6·5	1·3	6·6

* μmoles cytochrome *c* reduced per milligram per minute.
† μmoles NADH oxidized per milligram protein per minute.
‡ μmoles cytochrome *c* oxidized per milligram protein per minute.

At all times through the egg and larval stages, the four enzymatic systems were detectable. Shappirio and Williams (1957) reasoned that cytochrome *b* was the limiting factor associated with the disappearance of succinate–cytochrome *c* reductase activity of the wing epithelium in the diapausing pupa of the cecropia silkworm. It was apparent that cytochrome *b* was present in the egg and young larva of the tick in relatively constant amounts, and if, as suggested by Shappirio and Williams, it is the rate-limiting step in the succinate–cytochrome *b* reductase pathway, then the cytochrome *b* component increased by a corresponding factor of 2 about 1 week after hatching and remained at about this level through to day 36.

Of the two oxidative pathways of NADH, one via cytochrome *b* is antimycin-sensitive, and the other proceeds via cytochrome b_5, both converging on cytochrome *c*. NADH–cytochrome b_5 system provides the major pathway for electron transport

through to cytochrome *c* while the NADH–cytochrome *b* succinate–cytochrome *b* systems contribute a lesser percentage of the total reductase activity (Fig. 1). It is apparent, however, that some balancing mechanism must govern the rate of each pathway so that a percentage increase in succinate–cytochrome *c* reductase leading up to day 21 can be compensated for by change in activities of other components of the respiratory system.

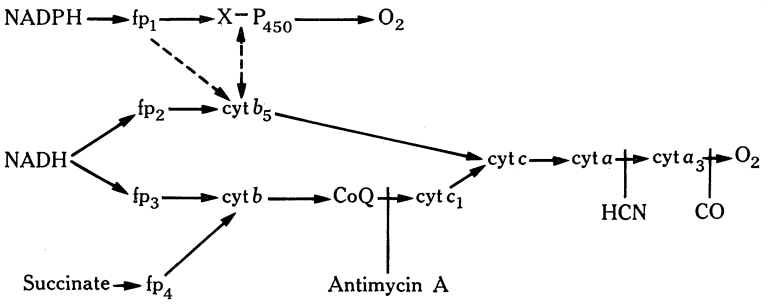

Fig. 4.—Pathways of electron transport in mitochondrial and microsomal particles (modified from Shappirio and Williams 1957). Sites of inhibiting action of antimycin A, cyanide, and carbon monoxide indicated by vertical lines. In the mitochondria, the NADH–succinate pathway via cytochrome *b* is sensitive to antimycin A (Dickens 1961). In the microsomes, the pathway for NADPH or NADH incorporates the cytochrome P_{450}–cytochrome b_5 relationship as suggested by Estabrook and Cohen (1969).

The addition of reduced cytochrome *c* and its subsequent oxidation by the homogenate gave a measure of the cytochrome *c* oxidase activity, i.e. cytochromes $a + a_3$, which are present through all stages of the life cycle, increase in activity corresponding to, or as a result of, increases in cytochromes b_5 and *b*.

The connecting link through which the electron transport must pass is cytochrome *c*. Its addition to NADH oxidase produced about a 2·5-fold increase in activity up to day 8. After this time, the stimulatory effect of exogenous cytochrome *c* was almost totally cancelled out by the synthesis within the mitochondria. The implication consistent with this sharp change in cytochrome *c* concentration is that the animal has reached a high plateau of respiratory chain development and only an outside stimulus, such as attachment to its host or engorging or both, will cause any further change in the cytochrome *c* concentration.

O'Kelly and Seifert (1969) found differences in the tick count when they infested cattle with larvae 2 or 3 weeks old. The highest tick counts were recorded for 2-week-old larvae, and they suggested that these larvae were at a more viable stage of development. It may be significant that this period (at about day 14) corresponds with a peak in oxidase activity (Fig. 2).

The larvae were maintained continuously at 27°C in the incubator prior to homogenization. Larvae in the field, where they are subjected to fluctuating diurnal temperatures, are known to survive longer than 36 days (Harley 1966). However, Utech (personal communication) has indicated that the life span of the majority of larvae was much shorter during summer conditions than at winter temperatures.

Although all larvae used were alive up to the time of homogenization on day 36, the normal nutritional substrates carried over from the egg sac would be largely depleted. The high oxidase activity at this time (Fig. 2) has not been explained but may have resulted from increased muscular activity, calling on the mitochondrial system, prior to death. At this stage all available metabolites may have been mobilized.

The activities of the enzyme system in different strains remain to be considered. The results presented in Table 2 are insufficient for full comparative studies. However, the most notable differences obtained on day 8 were the low respiratory activity of the Biarra strain and the high activity of the Mackay strain. Estabrook and Cohen (1969) have indicated that cytochrome b_5 is probably the second electron donor involved in hydroxylation reactions flowing through the terminal oxidase cytochrome P_{450} (Fig. 4) associated with drug metabolism. In their review of tick resistance to chemicals, Wharton and Roulston (1970) summarized some of the reported work demonstrating the difference in ability of strains of larvae to metabolize acaricides leading to the development of resistance.

As cytochrome b_5 is associated with the NADH–cytochrome *c* reductase pathway, and probably with cytochrome P_{450}, and in view of some of the marked differences of results with day 8 larvae, further study could be directed to examining the response of the respiratory system of various strains exposed *in vivo* to sublethal doses of acaricide.

V. Conclusion

The enzyme activities of succinate–cytochrome *c* reductase, NADH–cytochrome *c* reductase, NADH oxidase, and cytochrome *c* oxidase were detectable in all stages of development of the egg and larva of the cattle tick. Comparatively low but constant rates of activities occurred in the egg. At hatching and thereafter, each of the four enzyme systems recorded increasing activities, the most notable increases occurring on different days. The peak of the total oxidoreductase activity occurred on day 12. However, the succinate–cytochrome *c* reductase pathway continued to increase its percentage of the total reductase activity which offset the fall in the NADH–cytochrome *c* reductase activity via cytochrome *b*.

Changes in the cytochrome system may reflect stages of development of the larvae, such that the critical time in their life cycle may occur about day 9 when they are seeking the cattle host or engorging.

Four strains of *B. microplus* larvae (Yeerongpilly, Biarra, Ridgelands, and Mackay) were examined for respiratory activity on day 8 of their life cycle. Preliminary data showed that the Biarra strain exhibited comparatively low, and the Mackay strain relatively high, enzyme activities.

VI. Acknowledgments

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