SOME STUDIES ON THE CARBOHYDRATE METABOLISM AND ELECTROPHYSIOLOGY OF THE LOCUST (*Schistocerca gregaria*) CENTRAL NERVOUS SYSTEM *IN VITRO*

by

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2. Some aspects of the carbohydrate metabolism of the thoracic ganglia of the locust Schistocerca gregaria.

   Strang, R.H.C., Clement, E.M. and Rae, R.C. (1979)
   Comparative Biochemistry and Physiology B 62, 217-224.

3. The relative roles of glucose and trehalose in the nutrition of the nervous system of the locust Schistocerca gregaria.

   Strang, R.H.C. and Clement, E.M. (1979)
   J. Insect. Biochem. (In Press).

ABBREVIATIONS

ADP	Adenosine 5' - pyrophosphate
AMP	Adenosine 5' - phosphate
ASP	Aspartate
ATP	Adenosine 5' - triphosphate
BSA	Bovine Serium albumin
CNS	Central nervous system
DHAP	Dihydorxyacetone phosphate
Fp	Flavoprotein (oxidized)
G 1-P	Glucose 1 - phosphate
G 6-P	Glucose 6 - phosphate
Glu	Glutamate
α-GP	α - Glycerolphosphate
α-OG	α- oxoglutarate
Mal	Malate
NAD^+	Nicotinamide-adenine dinucleotide, (oxidized)
NADH	Nicotinamide-adenine dinucleotide, (reduced)
$NADP^+$	Nicotinamide-adenine dinucleotide phosphate, (oxodized)
NADPH	Nicotinamide-adenine dinucleotide phsophate, (reduced)
OAA	Oxaloxcetate
Pi	Orthophosphate
PPi	Pyrophosphate
Q10	Temperature coefficient
TCA	Tricarboxylic acid cycle
UDP	Uridine 5' - pyrophosphate
UDPG	Uridine 5' - pyrophosphate glucose
UTP	Uridine 5' - triphsophate

SUMMARY

This thesis is concerned with the basic carbohydrate metabolism, control of aerobic and anaerobic glycolysis and role of various carbohydrates as metabolic fuels for the locust meso- and metathoracic ganglia. These biochemical studies were accompanied by electrophysiological measurements on the metathoracic ganglion. The technique of measuring the concentrations of key metabolites, trehalose, glycogen, glucose, pyruvate, lactate, arginine phosphate, ATP, ADP and oxygen uptake of thoracic ganglia was applied throughout this work to investigate the control of aerobic and anaerobic glycolysis. In addition, the maximum specific activity of hexokinase was estimated to investigate its role in controlling glycolysis. The technique of immobilising locusts at -20^{o}C for measuring steady state metabolites worked well in contrast with the situation with mammals where this technique is not feasible. The technique of using micro-suction electrode was used to investigate the electrophysiology of metathoracic ganglion.

In the present study a superfusion technique which allows simultaneous recording of spontaneous activity and oxygen uptake of isolated meso- and metathoracic ganglia, has been developed. Locust ganglia were incubation in 3 ml of modified Hoyle's Saline (1953) containing 10mM glucose which was saturated with 100% O_2 and the temperature maintained at 35-37^{o}C. Under these conditions of incubation, the locust

central nervous tissues offer a useful preparation and have more merits than well documented mammalian central nervous tissues (McIlwain and Bachelard, 1971; McIlwain, 1975). Their advantages include:

(a) lack of vascular system, which means that even in vivo metabolites must diffuse from outside the ganglia;

(b) a well defined blood-brain barrier analogous to mammalian CNS, which would allow the tissue to be tolerant to certain changes in the medium;

(c) ease of isolation as compared to mammalian preparations and therefore lesser chances of disruption;

(d) comparing the concentrations of glycogen, glucose, ADP, ATP, arginine phosphate, pyruvate, lactate present in the locust ganglia during ncubation in vitro, with that in mammalian cerebral cortex slices (Rolleston and Newsholme, 1967a, 1967b), the regulatory effect of the ratio of ATP/ADP on hexokinase/phospho-fructokinase system and pyruvate kinase/glyceraldehyde-3-phosphosphate dehydrogenase are probably in general agreement, however, the low ratio of lactate/pyruvate present in vivo and which was maintained throughout the incubation, in contrast to higher lactate/pyruvate ratio present in mammalian cortex slices, probably makes locust central nervous tissues more aerobic. As oxygen uptake is an index of metabolic activity, therefore higher metabolic activity of locust nervous tissues as compared to that of mammalian tissues which has been found that locust nervous tissues showed a

higher oxygen uptake and a greater recovery of these metabolites *in vitro* as compared to that *in vivo* in contrast to mammalian cerebral cortex slices. The evidence also comes from the measurement of rate of flux of glucose (based on measurement of O_2 uptake and lactate production in the medium) in the locust ganglia. It is 3-7% of the total glucose utilized to lactate while the rest, via pyruvate to TCA cycle, whereas in mammalian central nervous tissues 20-40% of the total glucose utilized, is converted to lactate. The results of lower $\frac{\text{Lactate}}{\text{Pyruvate}}$ ratio and lower output of lactate in contrast to mammalian nervous tissues, probably reflect that locust central nervous tissues have a rate limitation at lactate dehydrogenase stage, in contrast to mammalian central nervous tissues whose lactate dehydrogenase is at equilibrium and is not a controlling enzyme.

(e) possession of very high spontaneous activity which can be monitored easily by usual electrical apparatus and which may be correlated with the energy metabolism, in contrast to cerebral cortex slices which do not show any endogenous activity *in vitro*. This makes the locust central nervous tissue a good model for studying the electrophysiology and biochemistry under different incubation conditions. The evidence comes from the present preliminary work of the effect of certain pharmacological agents on the

spontaneous activity which was modified in a predictable manner. Comparing the spontaneous activity *in vitro* with that *in vivo*, the activity was reduced to 30-50% in hypo-osmotic saline. It rose, however, to 60-75% in presence of isosmotic saline and therefore the adjustment of the modified Hoyle's saline to same osmolarity as present in haemolymph is necessary to raise the spontaneous activity close to that found *in vivo*. Under these conditions of incubation spontaneous activity remains steady for 3 hrs., begins then to decrease and finally disappears between 3-3½ hrs.

The present results showed that glucose, glycogen and trehalose could support the oxygen uptake and hence all these three substrates could act as metabolic fuels for locust CNS. Although glycogen is a large potential store of energy, *in vitro* much of it is lost from the tissue as glucose and trehalose. This makes it difficult to be certain as to its role in the living insect.

Due to the low maximum activity of hexokinase in locust central nervous tissues, in contrast to mammalian central nervous tissues, it was thought that hexokinase might be rate limiting upon glucose utilization. The present results of measurements of metabolites in anoxic conditions have failed to throw light on this question. Therefore it is not possible to conclude at the moment whether or not the activity of hexokinase imposes a limitation on the

rate of glucose utilisation by locust central nervous tissues. The locust central nervous tissues behaved differently compared to mammalian central nervous tissues in anoxic conditions. Glycogen starts disappearing before the depletion of glucose in the tissue and its disappearance is the same even in medium containing 10mM glucose. The formation of lactate is higher during the first 5 min compared to subsequent 15 minutes and this parallels the glycogen disappearance. Not all the glycogenolysis can be accounted for as lactate formation. The metabolic effects of anaerobic conditions probably reflect the compartmentation of locust ganglia with regard to glycogen stores.

PLAN OF THE THESIS

This thesis has been divided into four chapters:

Chapter 1: This chapter deals with the general introduction relating previous work on insect tissues and stressing the importance of insect central nervous tissues, to the present. In addition, the aims of this work and conclusions have also been mentioned.

Chapter 2: Deals with the materials used during the course of present work and a critical description of methods, relating those methods as used by other workers (where required) with particular reference to advantages and disadvantages. At the end important conclusions have also been mentioned.

Chapter 3: This chapter gives the report of the findings on oxygen uptake, carbohydrate metabolism of meso- and metathoracic ganglia and spontaneous activity of metathoracic ganglion. This has been divided into four sections: section 'A' deals with the results of oxygen uptake and spontaneous activity, section 'B' gives the results of measurement of metabolites, section 'C' deals with a comparison and components of physiological saline (originally used) and haemolymph of locust, and finally section 'D' gives the results of anaerobic glycolysis and activity of hexokinase.

Chapter 4: This chapter deals with the overall discussion of the results of Chapter 3 and the conclusions drawn from these results. This chapter is followed by the references used in this thesis.

CHAPTER 1

Chapter One

INTRODUCTION

1. Selection of locusts over other animals of Arthropoda

At present invertebrate and in particular insect nervous systems offer an investigator a promising preparation for the integrated study of four major fields, neuroanatomy, neurophysiology, neurochemistry, and behavioural studies. However, due to the enormous range of invertebrates both as regards the number and size of cells in their nervous systems and their behaviour, it is difficult to decide which animals are best studied. A case can be made for the study of almost every organism, but the strongest is that in favour of some arthropods. Currently there are a series of interesting studies being made on this group. Among these there have been the combined behavioural and electrophysiological investigations of Horridge (1962 , 1965); Eisenstein and Cohen (1965), which have increased our understanding of electrophysiological correlates of insect reflexes and learning. An area in particular need of investigation is carbohydrate metabolism of the insect central nervous system and an aim of the present work has been to investigate some of the basic aspects of carbohydrate metabolism *in vitro* and attempt to correlate any changes in electrical activity with changes in the pattern of carbohydrate metabolism and finally to compare with those *in vivo*.

The present work is concerned with the carbohydrate metabolism of meso- and metathoracic ganglia and electrophysiology of the metathoracic ganglion of the desert locust, Schistocerca gregaria. The selection of this locust over other animals of this group of arthropods, is based on the following three features:

(a.) locusts (S. gregaria) are easily obtained commercially and their relative larger size compared to cockroaches, which have been used very often by other workers, makes them easier to handle.

(b.) there have been extensive studies of the carbohydrate metabolism of other locust tissues, in particular flight muscles. It was hoped that this work would provide the basic features of carbohydrate metabolism in locust nervous tissues. This work has been reviewed by Newsholme and Start (1973).

(c.) the results of Treherne and Moreton (1970) suggest that the insects possess a well developed blood-brain barrier analogous to that of mammals. It was hoped that this barrier would make insect ganglia a useful preparation for in vitro work as it would be tolerant of a wide variation in the composition of the supporting medium.

2. Selection of meso- and metathoracic ganglia

The meso- and metathoracic ganglia were selected because they are easily removed from the animal with minimal damage and they provide a relatively large piece of homogenous nervous tissue. These ganglia have been extensively studied from the point of view of their anatomy

and electrophysiology and it was hoped that this knowledge would provide a basis for interpreting and correlating the relationship between the Biochemistry, Neuroanatomy and Neurophysiology of the insect central nervous system. Because of this, these areas are briefly reviewed in the following sections to provide a foundation for the aims of this work.

3. Structure of central nervous system of the locust

A simplified diagram of the locust nervous system is shown in Figure 1. This is based on the structure described by Albrecht (1953). The central nervous system consists of an anterior dorsal brain, connected by circum-oesophageal connectives to a double ventral nerve cord. At certain sites on the ventral nerve cord groups of neurons, which number in thousands, are organized into discrete ganglia. There are three ganglia known as the pro- meso- and metathoracic ganglia in the thoracic region. Meso- and metathoracic ganglia are relatively bigger in size compared to other ganglia of the nerve cord and have been exploited in this work because of the size and ease of dissection.

4. Structure of the ganglia

Both meso- and metathoracic ganglia are very alike in structure. The results of Boulton and Rowell (1968) and that of Lane and Treherne (1973) showed that each ganglion is covered by an acellular layer known as the neural lamella. This acts as a protective layer and is covered by fatty tissue which forms a continuous sheath covering

Simplified diagram of the locust ventral nerve cord

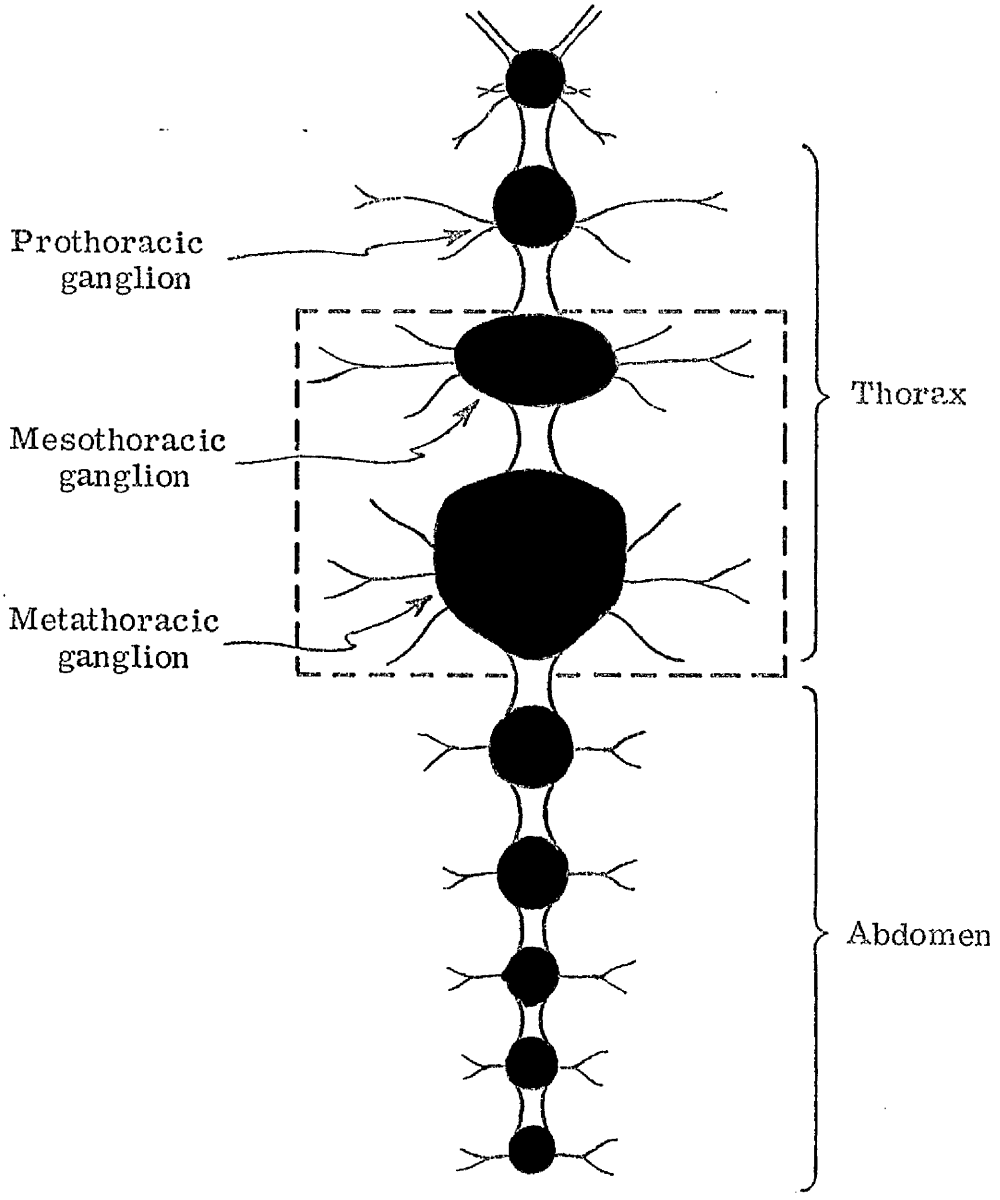


Figure 1.

the entire ganglia. This fat body sheath is covered by connective tissue which is essentially similar to that of underlying neural lamella. Beneath the acellular neural lamella lies a modified glial layer called the perineurium (Treherne et al., 1970). The cells of this layer interdigitate with one another in a highly complex fashion and the intercellular clefts formed between their lateral borders are kept to a minimum at the basal region of the cells by separate desmosomes, gap junctions and tight junctions, (Lane and Treherne, 1972). This cellular layer acts as a blood-brain barrier very like the vertebrate blood-brain barrier. The evidence for this comes from the histological studies of Lane and Treherne (1971); Wigglesworth (1960) and Elderfrawi et al. (1968) and electrophysiological studies of Pichon and Treherne (1973) and Treherne et al. (1973). The histological studies indicate that high molecular weight molecules like peroxidase (mol.wt.40,000) and Indian ink can penetrate the neural lamella following the injection of these substances into the haemolymph, but they are stopped by perineurial layer. Electrophysiological findings indicate that resting and action potentials are not affected by changing the concentration of either sodium or potassium ions in the bathing medium when it has an intact nerve sheath. However, by surgical removal of the nerve sheath or by bathing in hypertonic urea solution, the resting and action potentials are affected dramatically by decreasing the Na^+ concentration or increasing the K^+ in the

environment close to the neurones. Histological studies after treatment with urea solution suggest that it penetrated the perineurium and disrupted the blood-brain barrier which may result from a selective change in the permeability of the perineurial membrane or tight junctions. This idea of a blood-brain barrier is also supported by the fact that cockroach blood contains very high concentration of potassium and magnesium, yet the intercellular area contains high sodium but low potassium (Treherne and Moreton, 1970). Underneath this cellular layer, the perineurium, lie neuronal and glial cells. Wigglesworth (1960), Smith and Treherne (1963), have recognised two rather ill-defined regions in the glial system, the peripheral layer which contains cells that invest the neuronal cell bodies and an inner region of glial cells, adjacent to the surface of neuropile. The neuronal cell bodies send their dendrites into this central area, the neuropile. This is the site of synaptic transmission and integration. Its complete anatomy and function is still uncertain.

A whole mount of metathoracic ganglion stained with Nile Blue is shown in Figure 2. This staining process made nerve cell bodies visible. It can be seen that the cell bodies (the dark areas) are only found in certain parts of the ganglion and electrophysiological and histological evidence indicate that the position of specific neuronal cell bodies is constant (Burrows and Hoyle, 1973). The mapping of the positions of the neuronal cells in ganglia has opened up the possibility of linking biochemistry and

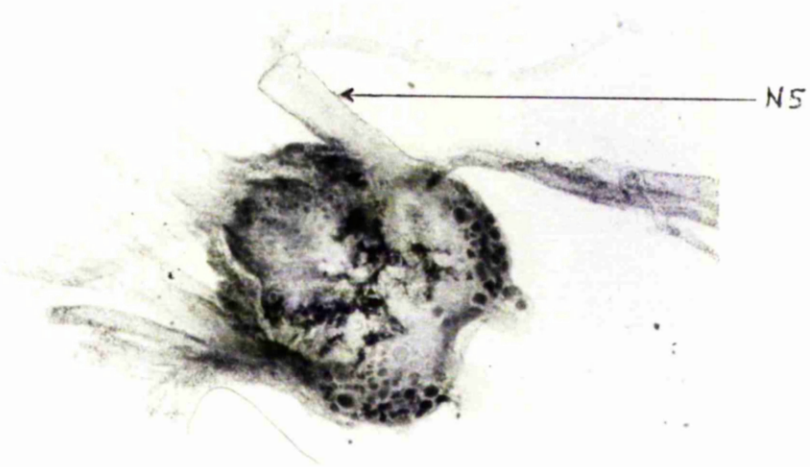


Figure 2.

electrophysiology at single cell level (Emson et al., 1974). This figure also shows the location of motor nerve, N5 (classified according to Pringle (1939), which has been used in the present work for electrical recordings. The large diameter and length of this nerve, render it an ideal system for studying its electrophysiology *in vitro* and *in vivo*.

5. *Electrophysiological and behavioural studies of Insect CNS*.

The basic electrophysiology of the insect nervous system is similar to that of other invertebrates and vertebrates (Wiersma, 1967). Transmission of nervous impulses involve both action potentials and chemical transmission across synapses as in the mammalian systems. Recording of synaptic potentials were made by Yamasaki and Narashi (1960) at the level of sixth abdominal ganglia of *periplaneta americana* with external electrodes placed as near as possible to the site of electrogenesis. Following the stimulation of the cerebral ganglion, they observed a potential exhibiting the characteristics of an excitatory post-synaptic potential in the abdominal ganglion. Hoyle and Burrows (1970) recorded intracellularly the excitatory post-synaptic potentials and inhibitory post-synaptic potentials in identified neurons of locust, *S. gregaria*.

As has already been mentioned, the locust can be used in studies of behavioural phenomena. Thus Horridge (1962), working with the nerve cords of cockroaches, and Eisenstein and Cohen (1965) working on the prothoracic ganglia, showed

that a decapitated insect could learn to keep its leg raised if given a shock whenever the tarsus fell below a certain arbitrary level. Insects receiving random stimuli did not show this learning behaviour. Similarly, Hoyle (1965) has demonstrated an electrophyiological correlate to a learning situation in a headless locust. He showed that if the leg was stimulated every time the frequency of discharge to the anterior coxal adductor muscle (which lifts the leg) fell below a preset level, then the discharge rate of this neuron could be tripled and remained high for many hours. In animals receiving stimuli unrelated to the low frequency of discharge in the neuron there was no increase in the frequency. This type of work and its future continuation points towards an understanding of higher behaviour in basic electrophysiological terms. Thus in future it may be possible to use a well understood insect ganglionpreparation to investigate biochemical aspects of higher behavioural patterns.

6. Carbohydrate metabolism of Insect tissues

There have been few studies of the carbohydrate metabolism of the insect nervous system, although carbohydrate metabolism has been well investigated in certain insect flight muscles. It is possible that many of the basic features of muscle carbohydrate metabolism will occur in the CNS. Biochemical studies of the insect flight muscle have been reviewed by Sacktor (1965); Newsholme and Start (1973). It has been confirmed that insect flight muscles are more aerobic than mammalian skeletal muscles and since the

metabolic rates are much higher in insect muscle preparations, these preparations offer a useful tool for studying the regulation of glycolysis and tricarboxylic acid cycle (Beis and Newsholme, 1975). This immediately led to the question of whether insect nervous tissues are also more aerobic, and these previous findings initiated the present work on control of aerobic and anaerobic glycolysis of insect central nervous tissues and to compare it with well documented mammalian central nervous tissues (McIlwain and Bachelard, 1971). It was hoped that such a comparison could highlight any major similarities or differences in the patterns of metabolism of the two tissues, such as, for instance the widely different concentrations of glycogen in mammalian and insect nervous systems (Strang and Bachelard, 1973; Steele and Robertson, 1963). There are four approaches to study the control of a metabolic pathway (Newsholme and Start, 1973):

(a) analysis of maximal activities of enzymes.

(b) analysis of properties of enzymes.

(c) supplementation experiments.

(d) intermediate concentrations and controlling enzymes.

In the first two methods, the properties of enzymes are analysed in conditions very far removed from those in which they function in pathways, and without knowledge of factors such as substrates, product activators or inhibitors concentration in the tissue. In the 2nd method in particular the basic premise, that enzymes possessing certain properties

are necessarily controlling enzymes is probably not justifiable (Ferdinand, 1966 ; Monod et al., 1955). Supplementation of pathways with intermediates, activators, or inhibitors is probably not generally applicable due to permeability barriers at both cellular and subcellular levels. This technique was first used by Potter and Niemeyer (1959), who showed that addition of $NADP^+$ to a particle-free preparation from rat brain inhibited glycolysis and concluded that this was due to inhibition of phosphoglucoisomerase by accumulation of 6-phosphogluconate. Similarly, Dipietro and Weinhouse (1959) were able to stimulate the hexose monophosphate shunt in brain homogenate by addition of $NADP^+$, but could not show activity in the absence of $NADP^+$ or in intact cerebral cortex slices. Consequently, despite the number of valuable, and often unimpeachable theories put forward on the basis of analysis of maximal activities, or enzymes properties or the effects of supplementation, it was decided to investigate the control of glycolysis in insect nervous tissues by measuring the concentration of intermediates. This method has considerable advantages over the others. In particular, it provides the experimental systems in which analysis are made on the tissue preparation in its functional state, and was probably first used by Cori (1942) who observed an accumulation of hexose monophosphate under certain conditions without corresponding accumulation of fructose diphosphate or lactate, and concluded that phosphofructokinase was a controlling enzyme for glucose oxidation in muscle. With the advances in techniques and

commercial availability of enzymes as analytical tools, this general approach has been extensively used in recent years. Examples of its use are the identification of phosphorylation sites in the electron transport chain (Chance and Williams, 1956); and identification of controlling enzymes for glycolysis in heart, diaphragm (Newsholme and Randle, 1961, 1964) and brain (Lowry et al., 1964).

Glycolysis is defined as the splitting of glucose or glycogen to pyruvate and lactate by Embden-Meyerhof pathway (Harper, 1975). It has been customary to separate carbohydrate metabolism into anaerobic and aerobic phases. However, this distinction is arbitrary since the reactions in glycolysis are the same in presence or in absence of oxygen, except in extent of end products. When oxygen in in short supply, reoxidation of NADH formed during glycolysis is impaired. Under these circumstances NADH is reoxidised by being coupled to the reduction of pyruvate to lactate, the NAD^+ so formed being used to allow for further glycolysis to proceed. Glycolysis can thus take place under anaerobic conditions, but this limits the amount of energy liberated per mol of glucose oxidised. Consequently to provide a given amount of energy, more glucose must undergo glycolysis under anaerobic as compared with aerobic conditions. It has also been very well established by other workers that of the glucose utilized by the mammalian central nervous tissue at rest and under optimum conditions of oxygen and temperature, 20-30%

would be converted to lactate and remainder oxidized to CO_2 and water (McIlwain and Bachelard, 1971). In the light of these previous findings, the measurement of concentration of glycogen, glucose, trehalose, pyruvate, lactate, arginine phosphate, ATP, ADP and oxygen uptake were selected as parameters to study the control of aerobic and anaerobic glycolysis in the present work. In addition to the measurement of concentration of metabolites as a mean of studying the control of glycolysis, the maximal activity of hexokinase was also found. This enzyme which catalyzes the first step in glycolysis is frequently a point of control.

7. The role of glucose, glycogen and trehalose as metabolic fuels in insect tissues.

A major feature of insect carbohydrate metabolism is the importance of the disaccharide trehalose. In insect haemolymph trehalose is a major sugar. Thus Clegg and Evans (1961) and McIlwain (1953) respectively found 60mM trehalose and 1 mM glucose present in blowfly haemolymph, and an average value of 5.0mM glucose present in mammalian blood. Although it is known that trehalose rather than glucose can be an important fuel for locust flight muscle, yet it is generally believed that the functioning of mammalian central nervous system has an absolute requirement for glucose (Elliott, 1955). Therefore, the involvement of glucose and trehalose as the metabolic fuel for insect central nervous tissues might provide an interesting study. Another aim of the present work was

to look at the role of these two sugars as metabolic fuels in insect nervous tissues. As mentioned before, mammalian and insect central nervous tissues differ dramatically with regard to glycogen concentration and therefore the role of this sugar as a metabolic fuel was also studied.

8. Advantages of working on insect CNS

In this section, the various advantages of working on insect CNS are summarized:

1. Simplicity:

This is the major advantage when working on insects. Its use of neurons is economical compared to mammalian CNS (the insect CNS contains neurons numbered in thousands while the mammalian CNS has millions of such cells). In addition to this, the insect CNS probably contains fewer uncertain metabolic compartments compared to that of mammals (Balázs and Cremer, 1971).

2. Neuroanatomy and physiology:

The insect CNS has long been exploited by the physiologists, with the result that the anatomy and physiology of the system are known to some extent.

3. Suitability of ganglia for *in vitro* work:

The individual ganglia are by their construction well suited to *in vitro* work. They lack a vascular system and yet possess in their neural lamella a mechanical protection and in the cells of the perineurium a blood-brain barrier. In addition to this, the isolated metathoracic ganglion

possesses a high level of spontaneous activity which may be quite easily monitored as action potentials from one of the major nerves (Weiant, 1958).

9. Disadvantages

Having mentioned the advantages of working on locust CNS, nevertheless, there are potential disadvantages which are:-

(1) Tissue weight measurements.

Due to small size of ganglia, difficulties might be encountered in estimating the weight of ganglia, and in the amount of metabolites available for estimation.

(2) Oxygen uptake and tracheal system.

There is a tubular system known as tracheal system throughout the ganglia through which exchange of gases occur between the ganglion cells and the atmosphere. These tubes permeate the whole ganglia, even into the neuropile. The isolation of ganglia for *in vitro* work, would disrupt the tracheal system. However, this may be at least partly offset, by increasing the concentration of dissolved oxygen in the medium.

Conclusions and aims:

The following can be concluded from the introduction to this thesis:

1. the meso- and metathoracic ganglia offer an ideal system for studying metabolism *in vitro* due to their easy isolation, possession of blood-brain barrier and lack of vascular system. On the other hand the metathoracic ganglion offers an ideal system for studying electrophysiology *in vitro* due to its possession of motor nerves of large diameter and length, and of the findings of other workers that electrical activity can be monitored both *in vivo* and *in vitro*.

2. the well known neuroanatomy, neurophysiology, behavioural studies, carbohydrate metabolism of insect flight muscles and well documented metabolic studies of mammalian central nervous tissues, favour strongly the aims of this work which are:

A. to investigate some of the basic aspects of carbohydrate metabolism *in vitro* and to attempt to correlate any changes in electrical activity with changes in the pattern of carbohydrate metabolism, and to compare them with those *in vivo*.

B. to study the control of aerobic and anaerobic glycolysis of locust central nervous tissues and to compare it with the well documented mammalian central nervous tissues.

C. to elucidate the role of glucose, trehalose and glycogen as metabolic fuels for locust nervous tissues.

3. the control of aerobic and anaerobic glycolysis can be best studied by measuring the concentrations of key metabolites which are glycogen, trehalose, glucose, pyruvate, lactate, arginine phosphate, ATP, ADP and rates of oxygen consumption. However, the maximum activity of hexokinase was estimated to investigate the control of glucose utilisation.

CHAPTER 2

Chapter Two

MATERIALS AND METHODS

MATERIALS

1. Locusts: Adult locusts (S. gregaria) of both sexes, were used in these studies. They were obtained from Larujon Locust Suppliers, c/o Welsh Mountain Zoo, Colwyn Bay, North Wales. The locusts were kept in a cage which was illuminated by an electric lamp to keep temperature between 30-32°C. They were fed on a diet of bran and water.

2. Electrical Apparatus: Oxygen electrode was supplied by Rank Bros. Bottisham, Cambridge, (U.K.). Preamplifier, type 101, was supplied by Iselworth Electronics, Frederick Street, Waddesdon, Bucks, England, and the audio amplifier was assembled in the department. The cathode ray oscilloscope type CD 1400, was supplied by the Solarton Electronics Group Ltd., England. Scalar type 1700/F, fitted with a pulse height discriminator, was supplied by Isotope Developments Ltd., England. The pen recorder for measuring oxygen uptake was manufactured by Kipps-Zonen Ltd., England. This recorder could give a response to a potential difference as low as 0.1 mV. Pen recorder for recording nervous impulses was supplied by Evershed and Vignols Ltd., Acton Lane Works, London. Peristaltic pump, type P8 and used for the replacement of the medium, was supplied by Pharmacia, Sweden. Recording suction electrodes were prepared by

a plastic tubing supplied by Portex Ltd., Hythe, Kent (U.K.).

3. Reagents: All the enzymes were obtained from Boehringer Mannheim Corporation, Limited, London, except arginine kinase and trehalase (α, α - trehalose glucohydrolase; 3.2.1. 28). Arginine kinase was obtained from Sigma Chemical Company, St. Louis, U.S.A. and Trehalase was extracted from the slime mould Dictyostelium discoideum and purified according to Friedman (1960) and Ceccarini (1966). The enzymes used are as follows:

(A) Hexokinase (E.C.2.7.1.1)

(B) Glucose 6-phosphate dehydrogenase (E.C.1.1.1.49)

(C) α1,4-1, 6 Amyloglucosidase (E.C.3.2.1.-)

(D) Pyruvate kinase (E.C.2.7.1.40)

(E) Myokinase (E.C.2.7.4.3)

(F) Lactate dehydrogenase (E.C.1.1.1.27)

(G) Catalase (E.C.1.11.1.6)

(H) Arginine kinase (E.C.2.7.3.3)

(I) Creatine phosphokinase (E.C.2.7.3.2)

Organic and inorganic substances used in these studies were obtained from BDH Chemicals, Poole, Dorset, (U.K.) and Boehringer Corporation Limited, London except bovine serum albumin which was obtained from Armour Pharmaceutical Company, Eastbourne, (U.K.).

METHODS:

1. Incubation Medium

The medium used in these experiments was that of Hoyle (1953) except that $MgCl_2$ was omitted and $NaHCO_3$ replaced by a phosphate buffering system (Burns, M., personal communication). In addition to this, it has been found by other workers (Smith et al., 1975 ; Barker and Gainer, 1975) that endogenous spontaneous activity in molluscan neurons is inhibited in presence of magnesium ions, calcium ions, however, are essential for obtaining the spontaneous activity. This saline contains about the same ionic concentration as is present in locust haemolymph. Different concentrations of glucose were added as exogenous energy sources. A range of salines were employed in the course of this work. The composition of media referred to as "basal" saline, "glucose" saline, "iso-osmolal" saline and "trehalose" saline are shown in Table 1. All the stock solutions of "basal" saline were kept at $4^{o}C$. When the "basal" saline was prepared for use, care was taken to add the calcium chloride at full dilution to prevent salting out. Trehalose and glucose were added to the ice cold "basal" saline according to the requirement and preparation. Initially care was taken to ensure that the saline was sterilized by heat or by filtration, but this was found to be unnecessary in the time scale of the reported experiments.

TABLE (1)

COMPOSITION OF SALINES

Components	Basal Saline mM	Glucose Saline mM	Iso-osmolal Saline mM	Trehalose Saline mM
KCL	10	10	10	10
NaCl	140	140	140	140
Na_2HPO_4	6	6	6	6
NaH_2PO_4	4	4	4	4
$CaCl_2$	2	2	2	2
Glucose	-	10	10	4
Sucrose	-	-	100	-
Trehalose	-	-	-	50

Salt solutions were stored separately in concentrated form, mixed and diluted to about required volume before the addition of $CaCl_2$, to prevent salting out.

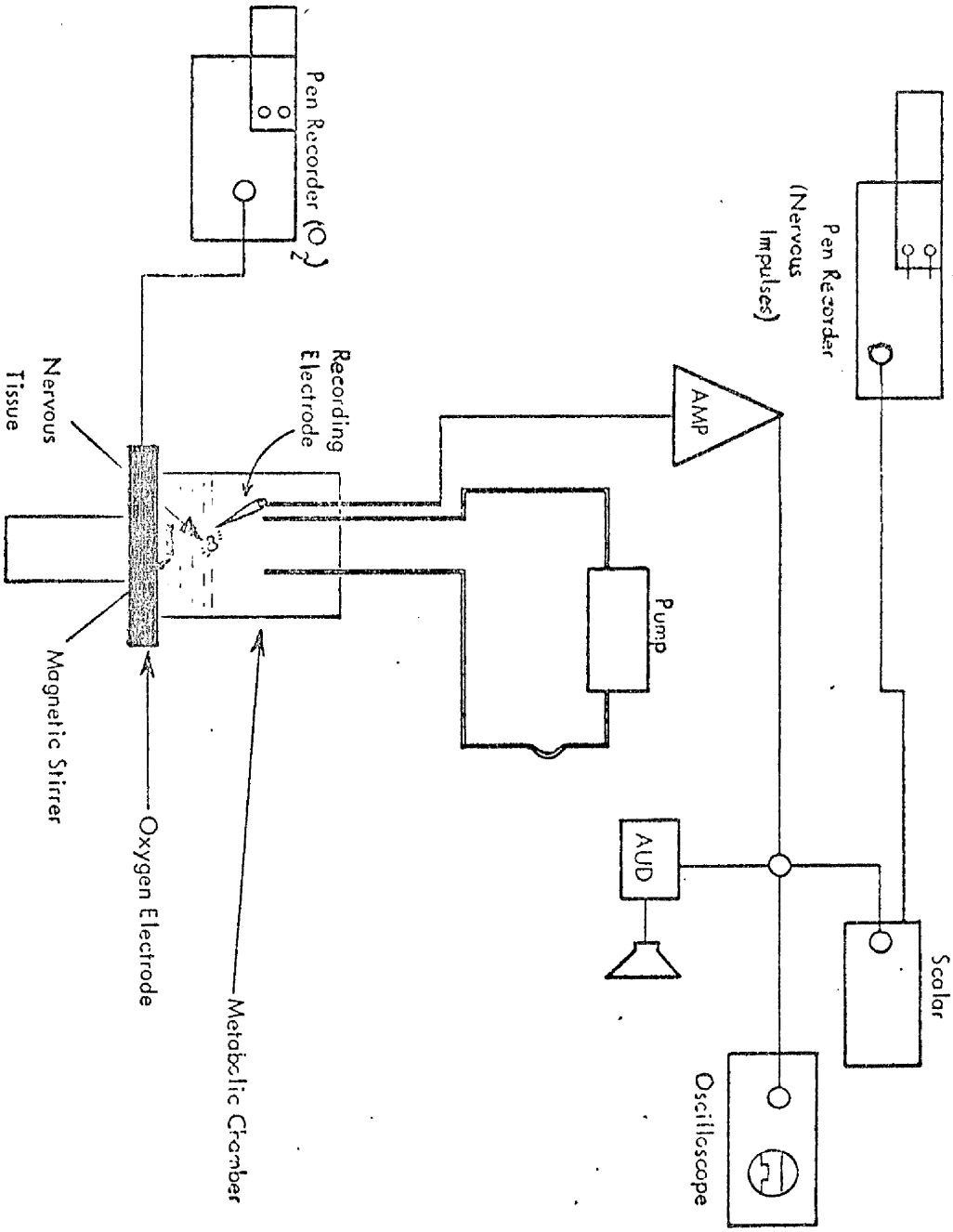
pH at room temperature of all the above salines was adjusted to 6.85.

2. Metabolic Chamber and Recording Apparatus.

The metabolic chamber and recording apparatus used in these studies are shown in Figure 3. The apparatus was based on an oxygen electrode which was fitted with a modified stopper. The stopper was perforated to accommodate a plastic recording suction electrode and tubes, which, with the aid of peristalic pump, allowed the intermittent or continuous replacement of the medium. Normally, the medium in the oxygen electrode chamber is stirred magnetically, but this caused an interference with the recording of spontaneous activity. This difficulty was overcome by using a glass stirrer attached to a nylon fibre and driven by a current of compressed air controlled by a flow meter. The stopper was perforated to allow the passage of this nylon fibre. The temperature of the chamber was controlled by a water jacket attached to a water bath. The signal from the recording electrode passed to a preamplifier and from the output of preamplifier, there passed three signals, one to the scalar, one to the cathode ray oscilloscope and one to the audio amplifier. The signal from the scalar went to the pen recorder which gave a quantitative measurement of nerve impulses in terms of action potentials per unit time. The signal from the oxygen electrode was connected to a pen recorder which gave quantitative measurements of oxygen uptake. The oxygen electrode, peristaltic pump and preamplifier were all placed in an aluminium Faraday cage to lessen electrical interference. The layout of all this apparatus is shown in

FIGURE 3

Diagrammatic representation of the modified O_2 electrode and electrical apparatus.



METABOLIC CHAMBER AND RECORDING APPARATUS

Figures 4 and 5.

3. Estimation of fresh wet weight and dry weight of ganglia:

The fresh wet and dry weights of the thoracic ganglia were measured by using a Microbalance, type BE 22, supplied by Mettler Instruments, Greifense -Zurich, Switzerland. Fresh wet weight of meso- and metathoracic ganglia varies from 2-3 mg. In order to measure the dry weight, the ganglia were lyophilized for 4 hours (this is the time period when a constant weight was obtained) and reweighed. Table 2 gives the measurement of dry and wet weight of ganglia. An average ratio of $\frac{\text{Wet weight}}{\text{Dry weight}}$ ratio is 9.68 which indicates that wet weight of the ganglia is, on average 9.68 times of dry weight. Most of the results encountered in the literature are on a fresh wet weight basis. However, certain results are encountered on a dry weight basis, e.g. Larrabee (1958), working on superior cervical ganglia of rat, has quoted all his results on a dry weight basis. Although all the results in this thesis have been quoted on a fresh wet weight basis, they were actually derived from the figures for dry weight by dividing 9.68. The measurement of wet weight was avoided because it was found to be extremely variable. An example of steady state ATP levels (as shown in Figure 20 of chapter 3) is taken here to clarify the procedure of dividing by a factor of 9.68. The actual ATP levels were 26.13 $\pm$ 2.9 (Mean $\pm$ S.D.) μmol../gm in terms of dry weight and therefore in terms of wet weight it would be 26.13 $\pm$ 2.9 $\div$ 9.68 = 2.7 μmol../gm.

FIGURE 4

Diagrammatic representation of the modified O_2 electrode used in most of the reported experiments.
The apparatus is not to scale.

FIGURE 4

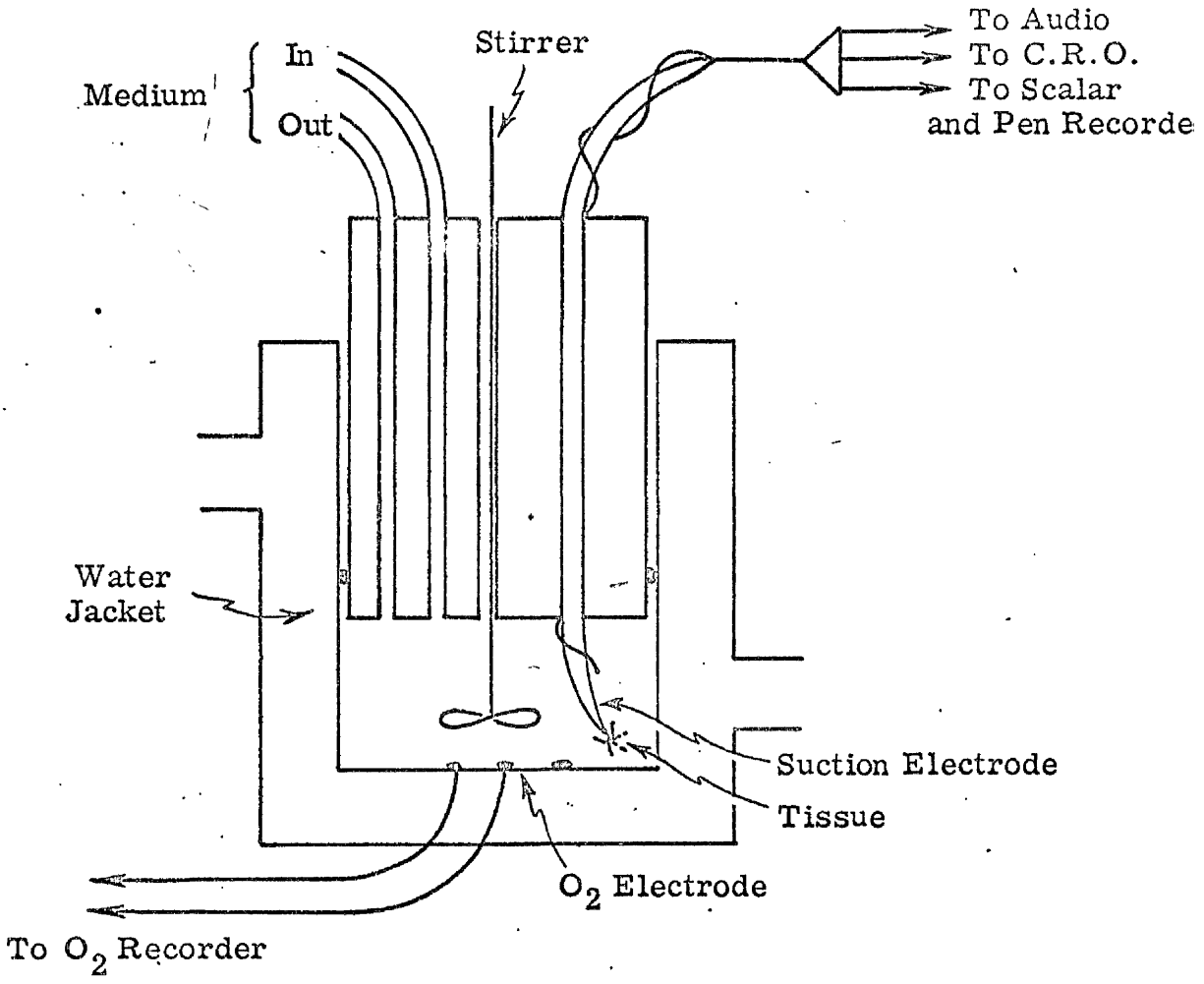

To Audio
To C.R.O.
To Scalar
and Pen Recorde
Stirrer
In
Medium
Out
Water
Jacket
Suction Electrode
Tissue
O_2 Electrode
To O_2 Recorder

FIGURE 5

Setting up of the metabolic chamber and electrical apparatus to record O_2 uptake and bioelectrical activity of the thoracic ganglion of the locust.

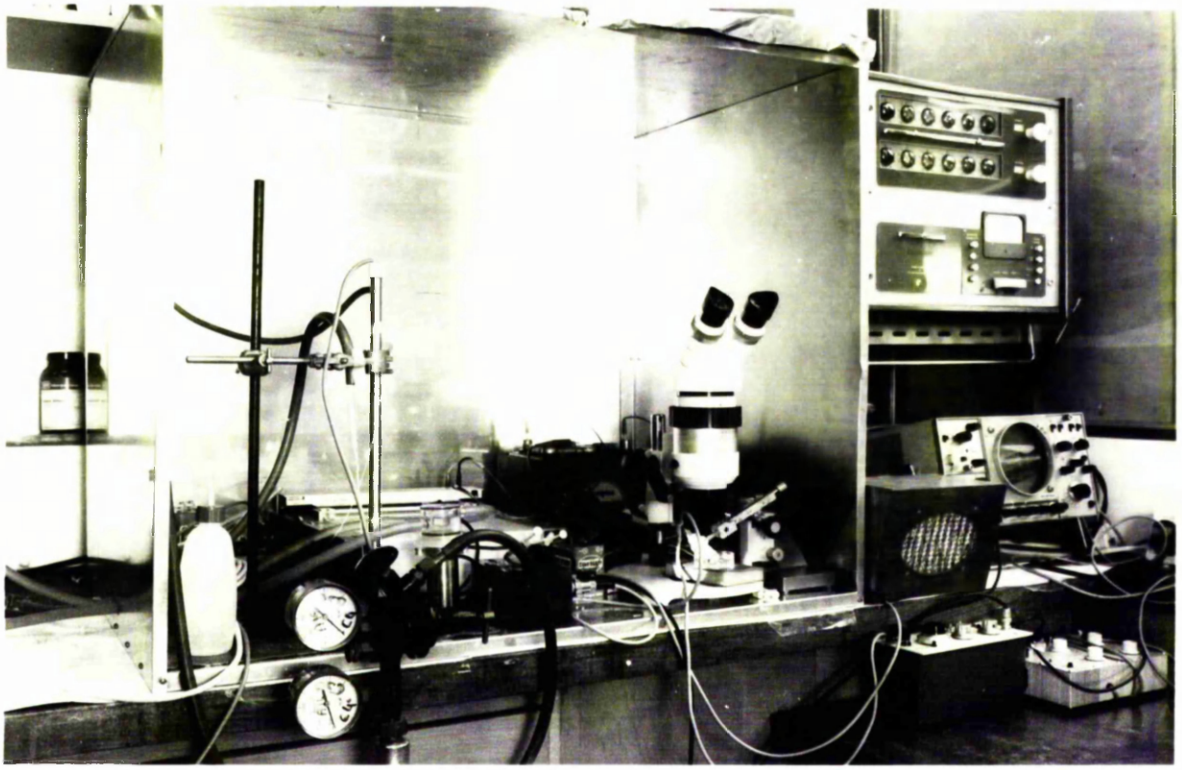


TABLE (2)

RELATIONSHIP OF THE DRY TO THE WET WEIGHT OF THE GANGLIA.

No. of ganglia	Wet weight (mg)	Dry weight (mg)	Wet weight / Dry weight
2	2.16	0.24	9.00
2	2.70	0.30	9.00
2	2.43	0.27	9.00
2	2.50	0.29	8.62
4	5.80	0.58	10.00
4	5.75	0.60	9.58
4	6.20	0.62	10.00
4	5.90	0.58	10.10
6	8.60	0.86	10.00
6	7.83	0.91	8.60
6	9.68	0.88	11.00
6	8.90	0.79	11.26
8	10.8	1.13	9.55
8	11.1	1.11	10.00
8	11.6	1.18	9.83
8	10.5	1.11	9.45

$$\frac{\text{Wet weight}}{\text{Dry weight}} \pm \text{S.D.} = 9.68 \pm 0.73$$

$\therefore$ Conversion factor for Dry weight = wet wt $\div$ 9.68

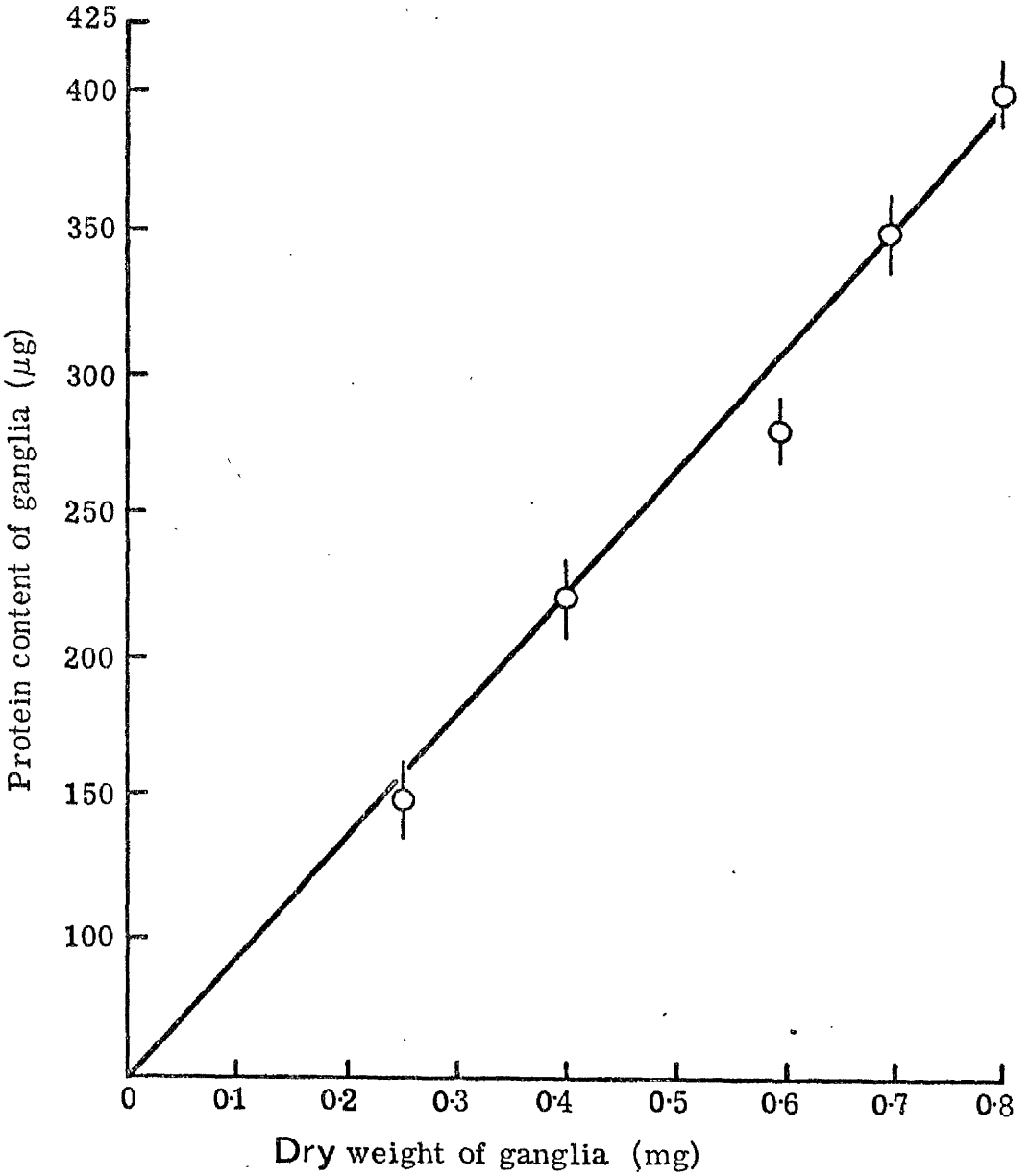
The ratio of fresh wet weight to dry weight can be compared with other nervous tissues. Previous results of Larrabee (1958) working on superior cervical ganglia showed that ratio of $\frac{\text{Wet weight}}{\text{Dry weight}} = 4.7 \pm 0.2$, on average of 4 individual results and water content represented 76.1% of wet weigh whereas present results on thoracic ganglia showed an average ratio of 9.68 and therefore water contents are 89.8% of wet weight. This difference could be due to large intercellular spaces in the thoracic ganglia (filled with water) compared to superior cervical ganglia of rat.

It was found that there was good correspondence between the dry weight and protein content of ganglia. This is shown in Figure 6. Due to small amount of tissue used and a need of keeping it at low temperature, it was not convenient to weigh the tissue prior to homogenization. Instead, protein present in portion of the homogenate was estimated and dry weight of the tissue was derived by referring to a previously drawn curve relating protein contents to the dry weight of the tissue. This method of deducing the wet weight from measured proteins is open to the criticism, due to changes in the extracellular and intracellular fluid and protein content might have taken place in the course of incubation. However, the fluid changes are eliminated by taking into consideration the dry weight. Protein content might have changed during the course of incubation and this would make it invalid to relate the measured proteins to the initial dry weight, i.e. before incubation. This problem was solved by

FIGURE 6

Relationship of protein contents to dry weight of isolated thoracic ganglia of the locust. Each point is the average of at least 6 individual estimates made on a number of ganglia ranging from 2-10.

Vertical lines represent ± S.D.


425
400
350
300
250
200
150
100
Protein content of ganglia (μg)
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
Dry weight of ganglia (mg)

measuring the protein contents after various times of incubation and relating with dry weight. The results showed that there was no change in the relationship between protein contents and dry weight. This is shown in Figure 7 and Table 3. Comparing these results of protein contents to weight to those at zero time of incubation, it is revealed that protein content of the thoracic ganglia do not significantly change. These results make the deduction of weight from protein measurements of incubated ganglia quite satisfactory.

To clarify these conversion factors a hypothetical example is taken. Consider a certain sample of thoracic ganglia contains proteins, 142.0 µgs and a dry weight deduced from standard curve as shown in Figure 6, would be 0.24 mg. Applying the conversion factor of 9.68 as shown in Table 2, we get wet weight equal to 2.32 mg. Suppose ATP concentration is equal to 6.48 µmol. Expressing this ATP concentration on a dry wt. basis, it would be 6.848÷0.24 or 27 µmol/g dry wt. On converting it into wet weight basis, it would be 27÷9.68 µmol/g wet wt. or 2.79 µmol/g wet weight.

4. Analytical Methods

4.1. Estimation of metabolites:

The metabolites, glycogen, glucose, lactate, arginine phosphate, ATP, ADP, pyruvate and trehalose, were all measured by using enzymic method of Lowry and Passonneau

FIGURE 7

Represents ratio of protein contents to dry weight of thoracic ganglia during the course of incubation *in vitro*. Each point is the average of 4 individual estimates made on a number of ganglia ranging from 2 - 6.

Vertical lines represent ± S.D.

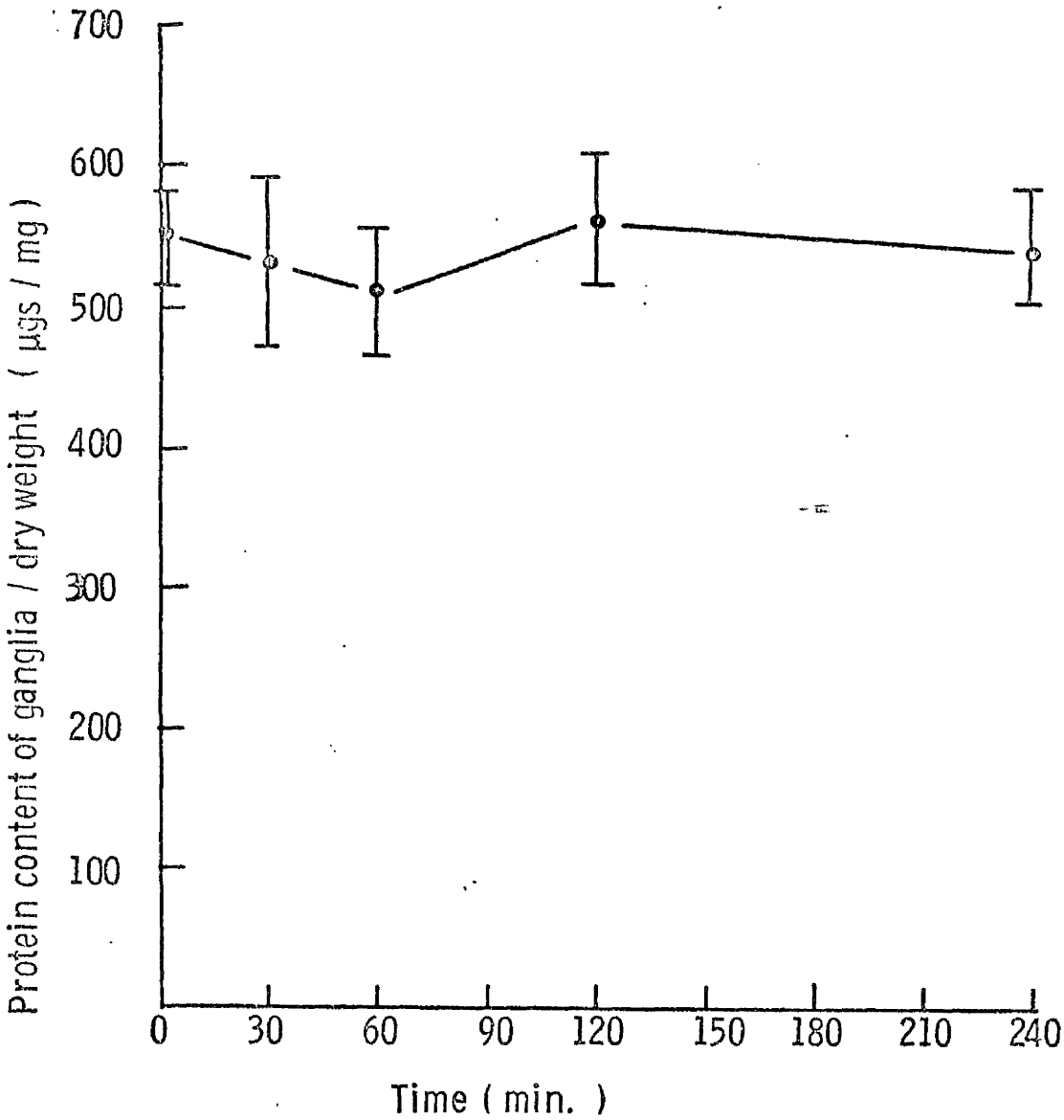

700
600
500
400
300
200
100
Protein content of ganglia / dry weight (µgs / mg)
0
30
60
90
120
150
180
210
240
Time (min.)

TABLE (3)

MEASUREMENT OF PROTEIN CONTENTS AND DRY WEIGHT OF THORACIC GANGLIA AFTER VARIOUS TIMES OF INCUBATION IN VITRO.

No. of ganglia	Incubation time (min)	Proteins (µgs)	Dry Wt. (mg)	Proteins µg/mg ± S.D. Dry wt.
2 2 4 4	0	142.5 150.0 307.5 315.0	0.24 0.27 0.60 0.58	* 551.02 ± 33.95 (4)
6 6 6 6	0 - 30	400.0 360.0 480.0 394.0	0.85 0.75 0.80 0.69	530.38 ± 64.86 (4)
4 6 6 4	0 - 60	300.0 373.5 360.0 263.0	0.62 0.65 0.72 0.52	515.65 ± 40.41 (4)
4 4 4 6	0 - 120	230.0 228.0 270.0 320.0	0.38 0.40 0.50 0.62	557.75 ± 38.47 (4)
2 2 4 2	0 - 240	149.0 153.0 270.0 150.0	0.28 0.27 0.50 0.30	534.69 ± 27.42 (4)

* There is no significant difference of ratios of Proteins to dry weight during the course of incubation.

Figures in brackets indicate the number of individual results for each estimated mean.

(1972). These may be summarized as follows:

(A) Glycogen and glucose:

$$\text{Glycogen} \xrightarrow{\alpha 1,4\text{-}1,6\ \text{Amyloglucosidase}} (\text{glucose})_n$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^{\oplus} \xrightarrow{*} \text{phosphogluconolactone} + \text{NADPH}$$

* glucose 6-phosphate dehydrogenase.

Soluble and insoluble glycogen were partially purified by the method of Bachelard and Strang (1974).

(B) ATP, ADP and Pyruvate:

$$\text{AMP} + \text{ATP} \xrightarrow{\text{Myokinase}} 2\ \text{ADP} \quad \text{(i)}$$

$$\text{ADP} + \text{Phosphoenol pyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{ATP} + \text{pyruvate} \quad \text{(ii)}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}^{\oplus} \quad \text{(iii)}$$

All these three metabolites were measured within the same sample by adding NADH, phosphoenol pyruvate and AMP, followed by addition of related enzymes.

(C) Lactate

$$\text{Lactate} + \text{NAD}^{\oplus} \xrightarrow[\text{hydrogenase}]{\text{Lactate de-}} \text{pyruvate} + \text{NADH}$$

$$\text{Pyruvate} + \text{hydrazine} \longrightarrow \text{Pyruvate hydrazone.}$$

(D) Arginine phosphate

$$\text{Arginine phosphate} + \text{ADP} \xrightarrow{\text{Argininekinase}} \text{Arginine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} + \text{NADP}^{\oplus} \xrightarrow[\text{G-6-P-dehydrogenase}]{\text{Hexokinase}} \text{NADPH} + \text{Phosphoglucono-lactone}$$

(E) Trehalose

$$\text{Trehalose} \xrightarrow{\alpha,\alpha\text{-Trehalose glucohydrolase}} 2\ \text{glucose}$$

$$\text{Glucose+ATP+NADP}^{\oplus} \xrightarrow[\text{6-phosphate dehydrogenase}]{\text{Hexokinase/G-}} \text{ADP+NADPH+phosphoglucono-lactone}$$

All these metabolites were measured fluorometrically at 340 n m and 460 n m , activating and fluorescent wavelengths respectively, using appropriate buffers. The measurement of metabolites by fluorometry is based on the fact that nicotinamide co-factors fluorescence in reduced form when present in buffer solutions near neutrality. This method allowed measurements of amounts of metabolites as low as 1 n mol .

4.2. Glucose and trehalose present in haemolymph and in thoracic ganglia.

Haemolymph from locusts was collected according to method of Walker et al. (1970). A diagonal cut was made across the head of the locust and the animal was then placed head down in a conical centrifuge tube which was preweighed. The conical tube was then spun gently for 5 minutes at about 250 g. The locust was then removed and tube plus

haemolymph weighed. If the contents were contaminated with gut contents, the samples were discarded.

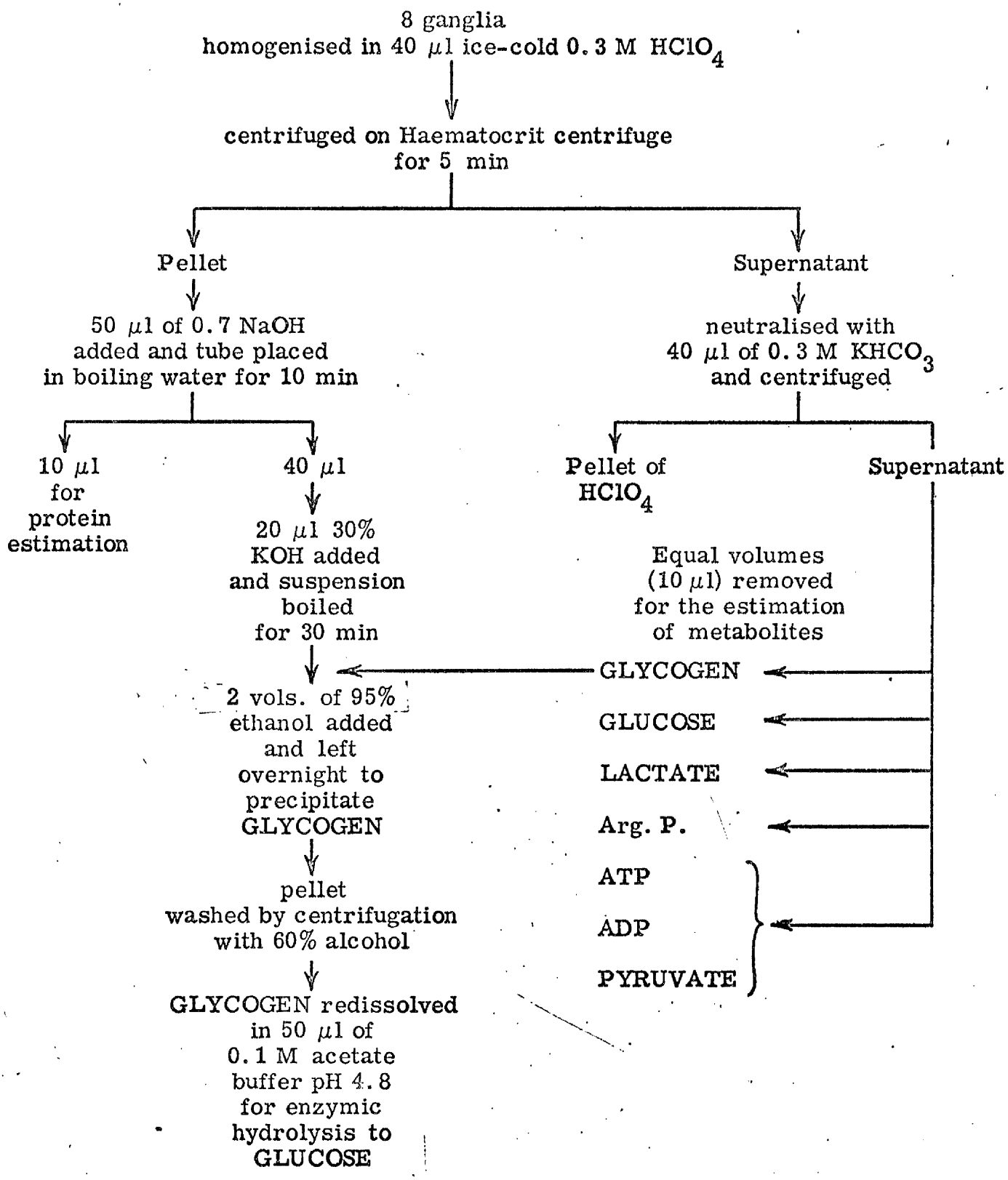
Preliminary studies of collecting haemolymph sufferedfrom contamination with gut contents and therefore a new technique was developed. Haemolymph was obtained by cutting the metathoracic legs and then covering the abdominal region of the animal by a disposable pipette tip to prevent contamination of haemolymph. Animals, thus covered, were placed in a conical glass tube and centrifuged at 250g for 5 minutes using a bench centrifuge. This method improved the situation, firstly by avoiding the contamination of collected haemolymph and secondly, the collected volume of haemolymph was doubled. The 50-60 µl of haemolymph thus obtained from each locust were deproteinized by using 2 volumes of 90% alcohol as described by Treherne (1960). Similarly, thoracic ganglia were homogenized in tris/HCl buffer pH 7.0 as described later. The glucose present in the deproteinized samples of haemolymph and in supernatant fractions of thoracic ganglia were measured by the enzymic method described. Trehalose was hydrolyzed to glucose by the enzyme trehalase (α, α — trehalose glucohydrolase) and glucose was estimated by the method described before.

4.3. Protein estimation:

Locust ganglia were homogenized in ice cold 40 µl 0.3M $HClO_4$ and then centrifuged on Haematocrit centrifuge at 3000g for 15 min. (as shown in Figure 8). The pellet

FIGURE 8

Summary of the methods of tissue preparation, and estimation of metabolites.


8 ganglia
homogenised in 40 µl ice-cold 0.3 M $HClO_4$
centrifuged on Haematocrit centrifuge for 5 min
Pellet
Supernatant
50 µl of 0.7 NaOH added and tube placed in boiling water for 10 min
neutralised with 40 µl of 0.3 M $KHCO_3$ and centrifuged
10 µl for protein estimation
40 µl
Pellet of $HClO_4$
Supernatant
20 µl 30% KOH added and suspension boiled for 30 min
Equal volumes (10 µl) removed for the estimation of metabolites
GLYCOGEN
GLUCOSE
LACTATE
Arg. P.
ATP
ADP
PYRUVATE
2 vols. of 95% ethanol added and left overnight to precipitate GLYCOGEN
pellet washed by centrifugation with 60% alcohol
GLYCOGEN redissolved in 50 µl of 0.1 M acetate buffer pH 4.8 for enzymic hydrolysis to GLUCOSE

was homogenized in 50 µl 0.7M NaOH and placed in boiling water for 10 min. A sample (10 µl) of this solution was taken for estimation of protein based on the method of Lowry et al. (1951). This sample was added to 0.1 ml of 0.7 MNaOH and placed in boiling water bath for 10 min. to ensure that proteinswere dissolved and to this was added 0.4 ml of 0.7M NaOH. To this solution was added 0.5 ml of Lowry reagent consisting of 0.466 ml of 9% Na_2CO_3 (W/V), 0.017 ml 4% sodium potassium tartarate (W/V) and 0.017 ml of 2% $CuSO_4$ $12H_2O$ (W/V) (27.4:1:1) which was made up freshly for each experiment. Ten minutes later 0.2 ml of 25% (V/V) Folin and Ciocalteu's phenol reagent was added. The absorbance was measured against reagent blank at 750 n m after 30 min. and BSA samples were used as standards in parallel.

4.4. Estimation of hexokinase:

The activity of hexokinase was estimated according to the method of Crabtree and Newsholme (1972) and also by the method of Bachelard and Goldfarb (1969). The difference between the two methods is that, in the former the assay mixture contains an ATP - regenerating system consisting of creatine phosphate and creatine phosphokinase.

4.5. Measurement of osmolarity:

The measurement of osmolarity of haemolymph and saline was made by a digital osmometer supplied by MSE Scientific Instruments, Manor, Royal Crawley, West Sussex (U.K.). This osmometer requires a minimum of 100 µl volume to

measure the osmolarity of a solution. Therefore, haemolymph obtained from two animals was pooled to fulfil this requirement.

5. Calibration of Oxygen electrode:

The oxygen electrode was calibrated by the method of Robinson and Cooper (1970), which has an advantage over the previous methods of Eastabrook and Mackler (1962). The calibration method of these authors most often applied to oxygen electrode studies, in which oxygen is consumed in the sto chio metric oxidation of a specified amount of NADH, catalysed by lysed mitochondria; the change in electrode current is thus equivalent to a known uptake of oxygen from solution. In this method the preparation of mitochondria has the disadvantage of being unduly lengthy (more than 4 hours) for routine use. An additional restriction is that the medium under investigation must support mitochondrial oxidation. In the method of Robinson and Cooper, the mitchondrial preparation of the above method is replaced by the compound N-methylphenazonium methosulfate (PMS) - the reactions involved are:-

$$PMS + NADH + H^{\oplus} \rightleftharpoons PMSH_2 + NAD^{\oplus}$$

$$PMSH_2 + O_2 \longrightarrow PMS + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + \frac{1}{2} O_2$$

Net Reaction: $NADH + H^{\oplus} + \frac{1}{2} O_2 \longrightarrow NAD^{\oplus} + H_2O$

This calibration of oxygen electrode by using specified amounts of NADH, PMS and catalase in 3 ml. of medium used, was essential to find the oxygen concentration corresponding to the 100% setting on the recorder. The instrument was also set to zero by using sodium dithionite which reduces the oxygen present in the medium.

6. Preparation of tissue samples for measuring concentration of metabolites:

According to Newsholme and Crabtree (1973); Crabtree and Newsholme (1975), steady-state concentration of metabolites of a certain pathway are defined as those concentrations of metabolites which are obtained by keeping the flux (that is, the rate of flow of material along the pathway) constant. As long as the flux rate is constant, so are the concentrations of intermediates. Obviously, a method which can stop the enzyme catalyzed reactions in a certain pathway as soon as possible can allow a worker to estimate the concentrations of steady-state metabolites close to that in vivo. According to the previous results of Wollenberger et al. (1960) and Faupel et al. (1972) the immersion of a tissue in liquid nitrogen reduces the temperature from 37°C to -90°C in a few seconds. This method of killing the animals to estimate the steady state levels has been used often by other authors, (McIlwain and Bachelard, 1971) working on mammalian tissues. This method of killing the animals has become a standard method.

A general survey of the literature on experiments of

locusts *in vivo* and *in vitro* (Treherne, 1960; Church, 1960) shows that CO_2, diethyl ether and chloroform have also been used to kill, and to anaesthetize the locusts. However, the effects of these anaesthetics have not been studied in detail with a view to their effect on concentration of metabolites in the CNS. In relation to the previous work of Lowry et al. (1964) and Mayman et al. (1964), the glucose content of mouse brain is increased more than two fold during anaesthesia with chloroform and ether. It has also been found by other authors that anaesthetics like CO_2, chloroform and ether have an effect on energy metabolism by inhibiting the electron transport chain (Chance and Hollunger, 1963 ; McIlwain, 1966). In view of these previous findings of the effect of anaesthetics on carbohydrate and energy metabolism, it is likely that the various methods of killing the animals (Table 4), would have some effect on the steady-state concentration of metabolites of the thoracic ganglia of locust. An attempt was made to see which is the best method of killing the animals which would allow measurements of the concentrations of metabolites close to that *in vivo*. Concentrations of glycogen, glucose and ATP of thoracic ganglia were selected as parameters to study the effect of CO_2, a mixture of chloroform/diethyl ether (1:1 $^{v}/_{v}$), dropping the animals into liquid nitrogen and immobilising at - 20°C for 10 minutes. It was found from the preliminary experiments that the locusts were immobilized completely in 7-10 minutes at -20°C, 5-7 minutes in ether/chloroform, 3-5 minutes in CO_2 and 0.5-1 minutes in liquid N_2. In order

to eliminate the time factor of the duration of anaesthesia, the locusts were kept for 10 minutes in anaesthesia and 10 minutes at -20°C to immobilising completely and 10 minutes immersion in liquid nitrogen. Anaesthetized and frozen animals were dissected at 0°C to remove the thoracic ganglia.

These isolated ganglia were kept cold in solid CO_2 at -80°C before the analysis of glycogen, glucose and ATP. The ganglia (5-6) were homogenized in a thick walled, ice cold, glass tube. This glass tube was 50 mm in length and 3 mm internal diameter. Ganglia were homogenized with the aid of a dental drill, fitted with a teflon pestel. Thereafter the extract was processed and the metabolites estimated according to Figure 8. Metabolites were estimated enzymically (as described before). The results of glycogen, glucose and ATP present in thoracic ganglia after anaesthetizing and immobilising at - 20°C and immersion in liquid nitrogen are shown in Table 4. The statistical treatment of these results of glycogen (as glucose), glucose and ATP of two groups I and IV, indicates that there is no significant difference between these measured metabolites of these two groups, whereas the results of groups II and III showed a sharp reduction in glycogen and a rise in glucose concentrations as compared to group IV results. The difference in concentration of ATP between groups III and IV is significant, whereas the difference is not significant between groups II and IV.

The different concentrations obtained of these metabolites

TABLE (4)

Measurement of metabolites present in locust ganglia after killing the animals by various methods:

Group No.	Method of killing the animals:	Glycogen (as glucose) µmol/g ± S.D.	Glucose µmol/g ± S.D.	ATP µmol/g ± S.D.
I	Immobilising for 10 min at -20°C	10.96±0.67 (5)	1.60±0.21 (5)	2.72±0.44 (6)
II	Anaesthetizing for 10 min in atmosphere of CO_2	8.91±0.77 (6)	2.33±0.31 (6)	2.43±0.31 (5)
III	Anaesthetizing chloroform/ether (1:1 v/v) for 10 min.	7.95±0.69 (5)	4.7 ±0.46 (5)	1.51±0.28 (6)
IV	Dropping into liquid N_2	11.0 ±0.53 (6)	1.82±0.0 (5)	2.58±0.26 (5)

Figures in brackets indicate the number of individual results for each estimated mean.

Statistical treatment of the above results:

Metabolite	Group No: 1 & IV	Group No: II & IV	Group No: III & IV
Glycogen	$P > 0.05$	$P < 0.01$	$P < 0.01$
Glucose	$P > 0.05$	$P < 0.01$	$P < 0.001$
ATP	$P > 0.05$	$P > 0.05$	$P < 0.01$

indicates that anaesthetics have some effect on locust thoracic ganglia. The results obtained by immobilising for 10 minutes at -20°C are identical to those by dropping into liquid N_2 which suggests the former method yields metabolites concentrations similar to those *in vivo*. These results are interesting and can be justified in relation to the findings of Church (1960), who found that locusts (*S. gregaria*) do not have any special mechanism of maintaining their body temperature except cessation of flight to protect from overheating. Their body temperature usually stays near that of its immediate surroundings. Behavioural and metabolic activity is controlled by surrounding temperature. Probably, the central nervous tissues of locust behave in the same way and therefore metabolic rate is not increased due to lack of extra energy demand in maintaining the body temperature when kept at -20°C. The immobilisation of locusts took 7-10 minutes, but the concentration of metabolites identical to dropping into liquid N_2, suggests the turnover of these metabolites (ATP, glucose and glycogen) remained steady up to 10 minutes. The lower levels of glycogen (as glucose), obtained by anaesthetizing in chloroform/ether as compared to liquid nitrogen treatment, suggests that either synthesis or breakdown of glycogen was affected by anaesthesia. These findings of a lower concentration of glycogen are in agreement with the effect of halothane anaesthesia on rat skeletal muscles (Rosenberg et al., 1977). These authors found that glycogen synthesis was inhibited and glycogenolysis was increased.

Therefore an increase in glucose concentration could be due to either an increase in uptake of glucose or inhibition of glycolysis at a certain step by ether/chloroform anaesthesia. These results showing increase in glucose concentration are in agreement with the previous findings of Mayman et al. (1964), working on mice brains. They found that glucose concentration was increased by ether, chloroform and phenobarbitone anaesthetics respectively. The lower concentration of ATP in chloroform/ether anaesthesia as compared to dropping into liquid nitrogen indicates that either aerobic glycolysis or the electron transport chain was impaired. These results are in agreement with the previous results of Rosenberg et al. (1977) and Brunner (1969), working on skeletal muscles of the rat. They found a decline in concentration of ATP after exposure to halothane and ether anaesthesia.

A study of previous findings on CO_2 anaesthesia shows that mechanism of its action is still not clear. However, the results of Folbergrova et al. (1974) showed that none of the groups of rats exposed to 50% CO_2 for, 5, 15, 45, 90 and 180 minutes showed significant changes in ATP, ADP and AMP concentrations. There was a decrease in glycogen concentration for 5 min. but normalization occurred subsequently, but glucose concentration increased 2 fold. Similarly, the results of Miller et al. (1975), working on rat brain showed that the rate of glucose utilization was reduced to half of its control level, when exposed to 20% CO_2 for 5 min and remained constant for 60 min. As

there was no significant difference in ATP concentration of that obtained by exposure of locusts to CO_2 for 10 minutes, as compared to dropping into liquid N_2, these results are in agreement with the previous results of Folbergrová et al. (1974). The higher concentration of glucose obtained after exposure to CO_2 for 10 minutes is again in agreement with the results of Folbergrová et al. (1974) and Miller et al. (1975).

The difference in glycogen concentration after 10 min. exposure to CO_2 is not consistent with those of the other metabolites. The difference could be due to a difference in percentage of carbon dioxide, as in the present work 100% CO_2 was used whereas other workers used 50% CO_2.

In conclusion, glycogen, glucose and ATP are affected more by CO_2 and chloroform/ether anaesthetics than by dropping into liquid nitrogen and by immobilizing at -20°C. The results are identical both in liquid N_2 and immobilizing at -20°C, and therefore these methods are equally effective. Locusts are much lighter in weight than experimental mammals and have wings which cause a problem when dropping them into liquid N_2, and sometimes take a longer time than mammals to be immobilized completely. Therefore, this method is not particularly useful for locusts. Hence, the method of immobilizing at -20°C for 10 minutes has been followed throughout for measuring the steady-state metabolites. For measuring the steady-state metabolites, locusts of both sexes were used. These animsls were kept in a plastic container with a lid on

at -20°C for 10 min. and then dissected at 0°C, removing meso- and metathoracic ganglia partly, freed of fatty tissues and tracheoles. These ganglia were kept cold in solid CO_2 at -80°C before analysis of metabolites.

For in vitro preparation, the insects heads were cut off and the bodies put on a plasticine block present in a petri dish. They were dissected to remove ganglia freed of fat body and tracheole system, while keeping ice-cold. Isolated ganglia were kept in ice-cold saline in a small bag of nylon gauze prior to use. This gauze, when placed in oxygen electrode chamber, prevented the ganglia being sucked into outlet tubes while the medium was replaced, while allowing the medium to circulate freely around the ganglia. The time of removing ganglia from one animal ranged 2-4 minutes and these ganglia were left in ice-cold saline for not more than five minutes prior to putting them in an oxygen electrode. Ganglia were removed from the metabolic chamber at different time intervals and kept frozen on solid CO_2 before homogenization.

An outline for the treatment of tissues prior to the estimation of metabolites is shown in Figure 8.

7. Spontaneous activity:

Introduction:

Graham and Gerard (1946), first, developed the technique of penetrating frog muscle fibres by an extremely fine glass tube filled with saline which thus served as an internal

microelectrode, by which the potential of the interior of the fibre could be measured, relative to its exterior. This glass microelectrode technique has been extended successfully to several other excised tissues: giant axons of sepia (Weidmann, 1951) and frog and mammalian nerve fibres (Woodbury and Woodbury, 1950; Brooks et al., 1952). A technique of inserting steel and platinum microelectrodes, insulated to the tip, into a motor nucleus in the spinal cord, has been developed (Eccles, 1950; Brooks and Eccles, 1947a, 1947b).

In recent electrophysical studies, the technique of recording from pacemaker cells by using glass microelectrodes filled with 2.5 M KCl, has been used to great advantage. Thus Anderson (1976) recorded action potentials from neurons of mollusc, Tritoma diomedia. Similarly, Okamoto et al. (1976) recorded action potentials (spikes) from the superior vermis portion of cerebellar slices of guinea-pig with an extracellular glass microelectrode filled with 2.5 M KCl placed at a depth of 300-350 μm from the surface. The position of the nerve N5 on metathoracic ganglion of cockroach (as classified by Pringle (1939) and also described in locust by Hoyle (1955, 1966a) is shown in Figure (2) of the first chapter. This nerve can be easily traced by the naked eye when removing the ventral cuticle near the metathoracic ganglion. Keeping in mind the construction of the metabolic chamber with stopper (as shown in Fig. 4 of the previous chapter) none of these electrodes (as described before) were particularly suitable. Any of

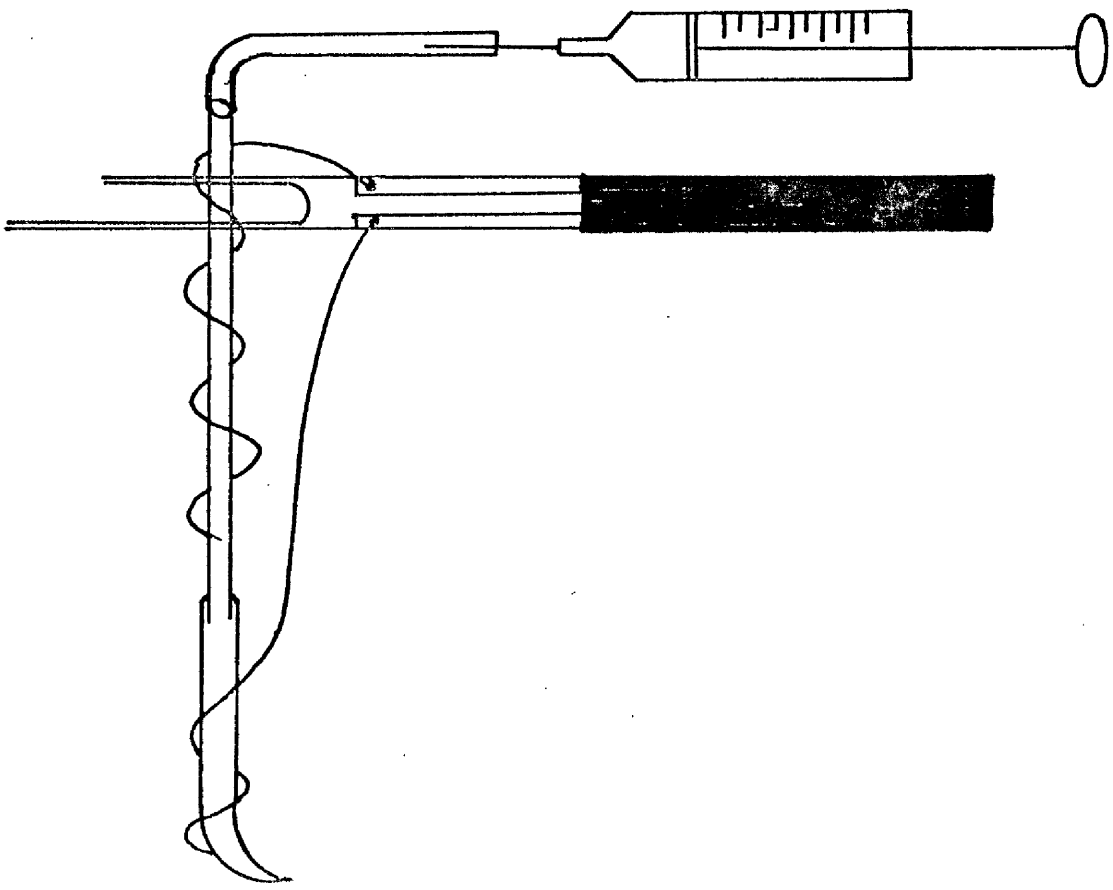
these electrodes could be used in vivo studies where just touching with the particular nerve is required whereas in metabolic chamber where the medium is continuously stirred the use of thin silver or platinum wire as electrode was not satisfactory. Also, this electrode as compared to other reference electrodes could not be easily insulated in saline to measure the potential difference. Glass microelectrodes also posed other problems of handling in the chamber. In the present study a plastic suction electrode has been devised. Basically, this is just as an extension of platinum electrode. Its construction is described here. A brass rod of 8 cm long and 1.5 cm diameter with a small groove on one side, was used. The two wires from the same input of the preamplifier, were attached on this brass rod separately by 'Araldite' with a gap of about 3-5 mm and two platinum wires of 200μ in diameter and 5 cm in length were soldered with these wires, coming from the lead of the preamplifier. Both the platinum wires were wrapped separately in very narrow plastic tubes leaving 2 cms naked at the tip. This plastic tubing served as an insulator. The naked length of one platinum wire was connected to a hollow steel tube, 3mm in diameter and 40 mm in length, simply by winding around the hollow tube. This hollow tube was attached to the brass rod in between the groove by means of 'Araldite'. The other platinum wire was wrapped around the plastic suction electrode. This brass rod was screwed in the micro-manipulator which can move the electrode both in horizontal and vertical directions. To the one end of the hollow tube, a plastic tube of 12 cm in length was attached, and this

plastic tube was attached to the needle of a disposable plastic syringe. To the other end, a plastic suction electrode (made by pulling the plastic tube while keeping hot on a soldering rod) was attached. The tip of the suction electrode was made of such a diameter to match the nerve N5. For *in vivo* experiments, the N5 was sucked into the recording electrode and reference electrode was put into the haemolymph of the exposed animal. For *in vitro* preparation, both the electrodes were passed through different holes of the stopper of the metabolic chamber. The metathoracic ganglion was sucked via nerve N5 and the other electrode was kept in the medium through the stopper. This plastic suction electrode technique worked well to its promise. The use of suction electrodes is very advantageous, as it is insulated from the reference electrode in the medium, and also not breakable while handling and putting in the metabolic chamber. The combined electrode gives the potential difference between the inside and outside of the nerve fibre. A simplified diagram of this electrode combination is shown in Figure 9.

7.1. Qualitative and quantitative measurement of spontaneous activity:

Spontaneous activity as recorded by the suction electrode was displayed on an oscilloscope, scalar and on an audio-amplifier. This arrangement gave a quantitative measurement of activity by displaying a visual and audible view of impulses by amplitude of actions, potentials and difference in noise over background noise. Some of the

Figure 9 Simplified Diagram of Suction-Electrode



traces from the oscilloscope were photographed by using a polaroid camera but in overall, records were made from the scalar fitted with a pulse height analyzer and a pen recorder. These pieces of equipment have been described by Stein, (1968). The pulse height analysing unit allowed the discrimination of a number of spikes of different amplitudes. The input to this unit was taken from the output of the preamplifier. The digital unit of it summed the number of impulses in a unit time and passed a voltage (proportional to the number of input events occurring in a unit time) to a pen recorder which operated on a range of 0.1 - 1mA current. The resultant display was a plot of average frequency over a certain period. The electrical activity measured in the present work was from a number of motor neurons, as nerve N5 contains nerve fibres from a number of motor neurons.

7.2. Measurement of spontaneous activity *in vivo* and *in vitro*

In vivo, spontaneous activity was measured by a plastic suction electrode, via a major nerve, N5 (as classified according to Pringle (1939) and Hoyle (1955) from a restrained but still living locust. The ventral thoracic cuticle was removed, taking care not to damage the tracheal system.

In vitro, the spontaneous activity was measured via the same nerve of metathoracic ganglion sucked into the recording suction electrode and suspended in the incubation medium through the stopper of metabolic chamber.

Spontaneous activity was measured in terms of action potentials, in both the cases. A preparation *in vitro* is shown in Figure 10, prior to putting the stopper in the oxygen electrode.

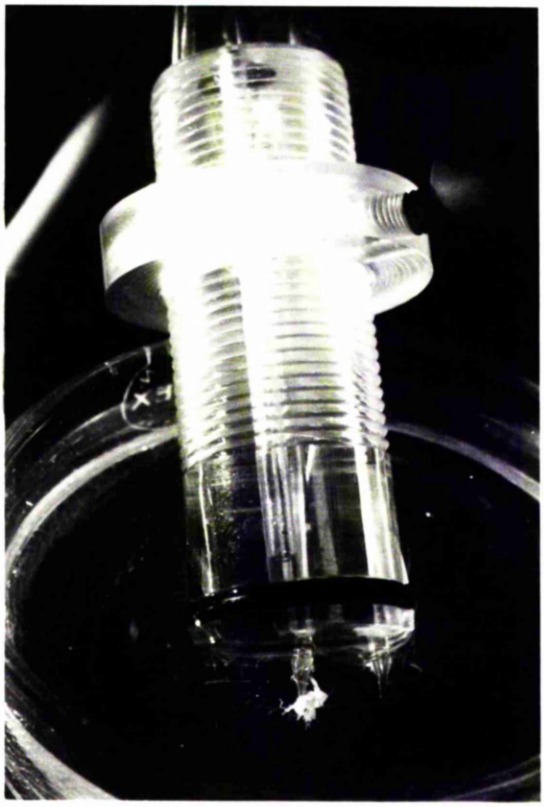
8. Expression and Statistical treatment of Results:
All the results mentioned in this thesis are expressed in terms of wet weight of the tissues because this is the form most commonly encountered in the literature.

The average and standard deviation of a minimum of 3 results was found and the difference between the two group of results was found by using students 't' test and the value of P was found from Statistical Tables of Fisher and Yates (1963).

Conclusions:
The following can be concluded from this chapter:
1. The layout of the apparatus as shown in Figure 4 and assembled in the present work could be used simultaneously for metabolic and electro-physiological investigations without any interference of either recording of electrical activity of metathoracic ganglion or oxygen uptake by meso- and metathoracic ganglia.
2. The derivation of dry weight of meso- and metathoracic ganglia by measurement of proteins and then interpretation from previously drawn curve of proteins versus dry weight is valid.
3. The method of obtaining haemolymph of locusts by

FIGURE 10

A typical preparation of metathoracic ganglion *in vitro*, prior to putting the stopper in the O_2 electrode. The ganglion has been sucked into the plastic suction electrode via a major nerve N5.



cutting off the metathoracic legs improved over the previous method of Walker et al. (1970), who obtained this by making a diagonal cut across the head of the locust.

4. For measuring the steady-state concentrations of metabolites of locust central nervous tissues, immobilizing the locusts at -20°C has considerable advantages over previous methods using anaesthetics and also over quick freezing in liquid N_2.

5. The use of a plastic suction electrode for measuring electrical activity through nerve N5 of metathoracic ganglion *in vivo* and *in vitro*, has considerable advantages over other microelectrodes.

CHAPTER 3

Chapter Three:

SECTION 'A'

A copious literature suggests that respiration by intact insects and oxygen uptake by isolated flight muscles has been studied in detail (Ruderman et al., 1975; Sacktor, 1965; Candy, 1970). This has concluded that the overall level of metabolism in working flight muscle of insects is particularly intense and their respiration rate is one of the highest so far reported, and also is most highly controlled. There has been a great deal of study of oxygen uptake of isolated mammalian nervous tissues, Larrabee (1958); McIlwain and Bachelard (1971), but insect nervous tissues have been always neglected in this respect. The limited literature of oxygen uptake of insect nervous tissues, certainly gives much more importance to the study of oxygen uptake by isolated meso- and metathoracic ganglia of the locust. In addition to this, as the aims of this work were to study some of the basic features of carbohydrate metabolism and control of aerobic glycolysis, oxygen uptake is directly related to the TCA cycle which is a major part of carbohydrate metabolism where most of the ATP are synthesized by coupling with electron transport chain and therefore an oxygen uptake study would give some idea of control of the TCA cycle. It may also be of interest to examine the rate of utilization of oxygen by insect central nervous tissues as an index

of its overall metabolic activity. The next question that arises is what type of studies of oxygen uptake can be done? One of the aims of this work, was to look at the role of glucose and trehalose as metabolic fuels in nervous tissues. As mammalian nervous tissues are very sensitive to oxygen concentrations, it was thought that a study of the O_2 uptake of isolated locust nervous tissue might make an interesting comparison. The results of oxygen uptake in trehalose-glucose medium are shown in section 'C' of this chapter. In addition to this, firstly, the isolation of ganglia <u>in vitro</u>, results in the disruption of the tracheal system (as described in the first chapter) and therefore it is likely that concentration of oxygen present in the medium would have some effect on uptake of oxygen by thoracic ganglia. Therefore, this study of oxygen uptake by variation in oxygen tension and glucose concentration should reveal the role of glucose in support of oxygen uptake and sensitivity of ganglia to the lack of oxygen due to tracheal disruption.

Secondly, the previous results of Larrabee (1958) on superior cervical ganglia of rats showed that the oxygen uptake is affected by variation in temperature and previous findings of Church (1960) suggest that locusts do not have any mechanism of control of their body temperature. Therefore it would be worthwhile to study the effect of temperature on oxygen uptake by isolated ganglia of the locust.

As regards electrophysiology of nerve N5, the previous results of Barker and Gainer (1975); David et al. (1974); Salánki et al. (1973); Wachtel and Wilson (1973), have shown that endogenous activity of molluscan neurons is largely temperature dependent and is abolished at low temperature. Similarly, the results of Libet and Gerard (1939), working on isolated frog brain and of Adrian and Buytendijk (1931) on isolated brain stem of goldfish are also in favour of temperature dependence of spontaneous activity. Therefore, it would be worthwhile to study the effect of temperature on spontaneous activity of isolated metathoracic ganglion. The question then arises as to which temperatures should be selected to study the oxygen uptake of meso- and metathoracic ganglia and spontaneous activity of metathoracic ganglion. In relation to this, the results of Church (1960) are interesting and worth mentioning here. He found, working on flying locusts, S. gregaria, that the flying efficiency of these animals is temperature dependent. Thus few locusts could fly effectively for more than a few minutes at 25°C, but at 30°C good fliers often kept going strongly and steadily for six or eight hours and at 35°C flight was at least as strong as at 30°C although not quite as strongly. At 40°C, although some specimens flew steadily for two or three hours, many would fly for no more than 10 or 15 minutes and if forced to keep trying, became paralyzed. At 45°C continuous flight was impossible. It would be interesting to see the effect of those temperatures where

animals show some activity and omitting very low and high temperatures where animals do not show any activity. This section gives the report of the findings of oxygen uptake and spontaneous activity.

1. Metabolic Chamber and Recording Apparatus:

The metabolic chamber as described in chapter 2 and shown in Figure 4 was widely used in this work. This device, firstly, avoided using two separate incubation chambers, one for electrophysiology and other for metabolic studies, and secondly it also economized the use of ganglia.

2. Oxygen uptake:

The unstimulated rates of oxygen uptake by meso- and metathoracic ganglia *in vitro* were investigated under conditions in which the temperature, oxygen tension and glucose concentration of the medium were varied.

2.1. Effect of temperature:

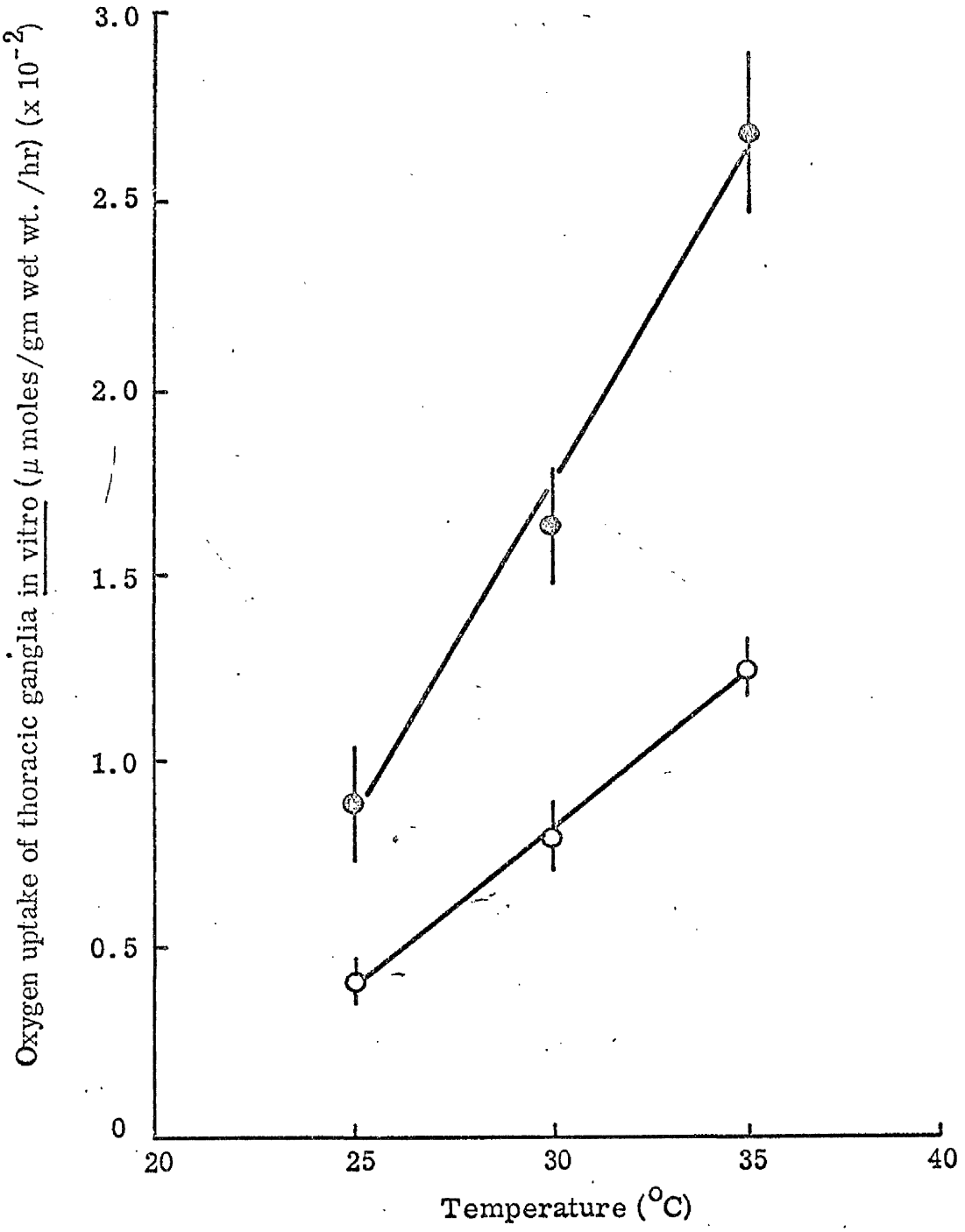
The oxygen uptake of the ganglia *in vitro* was studied at 25°, 30° and 35°C in 10mM glucose saline. The results are shown in Figure 11. The levels of oxygen uptake in the medium without saturation with100% oxygen are 42 and 124 μmoles/g/hr at 25°C and 35°C respectively and Q_{10} is 2.95. The results of oxygen uptake in the medium saturated with 100% oxygen are 84.0 and 267.0 μmoles/g/hr at 25°C and 35°C respectively. Therefore the Q_{10} value is 3.17. The oxygen uptake becomes more than doubled in the medium saturated with 100% oxygen as compared to that in the medium without saturation at 35°C.

FIGURE 11

Relationship of O_2 uptake to temperature for the isolated, unstimulated thoracic ganglia of the locust. Measurements were made in 10mM glucose saline, and are initial rates. The medium was previously equilibrated with air (O), or with 100% O_2, (●)

Vertical lines represent ± S.D.

FIGURE 11


Oxygen uptake of thoracic ganglia in vitro (μ moles/gm wet wt. /hr) (x 10^{-2})
3.0
2.5
2.0
1.5
1.0
0.5
0
20
25
30
35
40
Temperature (°C)

The levels of oxygen consumption and the temperature coefficients of locust ganglia with some comparative data of rat tissues taken from Larrabee (1958), have been shown in Tables 5 and 6, respectively.

2.2. Effect of oxygen tension:

Isolated ganglia were incubated in 3 ml · 10 mM glucose saline which was saturated with 100% O_2 and maintained at 35-36°C. O_2 uptake was determined at regular intervals in conjunction with the changes in concentration of oxygen present in the medium from chart recorder. The results of oxygen uptake against concentration of oxygen are shown in Figure 12. The curve shows that when the concentration of 750 nmoles of oxygen per millilitre present in the medium, this is no longer rate limiting. This concentration was achieved by saturation of the medium with 100% O_2.

2.3. Effect of glucose concentration:

In the studies of temperature and O_2 tension, 10mM glucose was used in the saline as an exogenous energy source. The purpose of these studies was to see how variation in glucose concentration affects the oxygen uptake. The measurements of oxygen uptake at different concentrations of glucose present in the saline, at 35-36°C and saturating with 100% O_2, were made and are shown in Figure 13. It is obvious from the curve that at concentrations above 10mM, glucose is no more rate limiting to oxidative metabolism.

TABLE (5)

Rates of oxygen consumption by various tissues in vitro, without stimulation.

Tissue	Condition	Temp.	µmol../g/hr (wet wt.)	Source
Rat cerebral Cortex	5.5 mM glucose/ saline	35-37°C	100.0	Elliott,1955
Rat sympathetic ganglia	"	35-37°C	60.0	Larrabee, 1958
Locust thoracic ganglia	10 mM glucose/ saline	35-37°C	267.0	This thesis.

TABLE (6)

Temperature coefficients of rates of O_2 consumption by various nervous tissues.

Tissue	Q10	Temp. range	Source
Rat cerebral cortex	2.1	10-35°C	Field et al. 1944
Rat sympathetic ganglia	2.3	24-36°C	Larrabee, 1958
Locust thoracic ganglia	3.17	25-35°C	This thesis.

FIGURE 12

Relationship of O_2 uptake to O_2 concentration in the medium for the isolated, unstimulated thoracic ganglia of the locust. Measurements were made at 35°C, in 10mM glucose saline. Filled symbols (●) denote the O_2 uptake at regular intervals of time during one experiment.

Superimposed upon the curve are the averages (± S.D.) of many measured rates made with the medium saturated with atmospheric, and 100% O_2 (O).

FIGURE 12

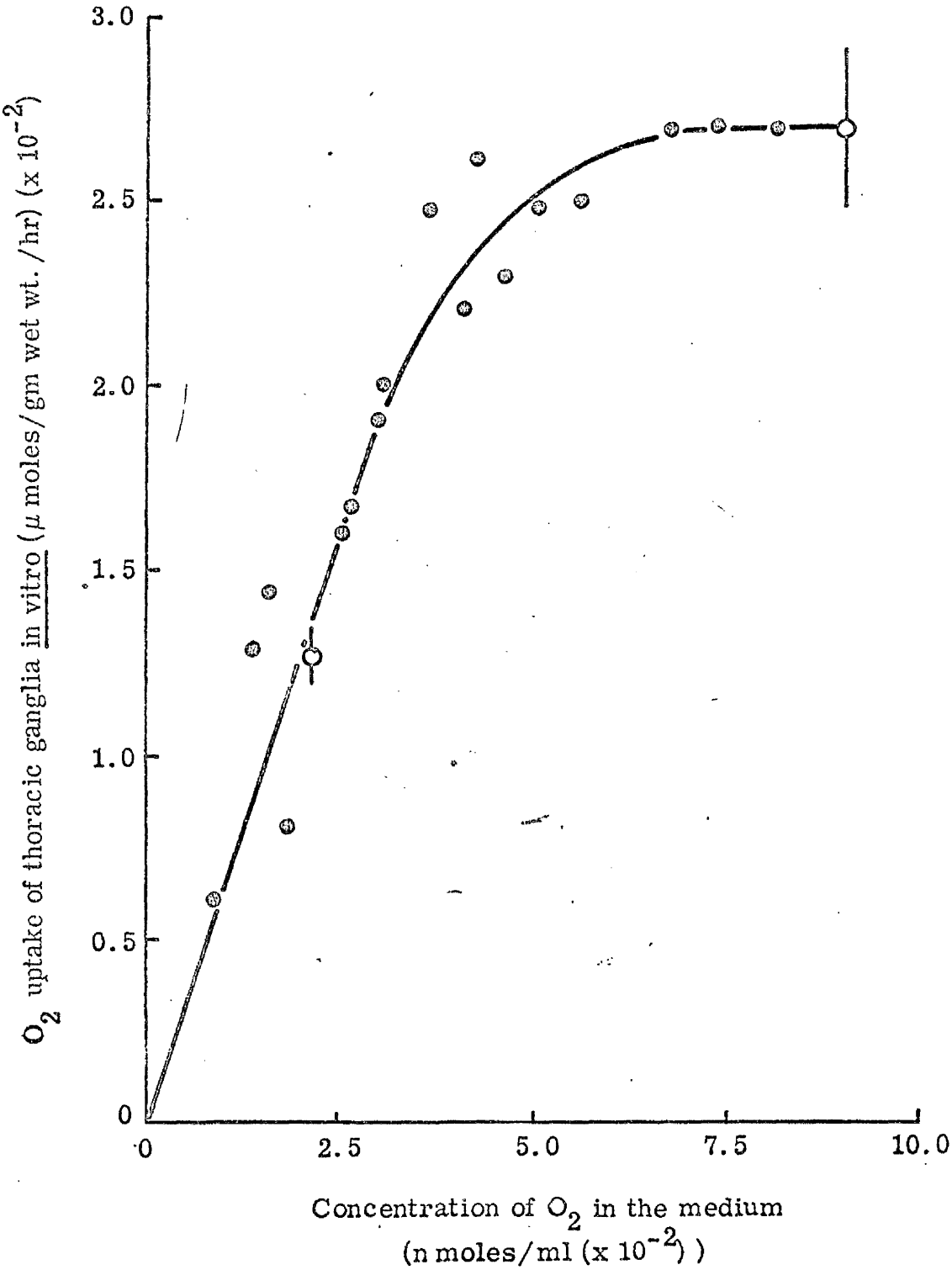
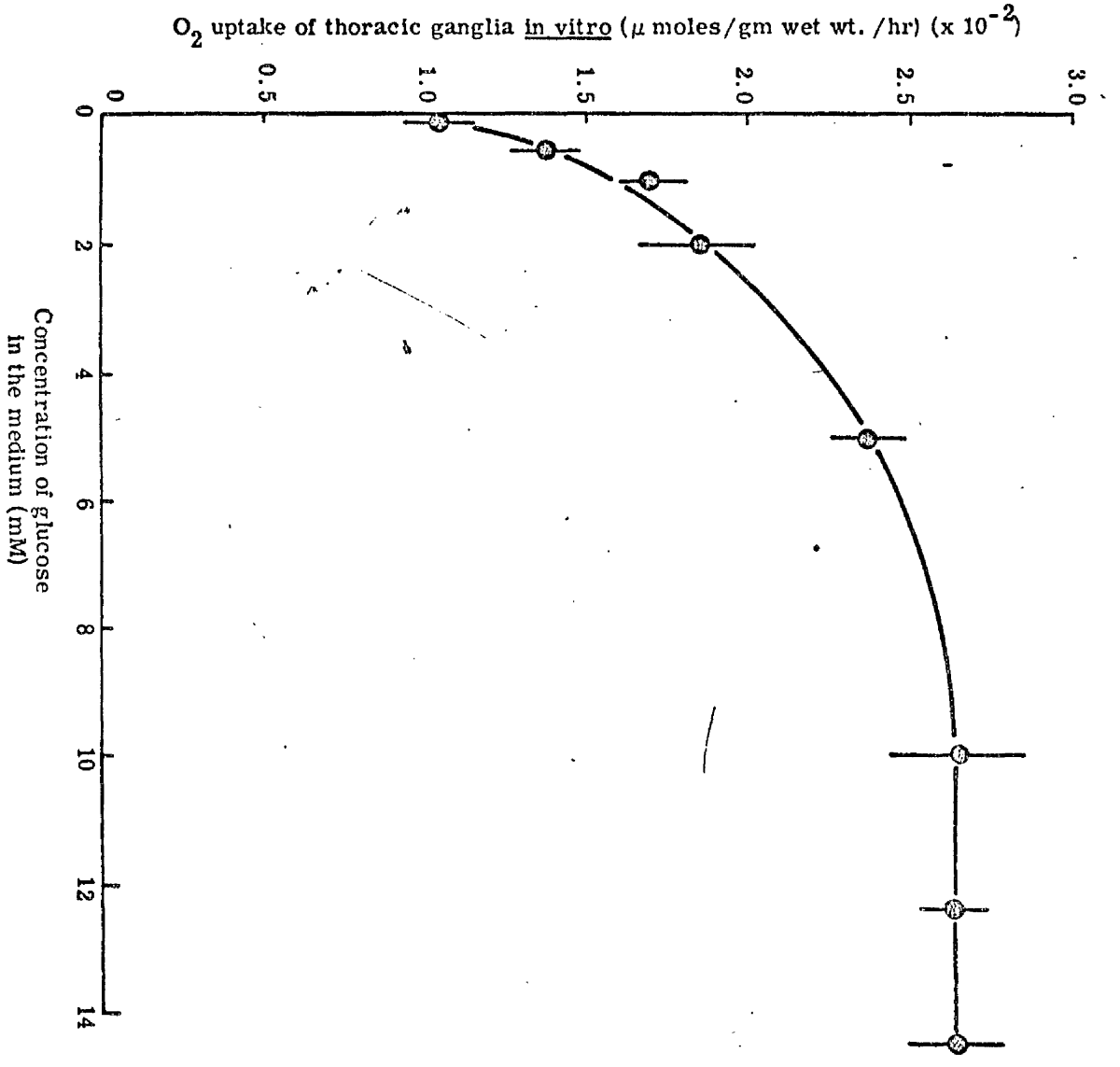

3.0
2.5
2.0
1.5
1.0
0.5
0
0
2.5
5.0
7.5
10.0
O_2 uptake of thoracic ganglia in vitro (μ moles/gm wet wt. /hr) (x 10^{-2})
Concentration of O_2 in the medium
(n moles/ml (x 10^{-2}))

FIGURE 13

Relationship of oxygen uptake to the concentration of glucose in the medium, for the isolated, unstimulated thoracic ganglia of the locust.

Each point represents the mean of at least 4 separate estimates made in saline saturated with 100% O_2 at 35°C. Vertical lines represent ± S.D.

FIGURE 13


O_2 uptake of thoracic ganglia in vitro (μ moles/gm wet wt. /hr) (x 10^{-2})
0
0.5
1.0
1.5
2.0
2.5
3.0
Concentration of glucose in the medium (mM)
0
2
4
6
8
10
12
14

3. Measurement of spontaneous Activity:

Spontaneous activity was measured in terms of impulses per second. Effects of variation in te mperature, and glucose concentration on spontaneous activity *in vitro* were studied. Electrical activity *in vivo* was also recorded and then compared with that *in vitro*.

3.1. Electrical activity in vivo:

Endogenous activity was recorded from a nerve,(N5)of metathoracic ganglion (by removing a small portion of ventral cuticle, taking care not to damage the tracheal system) for 95 minutes while the nervous system was almost intact and the locust alive, but restrained. Records of four individual results are shown in Table 7 and a typical oscilloscope trace is shown in Figure 14 which was displayed on oscilloscope between 60 and 95 minutes.

3.2. Effect of temperature:

The spontaneous activity *in vitro* was measured at two temperatures i.e. 25-27^{o}C and 35-37^{o}C for 100 minutes after isolation of ganglia and under conditions of 10mM glucose saline saturated continuously with 100% O_2. Records (as obtained from scalar) of spontaneous activity, at two different temperatures, of 4 individual results are shown in Table 8 and typical oscilloscope traces (photographed between 60 and 100 minutes) are shown in Figures 15 and 16, respectively. In order to make a comparison of these results, spontaneous activity (recorded between 60-95 mins. in terms of impulses/sec) is plotted against time for four individual results and is shown in Figure 17.

TABLE (7)

Recording of bioelectrical activity *in vivo*.

Preamplifier setting
Gain 1000, H.F. cut 1 KC/S:
L.F. cut 200 C/S.

Scalar setting
Discriminator Bias 20,
Input x 200, Channel width off

Impulses/Sec.

Time after isolation of ganglion	I	II	III	IV
0 min	2800	2000	2500	3000
5	3400	2600	2000	4000
10	4000	4000	2300	3500
20	5000	5300	3200	5000
30	4500	4300	4000	5000
40	4200	6000	2300	5600
50	3900	6500	2000	4500
60	3950	4350	3400	4250
70	4250	3770	4000	4000
80	4145	5000	4500	3750
90	3870	4400	5000	3900
100	2950	3900	4500	3850

TABLE (8)

Recording of spontaneous activity at two temperatures 25-27°C and 35-37°C *in vitro*.

Impulses/Sec.

Time after isolation of ganglia	I	II	III	IV	I	II	III	IV
	25 ——— 27°C				35 ——— 37°C			
0 min.	200	300	400	200	450	700	280	600
5	10	3	10	50	100	10	10	10
10	15	10	200	10	150	20	20	100
15	20	5	10	15	15	100	10	150
20	5	15	15	20	180	15	20	200
25	4	10	20	5	200	200	100	50
30	10	20	150	10	400	150	150	60
35	30	100	250	200	600	180	40	400
40	50	250	350	150	800	700	600	700
45	200	300	300	210	460	650	700	880
50	350	200	280	300	500	760	900	900
55	400	210	400	190	800	800	1100	980
60	300	220	300	360	900	910	1050	1050
65	220	300	300	400	1000	1100	1000	1080
70	220	200	410	410	900	1050	1050	1050
80	360	200	360	500	900	1050	950	900
90	310	260	380	485	1000	1000	900	1200
100	300	350	390	450	1200	950	1000	1100

FIGURE 14

A typical oscilloscope trace of the activity recorded from Nerve 5 for the metathoracic ganglia *in vivo*. The scanning speed of the oscilloscope trace was 2 ms/cm.

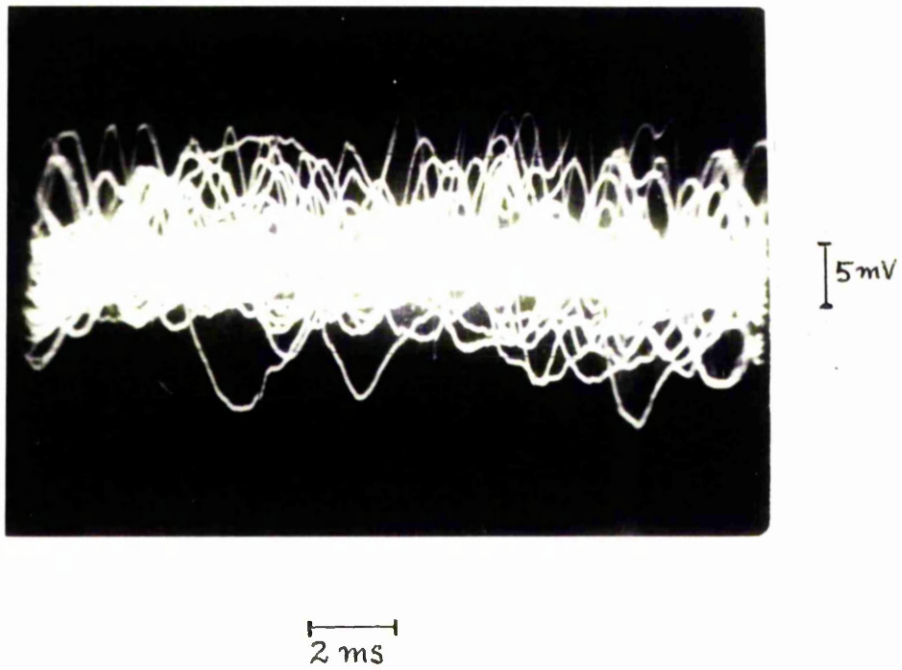

5mV
2 ms

FIGURE 15

A typical oscilloscope trace of the activity recorded from Nerve 5 for the metathoracic ganglia *in vitro* at 25-27°C. The scanning speed of the oscilloscope trace was 5 ms/cm.

figure 15

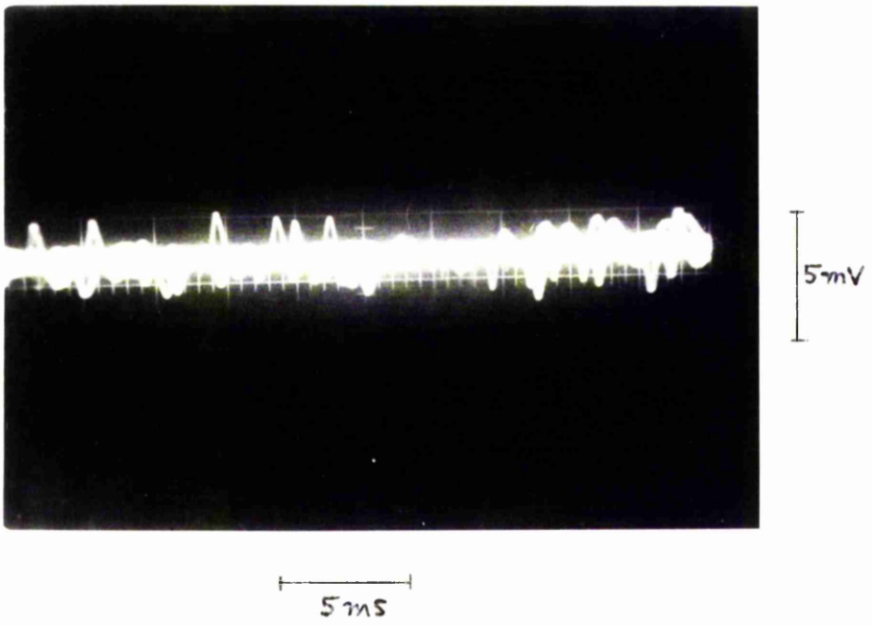


FIGURE 16

A typical oscilloscope trace of the activity record from Nerve 5 for the metathoracic ganglia *in vitro* at 35-37°C. The scanning speed of the oscilloscope trace was 5m$ /cm.

FIGURE 16

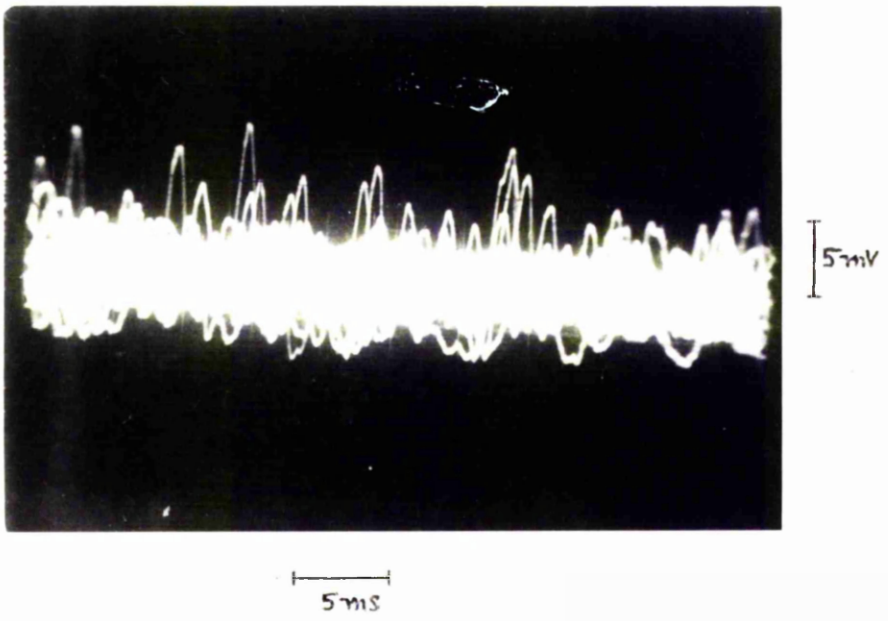
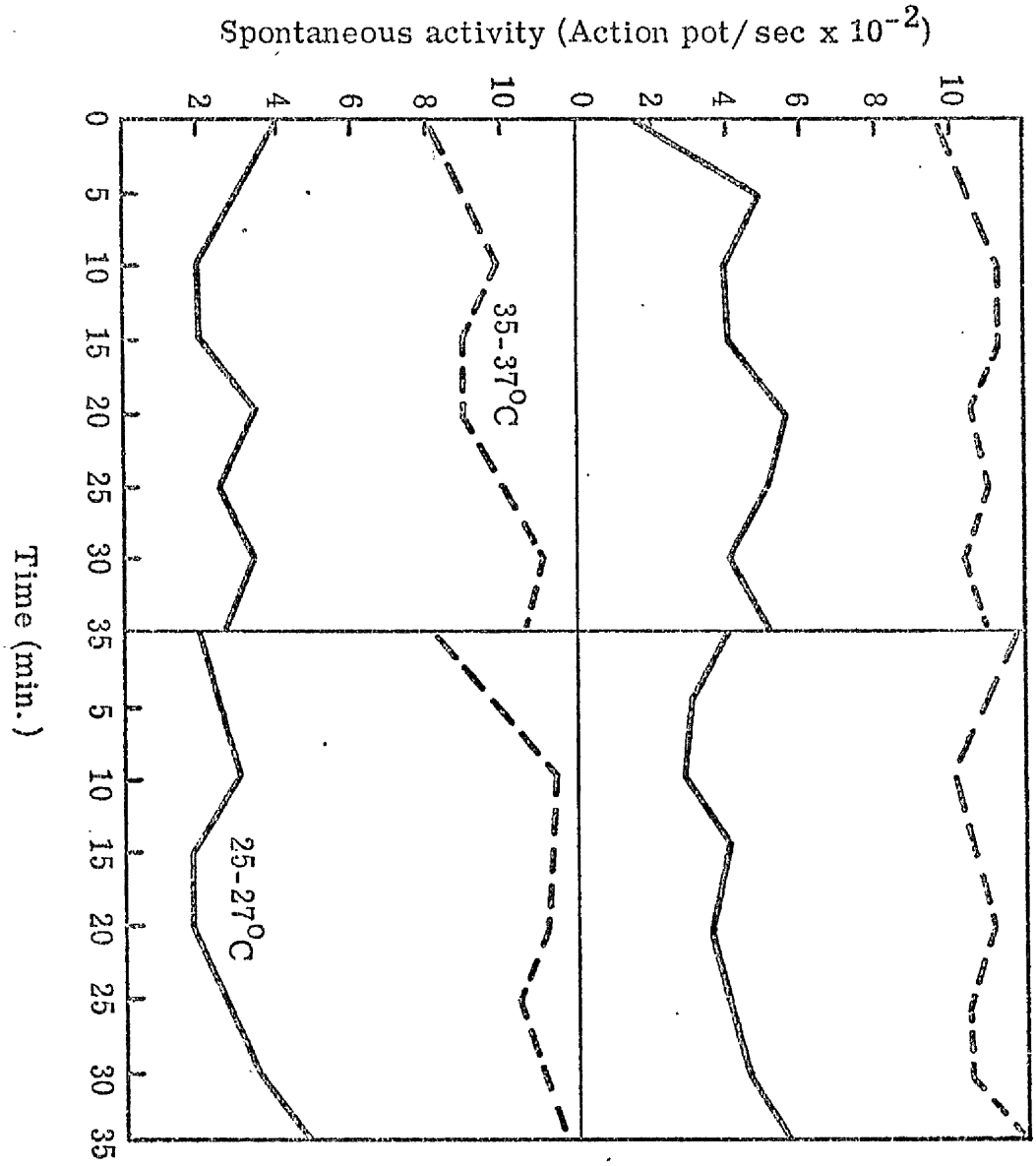


FIGURE 17

Comparison of spontaneous activity at two range of temperatures, 25-27°C and 35-37°C. Closed lines (_·_____) represent recordings made from Nerve 5 of the metathoracic ganglian of 4 separate locusts at 25-27°C and open lines (__ __ __) at 35-37°C.

FIGURE 17


Spontaneous activity (Action pot/sec x 10^{-2})
0
2
4
6
8
10
35-37°C
25-27°C
Time (min.)
0
5
10
15
20
25
30
35

3.3. Effect of Glucose in the medium:

Spontaneous activity was not affected by absence or presence of glucose in the medium for 90 minutes after isolation of ganglia in vitro. However, in the absence of glucose, activity started decreasing at 90 minutes and disappeared completely between 90-105 minutes, whereas in presence of 10mM glucose, it lasted for an average time of 180 minutes. It disappeared completely between 180-195 minutes. The records of spontaneous activity in presence and in absence of glucose are shown in the Table 9.

3.4. Spontaneous activity in Vitro:

There was inevitably a good deal of variation found between individual ganglia, in terms of the impulses/sec from one tissue to another. Nevertheless there was a common recognizable pattern, of low activity in first 45 min. after isolation, rising between 45-60 min. and then followed by a maximum for at least 3 hours, with some variation. There then followed a variable period of stability, before a decline and final extinction of spontaneous activity. Most of the tissues had ceased to show any activity 3½ hours after they had been removed from the animal. It was found that taking percentage of the maximum of spontaneous activity at various times of recording, gave a good picture for comparison of individual results and such results of 4 individual metathoracic ganglian are shown in Figure 18.

TABLE (9)

Recording of spontaneous activity in absence and presence of 10mM glucose present in the medium at 35-37°C.

Time	10mM glucose Medium I	II	III	IV	0.0mM glucose Medium I	II	III	IV
0 min	450	700	280	600	150	300	500	700
5	100	10	10	10	10	20	10	10
10	150	20	20	100	50	150	20	30
15	15	100	10	150	20	100	100	150
20	180	15	20	200	30	20	200	40
25	200	200	100	50	150	30	200	150
30	400	150	150	60	60	300	400	400
40	800	700	600	700	200	200	450	800
50	500	760	900	900	900	1000	900	900
60	900	910	1050	1050	1000	1100	1000	1000
70	900	1050	1050	1080	950	1050	1100	950
80	900	1050	1050	900	880	900	950	900
90	1000	1000	900	1200	1000	100	870	980
100	1100	950	1000	1100	20	10	10	20
110	1200	1000	1100	900	5	20	50	20
120	960	1100	1150	960	10	10	-	-
130	900	950	1200	1000	-	-	-	-
140	950	900	950	1050	-	-	-	-
150	860	890	900	1100	-	-	-	-
160	920	900	860	900	-	-	-	-
170	1000	1000	1000	950	-	-	-	-
180	600	200	150	300	-	-	-	-
190	500	100	200	50	-	-	-	-
195	100	10	10	60	-	-	-	-
200	50	20	10	10				

3.5. Comparison between the activity recorded *in vitro* with that in *vivo*.

Figure 19 gives 4 examples of such comparisons made on the same ganglia first in the almost intact insect, then *in vitro*. Clearly the number of impulses recorded *in vivo* is greater and much more variable than that made *in vitro*. Nevertheless, the level of the activity in the latter situation averages between 30-50% of that found *in vivo* under condition of minimal stimulation.

FIGURE 18

Four examples of the spontaneous activity of the thoracic ganglia *in vitro*.

The activity of 4 individual metathoracic ganglia is shown, in terms of action potentials recorded from Nerve 5 as a % of the maximum for each tissue.

FIGURE 18

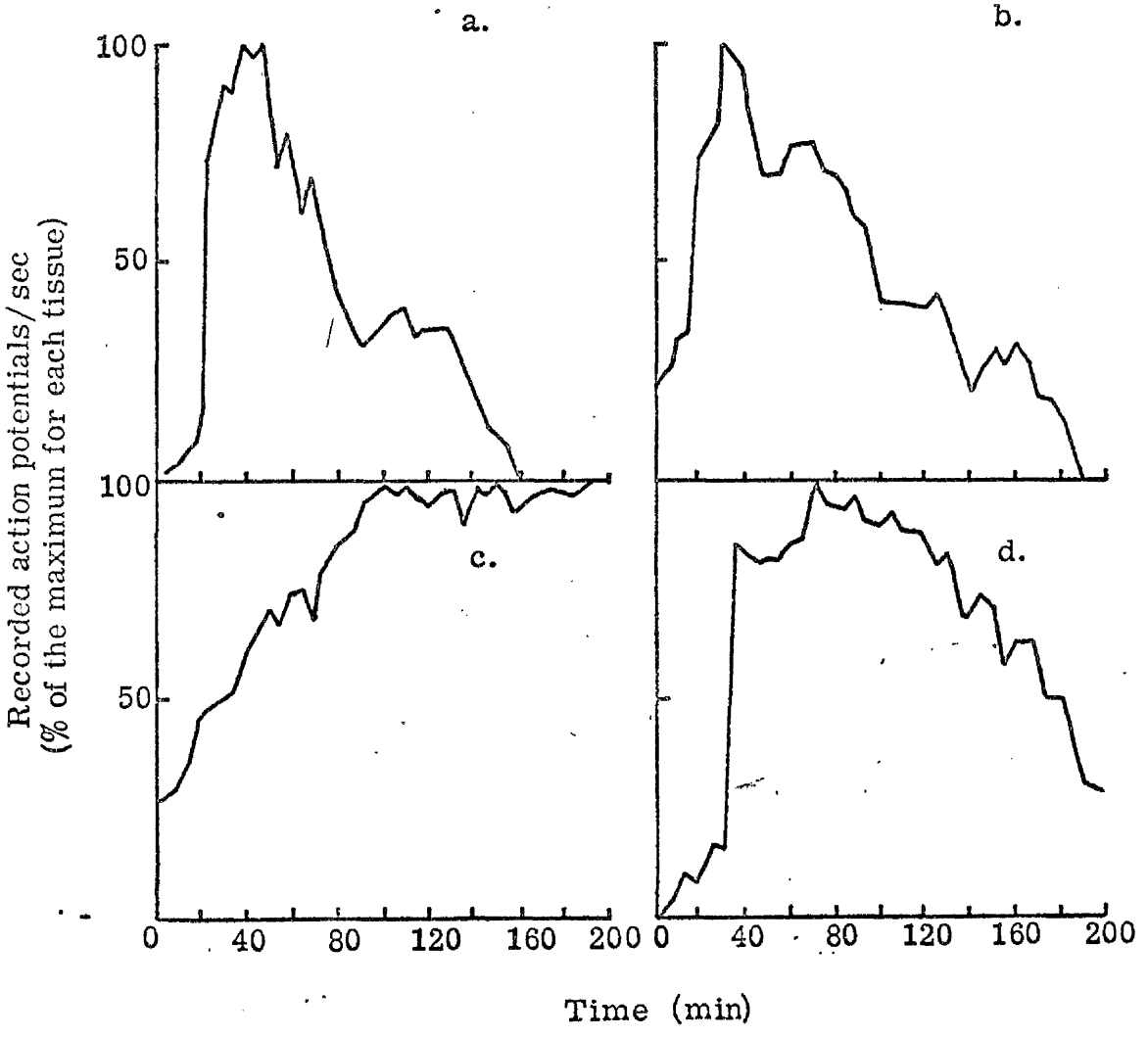
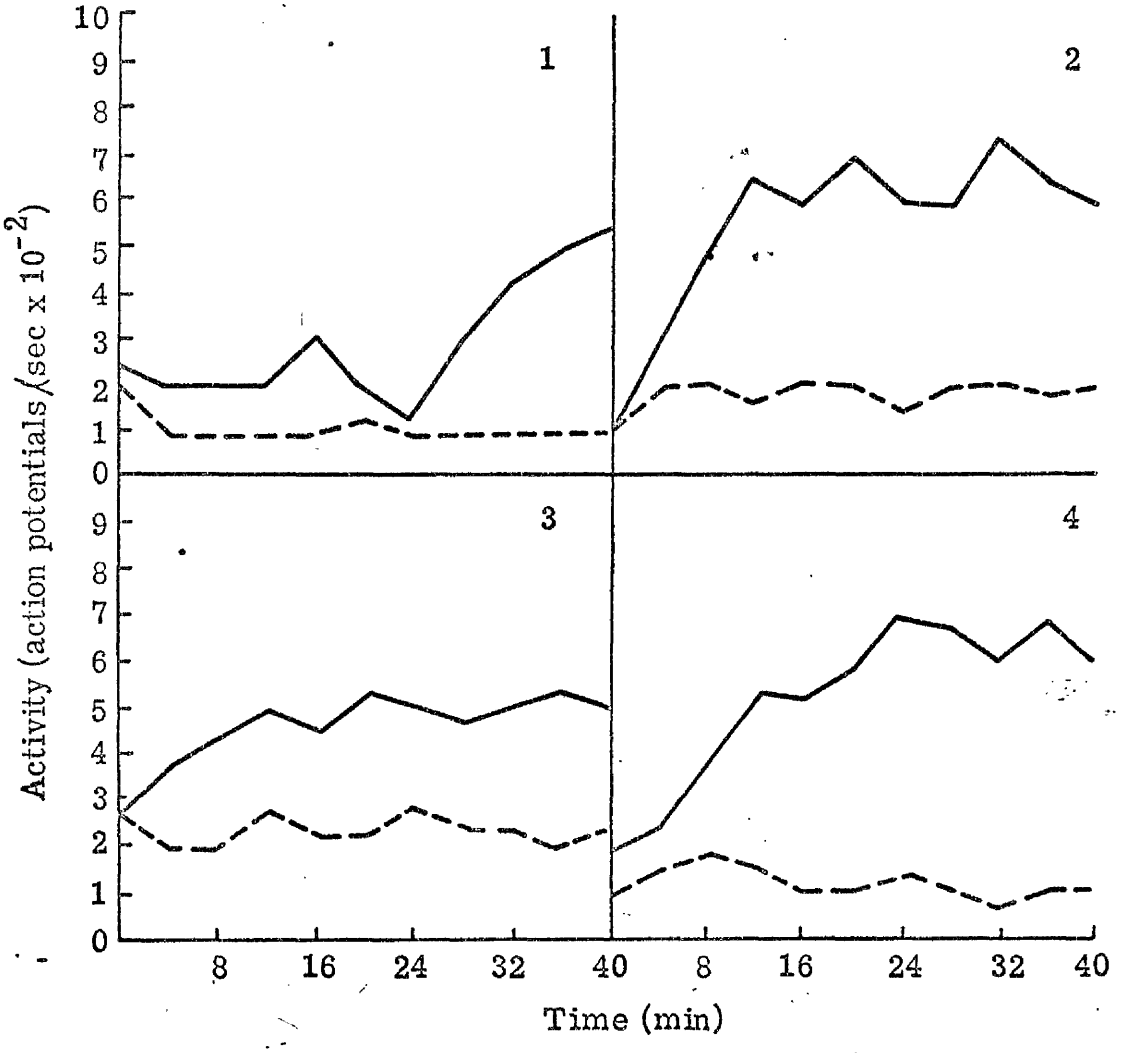

a.
b.
c.
d.
100
50
100
50
0
40
80
120
160
200
0
40
80
120
160
200
Recorded action potentials/sec
(% of the maximum for each tissue)
Time (min)

FIGURE 19

Comparison of the nervous activity *in vitro* with that *in vivo*. The figure shows recordings made from Nerve 5 of the metathoracic ganglion of 4 separate locusts, first *in vivo* (_____) then *in vitro* (___ ___ ___ ___).

FIGURE 19


Activity (action potentials/sec x 10^{-2})
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
8
16
24
32
40
Time (min)

SECTION 'B'

4. Measurement of steady-state concentrations of metabolites present in locust ganglia, with some comparative data from rat brain:

The levels of glycogen, glucose, trehalose, ATP, ADP, arginine phosphate, lactate and pyruvate were measured after immobilising the locust for 10 minutes at -20°C. In order to make a comparison, the results of Strang and Bachelard (1973); McIlwain and Bachelard (1971) have also been quoted as given in Table 10. Locust ganglia contain arginine phosphate whereas rat brain contains creatine phosphate as a labile phosphate energy reserve. In addition to this locust ganglia contain a large amount of trehalose whereas rat brain is devoid of this sugar. There is a dramatic difference in glycogen levels and lactate pyruvate ratio present in locust ganglia compared to rat brain. The levels of glycogen, μmol/g (as glucose) are 11.2 and 1.19 μmol /g present in locust ganglia and rat brain respectively. The ratio of lactate/pyruvate is 3.0 and 23.0 in locust ganglia and rat brain respectively.

5. Measurement of metabolites in the ganglia in glucose saline in vitro.

Having established the best conditions for measuring oxygen uptake and spontaneous activity in vitro, the major energy components of the ganglia were estimated.

5.1. Arginine phosphate, ATP and ADP:

All these metabolites as shown in Figure 20 were measured

TABLE (10)

Comparison of the concentrations of metabolites in rat brain and in locust thoracic ganglia.

Metabolites	Locust ganglia μmol /g ± S.D.	Rat brain μmol /g ± S.D.
Glycogen as glucose	11.2 ± 2.0 (6)	1.19 ± 0.07
Trehalose as glucose	12.8 ± 2.0	
Glucose	1.9 ± 0.5	1.00 ± 0.21
ATP	2.7 ± 0.3	1.56 ± 0.13
ADP	0.4 ± 0.1	0.37 ± 0.05
ATP/ADP	6.75	4.35
Arginine phosphate	3.15 ± 0.14	-
Arginine Phosphate/ATP	1.16	
Creatine phosphate	-	2.43 ± 0.25
Creatine phosphate/ATP	-	1.55
Lactate	1.8 ± 0.4	2.30 ± 0.55
Pyruvate	0.63 ± 0.1	0.10
Lactate/Pyruvate	3.0	23

The figure in the bracket indicates the number of individual results for each estimated mean.

both during a stage of low and maximum spontaneous activity, and after it had ceased over a period of 210 minutes, at intervals of 30 minutes. Zero time indicates the *in vivo* levels. The time period of initial 45 minutes after incubation, represents a state of low spontaneous activity. At 60 minutes, by which time the maximum spontaneous activity is achieved, the arginine phosphate and ATP concentrations rose and ADP decreased. Between 60-180 minutes, the levels vary, but these were not dramatically different as compared to steady-state levels. After 180 minutes, when the spontaneous activity had ceased, arginine phosphate and ATP levels fell dramatically from 2.8 to 0.9 and 2.3 to 0.65 μmol/g respectively. ADP concentrations were not measurable at this time.

5.2. *Glycogen and glucose*:

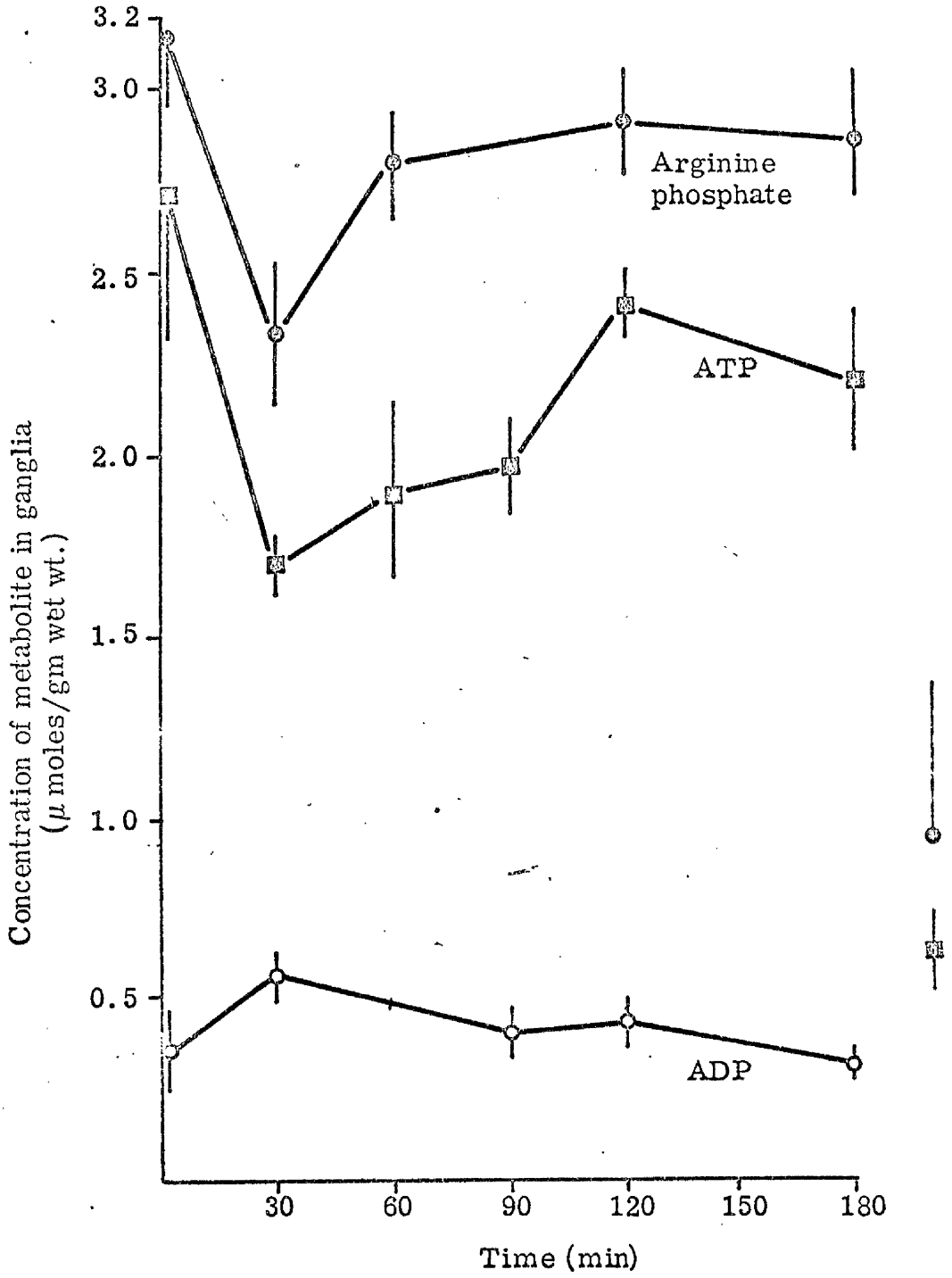
The greatest difference in concentration between steady-state levels *in vivo* and *in vitro* levels, was found in the case of glycogen, which fell from 11.0 μmol/g to between 7-8μmol/g during the initial period of incubation *in vitro*. Although it then stabilized, no recovery to that steady-state concentration was found. The concentration of glucose initially rose, perhaps in response to high glucose concentration in the medium, but thereafter fell to normal levels. No further changes in the concentrations of glucose and glycogen took place after the spontaneous activity had ceased. These results of

FIGURE 20

Represents the concentration of arginine phosphate, ATP and ADP, present in thoracic ganglia during the course of incubation *in vitro*.

The points are the averages (± S.D.) of 6 separate estimates of up to 8 ganglia. The points at zero time represent the concentrations found *in vivo*.

Detached points to the right of the graph represent the concentrations found after the spontaneous activity in the ganglia had ceased. Where no such points are shown, the concentrations were too low to measure.


3.2
3.0
2.5
2.0
1.5
1.0
0.5
Arginine phosphate
ATP
ADP
Concentration of metabolite in ganglia
(μ moles/gm wet wt.)
30
60
90
120
150
180
Time (min)

glycogen and glucose concentrations during incubation with steady-state concentration represented by zero time are shown in Figure 21.

5.3. Lactate and pyruvate:

The concentration of pyruvate and lactate vary over a period of 180 minutes. Although the lactate to pyruvate ratio differed from that found at steady-state at different times of incubation, there was no sustained increase in this ratio. The concentrations of lactate and pyruvate at different time intervals are shown in Figure 22.

6. Measurement of spontaneous activity and metabolites in saline containing no exogenous energy source in vitro.

In order to see what happens to the glucose and glycogen, the ganglia were incubated in saline and under the optimum conditions of oxygen and at 37°C. The measurements of the concentrations of glycogen, glucose, and ATP against time of incubation are plotted in Figure 23. The shaded bar at the top represents the duration of spontaneous activity. Spontaneous activity gradually increased up to 50 minutes until a steady-state of activity was achieved. It remained at maximum for 90 minutes and then started decreasing. It ceased between 100-120 minutes. Zero time indicates the levels of glycogen, glucose, trehalose and ATP at steady-state levels. The ATP concentrations remained constant up to 90-100 minutes and then fell dramatically between 90-100 minutes from 2.5 - 0.9 μmol/g. Glycogen and glucose

FIGURE 21

Represents the concentration of glycogen and glucose in the thoracic ganglia during the course of incubation *in vitro*. The points are the averages (± S.D.) of 6 separate estimates of up to 8 ganglia. The points at zero time represent the concentration found *in vivo*. Detached points to the right of the graph represent the concentrations found after the spontaneous activity in the ganglia had ceased. Where no such points are shown, the concentrations were too low to measure.

FIGURE 21

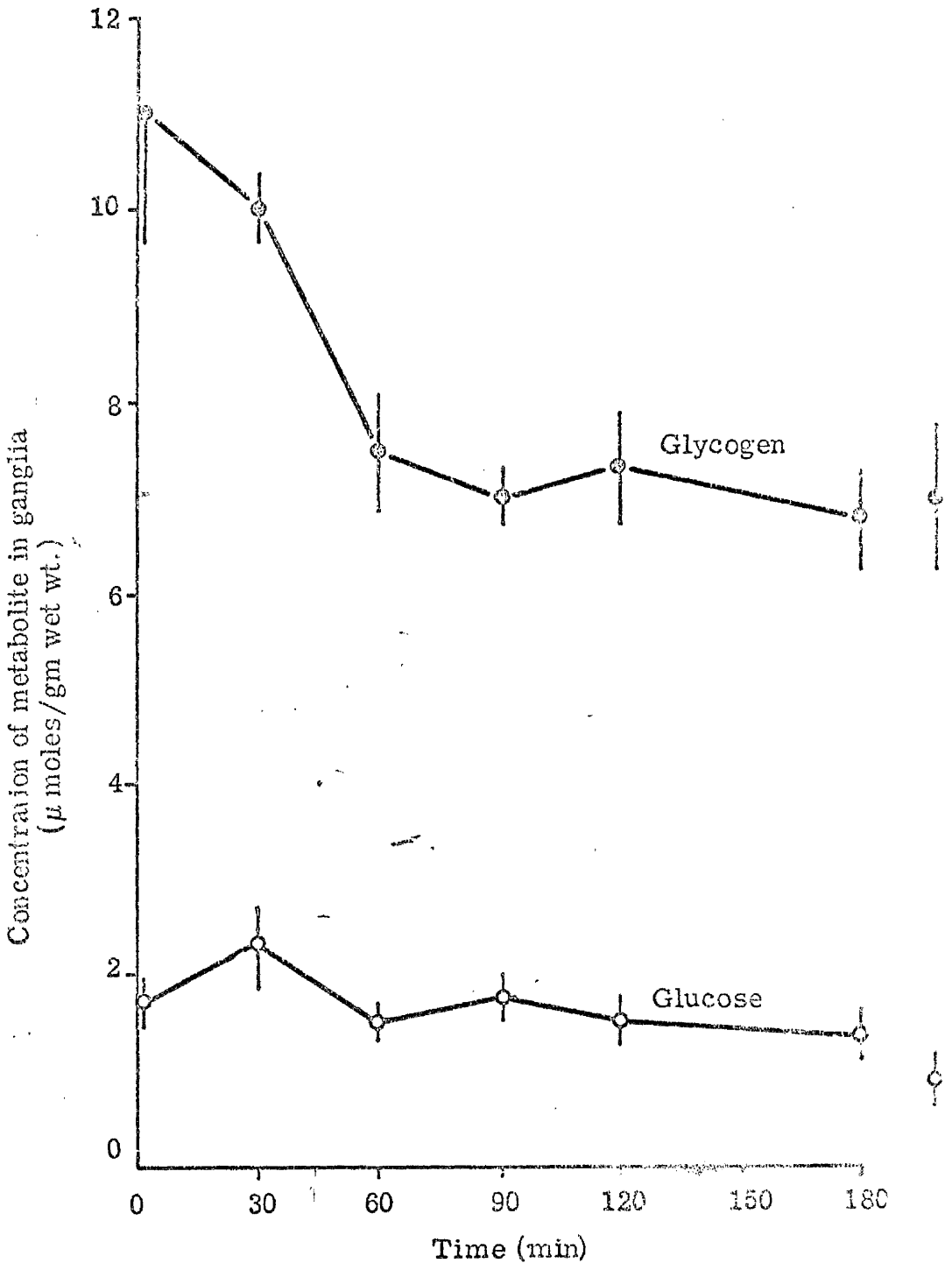
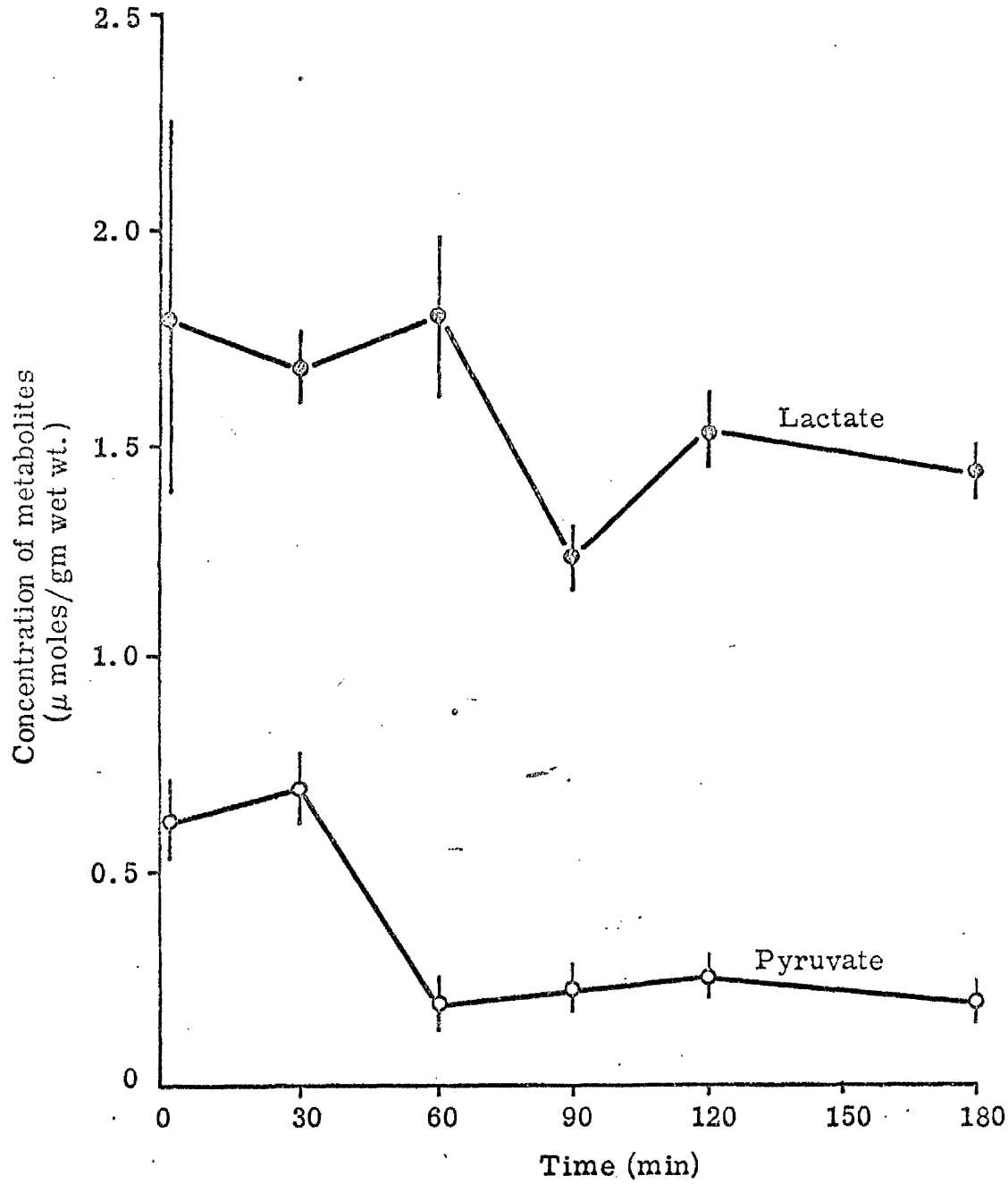

12
10
8
6
4
2
0
Concentration of metabolite in ganglia
(μ moles/gm wet wt.)
Glycogen
Glucose
0
30
60
90
120
150
180
Time (min)

FIGURE 22

Represents concentration of lactate and pyruvate in thoracic ganglia during the course of incubation *in vitro*.

The points are the averages (± S.D.) of 6 separate estimates of up to 8 ganglia. The points at zero time represent the concentration found *in vivo*.

FIGURE 22


2.5
2.0
1.5
1.0
0.5
0
Concentration of metabolites
(μ moles/gm wet wt.)
Lactate
Pyruvate
0
30
60
90
120
150
180
Time (min)

decline continuously and rates of their disappearance are shown in Table 11. The raee of disappearance of both glucose and glycogen is greater in the first 30 minutes compared to the following 60 minutes. The rate of disappearance of glucose in the tissue is at maximum between 90 and 100 minutes and disappears completely when spontaneous activity had ceased. On the other hand glycogen breakdown does not rise and a residual amount remains constant even when activity had ceased.

7. Production of lactate by thoracic ganglia in vitro.

The purpose of these studies was to see how much lactate was released in the medium by ganglia under aerobic conditions.

Thoracic ganglia were incubated in saline under optimum conditions of O_2 tension and glucose concentration and the temperature maintained at 35-37^{o}C. The incubation was continued for 180 min., and the medium was replaced at regular intervals by means of a peristaltic pump as already described in Materials and Methods. The results of two different experiments are shown in Figure 24. Total lactate production is plotted against time. During the first 50 minutes the spontaneous activity was low. It is clear from the curve that total lactate production increases linearly with the time. The time period between 60-180 minutes represents a state of maximum activity. The lactate production curve starts levelling off during this time and hence less lactate was produced in the

TABLE (11)

Rates of disappearance of endogenous glycogen and glucose from the ganglion in glucose-free saline.

Incubation time (min.)	Glycogen disappearance µ/mol /g/min.	Glucose disappearance µmol /g/min.
0-15	0.18	0.02
15-30	0.16	0.02
30-45	0.10	0.013
45-60	0.06	0.006
60-90	0.02	0.01
90-100	0.03	0.05
100-120	0.005	0.00

FIGURE 23

Represents concentration of glycogen, ATP and glucose in the thoracic ganglia and recording of spontaneous activity from Nerve 5 of metathoracic ganglion during the course of incubation *in vitro*. The ganglia were incubated at 35-37°C in glucose-free saline and the medium saturated with 100% O_2. The shaded bar at the top represents duration of spontaneous activity. The points are the averages ($\pm$ S.D.) of 5 separate estimates of up to 8 ganglia. The points at zero time represent the concentrations found *in vivo*. Detached points to the right of the graph represent the concentrations found after the spontaneous activity had ceased.

FIGURE 23

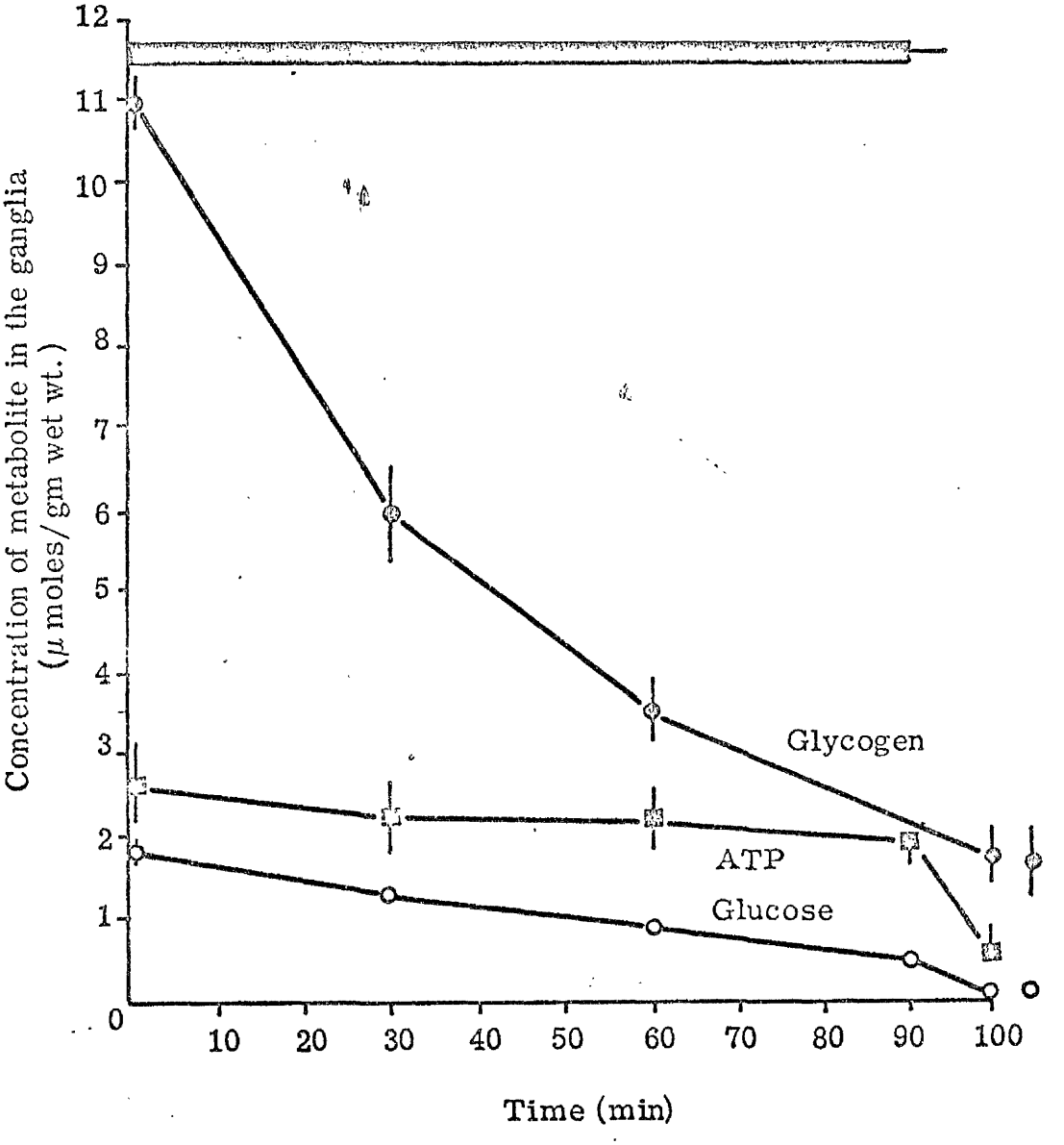
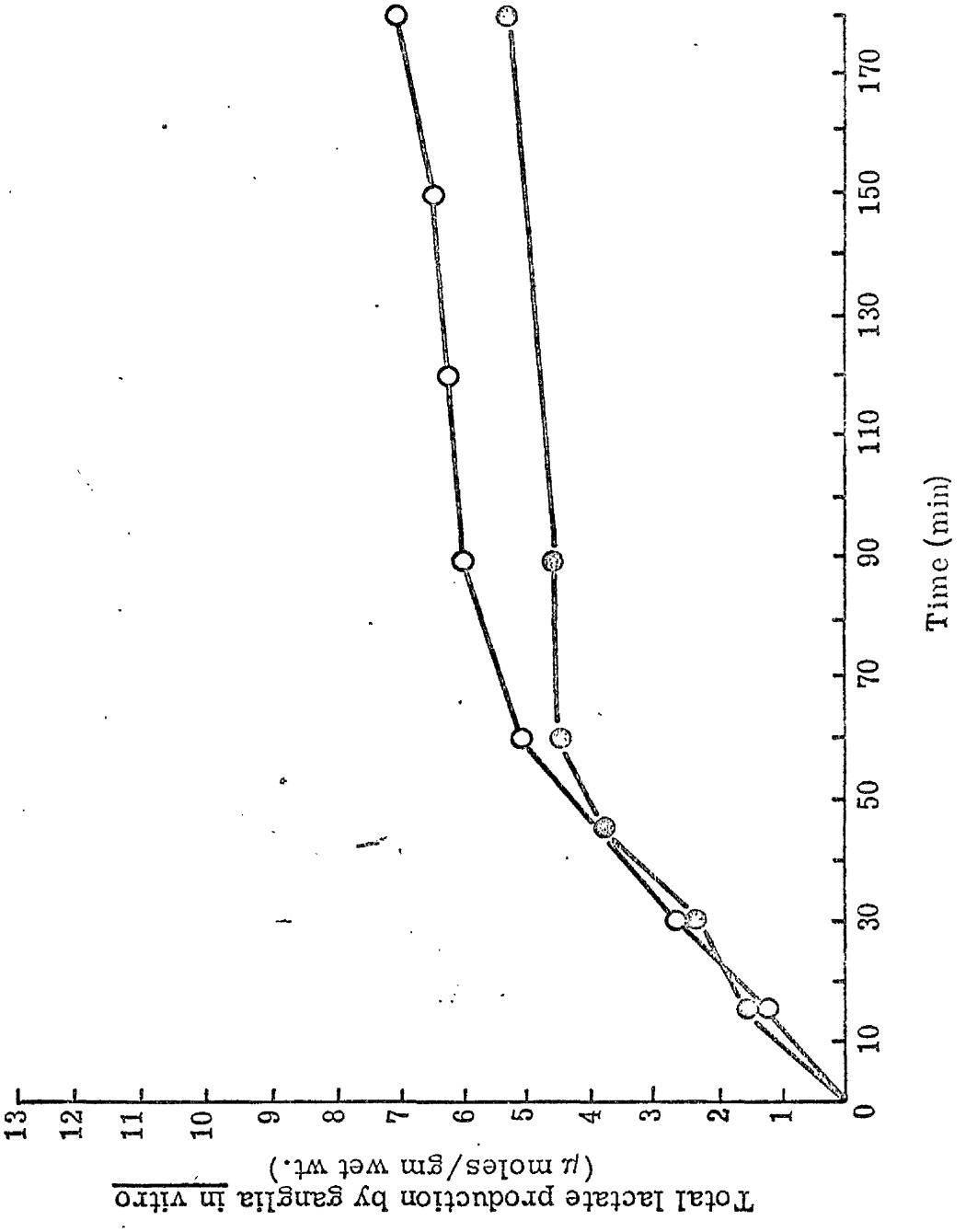

Concentration of metabolite in the ganglia
(μ moles/gm wet wt.)
12
11
10
9
8
7
6
5
4
3
2
1
0
Glycogen
ATP
Glucose
10
20
30
40
50
60
70
80
90
100
Time (min)

FIGURE 24

Represents the rates of lactate production by thoracic ganglia during the course of incubation *in vitro*. Incubation was carried out at 35-37^{o}C in 10mM glucose saline and with saturation of 100% O_2.

Filed (●) and open (O) symbols represent two different experiments respectively.

FIGURE 24


Total lactate production by ganglia in vitro
(μ moles/gm wet wt.)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
10
30
50
70
90
110
130
150
170
Time (min)

medium during this time. The rates of lactate production during low and high activity are 0.08 and 0.03 μmoles/g/min respectively.

SECTION 'C'

8. Introduction - osmolarity, glucose and trehalose:

It was shown in the previous section that spontaneous activity showed three phases *in vitro*, an initial state of low activity, then a state of high activity and finally a decline and disappearance of activity. This section gives the report of the attempt made to resolve the observation of slow state of spontaneous activity in initial 45 minutes.

It was though that this could be due either to energy states of the tissue or due to some differences between the physiological saline and the locust's haemolymph. The results of investigating the energy state of the locust ganglia *in vitro* were shown in the previous section and results of other aspects are described here. The physiological saline which has been used in the work described in the previous section 'A', contains the same ionic composition as is present in locust haemolymph (Hoyle, 1953). However, it was found that this saline differs from locust haemolymph with regard to two respects,
(a) osmolarity (b) addition of glucose alone as an exogeneous energy source instead of a combination of glucose and trehalose. Some comparative data of physiological saline and locust haemolymph are shown in Table 12. The technique of obtaining locust haemolymph and the measurement of osmolarity and sugars (glucose and trehalose) have been already described in Chapter 2. Although it was known from the previous results of other workers that

locust haemolymph contains very high concentration of trehalose (Newsholme and Start, 1973), yet only glucose was added to the saline. The purpose of adding glucose as a sole substrate was to investigate its role as metabolic fuel and this is also one of the aims of this research work. Locust haemolymph contains about 50 mM trehalose and 4mM glucose and its osmolarity is 400 mosmol/kg which is 1.33 times higher than that of saline used in all the work described until now.

It was thought that an initial state of slow spontaneous activity in vitro during 45 min. might be due to absence of these two factors in the physiological saline. In order to resolve this, osmolarity of the saline was adjusted close to that of locust haemolymph in one series of experiments and a combination of 4mM glucose and 50mM trehalose was used in the saline in another series of experiments in which oxygen uptake and spontaneous activity were studied.

8.1. Osmolarity:

To increase the osmolarity of the saline, 100 mM sucrose or mannitol was added and this increased the osmolarity to that of the average haemolymph. Thoracic ganglia were incubated in sucrose containing iso-osmotic saline and 10mM glucose as an exogenous energy source; oxygen uptake and spontaneous activity were recorded. The measurements of oxygen uptake by isolated ganglia during initial 60 min. and after 60 min. (these time periods

TABLE (12)

Comparative data of haemolymph and saline.

	Haemolymph ± S.D.	Glucose Saline	Trehalose Saline
Osmolarity	400 ± 6.0 mosm/kg (5)	300 mosm/kg	335 mosm/kg
Concn. of glucose	4.0 ± 0.21 mM (5)	10.0 mM	4 mM
Concn. of trehalose	50.0 ± 7.0 mM (4)		50 mM

(Figures in brackets indicate the number of individual results for each estimated mean).

are measured from the isolation of ganglia from the insect are given in Figure 25. In order to make the comparison, the results of oxygen uptake by ganglia in hypo-osmotic saline are also shown in Figure 25. The initial rates of oxygen uptake in hypo-osmotic saline are taken from Figure 11 as shown in Section 'A' of this chapter and rates after 60 minutes were obtained from the experiments performed along with iso-osmotic salines. The rates of oxygen uptake in hypo-osmotic salines are represented by bars I and II during initial 60 minutes and after 60 minutes respectively. The 't' test for these results clearly indicates that the rates are significantly higher during initial periods.

The rates of oxygen uptake in iso-osmotic salines are represented by bars III and IV during initial 60 minutes and after 60minutes respectively. The 't' test for these results indicates that difference is not significant.

In comparing the rates of oxygen uptake in hypo-osmotic and iso-osmotic salines during initial 60 minutes, the rates are significantly higher in hypo-osmotic salines. However, the rates are not significantly different after 60 minutes of incubation. The results of oxygen consumption in hypo-osmotic and iso-osmotic salines with some comparative data of vertebrate nervous tissues are shown in Figure 26. It is obvious from the data that locust ganglia have the highest rate of

FIGURE 25

Rates of oxygen uptake for the isolated, unstimulated thoracic ganglia in hypo-and iso-osmotic salines. Measurements were made in saline containing 10 mM glucose at 35-37^{o}C and which had been saturated with 100% O_2.

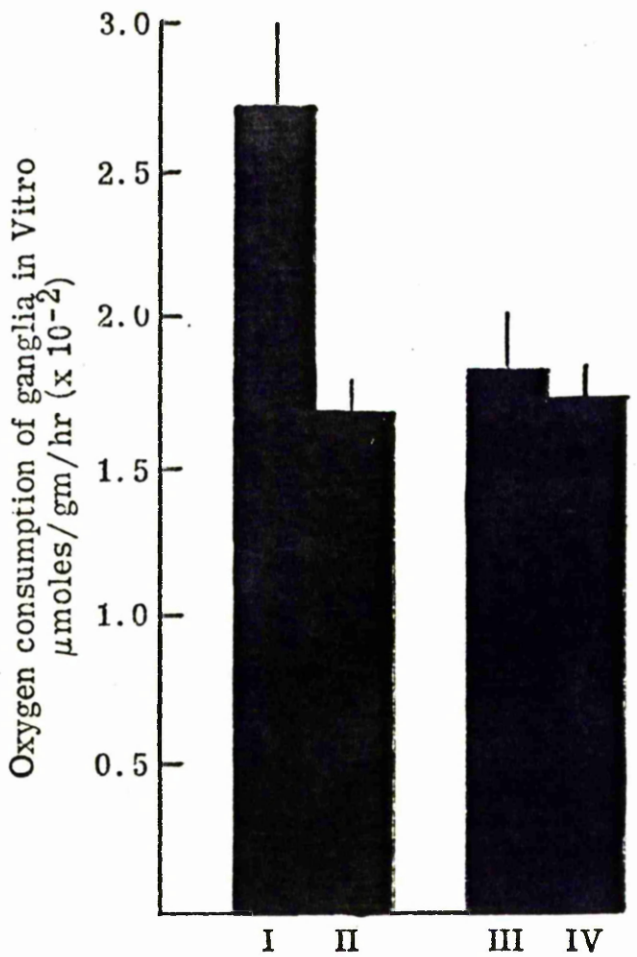
Bars I and II represent the rates in hypo-osmotic saline during first 60 min. and after 60 minutes respectively.

Bars III and IV represent the rates in isosmotic salines during first 60 minutes and after 60 minutes respectively. A vertical line at the top of each bar represents S.D.

The results represented by -

Bars I and II	($P < 0.001$)
Bars III and IV	($P > 0.05$)
Bars I and III	($P < 0.001$)
Bars II and IV	($P > 0.05$)

FIGURE 25


Oxygen consumption of ganglia in Vitro
μmoles/gm/hr (x 10-2)
3.0
2.5
2.0
1.5
1.0
0.5
I
II
III
IV

oxygen uptake.

The oscilliscope trace of the spontaneous activity of the metathoracic ganglion in iso-osmotic saline is shown in Figure 27 and spontaneous activity expressed in terms of impulses/sec and obtained from the records of the scalar is shown in Table 13. On comparing this activity in iso-osmotic saline with hypo-osmotic saline as shown in Table 9 of Section 'A' of this chapter, the initial state of low activity is also obtained in iso-osmotic saline, however, maximum activity is about twice as high as that in hypo-osmotic salines.

8.2. Trehalose and glucose:

In all the previous results already described 10 mM glucose was used as an exogenous energy source. This differs from the actual concentration and type of carbohydrates present in the haemolymph. As shown in Table 12, the haemolymph of locust contains 4 mM glucose and 50 mM trehalose.

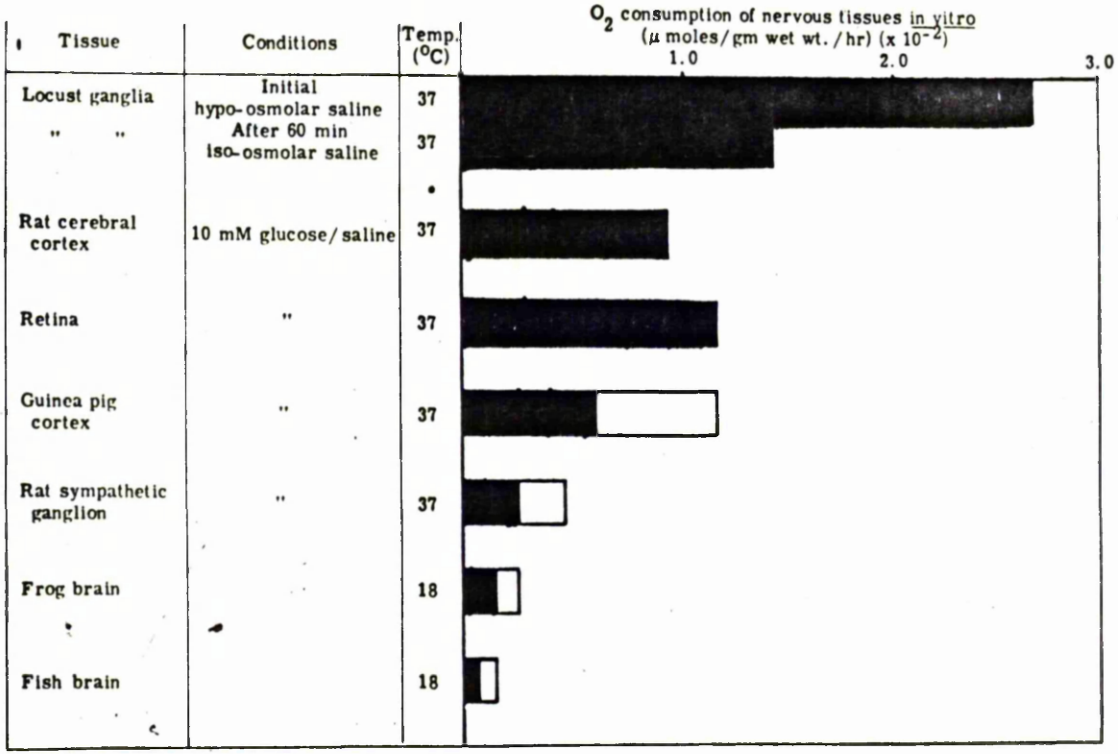
To reproduce *in vitro* the situation *in vivo*, the saline containing 4 mM glucose and 50 mM trehalose was used and oxygen uptake and spontaneous activity of the ganglia were recorded. The results of oxygen uptake with comparative data of hypo-osmotic saline are given in Table 14. There is no significant difference in the oxygen uptake between trehalose saline and 10 mM glucose saline.

This saline containing 4 mM glucose and 50 mM trehalose did not have any effect on the spontaenous activity of ganglia *in vitro*.

FIGURE 26

Comparison of the rates of oxygen uptake for the isolated, unstimulated thoracic ganglia of the locust in hypo- and iso-osmotic salines *in vitro* with other nervous tissues, stimulated and unstimulated.

Solid part of the bar represents the rate for unstimulated tissue, and the open bar the measurement on stimulation.



Comparison of O_2 consumption in vitro of various nervous tissues

FIGURE 26

FIGURE 27

A typical oscilloscope trace of the activity recorded from Nerve 5 for the metathoracic ganglia *in vitro* in iso-osmotic saline. The scanning speed of the oscilloscope trace was 5 ms/cm.

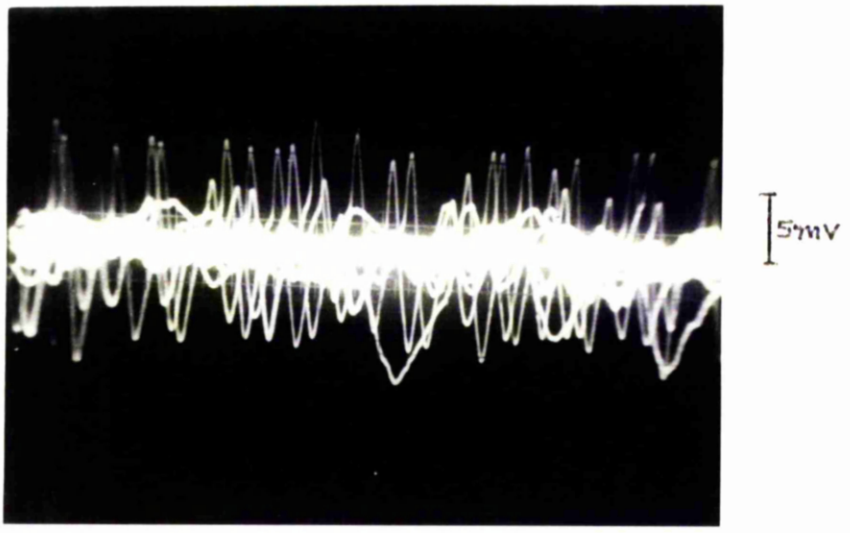

5mV
5 ms

TABLE (13)

Recording of spontaneous activity in iso-osmotic saline at 35-37°C

Preamplifier Setting: Gain 1000, H.F. Cut 1KC/S
L.F. Cut 200C/S

Scalar Setting: Disc. Bias 20
Input x 200, Channel width off

Time after Isolation of Ganglia	Impulses/Sec			
	I	II	III	IV
0 min.	300	450	280	150
5	150	10	20	15
10	15	30	100	30
15	20	70	300	15
20	20	100	400	30
25	10	30	700	100
30	30	80	800	200
40	80	200	1200	400
45	1000	900	1600	1000
50	1400	1200	1900	1300
60	1600	1400	2000	1600
70	1400	1600	1760	1700
80	1450	1650	1800	2000
90	1800	1800	1590	2010
100	1900	1350	1480	2220
110	1650	1800	1600	1980
120	1300	1550	1900	1800
130	2000	1600	2000	1930
140	1460	1470	1800	1800
150	1500	2000	1600	1750
160	1150	2010	1520	1600
170	1480	1610	1370	1800
180	1620	1500	1600	1900
190	250	1000	800	300
195	100	100	200	200
200	10	20	50	10

TABLE (14)

Oxygen consumption of ganglia in hypo-osmotic saline containing 10 mM glucose and in hypo-osmotic saline containing 4 mM glucose and 50 mM trehalose:

Incubation time (min.)	Conditions	Temp:	Oxygen Uptake µmol. /gm/hr ± S.D.
0-30	Hyposmotic saline, containing 10 mM glucose	37°C	267.0 ± 23.7 (5)
0-30	Saline containing 4 mM glucose + 50 mM trehalose	37°C	260 ± 22.1 (5)
60-90	Hyposmotic saline containing 10 mM glucose	37°C	174.6 ± 17.0 (5)
60-90	Saline containing 4 mM glucose + 50 mM trehalose	37°C	180 ± 16.3 (5)

The number in parenthesis indicates the number of individual results used for calculations.

SECTION 'D'

9. The effects of anoxia on the metabolism and spontaneous activity of thoracic ganglia.

9.1. Introduction:

The respiratory quotient of intact central nervous tissues in higher animals is 1 (Dickens and Simer, 1931; Gibbs et al., 1942; and Ketty, 1957). This indicates that glucose is the only fuel oxidized by the nervous tissues and volume of oxygen utilized is equivalent to carbon dioxide elimination. However, although the formation of other products from glucose and oxidation of alternative endogenous substances do occur, they do not do so to any subsequent extent. Thus, an exchange of glucose carbon between intermediates of glucose metabolism and endogenous compounds such as glutamate does take place, (Haslam and Krebs, 1963). Gibbs et al. (1942) and McIlwain (1966), working on isolated mammalian nervous tissues found that when glucose was sole substrate for nervous tissues, its utilization may be almost wholly for as CO_2 and lactate. This conclusion is also supported by the low rates of glycogen synthesis in cerebral cortex slices (McIlwain and Tresize, 1956; Kleinzeller and Rybova, 1957). Of possible alternative pathways for glucose, the hexose monophosphate

shunt has received the most attention. It has generally been concluded that this pathway is inoperative in the intact brain (Sacks, 1961). Thus, it is concluded that measurement of oxygen consumption and lactate formation by insect nervous tissues provides a reasonable indication of glucose utilization.

Under optimum conditions of glucose and oxygen concentrations, in the hypo-osmotic saline at 35-37°C *in vitro*, the oxygen uptake by thoracic ganglia is 4.45 µmol /g/min (Table 5, Section 'A' of this chapter). This is the highest rate of oxygen uptake obtained in any of the conditions used in the present work and the corresponding lactate production is 0.080 µmol /g/min. (Figure 24, Section 'B' of this chapter). Making the assumption that endogenous glucose is the sole substrate oxidized and keeping in mind the equation given below, glucose utilization

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

would be 4.45÷6 µmol /g/min. which is 0.74 µmol /g/min. via pyruvate to TCA cycle. Since one mol of glucose is converted to two moles of lactate, the corresponding amount of glucose utilized for lactate formation would be 0.04 µmol /g/min. In conclusion, the total glucose utilization is 0.74 + 0.04 = 0.78 µmol /g/min.

The details of the measurement of hexokinase activity present in thoracic ganglia are given in chapter 2. The levels of maximum specific activity of hexokinase are 1.5 µmol /g/min by the method of Bachelard and Goldfarb (1969)

and 3.03 μmol /g/min. as measured by the method of Crabtree and Newsholme (1972). The results along with the results of metabolism of thoracic ganglia and that of cerebral cortex slices are shown in Table 15. Although the activity of the enzyme is higher in the presence of a cycle for the regeneration of ATP, in its absence the rate is the same whether the concentration of ATP is 2.5 mM or 5.0 mM. It is possible therefore that the higher rate is due more to the absence of ADP which is an inhibitor of hexokinase (Sols and Crane, 1954), and thus the estimates made by the method of Bachelard and Goldfarb (1969) may reflect accurately the activity in vivo. If this is so, then the difference between the maximum rate of utilization of glucose in vitro by thoracic ganglia (estimated by the rate of oxygen uptake and lactate production) and hexokinase activity (1.5 μmol/g/min) is about 2 fold, in sharp contrast to the mammalian situation, (Bachelard, 1967) which is about 35-40 fold different. This suggests that hexokinase might be rate limiting in the locust nervous tissues.

It is clear from the results of MacMillan (1977) and other workers (McIlwain and Bachelard, 1971) that the anaerobic glycolysis in mammalian nervous tissues is increased 6-7 fold under anaerobic conditions and correspondingly lactate production in the medium is increased. This is the Pasteur effect; a stimulation by anaerobiosis of lactate

TABLE (15) Comparison of aerobic metabolism of thoracic ganglia of locust and mammalian central nervous tissues in vitro.

All the results are given as μmol. $^{-}$/g wet wt/min. except Km values .

Tissue	Oxygen Uptake ± S.D.	Glucose utilization ± S.D.	Lactate production ± S.D.	Hexokinase activity ± S.D.	Km for glucose (M)	Authors
Thoracic ganglia*	4.45 ±0.49	0.78 ±0.08	0.080 ±0.015	†1.5 ± 0.2 ‡3.03 ±0.4	2.2×10^{-4}	Present
Guinea pig Cerebral Cortex‖	1.40 ±0.12	0.68 ±0.096	0.60 ±0.16			Newsholme et al. Rolleston (1967a,b)
Guinea pig Cerebral Cortex				21-26	7.4×10^{-5}	Bachelard (1967)

* The results quoted above are the means ± S.D. of 6 individual observations.

‖ Results are given as Means ± S.D. of 12 independent observations.

† Estimation was made by the method of Bachelard and Goldfarb(1969)

‡ Crabtree and Newsholme (1972)

formation (Iwakawa, 1944). It is obvious that the activity of hexokinase is increased under anaerobiosis as compared to aerobic conditions. Therefore, one way to investigate whether or not hexokinase is rate limiting is to study the rate of glycolysis under anaerobic conditions. As one of the aims of this research work was to investigate the control of anaerobic glycolysis such studies would also fulfil this aim.

In the work described here, the control of anaerobic glycolysis was investigated by measuring the concentration of glucose, glycogen, ATP and lactate present in the thoracic ganglia after incubation in glucose-containing and glucose-free saline under anaerobic conditions and finally, hexokinase activity was interpreted in the light of the rates of glucose utilization. As another aim of the present work was to correlate spontaneous activity of metathoracic ganglion with the pattern of carbohydrate metabolism, spontaneous activity was also recorded simultaneously under anaerobiosis and described here.

9.2. Anaerobic glycolysis and endogenous activity of ganglia in glucose saline:

Ganglia were incubated in oxygenated 10 mM glucose saline for 60 min. During this time, they achieved a steady-state of spontaneous activity. They were then transferred to 10 mM glucose saline which had been saturated with 100%N_2

and incubated for 20 minutes. The results of spontaneous activity and metabolites (glycogen, glucose, lactate and ATP) are given in Figure 28. Glycogen was disappearing continuously during 20 minutes, and disappearance was faster in the first 5 minutes as compared to next 15 minutes. Glucose levels diminish slightly in first 5 minutes and then remain constant. ATP levels fall dramatically from 2.3 to 1.0 μmol in first 5 minutes and then remain constant. Lactate production in the medium parallels the glycogen breakdown, but is faster in first 5 minutes compared to next 15 minutes.

9.3. Anaerobic glycolysis and endogenous nervous activity of ganglia in glucose-free saline:

The purpose of these studies was to see what happens to the glucose present in the ganglia in anaerobiosis. When the ganglia attained a steady state of spontaneous activity, they were washed in glucose-free saline, and then transferred to the same saline which had been saturated with 100% N_2. The results of spontaneous activity and metabolites are shown in Fig. 29. All these results are the same except glucose levels diminish continuously up to 20 minutes. Although not diminished completely, levels are reduced from 2.5 to 1.5 μmol /g.

9.4. Rates of glucose and glycogen disappearance present in ganglia and production of lactate in the medium in anaerobiosis in vitro.

The rates of glycogen disappearance and lactate production in glucose saline are shown in Table 16. There is a higher rate of glycogen disappearance and lactate production during

FIGURE 28

Concentration of metabolites (glycogen, glucose, ATP), rates of lactate production by thoracic ganglia, and measurement of spontaneous activity of metathoracic ganglia during the course of incubation *in vitro* and under anoxic conditions.

Ganglia were incubated in saline, containing 10 mM glucose, at 35-37°C, which had been saturated with 100% N_2, following an initial incubation of 60 minutes in 10 mM glucose saline saturated with 100% O_2.

The points are the average ± S.D. of 5 separate estimates of up to 8 ganglia. The points at zero time represent the concentrations found after 45 minutes incubation in 100% O_2 saturated saline. The shaded part of the bar represents the duration of steady-state of spontaneous activity in 100% N_2 saturated saline.

FIGURE 28

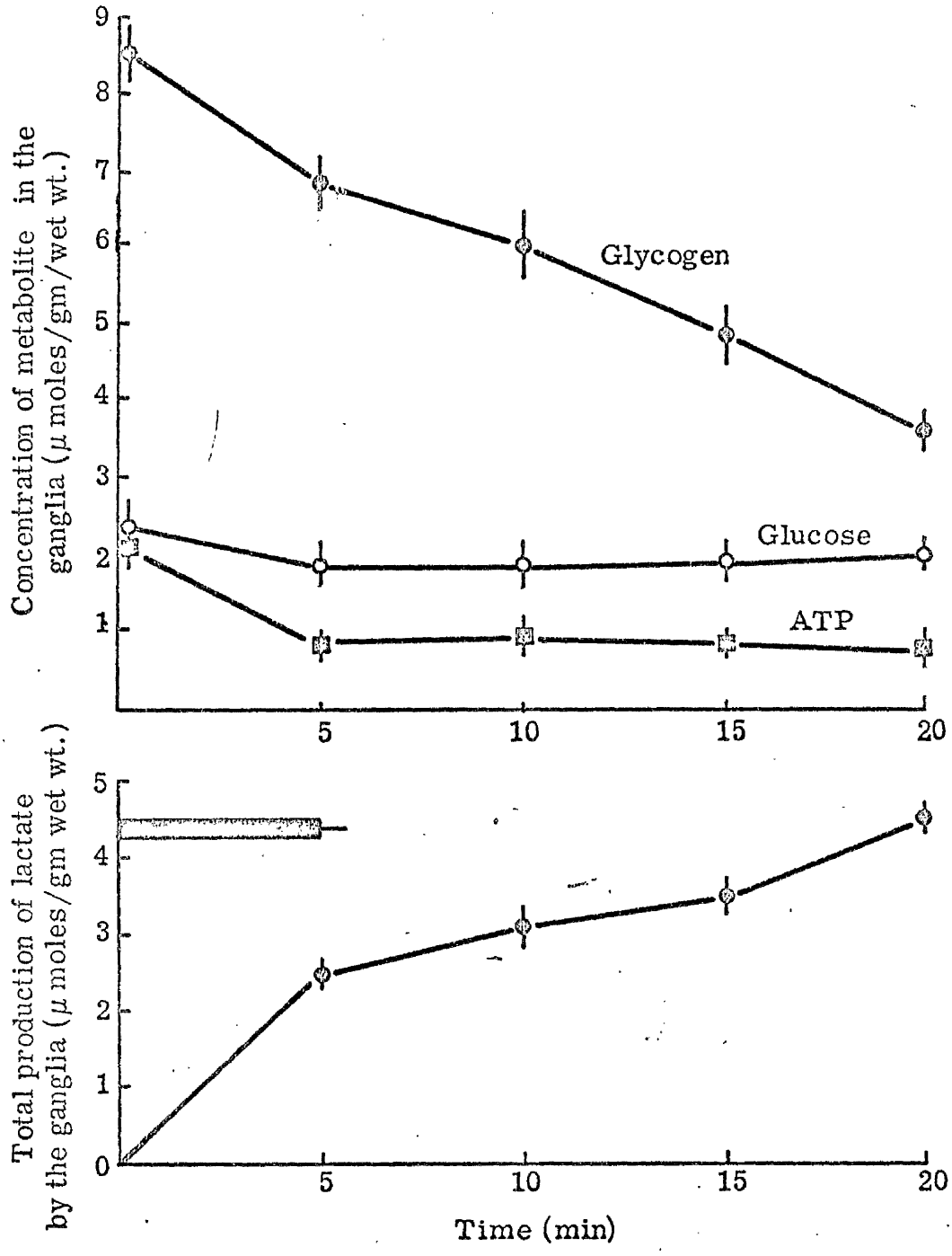

Concentration of metabolite in the ganglia (μ moles/gm/wet wt.)
Glycogen
Glucose
ATP
Total production of lactate by the ganglia (μ moles/gm wet wt.)
Time (min)

FIGURE 29

Concentration of metabolites (glycogen, glucose, ATP, rates of lactate production by thoracic ganglia, and measurement of spontaneous activity of ganglia during the course of incubation *in vitro* and under anoxic conditions. Ganglia were incubated in glucose-free saline, at 35-37°C which had been saturated with 100% N_2, following an initial incubation of 60 minutes in 10 mM glucose saline, saturated with 100% O_2.

The points are the average ± S.D. of 5 separate estimates of up to 8 ganglia. The points at zero time represent the concentrations found after 60 min. incubation in 100% O_2 saturated saline. The shaded part of the bar represents the duration of steady state of spontaneous activity in 100% N_2 saturated saline.

FIGURE 29

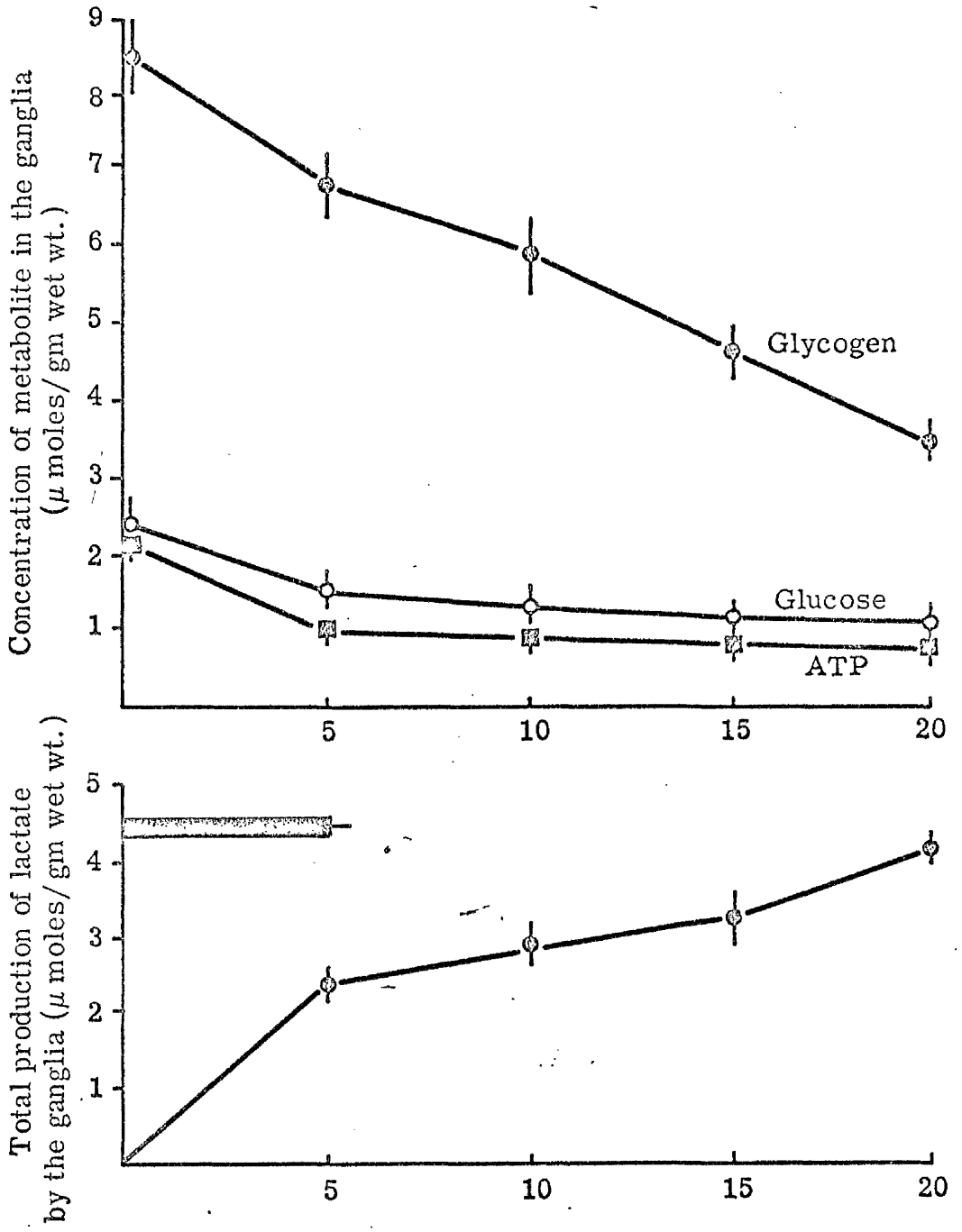

Concentration of metabolite in the ganglia
(μ moles/gm wet wt.)
9
8
7
6
5
4
3
2
1
Glycogen
Glucose
ATP
5
10
15
20
Total production of lactate
by the ganglia (μ moles/gm wet wt.)
5
4
3
2
1
5
10
15
20

TABLE (16)

Rate of glycogen disappearance from ganglia and lactate production in glucose saline in anaerobic conditions.

Incubation time (min)	Glycogen disappearance (glucose equivalent)	Lactate production (medium)
0 - 5	0.36	0. 5
5 - 10	0.13	0.14
10 - 15	0.23	0.06
15 - 20	0.21	0.24

Data shown in µmol. /g/min

TABLE (17)

Rates of glycogen and glucose disappearance and lactate production in glucose-free saline in anaerobic conditions.

Incubation time (min)	Glucose disappear-ance	Glycogen disappearance (glucose equivalent)	Lactate Production (medium)
0 - 5	0.18	0.40	0.44
5 - 10	0.034	0.20	0.12
10 - 15	0.080	0.16	0.10
15 - 20	0.020	0.25	0.18

Data shown in µmol /g/min

first 5 minutes of incubation as compared to next 15 minutes. The same is true in glucose-free saline. These rates of glucose and glycogen breakdown and lactate production are shown in Table 17.

10. Effect of the drugs, dexamphetamine, sodium phenobarbitone and chlorpromazine on endogenous nervous activity of ganglia in vitro.

The purpose of these studies was to see if endogenous nervous activity could be modified by drugs in a manner similar to higher nervous tissues.

The ganglia were incubated in oxygenated glucose saline and the spontaneous activity was recorded from a major motor nerve, N5, of metathoracic ganglia. Once a steady state was reached, to the medium was added the drug in a final concentration of 1 mM and effect on spontaneous activity was recorded. The results are shown in Table 18. Dexamphetamine acts as a stimulant in higher CNS and in case of locust ganglia it increased the spontaneous activity by 200%. Chlorpromazine is used as a tranquilizer and it depressed the spontaneous activity by 50%. Similarly, Na phenobarbitone, a depressant, decreased the spontaneous activity by 50%.

TABLE (18):

Effects of various drugs on spontaneous activity of ganglia in Vitro:

Drug	Concentration (mM)	Effect on spontaneous Activity	% Change	Effect of drugs on higher CNS
Dexamphetamine	1	↑	+ 200	Stimulant
Chloropromazine	1	↓	- 50	Tranquiliser
Na Phenobarbitone	1	↓	- 50	Depressant

CHAPTER 4

Chapter Four:

DISCUSSION

The aim of this research was to investigate the basic carbohydrate metabolism and control of aerobic and anaerobic glycolysis in locust nervous tissues. The first Chapter, the Introduction to this thesis, was concerned with the selection of locusts over other animals of arthropda and in particular the reasons for the suitability of meso- and metathoracic ganglia for the present work. This chapter has also been concerned with the experimental approach of investigating the control of aerobic and anaerobic glycolysis by measuring key metabolites. Ideally this experimental approach permits identification of those enzymes at which control can be exerted. However, this method does not permit one to make hypothesis either about the mechanism of regulation or the rate of rate-limiting enzymes. The second chapter was concerned with the materials and methods used in this research work. The previous methods used by other workers have been reviewed, and new methods developed in this research compared with other older methods. The methods which were developed and found to be useful for studying locust central nervous tissues are:

(A) combining electrophysiological and metabolic studies by using a metabolic chamber and replacing the magnetic stirrer by a glass stirrer driven by a current of compressed air (see Figure 4 in second Chapter);

(B) derivation of weight of thoracic ganglia from measurement of proteins;

(C) a better method of obtaining haemolymph from the locust is by cutting off the metathoracic legs and then spinning at low speed;

(D) an ideal method of killing locusts by immobilization for 10 min. at -20°C, in order to obtain steady-state concentration of metabolites.

The present chapter is concerned with the overall discussion of the results of this research work with particular reference to the results reported in the third chapter. These results are also discussed here in relation to the results of other workers. The discussion here is divided into four major headings which reflect the aims of this research work as discussed and mentioned in the introduction to this thesis.

1. Behaviour of the locust central nervous tissues *in vitro* under aerobic conditions and comparison with that *in vivo*.

As mentioned in chapter 3, oxygen uptake is an index of metabolic activity (Larrabee, 1958; McIlwain, 1953). In light of this criterion, the present results of measurement of oxygen uptake by thoracic ganglia (Figures, 11, 12 and 13 as shown in Section 'A' of Chapter 3) indicate that the addition of 10m moles of glucose to the physiological hypo-osmotic saline of Hoyle (1953), saturation with 100%

oxygen and maintaining the temperature between 35-37°C, are essential requirements for obtaining high metabolic activity in vitro. This requirement of high concentration of glucose certainly contrasts with the findings of McIlwain (1953), working with slices of mammalian cortex, who showed that a concentration of less than 5mM glucose was quite adequate. On the basis of the present information, it is impossible to decide on the reason for this difference between the two tissues in vitro. However, this in vitro requirement for a high glucose concentration is of interest in relation to the situation in the living locust where the levels of glucose in haemolymph are approximately 4mM (Table 12 , Chapter 3) and this low level of glucose has also been reported by other workers (Howden and Kilby, 1956; Treherne, 1958; Mayer and Candy, 1969). This would imply that glucose alone is an inadequate source of energy for the locust nervous system in vivo. In fact the predominant carbohydrate in haemolymph is trehalose, the concentration of which far exceeds that of glucose, (Table 12, Section 'C' of Chapter 3, Wyatt, 1967; Treherne, 1960), and this sugar would be expected to be the major contributor to the carbohydrate available to the nervous system for oxidation. Therefore, the requirement of a high concentration of glucose, when used as a sole substrate for isolated nervous tissues as compared to that in vivo, should not be surprising. This is also in line with the findings of Treherne (1960), for the cockroach nervous tissues, in which the greater part of the ^{14}C-labelled sugars entering the nerve cord, originated from the trehalose, only about 7%

being derived from the small amounts of glucose in the haemolymph. However, it is interesting to note that 10mM glucose present in the medium can support the same maximum rate of O_2 uptake of isolated ganglia as in the presence of a combination of 4mM glucose and 50mM trehalose, *in vitro* and these concentrations are found *in vivo* (Table 14, Chapter 3). This fact suggests that glucose is an equally important source of energy for the locust nervous system.

Having stated these facts which are in line with the present findings however there are some inconsistencies from the point of view of hexokinase activity. Hexokinase is the enzyme responsible for phosphorylation of glucose at the first step of glycolysis. The Km for glucose of this enzyme present in ganglia is 2.2×10^{-4} M (Table 15 shown in Section 'D', Chapter 3). As it is generally accepted, (Harper, 1975), the concentration of substrate about 6 fold higher than the Km value of the enzyme for that particular substrate should give a saturation with the enzyme. In light of this criterion, 1.33 mM of glucose present in the saline should be enough. The requirement of 10 m moles glucose (Figure 13) permits the hypothesis that either glucose entry was rate limiting or the measurement of the Km value of hexokinase does not reflect that *in vivo*. Similarly, there is a controversy already existing in the literature with regard to glucose oxidation by insect flight muscle and Km of hexokinase. Thus, Kerly and Leaback (1957), found a Km value of 1×10^{-3} M for hexokinase

present in locust flight muscle and therefore hexokinase should be maximally active at less than 10mM of glucose. On the other hand, Candy (1970) working on locust flight muscle found that 80 mM glucose is required in the perfusion medium before the oxidation reaches a maximum. Therefore, this implies that there is a transport barrier to glucose. This difference between the requirement of glucose and Km value determined on the basis of enzymic activity is another example of the fact that putting forward theories on the basis of analysis of maximal activities of enzymes can be misleading (as mentioned in Chapter 1).

It was thought before starting this research work that the disruption of the tracheal system would affect the oxygen uptake of locust nervous tissues <u>in vitro</u> (Chapter 1). It was found that the supply of oxygen in the saline can limit the oxygen uptake of thoracic ganglia, (Figure 12, Section 'A' of Chapter 3). However, at a concentration of 750 nmol/ml present in saline (obtained when it is equilibrated with 100% O_2), the oxygen uptake is no longer limited by O_2 diffusion. The oxygen uptake by locust nervous tissues under optimum conditions of glucose and oxygen concentrations present in hypo-osmotic saline and maintained at 35-37^{o}C, is 3.33 fold higher than the mammalian nervous tissues (Table 5, shown in Section 'A' of Chapter 3). This raises the question of why locust nervous tissues should have a higher oxygen uptake. This can be answered by some facts based on the present findings

and the findings of other workers. The results of oxygen uptake in hypo-osmotic saline during the first 60 min. and after 60 min (the time periods mentioned here after isolation of ganglia in vitro, as shown in Figure 25 of Section 'C', Chapter 3) showed that oxygen uptake dropped dramatically from 267 µmol/g/h to 175 µmol/g/h. On the other hand when cerebral cortex slices were incubated in iso-osmotic physiological saline for 90 min., there was no significant difference between oxygen uptake as measured during the initial 60 min. and consequent 30 min. (Rolleston and Newsholme, 1967a). This emphasises the role of osmolarity. The physiological saline used by these workers to investigate the oxygen uptake of guinea-pig cerebral cortex slices, was isosmotic with regard to the guinea-pig blood. This Ringer saline has also been used by other workers. The insect saline which has been used by physiologists and which has also been used in most of this research work, is hypo-osmotic as compared to locust blood (Table 12 shown in Section 'C' of Chapter 3). This raises the question of why this difference between the locust haemolymph and mammalian blood occurs with regard to osmolarity. This can be answered by reviewing the results of other authors (Bursell, 1970). The most striking difference between the insect haemolymph and mammalian blood concerns the concentrations of carbohydrates and free amino acids which are about 20 times as great in insect haemolymph as in mammalian blood. As in the present results (Table 12 as shown in Section 'C' of Chapter 3) the measurement of the concentration of

carbohydrates present in the locust haemolymph was made, and then the osmolarity of the saline was measured after reproducing these sugars in saline. It is obvious that the high concentration of trehalose present in the saline contributes by 35 mosmoles/Kg towards osmolarity as compared to saline containing 10mM glucose, whereas mammalian blood is devoid of this sugar. This present finding is in line with the previous findings of other authors (Bursell, 1970). As in the present results the measurement of free amino acids present in the locust haemolymph was not made, it is difficult to remark on the concentration of amino acids and their contribution towards osmolarity, compared to mammalian blood. It has been found that adjusting the osmolarity of this saline to about the same as in locust blood, the oxygen uptake is not significantly different during the initial 60 min. and consequent time periods. (Figure 25, in Section C, Chapter 3). As oxygen uptake is directly linked with mitochondria where most of the energy of a cell is synthesized, it is likely that higher oxygen uptake in hyposomotic saline during the initial 60 min., may be due to a higher demand of energy against adjusting the osmolarity in vitro. Therefore, the higher oxygen uptake of locust nervous tissues as compared to mammalian nervous tissues, may be due to osmolarity difference. This requires an explanation for how the locust ganglia were adjusted in hypo-osmotic saline and how the extra oxygen uptake was utilized. This recalls the results of Katzman

and Pappius (1973), who found that mammalian neurons act as excellent modified osmometers. If these cells must sacrifice volume or osmolarity, cellular volume is relatively preserved and osmolarity is altered to protect the brain function. Similarly, the results of Rymer and Fisherman (1973), working on brain and muscles showed that both brain and muscle volume are increased in terms of their swelling index (calculated from their increased water content) under hypo-osmotic conditions. However, the percent increase in muscle volume is twice to that of brain volume. There is a decrease in sodium and potassium content of both tissues due to dilution. There is a greater drop in brain potassium, about 20% of that in muscle potassium. This fall in brain potassium is adaptive, i.e. it contributes to the fall in the intracellular osmolarity and so limits the cellular swelling due to the entry of water. The fact that brain cells swell less than muscles is due to greater loss of intracellular potassium from brain cells than from muscles in response to drop in plasma osmolarity (Siesjö and Plum, 1973). The other workers (Nicol, 1967), working on marine animals have found that all processes involving active transport of water and salts against osmotic gradients necessitate the expenditure of energy by the organism in the form of osmotic work, and theoretically this should be capable of detection in the form of a corresponding increased level of oxygen consumption. In light of these previous findings, it is likely that extra oxygen uptake by locust central nervous tissues in hypo-

osmotic saline may be either due to an active transport of water or potassium ions out of the tissue against the osmotic gradient. The higher oxygen uptake by locust ganglia in hypo-osmotic saline is certainly in line with the findings of other workers (Nicol, 1967). However, the observation still remains that the locusts nervous tissues had shown significantly higher oxygen uptake compared to other nervous tissues, even in iso-osmotic saline (Figure 26, Section 'C' of Chapter 3). This difference is significant even when locust ganglia are compared to artificially stimulated nervous tissues. The explanation, probably lies in the mechanism of control of glycolysis and TCA cycle. It has been well established that glycolysis in mammalian central nervous tissues is controlled by two regulatory systems, the hexokinase/phosphofructokinase system and the glyceraldehyde-3-phosphate dehydrogenase/pyruvate kinase system (Rolleston and Newsholme, 1967a, 1967b; Lowry et al., 1964; Twakawa, 1944; Cori, 1942). It has also been established, firstly that even under optimum conditions of temperature, oxygen and glucose concentrations, the 20-30% of glucose utilized by mammalian nervous tissues at rest is converted to lactate, and the remainder is oxidized via pyruvate to TCA cycle; and secondly, that lactate dehydrogenase is an equilibrium enzyme ([illegible], Newsholme and Start, 1973: McIlwain and Bachelard, 1971). As in the present work the concentrations of fructose-6-phosphate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate and 1,3-diphosphoglycerate were not measured, it is difficult to

compare the control over these two enzyme systems in the locust ganglia. However, it is probable that the control of glycolysis in locust central nervous tissuesis modulated by these two enzyme systems as in mammalian nervous tissues. One piece of evidence comes from the results of rates of glucose utilization by thoracic ganglia and cerebral cortex slices (Table 15, Section 'D' of Chapter 3), as found there is no siginficant difference between the two tissues in this respect.

There are probably two differences which are based on the present findings and the results of other workers, working on mammalian and insect tissues, respectively. In the present results of incubation of locust ganglia under aerobic conditions, between 60 and 180 min. (which probably represents the situation close to that in vivo), the oxygen uptake is 2.91 µmol/g/min., (Figure 25, Section 'C', Chapter 3) and the rate of lactate output is 0.030 µmol/g/min. (Figure 24, Section 'B' of Chapter 3). Assuming glucose to be the only substrate utilized (details given in Section 'D') the rate of glucose flux via pyruvate to TCA cycle would be 2.91÷6 µmol/g/min (based on oxygen uptake). As two moles of lactate are formed from one mole of glucose, therefore the rate of glucose flux to lactate would be 0.015 µmol/g/min. Now combining these two fluxes of glucose the total flux is:

0.485 μmol/g/min. (based on O_2 uptake)

\+ 0.015 μmol/g/min. (based on lactate output in the medium)

= 0.5 μmol/g/min.

Out of 0.5 μmol/g/min. of glucose utilized, only 0.015 μmol/g/min. is converted to lactate and the remainder is oxidized to CO_2 and H_2O and hence in percentage terms, only 3% of the glucose is converted to lactate and 97% is oxidized via pyruvate to TCA cycle, as compared to 20-30% of glucose utilized by mammalian central nervous tissues, converted to lactate and the remainder oxidized via pyruvate to TCA cycle. However, the total rates of glucose utilization by thoracic ganglia and cerebral cortex slices are not significantly different, as mentioned earlier. In addition to this, although the lactate to pyruvate ratio is variable between 60-180 min. (Figure 22, shown in section 'B' of Chapter 3), nevertheless, the ratio is on average is 5.0, whereas in mammalian nervous tissues it is 24.0, during 60-90 min. of incubation under aerobic conditions (Rolleston and Newsholme, 1967 a,b). It has also been found that locust ganglia possess a maximum of 10 μmole/g/min. lactate dehydrogenase activity (Strang R.H.C., personal communication) which is much lower than that possessed by mammalian nervous tissues (90-180 μmole/g/min., Lowry and Passonneau, 1964; Johnson, 1960; Balázs et al., 1968). In conclusion, both the central nervous tissues have the same total rate of flux of glucose to lactate and to TCA cycle via pyruvate; however, locust ganglia have a much lower flux of glucose to lactate in contrast to

mammalian central nervous tissues. The lower lactate output into the medium and the lower lactate dehydrogenase activity possessed by locust ganglia, in contrast to mammalian nervous tissues, probably reflect the fact that lactate dehydrogenase is a rate-limiting enzyme, responsible for lesser flux of glucose to lactate. The percentage of flux of glucose to lactate is up to 40% (Rolleston & Newsholme, 1967 a,b) in mammalian nervous tissues whereas locust ganglia only convert about 3% of the glucose consumption to lactate and the remainder is oxidized via pyruvate to TCA. This higher flux of glucose to TCA cycle present in locust ganglia, in contrast to mammalian central nervous tissues, certainly gives an explanation of why isolated locust ganglia possessed a higher rate of oxygen uptake as compared to that of mammalian tissues. Similarly, locust ganglia contrast with guinea-pig cerebral cortex, in having an endogenous activity known as spontaneous activity. This spontaneous activity was recorded as action potentials from a nerve (N5) of the metathoracic ganglion. The generation of action potentials is defined as a process by which nerve impulses are transmitted from one site to another (Whittam, 1967). In a resting cell membrane, in contrast to extracellular fluid, intracellular fluid usually has a high concentration of potassium and low concentration of sodium. Such unequal distribution is believed to result from an energy-requiring active transport process located in the cell membrane known as the sodium/potassium pump, (Whittam, 1967). This unequal distribution of ions results in an electrochemical potential which is —70 to — 90 mV; the inside of the membrane being ——ve with regard to outside.

When impulses pass along the membrane, the distribution is changed, the outside becomes negative and inside positive for a short time. This means depolarization occurs. This changes the potential to between +30 and +50 mV. This is followed by repolarization which is performed by Na^+/K^+ pump. The operation of this pump depends on the energy in the form of ATP, (Gorman and Marmor, 1970). As most of the ATP of a cell is synthesized by oxidative metabolism occurring in mitochondria, the transmission of the impulses is indirectly dependent on the oxidative metabolism and probably on ATP. This has been found by Whittam (1962), Whittam and Bond (1964), and Marchbanks (1970), that in vitro, 40-50% of the energy available from cerebral respiration of mammalian nervous tissues, may be expended in maintaining this differential distribution of sodium and potassium ions by a pumping mechanism. Similarly, the previous results of Heald (1953), McIlwain (1953), Larrabee (1958), Horowicz and Larrabee (1958), indicate that oxygen uptake of mammalian nervous tissues becomes doubled during induced electrical stimulation as compared to unstimulated ones. In light of these findings, it is likely that endogenous electrical activity possessed by locust central nervous tissues may be another factor which determines the higher oxygen uptake of these tissues compared to mammalian nervous tissues. In conclusion, it is probable that locust central nervous tissues have a higher rate of flux of glucose via pyruvate to the TCA cycle, as compared to mammalian central nervous tissues due to these two factors:

(a) the lower rate of lactate output due to the low activity of lactate dehydrogenase;

(b) possession of high spontaneous activity.

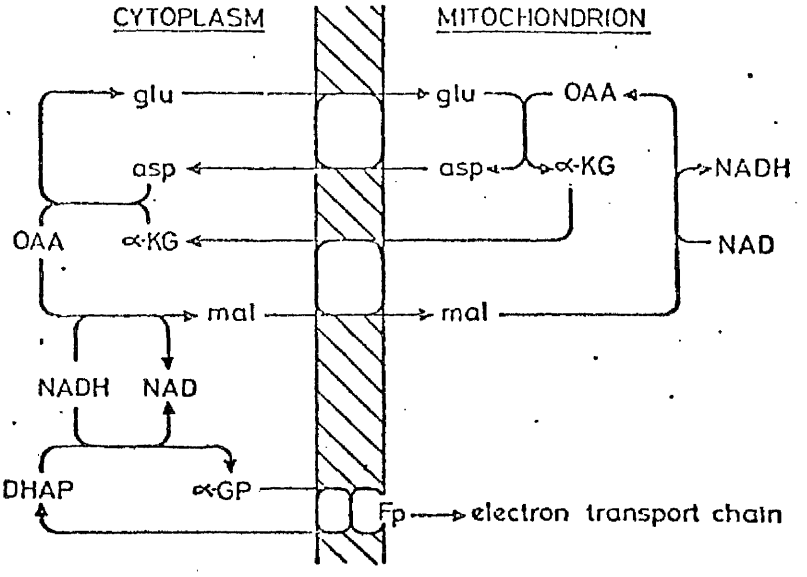
This raises the question of why locust central nervous tissues should have a control at lactate dehydrogenase whereas, mammalian central nervous tissues do not. This brings in the role of lactate dehydrogenase in tissues. The glycolytic pathway produces two moles of NADH for every mole of glucose oxidized to pyruvate and this reduction of $NAD^{\oplus}$ occurs at the glyceraldehyde 3-phosphate dehydrogenase reaction, whereas, glyceraldehydre-3-phosphate is converted to 1,3 -diphosphoglycerate. This NADH must be converted back to $NAD^{\oplus}$ if glycolysis is to continue and the supply of nicotinamide cofactors in the cyto-plasm is not to become limiting. Under anaerobic conditions NADH is reoxidised at the expense of conversion of glycolytically formed pyruvate to lactate by lactate dehydrogenase. However, in aerobic conditions a large amount of glycolytic pyruvate is oxidized in the TCA cycle and some other solution must be found. The available evidence indicates that the mitochordria is impermeable NADH and therefore reduced equivalents cannot be transferred directly to the electron transport chain (Greville, 1969; Sackter, 1970). To overcome this problem it has been proposed that reduced equivalents are transferred via the malate-aspartate shuttle, Borst, 1965; Chappell, 1968; or by the α- glycerol phosphate cycle, (Zebe et al., 1959; Sacktor and Dick, 1962) and these two cycles are shown in

Figure 30, in this chapter. The malate-aspartate shuttle requires malate dehydrogenase and glutamate -oxaloacetate transaminase. These enzymes are located in both the mitochondrial matrix and cytoplasmic spaces of a variety of mammalian muscle tissues (Klingenberg and Pette, 1962; Chappell, 1968 and Williamson et al.,1973). Furthermore the glycerolphosphate cycle plays a minor role in mammalian muscles (Williamson et al, 1971; Safer and Williamson, 1972) and mammalian brain (Lowry and Passonneau, 1964). On the other hand the α -glycerolphosphate cycle requires glycerolphosphate dehydrogenase, α -glycerolphosphate oxidase and mitochondrial flavoproteins. The cycle requires only catalytic amounts of dihydroxyacetone phosphate to oxidise the NADH being continuously formed by glycolysis (Sactor, 1965). Both these enzymes are present in insect flight muscles and show very high activity (Ringler and Singler, 1962; Delbrück et al, 1959), and furthermore insect flight muscles do not have malate-aspartate shuttle (Sacktor, 1970; Hansford, 1971). It is possible that a certain percentage of the reoxidation of NADH in mammalian brain is performed by the malate-aspartate shuttle while the rest is performed by lactate dehydrogenase. On the other hand the α -glycerolphosphate cycle present in locust central nervous tissues is much more efficient and therefore the role of lactate dehydrogenase is only minor. This certainly provides one reason why locust nervous tissues should have a lower activity of lactate dehydrogenase as compared to mammalian CNS.

FIGURE 30

An outline of malate-aspartate shuttle and α-glycerolphosphate cycle.

The malate-aspartate shuttle and the α-glycerophosphate cycle



The effect of temperature on the rate of biological reactions is usually represented by the temperature coefficient or Q10. (Harper, 1975). The term temperature coefficient is defined as the exact ratio by which the velocity of some process changes for a $10^{o}C$ temperature rise. The Q10 value, as obtained by incubation of locust central nervous tissue between a range of temperature of 25-$35^{o}C$ is 3.06. This is an average of two values, one obtained by equilibration of medium with air and the other by equilibration with 100% O_2. (Figure 11 as shown in Section 'A' of Chapter 3). Although the Q10 value is not significantly different between the air saturated medium and the oxygen saturated medium, the actual figures (Figure 11) become more or less doubled both at 25^{o} and $37^{o}C$, when the medium was equilibrated with 100% O_2. This reflects the fact that permeability of oxygen into the ganglia is increased when the oxygen concentration is increased in the medium. This requires an explanation for how the oxygen uptake was increased with the rise of temperature. As in the present work no measurement of metabolites was made at $25^{o}C$ and it is difficult to decide where the temperature effect was located. However, certain predictions can be made based on the findings of other workers. The most obvious one concerns the effect of temperature on enzyme catalyzed reactions. There is an optimal temperature at which the reaction is most rapid. Above or below this the reaction rate decreases. Since all the reactions of aerobic glycolysis are enzyme catalyzed, it is likely that

all the enzymes are activated with a rise in temperature until an optimal temperature is obtained and this results in an increased metabolism and hence an increased oxygen uptake. It may be that only the controlling enzymes consisting of hexokinase/phospho fructokinase system and glyceraldehyde dehydrogenase/pyruvate kinase systems were activated and therefore they allowed a greater flow of intermediates along the pathway to give an enhanced rate of flux of glucose via pyruvate to TCA cycle. Finally, it may be that glucose entry into the tissue is enhanced simply by decreasing the transport barriers at higher temperatures. The second question arises as to why locust central nervous tissues give Q10 = 3 whereas mammalian central nervous tissues give Q10 = 2.0, on average. (Table 6 shown in Section 'A' of Chapter 3). It is probable that the higher increase in oxygen uptake by locust central nervous tissues, in contrast to those mammalian nervous tissues mentioned in Table 6, was determined by spontaneous activity of ganglia. It has already been shown in Section 'A' of Chapter 3 that spontaneous activity was increased about 3 times with a 10^{o} rise in temperature ($25-37^{o}C$). As generation of spontaneous activity needs energy in the form of ATP, this would enhance the oxidative metabolism (as discussed previously) and hence increase the flux of glucose to TCA cycle. It has already been discussed and established that the rates of flux of glucose via pyruvate to lactate and TCA are 97% and 3% respectively and hence flux to TCA is more complete. In light of this,

it is more likely that glucose uptake from the medium by locust ganglia was enhanced at 37°C which resulted in increased flux via pyruvate to TCA.

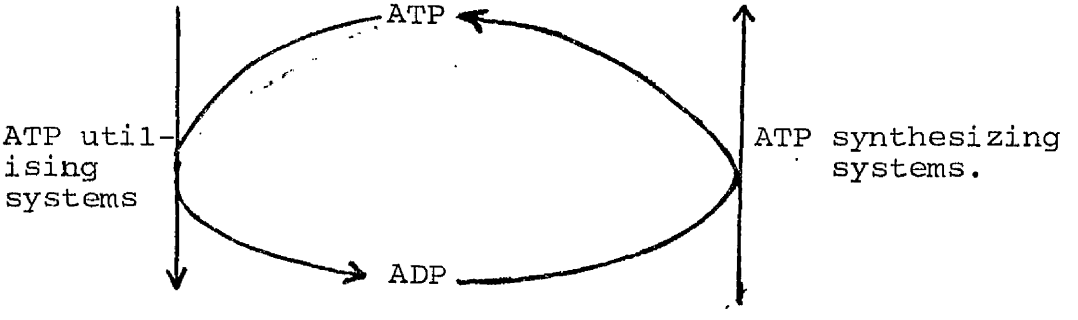
It is interesting to note that the present results of oxygen uptake by thoracic ganglia *in vitro* at different temperatures are in line with the results of Church (1960), who concluded that optimum activity of flying locusts was at 35°-37°C. This certainly suggests that the behaviour of the locust tissues *in vitro* may be close to that *in vivo*.

Under optimum conditions of incubation *in vitro*, the locust central nervous tissues showed four patterns of aerobic glycolysis with regard to high energy phosphates (Figure 20 as shown in Section 'B' of Chapter 3) as follows:

A. metabolism during an initial 30 minutes after isolation of ganglia *in vitro*;
B. between 30 and 60 minutes;
C. between 60 and 180 minutes;
D. after 180 minutes.

(A) During this time period, the concentrations of Arginine phosphate and ATP fell dramatically from 3.15 to 2.3 μmol.../g and 2.7 to 1.61 μmol.../g respectively, whereas ADP rose from 0.34 to 0.54 μmol.../g. The ratios of arginine phosphate/ATP and ATP/ADP are 1.42 and 2.98 at 30 min. as compared to 1.16 and 6.75 at zero time respectively. Why did the ratios of these high energy

phosphates change? This can be answered by taking into consideration how these ratios are maintained in a cell. It is generally believed that ATP is the immediate supply of energy for cellular function. (Davies, 1965; Peachey, 1968; Tregear, 1974). However, the amount of ATP found in a muscle cell (which contains a relatively high ATP concentration) can support contraction only for a short time. Thus, in an active muscle such as the insect flight muscle, the total ATP content would be exhausted in less than a second if it was not regenerated (Newsholme and Start, 1973). This regeneration of ATP occurs mainly in the mitochondria at the expense of oxygen and reduced cofactors. In order to maintain the ATP concentration at a steady-level, the rate at which ATP is utilized must be equal to the rate at which it was produced as shown below:



The oxygen uptake during the initial 60 min. was much higher than the remainder of the time period of incubation between 60-180 min. in hypo-osmotic saline (Table 19, this chapter). This together with the maintenance of ATP, arginine phosphate levels indicates that the ATP

synthesizing process in the mitchondria was not impaired. However, the oxygen uptake was not significantly different in iso-osmotic saline during this time period as compared to consequent time periods. This probably gives an indication that higher oxygen uptake in hypo-osmotic saline during the initial 60 minutes was in response to a higher demand of energy for osmotic work and that the increased oxygen uptake enhanced the rate of production of ATP. Although the measurement of the concentrations of high energy phosphates present in locust ganglia were made in hypo-osmotic saline, the results of Siesjö and Plum (1973) working on mammalian brain, showed that the actual concentrations of ATP and ADP were unaffected by changes in osmolarity, and therefore it is likely that the same ratios of ATP/ADP and arginine phosphate/ATP would have been obtained even in iso-osmotic saline. Evidence also comes from the results of McIlwain et al. (1951); Rolleston and Newsholme (1967b). These authors working on the mammalian cerebral cortex slices found that ATP/ADP and creatine phosphate/ATP ratios decreased during the initial 30 minutes of incubation using isosmotic physiological medium.

Under hypo-osmotic conditions, the extra oxygen uptake is probably used to fulfil the energy requirement of the cells; whereas in iso-osmotic saline no energy is used in adjusting osmolarity, and therefore no extra oxygen is needed.

The lower ratio of ATP/ADP probably indicates that there is

TABLE (19)

THE CONTROL CONDITIONS

Meso- and metathoracic ganglia were incubated in 3 ml medium containing 10.0 mM glucose, equilibrated with 100% oxygen and maintained at 35-37°C. The results are given as Rates (µmol /g. fresh wet wt/min). These were obtained from the data given in Chapter 3.

	Rates (µmol /g fresh wet wt/min)			
Time of incubation (min).	0-30	30-60	60-180	180-210
Oxygen Uptake (hyposmotic saline)	4.45	4.45	2.91	
Lactate formation	0.08	0.076	0.030	
Glycogen disappearance	0.03	0.085	0.01	-0.0076
Glucose utilization (hyposmotic saline)	0.74	0.74	0.49	
Glucose accumulation	0.02	-0.026	0.001	-0.019
Oxygen Uptake (isosmotic saline)	3.0	3.0	2.93	
Glucose utilization (isosmotic saline)	0.5	0.5	0.488	

an imbalance between the ATP synthesizing and utilization systems. This raises the question of why this imbalance was created. There are three possible reasons:

(a) trauma or injury of the tissue. As removal of the meso- and metathoracic ganglia from *in vivo* to *in vitro* preparation, demands the cutting off of all the nerves and connectives, it would probably demand very high energy to repair the injury *in vitro*;

(b) adjustment in a new ionic situation as compared to haemolymph;

(c) as has been discussed in the introduction to this thesis, locusts have a tracheal system which ramifies into the cells of ganglia to ensure rapid oxygen transport. It is likely that disruption of the tracheal system and putting the tissue into a new artificial situation would impair the supply of oxygen. This might result in the production of anoxic cores of the tissue. The evidence comes from the observation of relatively higher lactate formation during this time period as compared to between 60-180 minutes. (Table 19, this chapter). In conclusion, the demand for energy exceeds the ATP synthesizing systems during this period and therefore the ATP/ADP ratio is not maintained close to that *in vivo*. This is also supported by the observation of higher hydrolysis of arginine phosphate in locust central nervous tissues (Figure 20, chapter 3). Arginine phosphate acts as a store of high energy phosphate (Newsholme and Start, 1973) in locust flight muscles. This store is not mobilized

until the system is self-sufficient in regenerating its ATP in response to the demand. However, when the self-sufficient ATP generating system is not adequate, arginine kinase hydrolyses and undergoes the following reactions:

$$\text{Arginine - P} + \text{ADP} \underset{\text{ATP} \longrightarrow \text{ADP} + P_i^{\bullet}}{\overset{\text{arginine kinase}}{\rightleftharpoons}} \text{ATP} + \text{arginine}$$

Net Reaction:

$$\text{Arginine-P} \longrightarrow \text{arginine} + P_i^{\bullet}$$

The net result of the breakdown of arginine phosphate is an increase in arginine and inorganic phosphate while the levels of ATP and ADP remain fairly constant. Hence, under these conditions arginine phosphate may act as an energy donor for the synthesis of ATP. Decreased arginine phosphate/ATP ratio as compared to that *in vivo* probably indicates that regeneration of ATP in response to higher demand of energy, was met to some extent by mobilization of arginine phosphate present in the locust ganglia during this time. This requires an explanation, for what are the effects on the flux of glucose via pyruvate to lactate and to TCA cycle when the production of anoxic cores and lower concentrations of ATP and lower ratio of ATP/ADP are not repairable during the first 30 min. of incubation even under optimum conditions of *in vitro* preparation?

Assuming exogenous glucose is the sole substrate utilized (as described before) and therefore working out the glucose utilization on the basis of oxygen uptake, the utilization

would be 0.74 and 0.5 μmol./g/min. in hypoosmotic and isosmotic salines respectively. (Table 19 shown in this Chapter). It is obvious that 0.24 μmol/g/min. of glucose are utilized just to produce energy to adjust to the different osmolarity, and that the remaining 0.5 μmol./g/min are utilized to generate ATP for other functions. Excluding the factor of osmolarity and considering that two mols of lactate are formed per one mol of glucose utilized, total glucose utilization would be 0.50 (based on flux of glucose to TCA cycle) + 0.04 (based on flux of glucose to lactate) = 0.54 μmoles/g/min. Therefore, out of a total of 0.54 μmoles glucose per gram per minute, only 0.04 are converted to lactate and working out on a percentage basis it would be 7.0% of glucose metabolized through the flux of the glucose to lactate and 93.0% metabolized via pyruvate to TCA cycle. Comparing these percentages with consequent time periods during which the tissues were repaired and adjusted to a new steady-state, i.e. between 60-180 minutes, the glucose utilized through pyruvate to TCA cycle and through pyruvate to lactate is 97% and 3% respectively (Table 19, this chapter). But the results of oxygen uptake are not significantly different between the initial 60 minutes and consequent time periods which indicates that the increased lactate formation during initial 30 minutes may not be significant as glucose utilization was not significantly different during initial 30 min. and consequent time periods (Table 19), or that some endogenous substrate was utilized and metabolized as lactate. Another result of lower concentrations of ATP

and ATP/ADP ratio at 30 minutes probably affected the concentrations of glucose and glycogen present in the ganglia (Table 19). The glycogen, in terms of glucose equivalents has fallen from 11.2 to 10.0 μmol/g/min glucose has increased from 1.9 μmol/g (present at a steady state which is close to that in vivo to 2.35 μmol/g. Their rates of disappearance and accumulation are 0.03 μmol/g/min and 0.02 μmol/g/min respectively (Figure 21 shown in Section 'B', Table 19). This raises the question of why the glycogen disappearance and glucose accumulation occurred during this time period. The answer is that glycolytic pathways must have been affected by adenine nucleotides. The pathway can be affected by two factors:

(a) the rates at which the substrates are supplied and

(b) the activities of enzymes.

As the supply of substrate, 10mM glucose is adequate (Figure 13), therefore the activities of the enzymes must have been modulated by adenine nucleotides concentration. Recalling the previous results of Lardy and Parks (1956); Lowry et al. (1964); Lowry and Passonneu (1966); and Rolleston and Newsholme (1967a,b) it has been proposed that phospho-fructokinase (this enzyme is responsible for conversion of fructose 6-phosphate to fructose 1, 6-diphosphate) present in mammalian central nervous tissues is subject to control by the concentrations of ATP, ADP, cyclic AMP, AMP, Fructose 1, 6-diphosphate and orthophosphate. The prime controlling agent for this enzyme is ATP, which acts both as a substrate and as an inhibitor. This inhibition may be

relieved by fructose diphosphate, AMP, cyclic AMP, ADP and orthophosphate. The decreased concentration of ATP would enhance the activity of phosphofructokinase and as a result, this enzyme would decrease the concentration of glucose-6-phosphate which is an inhibitor of hexokinase. Therefore, the inhibition was relieved and increased activity of hexokinase probably increased the uptake of glucose. Similarly, it has been suggested that the glyceraldehyde-3-phosphate dehydrogenase/pyruvate kinase system acts as another regulatory system of mammalian central nervous tissues (Lowry and Passonneau, 1964; Velick and Furfire, 1963; McQuate and Utter, 1959; and Rolleston and Newsholme, 1967 a,b) The decreased concentration of ATP enhances the activity of pyruvate kinase. This in turn decreases the concentration of 1, 3-diphosphoglycerate which is an inhibitor of 3 phosphoglycerate dehydrogenase. Therefore the stimulation of pyruvate kinase by decreased concentration of ATP results in an increased activity of glycerol 3-phosphate dehydrogenase. In light of previous findings and on the basis of present results showing decreased ATP concentration and ATP/ADP ratio present in locust ganglia at 30 min. as compared to that <u>in vivo</u> at zero time (Figure 20, Section 'B' of Chapter 3), it is tempting to propose that the increase in concentration of glucose present in ganglia was probably due to enhanced transport of glucose into the ganglia in response to increase in flux of glucose through the glycolytic pathway by stimulation of the regulatory systems consisting of hexokinase-phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase/pyruvate kinase as present in

mammalian central nervous tissues. In conclusion, the control of glucose present in locust central nervous tissues during the first 30 min. is similar to that of mammalian central nervous tissues. As the stimulation of glyceraldehyde 3-phosphate dehydrogenase would increase the ratio of NADH/$NAD^{\oplus}$, the activity of lactate dehydrogenase might have increased and therefore slightly higher lactate formation took place. Out of 100% glucose utilized 7% was converted to lactate during initial 60 min; whereas in the subsequent time period, 3% glucose was converted to lactate. Although it has been previously proposed that slightly higher lactate formation could be due to certain anoxic cores produced in locust central nervous tissues, the stimulation of lactate dehydrogenase due to stimulation of glyceraldehyde 3 phosphate dehydrogenase even in aerobic areas might have also contributed to this increase in lactate formation. This leaves us with the second question of why the glycogen disappeared. The previous results of Morgan and Parmeggiani (1964); and Krebs et al. (1964); Larner et al. (1964) for mammalian muscles, Härkönen et al. (1969) for mammalian nervous tissues and the results of Newsholme and Start (1973), reviewed for locust flight muscles, have shown that phosphorylase (the enzyme responsible for breakdown of glycogen) is the major regulatory enzyme. Phosphorylase exists in two forms phosphorylase a and phosphorylase b. Phosphorylase b is an inactive form and can be converted to the active form, phosphorylase a by the action of phosphorylase kinase. It has been found by other workers

that in any situation in which the energy level of the cell is decreased or in which the glucose 6-phosphate level is decreased, phosphorylase b will be activated and glycogenolysis will result, (Newsholme and Start, 1973). In light of these findings, it is likely that the breakdown of glycogen during the initial 30 minutes was due to decreased concentrations of ATP and glucose 6-phosphate as a consequence of stimulation of phosphofructokinase. The present findings of a decrease in glycogen concentration during the initial 30 min. of incubation *in vitro* are in line with the previous results of McIlwain and Tresize (1956), and that of Rolleston and Newsholme (1967a,b) who found that glycogen contents of guinea-pig cerebral cortex slices decreased over the first 30 min.period of incubation. In conclusion, the condition of the locust central nervous tissue during the initial 30 min. period of incubation *in vitro* is quite different from that *in vivo* due to trauma of preparation, the new ionic situation, osmolarity and the disruption of the tracheal system probably causing anoxic cores. The results of decreased concentration of arginine phosphate, ATP, ADP, accumulation of glucose, increased lactate formation and decreased concentration of glycogen present in locust tissues at 30 min. are in line with the previous results of McIlwain *et al.* (1951); Rolleston and Newsholme (1967a,b) working on mammalian central nervous tissues, except the mammalian central tissues contain creatinine phosphate instead of arginine phosphate.

(b) Metabolism between 30 and 60 min period of incubation:

This period is marked by the recovery of high energy phosphates until the ratios of arginine phosphate/ATP and ATP/ADP are 1.25 and 4.5 respectively are obtained at 60 min. (Figure 20 shown in section 'B' of Chapter 3). These ratios are not dramatically different from that in vivo. The concentrations of arginine phosphate and ATP increased significantly between 30 and 60 min., whereas ADP decreased. As the rate of lactate formation and oxygen uptake were about the same as in the initial 30 min (Table 19 as shown in this Chapter), this probably indicates that flow rates along glycolytic and TCA pathways were the same. As regeneration of ATP occurs mainly in the mitochondria at the expense of oxygen and reduced co-factors, it is likely that the rate of ATP synthesizing system is the same as in the initial 30 minutes. This raises the question of why the ATP/ADP and arginine phosphate/ATP ratios recovered during this period of incubation. The answer is that the rate of ATP utilization might have decreased and hence ATP concentration increased and ADP decreased. Probably, locust ganglia have recovered from the trauma of preparation of tissue for in vitro work and they have adjusted to an artificial situation. The effect of recovery of ATP/ADP on glucose present in the ganglia (Figure 21, Section 'B', Chapter 3) is certainly in agreement with the inhibitory effect of adenine nucleotides on the hexokinase-phospho-fructokinase system and the glyceraldehyde 3-phosphate dehydrogenase/pyruvate kinase system as present in mammalian nervous tissues (Rolleston and Newsholme, 1967a,b).

A rise in ATP concentration during the 30-60 minute period of incubation probably inhibited the same regulatory enzyme systems of locust nervous tissues as present in mammalian nervous tissues and this would result indirectly in a decrease in glucose entry into the tissue. However, the maximum rate of disappearance of glycogen during this period of incubation (Table 19) is in contrast with the adeninine nucleotide situation. The increased concentration of ATP inhibits the activity of phosphofructokinase and as a result low rate of disappearance of glycogen should have occurred during this period. The dramatic decrease of glycogen (10- to -7.45 μmol/g, Figure 21, Section 'B' of Chapter 3), between 30-60 minutes is not in agreement with the previous results of McIlwain and Tresize (1956); LeBaron (1955); Kleinzeller and Rybova (1957); Rolleston and Newsholme (1967 a,b) who found that the content of glycogen present in mammalian central nervous tissues decreased over the first 30 minutes of incubation and then increased until the level was close to that in vivo. Hart and Steele (1973) found that much of the fall in glycogen present in cockroach central nervous tissues was during the first 60 minutes of incubation and glycogen lost from the tissue could be accounted for in terms of trehalose formed by the tissue and then lost from it into the medium. The present findings of glycogen disappearance are in agreement with these results, although the work in this thesis has not demonstrated the conversion of glycogen into trehalose.

As the measurement of metabolites was not made between the 30 and 60 minutes period of incubation in vitro, it is difficult

to decide the exact time when a new steady state close to that *in vivo* was achieved. However, a steady state of spontaneous activity was achieved between 50-60 min. (Figures 16 and 18, Section 'A', Chapter 3). As spontaneous activity is another index of metabolic activity, it is likely that this is the period when a new steady state with regard to metabolites was reached. This indicates that after the trauma of preparation, an initial period of 60 minutes incubation in the control conditions is sufficient for locust central nervous tissues to reach a steady state *in vitro*. Therefore, the various alternative conditions of incubation on the parameters measured could be tested after a 60 min., incubation period. These results from locust ganglia are not in agreement with the earlier conclusions of Rolleston and Newsholme, (1967a) who found that a 30 min. incubation was sufficient time for the intermediates in cerebral cortex slices to reach a steady state. In conclusion, over the first 30 to 60 min. period of incubation of locust ganglia *in vitro*, the percentage of conversion of glucose to lactate and to CO_2 and H_2O is about the same as in the initial 30 minutes; however the demand for energy has decreased due to adjustment of the tissue in the artificial physiological medium. This resulted in the recovery of the ATP/ADP arginine Phosphate/ATP ratios close to that *in vivo*. The evidence of attainment of steady state of spontaneous activity between 50-60 min. indicates that the period of the initial 60 minutes of incubation might be sufficient time for metabolites of locust central nervous tissues to achieve a steady state *in vitro*.

(C) Metabolism between 60 and 180 min. period of incubation:

This period of incubation represents a steady state for metabolites in locust ganglia *in vitro*. Although there is a variation in high energy phosphates, nevertheless the arginine phosphate/ATP and ATP/ADP ratios are 1.28 and 5.8 on average (Figure 20, section 'A' of Chapter 3). Comparing these ratios to the situation *in vivo* (Table 10, shown in Section 'B' of Chapter 3), the ratios are not significantly different. The oxygen uptake does not vary much during this period and also it is not significantly different from the initial 60 min. excluding the factor of osmolarity (2.91 as compared to 3.1 µmole /g/min. during an initial 60 min. of incubation, Table 19, in this chapter). The glucose utilization is also not significantly different during this period compared to the initial 60 min. of incubation, excluding the glucose utilization due to osmolarity adjustment (Table 19). The lactate formation decreased to 0.03 µmole /g/min from 0.08 on average during the initial 60 min. (Table 19 and Figure 24, Section 'C', Chapter 3). Lactate production of 0.03 µmol /g/min. would correspond to 0.015 µmole /g/min. of glucose. The glucose utilized based on the rate of oxygen uptake by locust ganglia is 0.488 (shown in Table 19 and as described before). Therefore the total rate of glucose uptake will be 0.503 µmol s/g/min. In terms of percentages, this means that out of 100% glucose utilized, 3% is converted to lactate and the remainder to CO_2 and H_2O. On comparing these results with those in the initial

60 min. the conversion of glucose to lactate is 3% as compared to 7% during the initial 60 min; however the difference is not significant. These results resemble those from mammalian cerebral cortex found by McIlwain et al., (1951); Rolleston and Newsholme (1967a), except that conversion of glucose to lactate is 20-40% instead of the 3-7% present in locust central nervous tissues and in the case of locust tissue the recovery is more complete.

The glycogen disappearance was very small during this time period (Table 19); however the levels of glycogen close to those in vivo were never achieved. This contrasts with mammalian central nervous tissues. Thus McIlwain and Tresize (1956); LeBaron (1955), Kleinzeller and Rybova (1957); Rolleston and Newsholme (1967a), all found that the glycogen content of cerebral cortex slices recovered a level close to that in vivo between 30-60 min. Furthermore the lactate and pyruvate levels were variable during this period of incubation (Figure 22, Chapter 3). However, the lactate/pyruvate ratio is 5.0 on average during this time. This ratio is not significantly different from that in vivo (5.0 as compared to 3.0 present in vivo, Table 10, Section 'B'). This again contrasts with the ratio of lactate/pyruvate present in mammalian central nervous tissues. Thus Rolleston and Newsholme (1967a, b) found that a ratio of 24 was obtained during steady state conditions of incubation of guinea-pig cerebral cortex slices. This raises the question of what the lactate/pyruvate ratio reflects in nervous tissues. The ratio of lactate to pyruvate has been used to give an indication of the redox

value of the cytoplasm of cells of mammalian nervous tissues <u>in vitro</u> (Rolleston and Newsholme, 1967a, Newsholme and Start, 1973). If the activity of lactate dehydrogenase was sufficiently high to establish equilibrium conditions, then the following relationship would be:

$$\frac{\text{Lactate}}{\text{Pyruvate}} = \frac{1}{\text{Keg}} \times \frac{[\text{NADH}]\ [\text{H}^+]}{[\text{NAD}^+]}$$

This relationship is only valid in the cell if the system is at, or close to, equilibrium, and this in turn demands a high activity of lactate dehydrogenase. As mammalian nervous tissues contain a very high activity of lactate dehydrogenase (90-180 μmol/min/g and this is an equilibrium enzyme (Lowry and Passonneau, 1964; McIlwain and Bachelard, 1971), the ratio of lactate to pyruvate can be used as an index of an adequate supply of oxygen to the mammalian nervous tissues. The lactate dehydrogenase has been found to be 10 μmol/g/min (Strang, R.H.C. personal communication) in locust ganglia and this is consistent with the very small flux of glucose via pyruvate to lactate as compared to the very high flux of glucose via pyruvate to TCA cycle, based on the present findings. In light of these results, it is likely that lactate dehydrogenase may be a non-equilibrium enzyme. Therefore the ratio of lactate to pyruvate should be treated with some caution, and cannot stand by itself as evidence of an adequate supply of oxygen to the isolated locust nervous tissues. Other authors (Newsholme and Start,

1973) have also suggested the use of the ratio

$$\frac{\text{Dihydroxyacetone phosphate}}{\alpha\text{-glycerophosphate}}$$

as an index of the redox value of the cytoplasm of cells. If the activity of glycerophosphate dehydrogenase was sufficiently high to establish equilibrium conditions, then the following relationship holds:

$$\frac{\text{Dihydroxyacetone phosphate}}{\alpha\text{-glycerophosphate}} = \frac{1}{Keq} \times \frac{[NADH]\ [H^+]}{[NAD^+]}$$

The α-glycerophosphate dehydrogenase has been found to be an equilibrium enzyme present in locust flight muscle (Newsholme and Start, 1973). It is likely that locust central nervous tissues also have high activity of α-glycerolphosphate dehydrogenase and therefore might be used as an index of redox value of cytoplasm of the cells of locust ganglia. In conclusion, the measurement of intermediates present in locust central nervous tissues during 60-180 min. of incubation of _in vitro_, indicates that the condition of the tissue is close to that _in vivo_ and therefore the effect of various alternative conditions of incubation on the parameters measured, could be tested during this time. The achievement of a steady state by locust ganglia from 60-180 min. is in agreement with the previous results of McIlwain et al. (1951). However locust central nervous tissues are more aerobic than mammalian nervous tissues and the flux of glucose via pyruvate to lactate in locust ganglia did not rise _in vitro_.

(D) Metabolism after 180 minutes.

The steady state of metabolites present in locust ganglia during 60-180 minutes are followed by a dramatic fall in high energy phosphates even under optimum conditions of temperature, a concentration of glucose and oxygen is present in the medium. The maximum period for this decrease is 30 minutes (Figure 21), but the reason for this dramatic fall in high energy phosphates was not investigated in this research work. Probably, after a period of 180 minutes, this preparation loses its usefulness. In conclusion, locust central nervous tissues probably behave *in vitro*, very much like mammalian central nervous tissues with regard to positive and negative feedback mechanisms of adenine nucleotides and probably the same enzyme systems comprising hexokinase/phosphorfructokinase, glyceraldehyde 3-phosphate dehydrogenase/pyruvate kinase and phosphorylase kinase are modulated by adenine nucleotides. However, several aspects of locust central nervous tissues metabolism are different to those of mammalian nervous tissues.

(a) The flux of glucose in locust ganglia is much higher compared to mammalian nervous tissues and probably, locust nervous tissues are more aerobic than mammalian nervous tissues. Due to the presence of very low activity of lactate dehydrogenase and a lower output of lactate, it is probable that locust ganglia have a rate limiting control at the lactate dehydrogenase stage whereas mammalian nervous tissues have relatively higher lactate output, and evidently do not have rate limitation at

the lactate dehydrogenase stage. The ratio of Lactate to pyruvate cannot be used as an index of adequate ozygen supply to locust central nervous tissues.

(b) The loss of glycogen was maximum between 30-60 minutes. This loss of glycogen was never recovered in locust nervous tissue, whereas in mammalian nervous tissue glycogen recovery was complete during the first 60 minutes.

(c) The steady state of the locust central nervous tissues *in vitro* was probably achieved between the initial 50 and 60 minutes, whereas in mammalian nervous tissues they achieved a steady state after 30 minutes. The recovery is more complete in the case of locust nervous tissues as compared to that *in vivo* in contrast to mammalian central nervous tissues.

2. The roles of glucose, glycogen and trehalose as metabolic fuels in locust central nervous tissues:

The rolesof these three carbohydrates as metabolic fuels for isolated locust central nervous tissues was investigated by measurement of oxygen uptake which is an index of the metabolic activity. The requirement of a very high concentration of the sole substrate, glucose, in contrast to the requirement for mammalian central nervous tissues, has been discussed under the previous heading of this discussion. Glycogen and trehalose seem to constitute the major stores of carbohydrate of locust CNS as compared to a very low concentration of glycogen and complete absence of trehalose in the mammalian CNS (Table 10, Section 'B'

Chapter 3). Other authors (Steele and Robertson, 1972); Hart and Steel, 1973) have found even higher concentrations of glycogen present in cockroach CNS. This raises the question of whether this major store can be used as an oxidizable substrate. In order to resolve this question, isolated locust ganglia were incubated in a medium containing no exogenous substrate but under optimum conditions (described in Chapter 3). During this time the glycogen suffered an exponential fall in concentration (Figure 23 as shown in Section C of Chapter 3). Although there was a decline in glucose concentration during this time, it was very modest and much slower than that of glycogen until the glycogen had almost disappeared, when its rate of disappearance quickened until the glucose could hardly be estimated. Now let us look at Figure 13, as shown in Chapter 3, which shows that oxygen uptake, by isllated ganglia in hypoosmotic saline containing no exogenous substrate, is 1.66 μmol/g/min. If glucose or glycogen were the sole substrate for locust tissue to be utilized (as described previously) 1.66 μmol/g/min of oxygen uptake would correspond to 0.27 μmol/g/min (the rate of substrate utilized). The glycogen loss on average during the initial 50 minute period, in terms of glucose equivalents is 0.14 μmol/g/min. (Table 11 as shown in Section 'B' of Chapter 3). The other source of oxidizable substrate was glucose and its disappearance rate was 0.017 μmol/g/min (Table 11). On summing up the rates

substrates, 0.157 μmol/g/min, a total rate of disappearance is obtained. Therefore, only 10.8% of the oxidizable substrate is coming from endogenous glucose and the remainder is accounted for by glycogen. This certainly contrasts with themammalian central nervous tissues. Other workers have found that glycogen stores are not utilized before the glucose concentrations are depleted (McIlwain and Bachelard, 1971). However, this total rate of 0.157 μmol/g/min (based on the loss of glucose and glycogen) of sugars utilized by locust ganglia is much lower than the theoretically worked out rate of 0.27 μmol/g/min (based on oxygen uptake). It has been found that the disappearance of another sugar, trehalose (which is present in high concentration in the ganglia) is very high, i.e. from 12.8 μmol/g *in vivo* to 3.8 μmol/g after a 30 minutes incubation in the medium (Strang, R.H.C. personal communication). Therefore the rate of disappearance is 0.3 μmol/g/min. Obviously, this can account for all the theoretically worked out rates of utilization of sugars. It is interesting to recall here, the previous findings of Hart and Steele (1973) who found that 84% of the glycogen loss in the cockroach nerve cord could be accounted for as trehalose and glucose diffused into the medium. Working solely on the basis of the present findings it is difficult to determine how much of the oxidizable substrate was contributed by either glycogen or trehalose. However, it can be concluded that much of the oxidizable substrate came from both glycogen and trehalose. Lactate was not taken into account,

as no estimates of lactate were made, in the absence of glucose in medium, but on the basis of previous results, it would not add much to the sum. The disappearance of trehalose is faster than that of glycogen during the first 30 minutes. As no estimates of trehalose were made after a 30 minutes incubation, it is difficult to say what is happening to trehalose during subsequent periods of incubation. However, the glycogen loss is continuous up to about 60 minutes and during this time the concentration of ATP was maintained. Glucose present in the locust ganglia is completely depleted after 90 minutes, and there are still 2 µmol/g glycogen in terms of glucose equivalents left in the tissue. The question arises of why this glycogen is not mobilized to maintain the ATP concentration for a longer time? This cannot be answered at present. On comparing the glycogen disappearance from ganglia in a 10mM glucose medium and a medium containing no exogenous substrate (Table 11, Section 'B', Chapter 3, and Table 19, Chapter 4). The glycogen loss is about 5-6 fold higher in medium containing no exogenous substrate during the first 30 min. The higherloss of glycogen in medium containing no exogenous substrate probably indicates that glycogen was utilized as an oxidizable substrate. The glycogen loss is about the same in medium containing no exogenous substrate, as compared to 10mM-glucose medium during the 30-60 min. period of incubation (Table 11 and 19). This is the period when tissues are recovering from the trauma of preparation and adjusting to

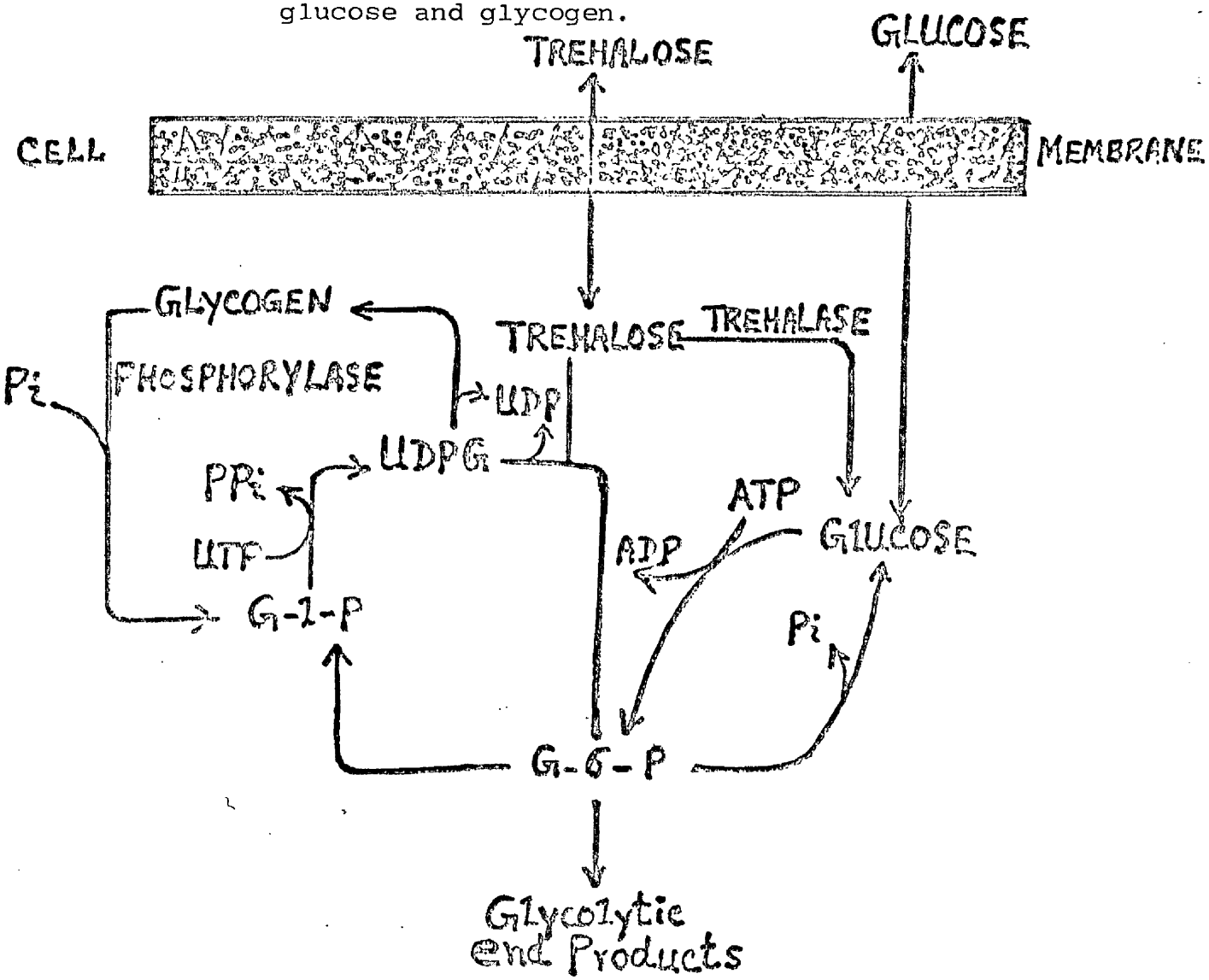
an artificial medium. Roughly the same oxygen uptake is obtained during this period compared to the initial 30 min. and the maintenance of ATP concentration certainly suggests that some other oxidizable substrate must have been utilized. Trehalose and glucose are the obvious candidates. The disappearance of glycogen during 60-90 min. is about double that occurring in 10mM glucose medium (Table 11 and 19). As an overall estimate, the glycogen in terms of glucose has fallen from 11 µmol.../g at 0 min to 2.7 µmol. /g at 90 minutes in a medium containing no exogenous substrate while glycogen falls from 11.00 µmol/g to 7.12 µmol/g in a 10 mM glucose medium, and therefore the rate of glycogen disappearance is 0.092 and 0.042 µmol../g/min. in glucose-free and glucose saline respectively. In conclusion the rate of disappearance of glycogen is doubled in glucose-free saline. Furthermore, the evidence that trehalose acts as an oxidizable substrate for locust central nervous tissues comes from the results of oxygen uptake by locust ganglia <u>in vitro</u> in a medium containing 4mM glucose and 50mM trehalose, (Table 14 as shown in Section 'C' of Chapter 3). The results of oxygen uptake during the 0-30 min. and 60-90 min. periods of incubation clearly indicate that the oxygen uptake is not significantly different as compared to a 10mM glucose medium. Referring to Figure 13, as shown in Section 'A' of Chapter 3, the concentration of glucose present in the medium is rate limiting to the oxygen uptake until 10 mmolar glucose is added. In a medium containing 4 mM glucose, oxygen uptake is significantly lower

than in 10mM glucose (Figure 13, Section 'A' of Chapter 3). This certainly indicates that trehalose does have a role in oxidation. If trehalose was utilized as an exogenous oxidizable substrate, this raises the question of how the trehalose entered the tissue, and how it was metabolized via glycolysis and TCA cycle. The results of other authors (Rees, 1977) have shown that trehalose present in the haemolymph enters the cells by simple diffusion rather than by active transport. After reaching the site of utilization, trehalose diffuses into the cell and is hydrolyzed into two molecules of glucose by trehalase (Figure 31, this Chapter). This enzyme has been identified in insect flight muscle and nervous tissue (House, 1974; Hart and Steel, 1973). The Km value of trehalase has been found by Gilby et al. (1967) to be 3mM and therefore trehalase should be maximally active at less than 30 mM trehalose. When 50 mM trehalose is used as exogenous substrate, this concentration is more than sufficient for the trehalase to operate at its maximum activity. The question arises of why the locust haemolymph should have a combination of two sugars, 4mM glucose and 50 mM trehalase whereas only 10 mM glucose is sufficient in vitro for isolated tissue to give the optimum metabolic activity. The answer is:

(a) the presence of trehalose as the major blood sugar has many advantages. Firstly, there is an obvious psmotic advantage in having disaccharide rather than a monosaccharide as the major blood sugar. Secondly, its

FIGURE 31

Simplified diagram showing interconversion of trehalose, glucose and glycogen.



non-reducing nature means that there is less chance of unwanted side effects at high concentration. Thirdly, there may possibly be the same advantage in being able to have a further control point at trehalose hydrolysis and finally, it means that there is a diffusion gradient for monosaccharide from the gut into the haemolymph, thus facilitating uptake of dietary sugar.

(b) the Km values of hexokinase for glucose and the requirement for glucose concentration present in the perfusion medium are still controversial, (as discussed earlier). However, in a medium containing 4 mM glucose, the oxygen uptake of isolated ganglia was rate limited hence a requirement of another sugar, trehalose in the presence of 4 mM glucose is essential in the living animal.

In conclusion, all the three substrates, glucose, glycogen and trehalose may act as metabolic fuels for locust central nervous tissues <u>in vitro</u> or <u>in vivo</u>. The requirement of the sole substrate, glucose, to get an optimum metabolic activity of locust central nervous tissues <u>in vitro</u>, certainly confirms the previously made conclusion by other authors ad given in the reviews. Greville, 1962, working on mammalian central nervous tissues, argued that the functioning of the central nervous system has an absolute requirement for glucose.

3. Behaviour of locust central nervous tissues under anoxic conditions:

The results of the measurement of metabolites, glycogen, glucose, ATP and lactate formation in anoxic conditions are shown in Figures 28 and 29 of Section 'D', Chapter 3. Under anoxic conditions of incubation in vitro, the locust nervous tissues showed two patterns of anaerobic glycolysis with regard to lactate formation, ATP, glucose and glycogen loss both in glucose containing and glucose-free medium:

(a) metabolism during the first 5 minutes in anoxia after a pre-equilibration period of 60 minutes in aerobic conditions;

(b) metabolism between consequent 5-20 minute periods in anoxic conditions.

(a) metabolism during the first 5 minutes: This period of incubation is marked by a dramatic fall in ATP concentration from 2.0 to about 0.95 µmol/g, glycogen falls from 8.4 to 6.9 µmol/g, an increase in lactate production and a small fall in glucose concentration both in glucose and glucose-free medium, (shown in Figures 28 and 29 Tables 16 and 17, in Section 'D'). The concentration of these metabolites at zero time are the concentrations obtained by 60 minutes pre-equilibration in optimum conditions (see Figures 20, 21 and 24 for reference as shown in Section 'B' Chapter 3). Although the ADP concentration was not measured at 5 minutes it is likely that as the adenine nucleotide system would have

behaved in the same way as aerobic conditions and hence the ADP concentration would have increased as the ATP concentration decreased. In other words, the ATP/ADP ratios would have decreased at 5 min compared to the ratio at zero time. What could be the explanation for this decrease in ATP concentration? The explanation lies in the fact that the ATP synthesizing system was impaired due to the non-operation of the TCA cycle and oxidative phosphorylation as a result of anoxia. (The TCA cycle is the major site of ATP synthesizing system as described previously). The present findings of a decrease in the concentration of ATP present in locust ganglia during anoxia, is in line with the previous results of other workers on mammalian central nervous tissues, (Plum, 1974). These findings are that anaerobic glycolysis seems to be inadequate to supply sufficient energy to maintain bio electricalfunction for more than a few minutes. It has been shown that the glucose utilization of mammalian nervous tissues increased 5-6 fold under anaerobic conditions (Härkönen et al., 1969; McIlwain and Bachelard, 1971), but this increase would yield only 30% of the normal ATP synthesis in aerobic conditions (Plum, 1974). If the dramatic fall in ATP levels cannot be restored in anoxia the question arises of what the effects are of this lower concentration of ATP on various metabolic rates. Recalling the previous results of Rolleston and Newsholme, 1967b; Lowry et al. 1964; Lowry and Passonneau, 1966, working on mammalian CNS and other authors working on phosphorylase kinase (Newsholme and Start, 1973), these authors concluded that the decreased concentration of

ATP stimulates the glycolytic pathway and glycogen breakdown by increasingthe activities of the controlling enzymes responsible for increasing the flow of the metabolites. This regulatory system consists of the hexokinase/ phosphofructokinase system and the glyceraldehyde-3-phosphate dehydrogenase/pyruvate kinase system for glycolytic pathways and phosphorylase b kinase for glycogen breakdown. Inthelight of the present findings of slight depletion in glucose and dramatic fall in glycogen and the above conclusions, it is likely that the decreased concentration of ATP stimulated the hexokinase/phospho-fructokinase and glyceraldehyde-3 phosphate dehydrogenase/ pyruvate kinase systems and this resulted in increased utilisation of glucose via glyclytic pathway. However, this is in contrast with the situation in aerobic conditions (Figure 21, as shown in Chapter 3), where decreased concentration of ATP$_{after}$a30 min incubation period, increased the glucose concentration. It is likely that glucose entering into the tissue was impaired under anoxia and only endogenous glucose was utilized due to stimulation of controlling enzymes. The dramatic fall in glycogen is in contrast to the small depletion of glucose and this probably reflects that the phosphorylase kinase was much more stimulated by the ATP concentration than that of the controlling systems of glycolytic pathways, and therefore as a result much more glucose 6-phosphate would have formed. This glucose 6-phosphate is an inhibitor of hexokinase and therefore hexokinase might have been inhibited. The evidence

also comes from the previous results of other authors working on perfused rat heart and rat superior cervical ganglia (Morgan and Permeggiani, 1964; Härkönen et al, 1969; McIlwain and Bachelard, 1971). These authors found that the conversion of phosphorylase b to phosphorylase a was much greater under anaerobic conditions. These authors also found that no glycogenolysis could be detected in isolated tissues when perfused with glucose and this was consistent with the fact that 98% of the phosphorylase was in the inactive b form. The present result of the same glucose depletion and glycogenolysis both in glucose and glucose-free saline are not explicable. However, this finding of glycogenolysis raises the question of what the role of glycogen is in anoxic conditions. The rate of lactate formation was increased 5-6 fold as compared to that in aerobic conditions (shown in Tables 16 and 17, and Figure 24 in Section 'B'). The depletion of glucose cannot be accounted for by this lactate formation, as the depletion is very small in comparison to lactate formation. However, the rate of disappearance of glycogen can account for all the rate of lactate production. Thus the rate of glycogen disappearance is 0.36 μmol /g/min in glucose saline and 0.50 μmol /g/min lactate is formed which would correspond to 0.25 μmol /g/min of glycogen in terms of glucose equivalents. Similarly, in glucose-free saline, the glycogen loss is 0.40 μmol /g/min and lactate formation 0.44 μmol /g/min. Considering the above assumption, 0.14 μmol /g/min would correspond to 0.22 μmol /g/min of

glycogen in terms of glucose. This probably means that most of the lactate formation was a result of glycogenolysis, but there is still about half of the total loss of glycogen which cannot be accounted for by lactate formation. The results of Hart and Steele (1973), (see Figure 21 as shown in Section 'B' of Chapter 3), show that 84% of the glycogen was lost as glucose and trehalose, and therefore it is likely that the remainder of the glycogen was lost as trehalose in the medium. The other possible candidate, responsible for the flux to lactate, in locust nervous tissue is trehalose which is present in very high concentration and can account for lactate formation. The contribution of this sugar is very small and would not add much to the sum, as most of the trehalose was lost during pre-equilibration conditions of aerobiosis during the first 30 min. (Strang, RHC, personal communication).

In conclusion, anoxic locust central nervous tissues behaved during the first five minutes very much like mammalian central nervous tissues with regard to lactate formation and fall in ATP concentration. Lactate formation was increased 5-6 fold in anaerobic conditions as compared to aerobic in both locust and mammalian nervous tissues. However, locust tissues contrast with mammalian tissues with regard to glycogenolysis, probably a source of glucose for lactate formation in glucose-saline. Whereas in mammalian central nervous tissues no glycogenolysis occurred when the tissue was perfused in glucose saline, there was a large fall in the glycogen content of locust ganglia. The question of why

locust central nervous tissues behaved differently with regard to glycogenolysis is still without a satisfactory answer.

(b) Metabolism between 5 and 20 min. of incubation in anoxic conditions:

This period is marked by a relatively lower rate of loss of glycogen as compared to that in the first 5 min., relatively low lactate formation in the medium and maintenance of ATP both in glucose and glucose-free saline. The depletion of glucose was negligible in glucose-saline and the depletion was slightly higher in glucose free saline (shown in Figures 28 and 29, and Tables 16 and 17, Chapter 3). The question arises of why the glycogen loss continued both in glucose and glucose-free saline while glucose levels were maintained. The answer is that the decreased concentration of ATP (as it had not recovered at any time during 5-20 min. to the level at zero time) would have stimulated the phosphorylase kinase, and as a result glycogenolysis remained continuous. The lowered concentration of ATP should have stimulated the hexokinase/phosphofructokinase system and the glyceraldehyde phosphate dehydrogenase/pyruvate kinase system and as a result increased the flux of glucose to lactate. The answer to this question is uncertain. It may be that glucose entry from the medium to the tissue was limited and this resulted in less glucose utilization. However, this is probably contradicted by the fact that endogenous glucose levels present already in the tissue are not depleted completely. The rate of lactate

formation again parallels the glycogenolysis both in glucose and glucose-free medium (Tables 16 and 17). The rate of lactate formation on average is 0.14 μmole/g/min and the rate of glycogen loss is 0.19 μmole/g/min during this period of incubation in glucose saline. Therefore, 0.14 μmole/g/min lactate would correspond to 0.07 μmole/g/min of glycogen in terms of glucose equivalents. The formation of lactate can be accounted for in terms of glucose units coming from glycogenolysis. The remaining glycogen could be accounted for as being lost into the medium as trehalose and glucose. As the loss of glucose from the tissue is negligible, glucose cannot be the major factor producing this lactate. Similarly, the rate of formation of lactate is 0.13 μmole/g/min on average and the rate of glycogenolysis is on average, 0.20 μmole/g/min during this period in glucose-free saline. Therefore, 0.13 μmoles/g/min of lactate formation would correspond to 0.065 μmole/g/min of glucose utilized. The rate of glycogenolysis is more than sufficient to account for this lactate output. The remainder of glycogen could have been lost into the medium as glucose and trehalose. The depletion rate of glucose is 0.044 μmole/g/min on average during this time. It is obvious that this rate is much lower than the theoretical glucose utilization rate (0.065 μmole/g/min) required for lactate formation. It may be that both glycogenolysis and glucose depletion could have contributed towards the lactate formation in glucose free saline. The lactate formation during this period is about 1-2 times higher than in aerobic conditions; this is much lower than

in the first 5 min and also contrasts with the previous results of other authors working on mammalian nervous tissues (McIlwain and Bachelard, 1971; Lowry et al., 1964; Härkönen et al., 1969,) who found lactate output increased 5-6 times during this period. The question arises as to how the ATP levels were maintained during this period, as most of the ATP is synthesized via oxidative phosphorylation in a cell under aerobic conditions. However, ATP is also synthesized via glycolysis (Harper, 1977). The locust central nervous tissues were exposed to anoxic conditions and therefore there is no question of ATP formation via oxidative phosphorylation. The only site of ATP synthesis left under these conditions, is via anaerobic glycolysis. There are two sites in the anaerobic glycolysis where ATP is synthesized:

(a) Conversion of 1, 3-diphosphoglycerate to 3-phosphoglycerate by the enzyme phosphoglycerate kinase;

(b) Conversion of phosphoenol-pyruvate to pyruvate by pyruvate kinase.

The phosphoglycerate kinase is an equilibrium enzyme whereas pyruvate kinase is a non-equilibrium enzyme and is subject to control by ATP concentration (Rolleston and Newsholme, 1976b). The decreased concentration of ATP at 5 min of incubation probably stimulated this enzyme which resulted in more flux of phosphoenol pyruvate to pyruvate. This also had a positive feedback effect on glyceraldehyde 3-phosphate dehydrogenase due to decreased concentration of 1, 3-di-

-phosphoglycerate which is an inhibitor of glyceraldehyde 3-phosphate dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase is a regulatory enzyme and has a product inhibition. Due to decreased concentration of 1, 3-diphosphoglycerate, this enzyme would be stimulated and this results in more flux of intermediates along the glycolytic pathway and more ATP synthesis. However, stimulation of glyceraldehyde-3-phosphate dehydrogenase, also results in a change in $NADH/NAD^+$ ratio. This higher ratio of nicotinamide nucleotides must be converted to a lower ratio by the regeneration nicotinamide nucleotides in the oxidiation state, if glycolysis is to continue as a source of ATP. Under aerobic conditions α-glycerophosphate cycle is the source for regeneration of nicotinamide cofactors of locust flight muscle (Newsholme and Start, 1973) and this cycle has also been strongly proposed for locust central nervous tissues (described previously). However this cycle cannot be operative in locust tissues under anaerobic conditions. The other alternative is conversion of pyruvate to lactate by lactate dehydrogenase. The loss of glycogen and continuous formation of lactate (as shown in the present results) probably reflects the fact that the glycogen in terms of glucose acts as a fuel for ATP synthesis in order to maintain the ATP concentration; and lactate formation ensures the operation of the anaerobic glycolysis during this period.

In conclusion, the locust central nervous tissues behaved like mammalian central nervous tissues with regard to

maintenance of ATP during 5-20 mins. The ATP conurbations never returned to the *in vivo* levels and were much lower than ATP levels in aerobic tissues. Therefore, locust central nervous tissue, like mammalian nervous tissue, cannot survive in anaerobic conditions. However, locust central nervous tissues contrast with the mammalian nervous tissues, in that the former show a rate of lactate formation 1-2 times higher in anaerobic than in aerobic conditions during the period from 5-20 mins, whereas the latter show a rate of lactate output 5-6 times higher. Another difference is that locust tissues showed glycogenolysis even in glucose-saline whereas no glycogenolysis was shown by mammalian nervous tissues in glucose saline. A third difference is that glucose entry into the tissue from medium might be rate-limiting in the case of locust nervous tissues whereas no such observation was made in the mammalian situation. The metabolism of locust central nervous tissues is quite different in the first 5 min and from 5-20 min during anaerobic conditions with regard to glycogen loss and lactate formation. This raises the question of why this difference exists in contrast to mammalian CNS. This probably can be answered by taking two considerations into account: the present findings of spontaneous activity and the previous results of other workers.

(A) Correlation of the spontaneous activity with the anaerobic glycolysis:

The endogenous spontaneous activity had ceased after 5 min in anoxic conditions regardless of the presence or absence of glucose in the medium. (Figures 28 and 29 Section 'D'). In addition, the concentration of ATP fell to half its value at 5 min and thereafter remained steady. This requires an explanation for how the spontaneous activity ceased in anoxic conditions after 5 min. This can be answered by reviewing the previous results of other workers. In aerobic conditions, one mol of glucose when completely oxidized yields 38 moles of ATP whereas in anaerobic glycolysis only two moles of ATP are formed per mol of glucose (Harper, 1977). Although anaerobic glycolysis is increased 5-6 fold under anaerobic conditions (McIlwain and Bachelard, 1971), yet this increase would yield only 30-35% of the normal ATP synthesis in aerobic conditions (Plum, 1974). To recall the previous results of Marchbanks (1970); Whittam, (1962); Whittam and Bond, (1964), working on mammalian central nervous tissues, these showed that in vitro, 40-50% of the energy available from cerebral respiration may be expected in maintaining the differential distribution of sodium and potassium ions. As the sodium/potassium pump is directly related to the action potentials in repolarization, cessation of activity must have been due to inadequate supply of energy in anoxic conditions. The question of why it lasted for 5 minutes still remains unanswered. The answer is that

the supply of fuel for this time period was adequate to cope with the requirement of energy for the maintenance of action potentials as compared to the remainder of the experiment. The evidence comes from the increased glycogenolysis and lactate output during the first 5 min as compared to the remainder of the experiment. As glycogenolysis is considered to be the source of lactate, therefore, increased loss of glycogen to lactate probably reflects that more ATP would have been synthesized to supply the energy to maintain the differential gradient of Na^+ and K^+ ions. Why did glycogen not disappear at the same rate as in the first 5 min and continue as a source of lactate and energy to the tissues for the maintenance of the Na^+ & K^+ differential gradient and maintenance of spontaneous activity, as glycogen stores are adequate at 5 min. (Figure 28 and 29)? This may be answered by taking into consideration a compartmentation present in locust central nervous tissues.

(B) Compartmentation of the thoracic ganglia:

What is a compartment in a metabolic sense? A compartment is a space, bounded by an observable membrane, into or out of which the free movement by diffusion or by active movement of certain substances is restricted (Balázs and Cremer, 1971). Several authors, De Robertis (1965); Berl (1971); and Balázs et al. (1970), working on mammalian nervous tissues have attempted to assign metabolic compartments to structures by comparing the characteristic

features of different morphological structures with the biochemical properties of the functional compartments. Much of the evidence is consistent with the view that there exist two compartments. The larger compartment is associated with neuronal structures and the smaller with glial cells. The classification of nervous tissues into large and small compartments is not dependent on the size or on the volume of the compartments, but depends entirely on the fluxes of certain metabolites. Thus, it has been found by Giacobini (1964) that 70% of the oxygen uptake of brain and perhaps more is neuronal, and that neurons make up the bulk of the volume. Similarly, the results of Sotelo (1969), suggest that aerobic glycolysis in the neurons is much more vigorous than in glial cells.

The results of other authors (Balázs & Cremer, 1971) showed that the neuronal cells contain a greater number of polyribosomes and mitochondrial fractions which suggests strongly that the sites of respiration and protein synthesis are present in abundance in neuronal cells. These facts also suggest that neurons cannot fit into the 'small' compartment category with regard to aerobic metabolism and protein synthesis. They are either entirely in the 'large' compartment category or are themselves compartmented. A number of arguments for glial involvement in the small compartment are given by Balázs et al. (1970).

The previous results of Haymaker (1969); Friede (1965; 1970);

Glees and Briepohl (1967); and Glees (1955), working on vertebrate nervous tissues, showed that glycogen granules are acuumulated in glial cells. Similarly, the results of Wigglesworth (1960) and Treherne (1960), have shown that glial cells present in the thoracic ganglia of cockroach and locust contain very high concentration of glycogen as compared to neuronal cells. In light of these findings, it is tempting to suggest that the thoracic ganglia of the locust might contain compartments consisting of glial cells and compartments of neuronal cells with regard to presence of glycogen granules. Probably, during five minutes, the glycogen present in the neuronal cells disappeared to maintain the spontaneous activity and metabolism of neuronal cells and thereafter the lactate production and glycogen loss is localized to glial cells. As the concentration of ATP was measured in whole ganglia, it is not possible to estimate what percentage was present in glial and neuronal cells. As it was found that ATP levels remained steady after a fall to half their initial level at 5 minutes, it is possible that the levels only reflect the state of glial cells which can maintain their concentration of ATP by anaerobic glycolysis due to their lower rate of metabolism compared to neuronal cells.

4. Spontaneous activity of metathoracic ganglia with the pattern of carbohydrate metabolism *in vitro*.

The previous work of Weiant (1958) provided the background against which the recording of spontaneous activity from metathoracic ganglion of locust was planned. He recorded the electrical activity from motor nerve, N5, of the metathoracic ganglion of the cockroach while it was intact and then recorded the activity after cutting the connectives between meso- and metathoracic ganglia and all the other connectives. However, in both cases the ganglion was bathed in the haemolymph of the insect under study. He found that spontaneous activity was greatly increased when connectives between meso- and metathoracic ganglia were cut as compared to that in the intact ganglion. The conclusion is that spontaneous activity in metathoracic ganglion of the cockroach is inhibited by the cerebral ganglia. The term spontaneous is used here to mean the electrical activity recorded from certain motor neurons without any sensory inputs. The purpose of recording spontaneous activity from the nerve, N5, of the metathoracic ganglion of locust was, firstly, to follow up these previous restults to see how locust ganglion behaves during incubation, in Hoyle's physiological saline *in vitro* and to compare it with the situation *in vivo*. Secondly, to see how this spontaneous activity was affected by variation in the conditions *in vitro*; and thirdly, to see what is the correlation of spontaneous activity with carbohydrate metabolism. In addition to

this, electrical activity forms an integral part of the physiology of the nervous system, therefore, measurements of spontaneous activity *in vivo* and *in vitro* would probably serve as an index of the activity of the locust CNS *in vitro*, compared to that *in vivo*.

The previous results of McIlwain (1951), working on guinea-pig and rat cerebral cortex slices showed that electrical stimulation increases metabolic activity. Similarly, in the whole animal, the electrical activity of the brain can be increased by electrical stimulation and this increased the metabolic activity accompanying it. What are the factors of the metabolism of mammalian CNS which accompany the electrical activity induced by electrical stimulation? This can be answered by reviewing the previous results of McIlwain et al. (1951); McIlwain (1953), working on mammalian central nervous tissues and Larrabee (1958); Horowicz and Larrabee (1958), working on sympathetic ganglia of the rat. The findings of these authors are that high energy phosphate derivatives, oxygen uptake and glucose consumption form important links between the electrical activity and metabolism, *in vitro*. Now, considering the locust central nervous tissues, the metathoracic ganglion shows an endogenous electrical activity which can be monitored as action potentials both *in vivo* and *in vitro* (Tables 7 and 8, Figures 14 and 16, Section 'A', Chapter 3), without any electrical stimulation, whereas electrical stimulation is an essential

requirement for cerebral cortex slices. This lack of an external stimulation requirement, probably gives this preparation an advantage over cerebral cortex slices. In addition to this, the artificially induced electrical activity might not be representative of the activity *in vivo* and the accompanying metabolism might be quite different from that *in vivo*. Therefore, the presence of endogenous activity is probably an advantage over induced electrical activity. As the electrical activity present in metathoracic ganglion was observed without any sensory inputs, the term spontaneous activity was used for *in vitro* records of electrical changes from metathoracic nerve (N5) whereas the term electrical activity is retained for action potentials recorded from the N5 nerve, while the system was almost intact and the animal alive but restrained.

The present results of measurement of spontaneous activity via the N5 nerve, while the ganglion was bathed in saline, indicated that maintaining the saline between 35-37^{o}C, saturation with 100% O_2, and an addition of 10mM glucose are essential requirements for obtaining a level of 30-50% of spontaneous activity compared to that *in vivo*. (Table, 7, 8 and 9; Figure 14, 15 and 16, Section 'A', Chapter 3). Athough in the present results of measurement of spontaneous activity, the effect of oxygen concentration present in the medium was not investigated, it was assumed that equilibration of the medium with 100% O_2 was an essential requirement for obtaining the suitable spontaneous activity. It was found that the concentration of oxygen present in the medium, was rate

limiting on oxygen uptake, however, saturation with 100% O_2 being an essential requirement for preventing this limitation. This was in light of two criteria:

(a) direct correlation of spontaneous activity with glucose present in the medium and with high energy phosphates (Figure 23 as shown in Section 'B' of Chapter 3);

(b) a proportional increase in oxygen uptake at two different temperatures was accompanied by an increase in spontaneous activity (Figures 11 and 17 as shown in Section 'A' of Chapter 3). The oxygen uptake was increased slightly more than 3 fold and spontaneous activity increased 3-4 times. The explanation of this rise in spontaneous activity and the correlation with the rise in oxygen uptake, possibly lies in:

(a) the nature of the source of the spontaneous activity of the metathoracic ganglion;

(b) the transmission of impulses from this source via the motor nerve, N5.

(a) Wyman (1966); Hoyle (1964); Ewing and Manning (1966); and Wilson (1966a), working on insect central nervous system, proposed that spontaneous motor output is due to a central neuronal oscillator responsible for generating patterned activity of the motor neurons. This oscillator is loosely coupled via internal motor pathways and sensory pathways. It is possible that this kind of oscillator is present in the metathoracic ganglion and is responsible for motor output. It is probable that the activity of this oscillator is increased roughly three times with a $10^{o}C$ rise in temperature and consequently higher energy in the form of

high energy phosphates is required. A threefold increase in oxygen uptake by ganglia (Figure 11, Section 'A' of Chapter 3) with a 10°C rise in temperature, probably fulfils this demand. However, the results of other authors (Wiersma, 1967) working on insect nervous tissues, suggest that these oscillators need a very small amount of energy as compared to the energy required for the Na^+ electrogenic pump. The Na^+ electrogenic pump or Na^+/K^+ pump is involved in the transmission of action potentials. These authors stated that the flux of Na^+ and K^+ ions during the activity is in the direction of the concentration gradients. The increased rate of flow of very small amounts of ions for extremely short periods of time must obviously require very little energy for the transient change of permeability. This process is fundamentally different with respect to ionic movement during recovery. The restoration of the original steady state requires extrusion of sodium ions and entry of potassium ions, against the concentration gradient. This process of repolarization is fulfilled by the Na^+/K^+ pump at the expense of energy obtained by hydrolysis of ATP. In light of these results, it is possible that both the activity of the oscillator of the thoric ganglia and the transmission of impulses increase with a rise of 10°C. However, a higher oxygen uptake in direct proportion to the spontaneous activity may be in response to the higher energy requirement of Na^+/K^+ pump whereas the oscillator's demand for energy is negligible. It is intesting to note that the present findings as to the Q10 value of spontaneous activity are in agreement with results of

Schoffeniels (1958b). These results from the isolated single electroplax of the electric organ of Electrophorus electricus, have shown that the Q10 of the action potentials were found to be around 3.6, whereas the present results from isolated ganglia have shown a Q10 of the spontaneous activity to be 3-4.

As most of the ATP is synthesized via the TCA cycle together with oxidative phosphorylation in the electron transport chain and mitochondria; and as the mitochondria are the sites where all these processes coupled with oxygen uptake occur, the more than threefold increase in oxygen uptake correlated with the measurement of spontaneous activity gives a clue that the ATP synthetic process must have changed with increase in temperature. The evidence of the correlation of spontaneous activity with high energy phosphates also comes from the present results showing the cessation of spontaneous activity at 90 min in absence of glucose, and a dramatic fall in ATP levels (Figure 23 as shown in Section 'B' of Chapter 3); whereas in the presence of glucose, the ATP levels are maintained for 3 hrs and spontaneous activity was also observed for 3 hrs. (Figures 18 and 20 as shown in Section 'A' and 'B' respectively). The cessation of spontaneous activity after 3 hrs even in presence of glucose is again correlated with high energy phosphates and the reason for its cessation after 3 hrs is beyond the scope of this present research work. Nevertheless, this could be one of the important future aims of this work. The present results, correlating spontaneous activity with

ATP levels are not in agreement with the previous results of Härkönen et al. (1969), working on the rat superior cervical ganglion, who found that ganglionic transmission failed in both resting and stimulated states in spite of the continued presence of very substantial levels of ATP. Failure seemed to be associated not with ATP depletion but rather with the complete loss of glucose and glycogen.

Under the optimum conditions of incubation the locust ganglia showed four patterns of metabolism. Similarly, spontaneous activity of the metathoracic ganglion showed four patterns:

(a) spontaneous activity during the initial 45 minutes; and
(b) between 45 and 60 minutes;
(c) between 60 and 180 min;
(d) after 180 min.

(a) This initial period is marked by a low spontaneous activity (Table 9 as shown in Section 'A' of Chapter 3). This raises the question of why the spontaneous activity was low during this period of incubation. The energy state of the tissue, probably gives a clue to the answer. As the ATP/ADP and arginine phosphate/ATP ratios are much lower during this period as compared to that <u>in vivo</u>, it is possible that the Na^+/K^+ electrogenic pump might have been inhibited due to lack of ATP. Hence, the mechanism of transmission of impulses might have been impaired.

The observations of high oxygen uptake by thoracic ganglia but lower spontaneous activity (Tables 9 and 19, shown in

Section 'A' of third Chapter and in this Chapter respectively, and Figure 18, Section 'A'), contradicts the present finding for thoracic ganglia which has shown that spontaneous activity directly correlates with oxygen uptake with regard to change in temperature of 10°C (Figures 11 and 17, Section 'A' of third Chapter). However, the result of this oxygen uptake in iso-osmotic saline indicated that the rates are significantly lower as compared to those in hypo-osmotic saline (Figure 25, Section 'B' of Chapter 3). On the other hand, the pattern of slow spontaneous activity is obvious in both the salines (Tables 8 and 13, Sections 'A' and 'C' of Chapter 3 respectively). This gives an indication that extra oxygen uptake in hypo-osmotic saline is due to energy required for osmotic work (described previously). Hence, there is no contradiction with regard to the correlation of spontaneous activity and oxygen uptake.

(b) This period represents a recovery of spontaneous activity (Table 9, Section A of Chapter 3). The ATP/ADP and arginine phosphate/ATP are also recovering. This again agrees with the argument that the recovery of high energy phosphates might have allowed the increased operation of Na^+/K^+ electrogenic pump and therefore, transmission of impulses might have increased.

(c) This period of incubation is marked by a steady state of spontaneous activity. It can again be correlated with a steady state of the ATP/ADP ratio (Figure 20, Section 'B' of Chapter 3). This is the period when tissue has recovered

from the trauma of preparation as indicated by arginine phosphate/ATP and ATP/ADP ratios close to those *in vivo* and lower rate of lactate formation (Figures 20 and 24, Section 'B'). This period probably represents a steady state situation of locust central nervous tissues with regard to metabolism and physiology in an artificial medium when the trauma of preparation and the adjustment to a new ionic situation has been overcome and represents a state close to that *in vivo*. However, comparison of spontaneous activity *in vitro* with that *in vivo* (Figures 14, 16 and 19 as shown in Section 'A' of Chapter 3) shows that spontaneous activity averages 30-50% of the levels found *in vivo* under conditions of minimal stimulation. The main difference is that the largest action potentials present *in vivo* are absent in isolated tissue. This requires an explanation of why the spontaneous activity of metathoracic ganglion was not reproduced *in vitro* as close to that *in vivo*; whereas metabolism was close to that *in vivo*. The explanation probably lies partly in differences due to osmolarity, as in iso-osmotic saline, the spontaneous activity was 60-75% as compared to that *in vivo*. Although the spontaneous activity was significantly higher, in iso-osmotic saline, as compared to that in hypo-osmotic saline, the oxygen uptake is still the same both in hypo- and in iso-osmotic salines (Figure 25, Section 'B' of Chapter 3). It is likely, that in hypo-osmotic saline, the oxygen uptake by ganglia fulfils the demand for energy required by osmotic work and by the Na^+/K^+ electrogenic pump. On

the other hand, in iso-osmotic saline, the same oxygen uptake only fulfils the demand of energy required by the Na^+/K^+ electrogenic pump and hence an increased operation of this pump and increased transmission of impulses were obtained. This certainly gives an explanation for why higher spontaneous activity was obtained in iso-osmotic saline.

(d) This period of incubation (180-210 min), Figure 20, Section 'B' of Chapter 3) is marked by a dramatic fall in ATP and arginine phosphate levels. The spontaneous activity ceased during this time and hence a direct relationship between spontaneous activity and high energy phosphates is still attained (described previously). It is possible that the transmission of impulses and motor output of the oscillator stopped, during this time, due to lack of ATP. The reason for this low concentration of ATP and arginine phosphate is not explicable. However, the supply of fuel and oxygen cannot be the cause for this dramatic fall in high energy phosphates, as there are sufficient amounts of glucose circulating in the medium and the medium was in equilibration with 100% oxygen. It may be that a change in pH of the medium caused by a release of CO_2 and formation of lactate by the tissue, would have affected the tissue <u>in vitro</u>. However, a low rate of lactate production (Figure 24, Section 'B', Chapter 3) is unlikely to cause any change in pH in the medium. In addition to this the pH measurement of the medium was made after 3½ hours and it was not significantly different from

the initial pH of the medium.

In conclusion, the endogenous spontaneous activity of the metathoracic ganglion showed four patterns during incubation which are correlated with the patterns of carbohydrate metabolism. The different patterns of spontaneous activity may be due to variation in transmissions of nerve impulses modulated by the Na^+/K^+ electrogenic pump dependent on ATP. The steady state of the spontaneous activity was achieved after 50 min, the initial period of incubation, and it lasted for 3½ hrs and this subsequent activity is close to that *in vivo* while using iso-osmotic saline. The effect of various alternative conditions of incubation on the spontaneous activity could be tested during this period.

The present results of measurements of spontaneous activity are not in line with the previous results of Weight, (1958), working on the cockroach metathoracic ganglion, who found that the spontaneous activity of the metathoracic ganglion as recorded from the N5 nerve, after cutting all the connectives, was much higher than the electrical activity via the N5 nerve, while the ganglion was intact. He concluded that the trigger responsible for the motor output is inhibited by some type of control located in the cerebral ganglia. Once the connection between the cerebral ganglion is abolished, an increase in spontaneous activity occurs due to release of inhibition. In the present results, the spontaneous activity was about 25-40% less than that *in vivo*, and no stimulation was found. It may be that the

oscillator present in the metathoracic ganglion of the locust is stimulated by the cerebral ganglia or that sensory inputs have a stimulatory effect on the oscillator. Therefore, in the absence of all the sensory and motor impulses, the motor output activity of the oscillator was decreased and this is why less activity was observed. The question arises of why locust metathoracic ganglion activity was different from that of the cockroach. The answer is probably due to

(a) Weiant (1958) used haemolymph as the bathing medium, whereas in the present work artificial medium was used. It may be that locust tissue behaved differently in this saline.

(b) The difference could be due to a difference in the insect used.

It may be that a neural model responsible for this motor output can be proposed, based on the present findings of spontaneous and electrical activity.

In Vivo:

Electrical activity = motor output of genetically set oscillator + efferent output due to sensory input impulses

OR

Electrical activity = increased motor output due to stimulation of some centres present in cerebral ganglia.

In Vitro:

Spontaneous activity = motor output of the oscillator

OR

Spontaneous activity = decreased motor output of the genetically set oscillator due to release of stimulation of cerebral ganglia.

Finally, a comparison can be made between the behaviour in vitro of the locust nervous tissues, and that of the most documented mammalian preparations. These are the superior cervical ganglion of the rat and guinea-pig cerebral cortex slices. The most obvious difference is the possession by the insect ganglia of a high level of spontaneous activity, resembling in this respect the isolated brain of the frog (Libet & Gerard, 1939), and goldfish (Adrian and Buytendijk, 1939). This removes the need for electrical stimulation. Although less controllable than artificially induced activity, it has long been known (in the cockroach) to respond in a consistent way to pharmacological agents (Roeder and Roeder, 1939). Other workers have made use of the spontaneous activity of the isolated nervous system for pharmacological studies (Finlayson & Osborne, 1970, 1977). The present results (Table 18, Section 'D', Chapter 3) have confirmed that such agents as phenobarbitone and amphetamines affect the rate of firing of isolated metathoracic ganglia.

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