Biochimica et Biophysica Acta, 449 (1976) 491–499

BBA 47189

THE ISOLATION OF MITOCHONDRIA FROM DIPTERAN FLIGHT MUSCLE

ELLA N. SLACK* and EINAR BURSELL

Department of Zoology, University of Rhodesia, Salisbury (Rhodesia)

(Received March 9th, 1976)

SUMMARY

Procedures for the isolation of mitochondria from dipteran flight muscle have been investigated in an attempt to determine the extent and to identify the causes of deterioration associated with isolation. In the light of the results obtained isolation procedures have been improved by minimising mechanical damage, avoiding the development of anoxic conditions, and by the use of an isolation medium of a more physiological nature, containing the potassium salt of an organic anion as the principal osmoeffector, phosphate as the principal buffer, and low concentrations of free Mg^{2+}. The oxidative capacity of mitochondria isolated by the improved method is adequate to support the in vivo requirements of the flight system.

INTRODUCTION

A great deal of work has been done on mitochondria isolated from insect flight muscle during the last twenty years (see review by Sacktor, ref. 1), but only exceptionally [2–4] have efforts been made to determine the extent to which isolation procedures may have caused a deterioration of mitochondrial function. Despite early claims by Chance and Sacktor [5] a wide discrepancy still exists between the respiratory capacity of in vitro systems and the corresponding in vivo consumption of oxygen during flight [6] suggesting that substantial impairment of oxidative capacity may have occurred during isolation; and the fact that rates of oxidation often vary greatly from one isolation to the next [6, 7] indicates that extraction procedures are not always adequately controlled. Similar variability was encountered during early phases of the present work, and it became clear that if biologically meaningful results were to be obtained it would be essential to make a careful evaluation of procedures with a view to the development of improved isolation methods.

EXPERIMENTAL PROCEDURES

Mitochondria were isolated from the flight muscles of tsetse flies (*Glossina morsitans* Westwood) and blowflies (*Sarcophaga nodosa* Engels) maintained in the

* Present address: National Institute of Child Health and Human Development, Baltimore City Hospitals, Baltimore, Md. 21224, U.S.A.

laboratory. Initially mitochondria were isolated by standard methods as described in the literature [8–12] in a medium containing 0.3 M sucrose, 5 mM Tris buffer at pH 7.4, 1 % bovine serum albumin ("essentially fatty-acid free") and 1 mM EDTA; these will be referred to as standard mitochondria to distinguish them from mitochondria isolated by improved procedures. In the finally adopted method insects were pre-chilled at 0 °C for 20 min before use; flight muscles from not more than 20 blowflies, or 40 tsetse flies, were squeezed from the thorax into 3 ml of isolation medium consisting of 0.16 M potassium D-aspartate, 5 mM phosphate buffer at pH 7.4, 1 % bovine serum albumin and a 2 mM EDTA buffer system with free Mg^{2+} at 10^{-7} M and free Ca^{2+} at 10^{-9} M. The muscles were partially disrupted by gentle action of a magnetic stirrer (about 200 rev./min with a 1 cm-long stirrer) for 4 min, and filtered through gauze to remove myofibrillar components. The mitochondrial suspension was carefully layered, in a round-bottomed centrifuge tube of 3 cm diameter, on isolation medium whose density had been increased by the addition of sucrose to a concentration of 40 mM, and the mitochondria were washed by centrifuging them through the denser medium at $1500 \times g$ for 7 min. The surface of the thinly dispersed mitochondrial pellet was then washed with fresh isolation medium and the mitochondria resuspended by gentle magnetic stirring for 3 min in 2 ml of storage medium containing 0.32 M sucrose, 5 mM phosphate buffer at pH 7.4, 1 % serum albumin and a 1 mM EDTA buffer system containing free Mg^{2+} at 10^{-9} M. Finally the suspension was filtered through a small gauze plug to remove clumped mitochondria. Care was taken to maintain all media at or near 0 °C throughout the isolation procedure.

Oxygen consumption was measured at 25 °C with an oxygen electrode (Beckman Oxygen Analyser) in a stoppered cell of 1.9 ml capacity containing assay medium with 0.16 M potassium aspartate, 5 mM phosphate buffer at pH 7.4 and a 1 mM EDTA system with Mg^{2+} at 10^{-5} M. Mitochondria, substrates and cofactors were added by Hamilton syringe through an injection port. For the assay of State 3 oxidation, substrates were added at a concentration of 5–10 mM and ADP at 0.5 mM; when α-glycerophosphate was the substrate Ca^{2+} was added to a concentration of 10^{-6} M. All reagents were supplied by Sigma Chemical Company.

Estimation of mitochondrial protein was based on the methods and results of Slack and Bursell [13]. A "Soniprobe" (Dawe Instruments Ltd., London, Type 1130A) was used for the sonication of mitochondrial suspensions, care being taken to maintain temperatures below +2 °C.

RESULTS

1. Criteria of mitochondrial quality

A variety of criteria have been used to assess the quality of isolated mitochondria, of which the respiratory control index is generally regarded as being the most sensitive [14, 15]. Others include the P/O ratio, depletion of cytochrome *c* or other endogenous components, ATPase activity and oxidative activity in relation to exogenous NADH or succinate. All of these were used in the present investigation, but in our experience a far more sensitive index of mitochondrial quality was provided by the level of State 3 respiration with the physiologically normal substrate (pyruvate for the blowfly, proline for the tsetse fly [16]), and by the stability of the State 3 respira-

tion during storage. These indices showed substantial change long before there was any loss of respiratory control, for example, and since they provided the most discriminating standard of mitochondrial intactness, they have been used as a basis for evaluation in most of the investigations to be described.

2. Mechanical damage

Full advantage does not yet seem to have been taken of the ease with which mitochondria can be isolated from dipteran flight muscle, and standard methods still involve some form of fairly harsh mechanical treatment. Fig. 1 illustrates the effect of gentle mortar and pestle treatment and it is clear that substantial damage is suffered. Pyruvate oxidation by blowfly sarcosomes is greatly depressed with increased duration of treatment, α-glycerophosphate oxidation less so, while the oxidation of exogenous NADH (and of succinate, not shown) increases. Similar effects, increasing in magnitude with increasing duration or intensity of treatment, can be produced by mild sonication, by Potter-Elvehjem homogenisation, or simply by vigorous shaking of the

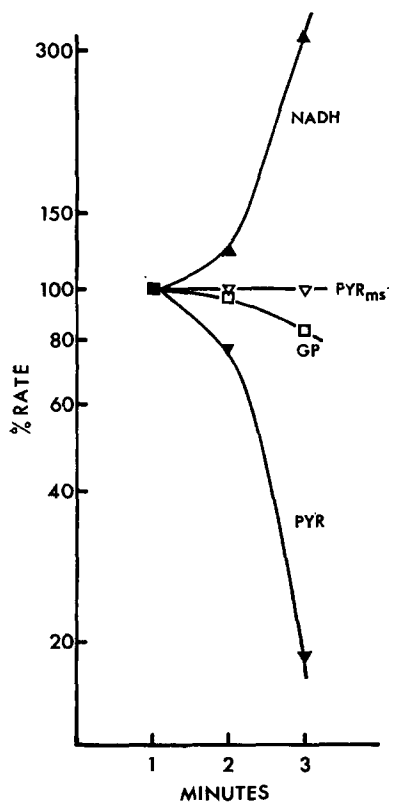

Fig. 1. The effect of mechanical disruption on oxidative capacity. Standard mitochrondria of the blowfly were prepared using increasing durations of mortar and pestle homogenisation. The State 3 respiration was recorded with NADH (▲); α-glycerophosphate (GP, □) and pyruvate (PYR, ▼). Pyruvate oxidation following magnetic stirring is also shown (PYR_{ms}, ▽). To accommodate increases and decrease on a convenient scale, respiratory rates, expressed as a percentage of the rate after 1 min mechanical treatment, have been plotted on a logarithmic scale.

mitochondrial suspension. The figure also shows that pyruvate oxidation is unaffected by gentle magnetic stirring, which provides some indication of the mechanical disruption that can be tolerated without damaging effect.

Subsequent isolations of mitochondria were accordingly made on the basis of magnetic stirring, and the State 3 oxidation of mitochondria isolated in this way was found to be very much greater than that of standard mitochondria. The use of gentler methods of disruption had the additional advantage that myofibrils remained largely intact; they could therefore be cleanly removed by filtration through gauze and the necessity for differential centrifugation was avoided. A loss in mitochondrial yield was inevitably associated with the gentler isolation technique, but this was amply compensated for by an increase in the oxidative capacity of the mitochondria recovered.

3. Effects of anoxia

During early phases of this work it was noted that concentrated suspensions of mitochondria, in excess of 1 mg mitochondrial protein per ml, tended to have lower rates of State 3 oxidation and to deteriorate more rapidly than dilute ones. The possibility was explored that the anoxic conditions which would tend to develop in concentrated suspensions might be responsible, and the results, shown in Fig. 2, indicate that storage of mitochondria under anoxic conditions has a profoundly damaging effect. The State 3 oxidation of pyruvate (▽) and of α-glycerophosphate (□) was greatly depressed after 1 h anoxic storage, and with neither substrate could the oxidative capacity be restored by re-oxygenation. The fact that Ca^{2+}-stimulated

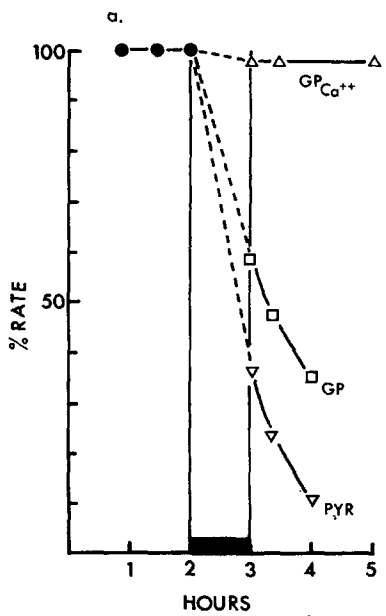

Fig. 2. Effects of anoxia on State 3 respiration. Mitochondria isolated from the flight muscles of blowflies were subjected to anoxic storage for one hour, as indicated by the black bar. Rates of respiration are plotted as a percentage of those recorded with mitochondria maintained throughout under aerobic conditions. ▽, State 3 pyruvate respiration; □, State 3 α-glycerophosphate respiration; △, Ca^{2+}-stimulated (10^{-3} M) α-glycerophosphate respiration.

oxidation of α-glycerophosphate (▽) was unaffected suggests that inhibition is at the level of the coupled electron transport system, rather than at the level of the dehydrogenases.

Even when mitochondria are maintained in dilute suspension there are two stages in the isolation procedure when they would be particularly prone to anoxia. One is during the early stages of extraction when substrates are present at concentrations adequate to maintain high levels of oxidation, and the layering procedure was introduced to minimise the risk of anoxia by ensuring rapid removal of mitochondria from contact with substrates. The second point at which anoxia would be liable to develop is during centrifugation with consequent formation of the mitochondrial pellet, in which there would be a depletion of oxygen associated with the oxidation of endogenous substrates, and damaging effects at this stage were found to be greater the bigger the mitochondrial pellet (see ref. 17 for details). To minimise deterioration the quantity of material used for isolations was limited, and extracts were centrifuged in round-bottomed tubes of large diameter to avoid the formation of a compact pellet.

4. The use of phosphate buffers

Tris has regularly been used as a buffer for the isolation of insect mitochondria, but in our experience its use, particularly at concentrations above 20 mM, is associated with inhibition of NADH-linked oxidations. Independent reports of the deleterious

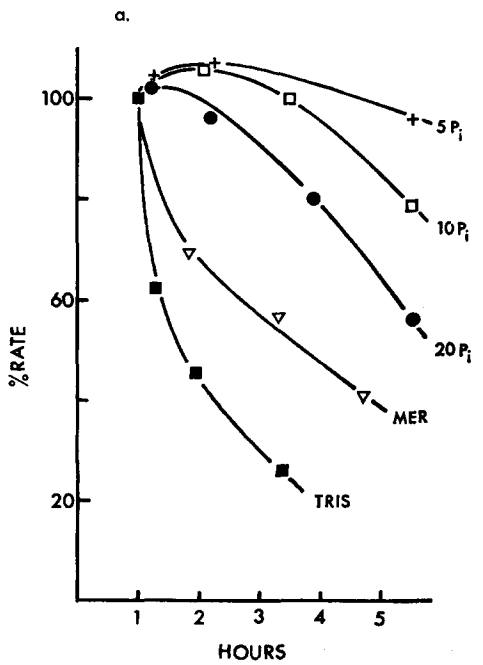

Fig. 3. The effect of phosphate on the maintenance of State 3 oxidation of pyruvate during storage of blowfly mitochondria. Mitochondria were isolated and stored in sucrose medium with Tris. Rates of oxidation are expressed as a percentage of the rate recorded immediately after isolation, and plotted against time since the start of isolation. ■, no additions; ▽, with 25 μM mersalyl; ●, with 20 mM phosphate; □, with 10 mM phosphate; +, with 5 mM phosphate.

effects of Tris [4, 18, 19] led us to explore the possible advantages of a phosphate buffer system. Fig. 3 shows that the State 3 respiration of pyruvate by blowfly mitochondria isolated and stored in a medium buffered with Tris (■) was markedly increased by the presence of low concentrations of phosphate in the storage medium (+); at higher concentrations of phosphate (□ and ●) a certain amount of swelling occurs during prolonged storage, and the protective action is reduced. In the absence of phosphate, addition of 20 μM mersalyl, which is a specific inhibitor of phosphate translocation at the concentration used [20], affords some protection against deterioration (△), suggesting that the presence of phosphate in the medium may act by buffering against the loss of endogenous phosphate. Depletion of intramitochondrial phosphate might result in a partial inhibition of the dehydrogenases, which are known to have a high phosphate requirement [21, 22].

Similar effects can be demonstrated with mitochondria isolated in a medium containing 5 mM phosphate and subsequently stored in the presence or absence of phosphate, except that under these circumstances the rate of respiration of the freshly isolated mitochondria is at a very much higher level (about 3.6 as compared with 2.9 μatoms O_2/min per mg), showing that the presence of phosphate in the isolation medium, as in the storage medium, has beneficial effects.

5. Isolation media

Sucrose and KCl continue to be the most widely used solutes for the maintenance of osmotic equilibrium in mitochondrial extracts, despite the fact that inhibitory effects of sucrose on oxidative systems have been widely reported [23–26] and that the use of KCl is associated with a loss of cytochrome *c* [27]. Similar effects were noted during the present investigation, and a search was accordingly made for possible alternatives to sucrose and KCl. Consideration was given to the fact that the main osmoeffectors in insect sarcoplasm are the potassium salts of organic anions, and it was found that a 0.16 M solution of the potassium salt of D-aspartate, which is not subject to mitochondrial oxidation, provided a better medium for the extraction of mitochondria than KCl or sucrose, as judged by the level of State 3 oxidation. However, sucrose proved superior for the maintenance of physiological integrity during prolonged storage, and best results were obtained with mitochondria that had been isolated in 0.16 M aspartate and stored in 0.32 M sucrose.

6. Divalent cations

The damaging effect of free Ca^{2+} on in vitro respiratory function has long been recognised [28–31] and calcium chelators are routinely incorporated in media used for the isolation of mitochondria to ensure that Ca^{2+} concentration is kept below 10^{-9} M. Careful adjustment of the concentration of Mg^{2+} was also found to be important for the maintenance of oxidative capacity; the optimum concentration for isolation was found to be between 10^{-7} and 10^{-8} M, but during storage free Mg^{2+} had to be maintained at levels of 10^{-9} M to avoid premature deterioration.

7. Oxidative capacity of mitochondria isolated by improved methods

Fig. 4 shows the level of State 3 oxidation of blowfly and tsetse fly mitochondria isolated by standard as compared with improved isolation procedures. The introduction of improved methods resulted in a four-fold increase in the oxidative capacity,

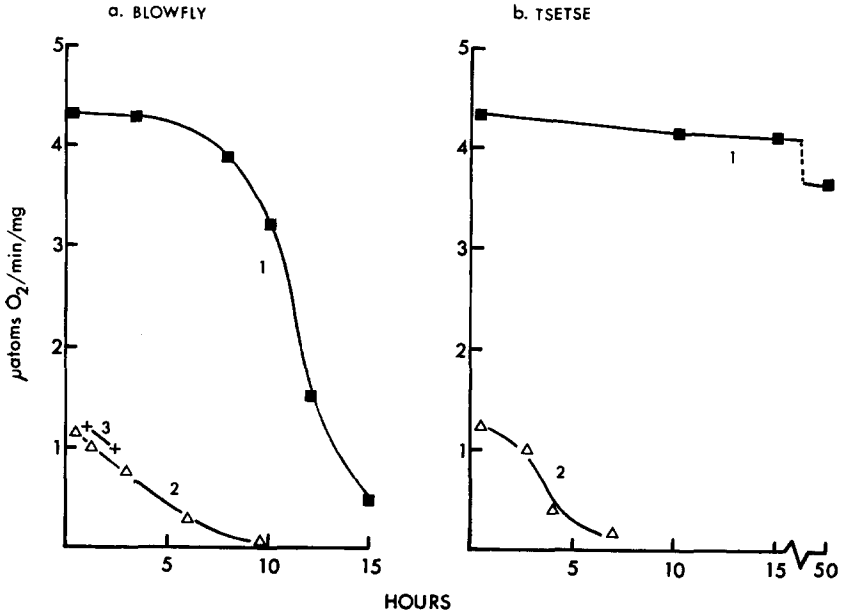

Fig. 4. The State 3 oxidation of mitochondria isolated by standard (△) and by improved (■) methods, as described under Experimental Procedure. (a) Blowfly mitochondria assayed with pyruvate as a substrate. Curve 1, improved; curve 2, standard; curve 3, data for *Phormia regina* from Childress and Sacktor [4]. (b) Tsetse fly mitochondria assayed with proline as a substrate. Curve 1, improved; curve 2, standard.

from levels similar to those currently reported for other dipteran sarcosomes [1, 4, 6, 7, 22] to values in excess of 4 μatoms O_2/min per mg mitochondrial protein, somewhat higher than previously reported values for the tsetse fly [16, 32]; in addition, the day-to-day variability was reduced to acceptable levels, not exceeding 10 %. The stability of mitochondrial suspensions was also greatly improved, and mitochondria of the blowfly were capable of being stored for 8 h, and of the tsetse fly for more than 24 h, without serious deterioration.

The very high oxidative capacity of dipteran sarcosomes provides a convenient opportunity for checking on the extent of deterioration associated with isolation procedures. If freshly isolated flight muscle is briefly washed in assay medium containing appropriate substrates and cofactors, the quantity of mitochondrial protein liberated is adequate for the assay of oxygen consumption, which can be initiated less than a minute after the mitochondria have first been exposed to the in vitro system. Estimates of mitochondrial concentration can subsequently be made on the basis of the optical density of the assay suspension, values being converted to mitochondrial protein through a previously established relation between the two variables. Such a check shows that with mitochondria isolated by the improved methods no significant deterioration has occurred during isolation in respect of the most sensitive criterion of mitochondrial quality, which is State 3 respiration. This conclusion is strengthened by the finding that the duration of exposure to each individual phase of the isolation procedure may be doubled, to give a total duration for the complete extraction procedure of 40 as compared with 20 min, without effect on the level of State 3 oxidation.

The possibility is still not excluded that substantial deterioration may occur at the moment of exposure to in vitro conditions, but a comparison of the oxidative capacity of isolated mitochondria with estimated requirements of the flight system provides no evidence of this. The flight muscles of an adult male of *G. morsitans* contain 1.1 mg of mitochondrial protein [33] which, on the basis of the results shown in Figure 4b, would be capable of supporting the State 3 oxidation of proline at a rate of 4.73 μatoms O_2/min, or 3178 μl O_2/h at 25 °C. The resting oxygen consumption of a mature male at this temperature is about 25 μl O_2/h [34], which means that the in vitro capacity would be sufficient to sustain a 120-fold increase in oxygen consumption during the transition from rest to flight. This is more than adequate to account for the observed increase in oxygen consumption during flight in Diptera generally [35]. As a further check it can be calculated that an oxygen consumption of 4.73 μatoms O_2/min would correspond to a proline consumption of 0.79 μmol/min [32] in good accord with observed rates of consumption during initial stages of flight at 27 °C, estimated at 0.70 μmol/min [36].

DISCUSSION

Present results have indicated that methods in general use for the isolation of flight muscle mitochondria may cause extensive physiological damage, most strikingly manifested as a drastic loss of oxidative capacity (see Fig. 4). Main causes of deterioration have been identified, the most important being mechanical damage to the mitochondrial membranes, which would lead to increased permeability and loss of endogenous material. Even when mechanical damage is minimized it is still necessary to buffer the isolated mitochondria against loss of endogenous components by incorporation in the isolation medium of phosphate and magnesium. Another damaging feature of standard isolation procedures was found to be the development of anoxia. Improvements in isolation procedure designed to reduce deterioration associated with these effects have enabled the isolation of stable mitochondrial suspensions exhibiting high levels of respiratory control, P/O ratios close to the theoretical values (refs. 16 and 32, and in preparation) and oxidative capacities which are commensurate with estimated requirements of the flight system. It seems reasonable to hope that results obtained with such mitochondria will be meaningful in relation to the normal physiological performance of dipteran flight muscle.

ACKNOWLEDGEMENTS

Our thanks are due to the Overseas Development Administration of the Commonwealth Office for generous financial assistance.

REFERENCES

1 Sacktor, B. (1970) Adv. Insect Physiol. 7, 267–347
2 Slater, E. C. and Lewis, S. E. (1954) Biochem. J. 58, 207–217
3 Balboni, E. R. (1965) J. Insect Physiol. 11, 1559–1572
4 Childress, C. C. and Sacktor, B. (1966) Science 154, 268–270
5 Chance, B. and Sacktor, B. (1958) Arch. Biochem. Biophys. 76, 509–531

6 Tribe, M. A. and Ashhurst, D. E. (1972) J. Cell Sci. 10, 443–469
7 Van den Bergh, S. G. and Slater, E. C. (1962) Biochem. J. 82, 362–371
8 Sacktor, B. and Cochran, D. G. (1958) Arch. Biochem. Biophys. 74, 266–276
9 Stevenson, E. (1966) Biochim. Biophys. Acta 128, 29–33
10 Van den Bergh, S. G. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), Vol. X, pp. 117–122, Academic Press, New York
11 Vaughan, H. and Newsholme, E. A. (1969) FEBS Lett. 5, 124
12 Walker, A. C. and Birt, L. M. (1969) J. Insect Physiol. 15, 305–317
13 Slack, E. and Bursell, E. (1972) Biochim. Biophys. Acta 256, 287–292
14 Lehninger, A. L. (1964) The Mitochondrion, p. 136, W. A. Benjamin, Menlo Park, Calif.
15 Chance, B. and Baltscheffsky, M. (1965) Biochem. J. 68, 283–295
16 Bursell, E. (1975) Comp. Biochem. Physiol. 52B, 235–238
17 Slack, E. N. (1975) Mitochondria from insect flight muscle, Ph.D. thesis, University of London
18 Stinson, R. A. and Spencer, M. (1968) Canad. J. Biochem. 46, 43–50
19 Kapoor, M. and Tronsgaard, T. M. (1972) FEBS Lett. 23, 349–351
20 Tyler, D. D. (1968) Biochem. J. 107, 121–123
21 Hansford, R. G. and Sacktor, B. (1970) J. Biol. Chem. 245, 991–994
22 Hansford, R. G. (1972) Biochem. J. 127, 271–283
23 Johnson, D. and Lardy, H. (1958) Nature 181, 701–702
24 Carafoli, E., Gamble, R. L. and Lehninger, A. L. (1966) J. Biol. Chem. 241, 2644–2652
25 Chappell, J. B. and Haarhoff, K. N. (1967) in Biochemistry of Mitochondria (Slater, E. C., Kaniuga, Z. and Wojtczak, L., eds.), pp. 75–91, Academic Press, London, Polish Scientific Publishers, Warsaw
26 Loh, H. H., Volfin, P. and Kun, E. (1968) Biochemistry 7, 726–736
27 Lenaz, G. and MacLennan, D. H. (1966) J. Biol. Chem. 241, 5260–5265
28 Slater, E. C. and Cleland, K. W. (1953) Biochem. J. 55, 566–580
29 Lester, R. L. and Hatefi, Y. (1958) Biochim. Biophys. Acta 29, 103–112
30 Lehninger, A. L. (1962) Physiol. Rev. 42, 467–517
31 Chance, B. (1965) J. Biol. Chem. 240, 2729–2748
32 Bursell, E. and Slack, E. (1976) Insect Biochem. 6, 159–167
33 Bursell, E. (1973) J. Insect Physiol. 19, 1079–1086
34 Rajagopal, P. K. and Bursell, E. (1966) J. Insect Physiol. 12, 287–297
35 Chadwick, L. E. (1953) in Insect Physiology (Roeder, K. D., ed.), pp. 615–636, John Wiley and Sons, New York
36 Bursell, E. (1963) J. Insect Physiol. 9, 439–452