THE ANAEROBIC METABOLISM OF THE COMMON SHORE CRAB, *CARCINUS MAENAS* (L.).

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Declaration.

I hereby declare that this thesis represents, except where a note is made to the contrary, work carried out by myself. It has not been previously submitted for any degree.

Andrew D. Hill

3rd September, 1989.

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ABBREVIATIONS.

The abbreviations recommended by the Biochemical Journal in its 'Instructions to Authors' (Biochem. J. (1987) 241, 1-24) have been used with the following additions:

AEC	adenylate energy charge.
Cco_2	concentration of total carbon dioxide (mM).
cpm	counts per minute.
dpm	disintegrations per minute.
GAP	glyceraldehyde 3-phosphate.
G-6-P DH	glucose-6-phosphate dehydrogenase.
GTP	guanosine triphosphate.
H/P sugars	hexose/pentose sugars.
HK	hexokinase.
HPLC	High Performance Liquid Chromatography.
$_kHO_2$	oxycaloric equivalent.
LDH	lactate dehydrogenase
LT_{50}	the time after which 50 % of the sample animals had died.
Mo_2	rate of weight specific oxygen consumption.
MK	myokinase.
$P_{a}o_2$	partial pressure of postbranchial haemolymph.
PCA	perchloric acid.
Pc	the critical oxygen tension at which respiratory independence is lost
Pco_2	partial pressure of carbon dioxide.
PEP	phosphoenolpyruvate.
PFK	phosphofructokinase.
PK	pyruvate kinase.
Po_2	partial pressure of oxygen.
RT	retention time.
S.D.	standard deviation.
SF	solvent front.
SID	strong ion difference.
TCA	tricarboxylic acid cycle.
$_tQ$	heat dissipation.
UMBSM	University Marine Biological Station Millport.
1,3-DPG	1,3-diphosphoglycerate (recently changed to 1,3-bisphosphoglycerate).

PLEASE NOTE: - Due to the limitations of the word-processing system used to produce this thesis, it has not been possible to print the character 'μ'. Because of this, the abbreviations umole; ul; ug and uJ have been used throughout the thesis to represent micromoles, microlitres, micrograms and microjoules respectively.

CHEMICALS.

All chemicals used in this study, unless listed below, were purchased from:

Sigma Chemical Co. Ltd.,
Fancy Rd.,
Poole,
Dorset, BH17 7TG.

Chemical	Supplier
D-[U-^{14}C]-glucose L-[U-^{14}C]-lactate	Amersham International plc, Amersham Laboratories, White Lion Rd., Amersham, Bucks, HP7 9LL.
Calcium nitrate Perchloric acid Phosphoric acid Citric acid Acetic acid Lanthanum chloride GTP	BDH Chemicals Ltd., Broom Rd., Poole, Dorset, BH12 4NN.
Nitrogen Carbon dioxide	BOC Ltd., Great West House, Brentford, Middlesex.
Tropic Marin	Tropical Marine Centre, Borehamwood, Herts.
pH precision buffers (S1500 & S1510)	Radiometer A/S, Emdrupve J.72, DK-2400, Copenhagen, Denmark.

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ABSTRACT.

The object throughout this study has been to investigate the importance of anaerobic metabolism in the decapod crustacean, *Carcinus maenas* (L.), as a result of exposure to environmental anoxia. An integrated approach was therefore adopted, spanning the subjects of biochemistry, physiology and field ecology.

The study was initiated by an investigation into diel and seasonal environmental extremes that occur within intertidal rock pools. It was found that the diel ranges shown by the various physico-chemical factors become larger during the summer months. The fluctuations in the partial pressure of dissolved gases within the rock pools were the result of algal photosynthesis elevating the oxygen levels during the day and respiration of the pools' fauna and flora, in the absence of photosynthesis, causing a depletion at night. This depletion was most pronounced during the summer, when the Po_2 of the rock pool, used in the field experiments, decreased to approximately 5 Torr.

Studies investigating the distribution of *Carcinus maenas* throughout the year, revealed that far greater numbers of crabs (mainly male), were present in exposed intertidal rock pools during the summer than during the winter. This peak in the number of crabs present on the littoral zone coincided with the time of year that the pools became most hypoxic. Severe hypoxia (Po_2 < 10 Torr) lasted up to 4 h and required the crabs to either employ a behavioural response to avoid these extreme conditions or to resort to anaerobic metabolism.

Field observations showed that, in common with several other intertidal species of decapod crustaceans, *Carcinus maenas* exhibited partial and full emersion responses, when exposed to hypoxic or anoxic conditions in rock pools. Laboratory experiments demonstrated that under conditions of severe hypoxia, the concentration of L-lactate increased in fully immersed crabs, but no accumulation was observed in those crabs

that had become partially or fully emersed. The possible advantages of these three different behavioural responses to environmental anoxia were briefly considered.

There are situations, however, in which either the crab is not able to become emersed (e.g. deep rock pool with steep walls) or in which it would be dangerous to do so (e.g. high predation risks). The physiological responses which enable *Carcinus maenas* to survive exposure to hypoxia were investigated. An increase in the frequency of beating of the scaphognathites (hyperventilation) during mild hypoxia, resulting in an increase of the ventilatory flow, was implied from measurements of the pH and Pco_2 of postbranchial haemolymph. Once the environmental Po_2 decreased to below the critical oxygen tension (Pc point), the heart rate declined and the crabs were unable to maintain a rate of oxygen uptake that was sufficient to sustain the normal rate of oxygen consumption.

As the degree of hypoxia became more severe, the biochemical responses associated with hypoxia and anoxia assumed a greater importance. The concentration of L-lactate started to increase, indicating the utilisation of anaerobic metabolism. During the early stages of severe hypoxia the transphosphorylation of ADP by phospho-l-arginine was observed to supplement the glycolytically derived energy.

Anaerobic metabolism is energetically very wasteful, owing to the incomplete oxidation of the carbohydrate precursor, resulting in a very much lower efficiency of ATP production than under aerobic conditions. If an animals' anoxic energy demand remains at the pre-anoxic rate, then the rate of glycolysis must be enhanced (i.e. 'Pasteur effect'), which would rapidly deplete the carbohydrate pool. The need for this Pasteur effect could be overcome by reducing the energy demand of the animal. In the present study, after 2 hours of anoxia, the metabolic rate in *Carcinus maenas* had decreased to 16 % of the rate under aerobic conditions. It appears that this reduction in metabolic rate is probably the most important response of *Carcinus maenas* to explain the crabs' high anoxia-tolerance. Although the

mechanism by which this depression occurs was not investigated in the present study, a brief review in Chapter 7 summarises some of the more recent work on this subject.

Although L-lactate undoubtedly accumulates during anaerobic metabolism, there had been no previous comprehensive study to investigate the occurrence of other anaerobic pathways in decapod crustaceans. A major aim of the present study was therefore to establish their relative importance. A stoichiometric comparison revealed that approximately 90 % of the catabolism of the carbohydrate pool (mainly glycogen and oligosaccharides), associated with exposure to anoxia, could be explained by the accumulation of L-lactate. High Performance Liquid Chromatography indicated that, despite a small increase in the concentration of fumarate in response to exposure to anoxia, organic acids were unimportant anaerobic end products in decapod crustaceans. Amino acids were also found to be of minor importance. Further evidence confirming the dominance of L-lactate as the only major anaerobic end product in decapod crustaceans, was obtained from radiolabelling experiments, in which D-[U-^{14}C]-glucose was observed to become rapidly incorporated into a 'weak acid' fraction, consisting mainly of L-lactate. A comparative review of the anaerobic pathways present in other invertebrate groups was also presented.

One of the initial aims of this project was to determine the fate of L-lactate following a period of exposure to anoxia. The existence of a gluconeogenic pathway was indicated, since a stoichiometric comparison revealed that about 89 % of the carbohydrate (mainly glycogen and oligosaccharides) accumulating during recovery could be explained by the depletion of L-lactate. This precursor/product relationship was later confirmed when it was demonstrated that L-[U-^{14}C]-lactate was incorporated into glycogen and later into amino acids during recovery. In addition, radioactivity from L-[U-^{14}C]-lactate, was observed to become

incorporated into carbon dioxide and bicarbonate, indicating the complete oxidation of L-lactate as a possible means of the elimination of this anaerobic end product. No excretion of labelled L-lactate by *Carcinus maenas* during recovery was detected. It appears, therefore, that end product elimination in decapod crustaceans relies mainly on the re-metabolism of the initial precursors, and to a limited extent, the complete oxidation of L-lactate to carbon dioxide. These results were found to be consistent with those reported in the literature.

Of particular interest in the present study, was that, in addition to the typical physiological and behavioural responses, the concentration of L-lactate in *Carcinus maenas* was observed to double during the first hour of recovery. Indirect calorimetric measurements indicated that the energy produced from this rapid accumulation of L-lactate, accounted for approximately 22 % of the total energy produced over this period. The possible reason for this anaerobic component of energy metabolism during this period was discussed.

CHAPTER 1 - GENERAL INTRODUCTION.

A prerequisite for life is a continual supply of energy, in the form of ATP molecules. Until the mid 1940's it was believed that, in multicellular tissues, energy was in general only produced by aerobic metabolic pathways. It has become apparent, however, that at certain times, some animals are unable to meet their energy requirements purely by the complete oxidation of carbohydrates, fats and proteins to CO_2 and H_2O. At such times, animals have to rely partly or exclusively on anaerobic metabolism.

Amongst air-breathing animals, anaerobic metabolism is usually confined to particular tissues that have been unable to meet, by aerobic metabolism, a transient increase in energy demand. This often occurs in actively contracting muscle fibres, in which the rate of oxygen metabolism is greater than the rate of oxygen delivery ('Functional anaerobiosis'). Aquatic animals experience anoxia more often than air breathers, since water contains far less oxygen per unit volume than air. Aquatic animals are, therefore, periodically exposed to hypoxic conditions that are potentially very serious ('Environmental anaerobiosis'). Although problems associated with exposure to hypoxia are experienced by all the major groups of invertebrates and vertebrates, the present study has been restricted to an investigation of the decapod crustaceans.

Decapod crustaceans encounter environmental hypoxia in a variety of habitats, e.g. organically enriched freshwater rivers; intertidal rock pools exposed at low tide (Truchot & Duhamel-Jouve, 1980; Taylor & Spicer, 1987) and the permanent burrows of the Thalassinidea (Atkinson & Taylor, 1988). Comparative studies have shown that those species that regularly live in these habitats, tend to demonstrate clear adaptive responses that enable them to survive exposure to hypoxic conditions (Teal & Carey, 1967; Pritchard & Eddy, 1979; Taylor & Spicer, 1987; Anderson,

1989). These adaptive responses can be divided into three separate functional levels - i) behavioural, ii) respiratory and iii) metabolic.

Behavioural responses may simply involve the movement of an animal away from an environment that is becoming progressively more hypoxic. Since many decapod crustaceans are capable of rapid locomotor activity, one might expect that this avoidance behaviour would be of great importance. In the intertidal rock pool species *Palaemon elegans* and *Carcinus maenas*, for example, partial and full emersion into the air has been observed, during periods of severe hypoxia, enabling the animals to respire aerially (Bohn, 1897; Taylor *et al.*, 1973; Taylor & Spicer, 1987). Willis & Berrigan (1977) observed a similar response in the freshwater *Macrobrachium rosenbergii*. There are occasions, however, when such an avoidance strategy is inappropriate (e.g. high predator risk) or impossible (e.g. deep steep sided rock pools preventing any type of emergence).

Despite early studies, which showed that many species had a poor oxyregulatory ability (reviewed by Wolvekamp & Waterman, 1960), more recent studies have shown that decapod crustaceans generally have considerable abilities to maintain respiratory independence, over a range of ambient oxygen tensions (Taylor & Butler, 1978; McMahon & Wilkens, 1983). The mechanisms by which this is achieved have been the subject of much research, involving the measurements of oxygen consumption and frequency of scaphognathite and heart beat (Taylor, 1976; Burnett, 1979: Bradford & Taylor, 1982; Anderson, 1989). The effectiveness of these respiratory responses have been assessed by measuring the Po_2 of post- and pre-branchial haemolymph, together with studies of the oxygen transport properties of the haemolymph (McMahon & Wilkens, 1975; Butler *et al.*, 1978). Respiratory independence, however, can only be maintained down to a critical oxygen tension (Pc), since below this environmental Po_2, maintaining the postbranchial haemolymph (P_aO_2) becomes energetically impractical. The Pc of decapod crustaceans varies considerably, with lower values occurring amongst the more

anoxia-tolerant thalassinids, than in other decapod species (Thompson & Pritchard, 1969; Felder, 1979; Hagerman & Uglow, 1985; Bradford & Taylor, 1982; Anderson, 1989). Under conditions of mild hypoxia, the frequency of heart and scaphognathite beat typically increases (Arudpragasam & Naylor, 1964; Morris & Taylor, 1985; Johnson & Uglow, 1987). If the environmental Po_2 decreases below the Pc point, however, the scaphognathite and heart rate decline, resulting in a reduction of the P_ao_2 (Taylor *et al.*, 1973, 1977; Taylor, 1976; Wheatly & Taylor, 1981). At this point the rate of oxygen consumption can no longer be maintained and animals begin to respire anaerobically.

More recently, attention has turned to the role of the haemolymph in oxygen transport. In particular, there has been considerable interest in the role of organic modulators of oxygen affinity. It has been shown, for example, that the hypoxia-induced increase in the concentration of L-lactate, increases the oxygen affinity of the haemocyanin, which, at least in functional anaerobiosis improves the ability of the haemolymph to take up oxygen (Truchot, 1980). Currently, research has centred on other modulators of oxygen affinity, including organic acids, urate (a product of purine catabolism) and more recently neurohormones (Booth *et al.*, 1982; Graham *et al.*, 1983; Bouchet & Truchot, 1985; Morris *et al.*, 1985; Morris & McMahon, 1989). As a result, our understanding of the modulation of the oxygen transport properties of the respiratory pigment is increasing rapidly.

Studies of the metabolic responses of decapod crustaceans to environmental hypoxia, have suggested that L-lactate is a major end product of anaerobic metabolism. An accumulation of this metabolite in haemolymph and tissue samples during hypoxic conditions has been reported by many authors (Teal & Carey, 1967; Pritchard & Eddy, 1979; Bridges & Brand, 1980; Zebe, 1982; Gäde, 1984; Albert & Ellington, 1985; Lowery & Tate, 1986; van Aardt, 1988). As hypoxia becomes progressively more severe, so anaerobic metabolism becomes increasingly important,

with greater rates of L-lactate accumulation. A secondary source of energy involves the transphosphorylation of ADP by phospho-l-arginine during the initial stages of exposure to hypoxia (Onnen & Zebe, 1983; Gäde, 1983).

There is a considerable literature on the responses of decapod crustaceans to hypoxic conditions. In contrast, however, there have been few studies of the respiratory and metabolic responses to very severe hypoxia and anoxia. The reason for the paucity of such studies is unclear, but is probably due to the fact that exposure to anoxia in the field has previously been regarded as very rare. There is growing evidence to suggest, however, that this may not be the case. Deep fjordic water in certain of the sea lochs of the west coast of Scotland is known to become anoxic during the summer months (Edwards & Edelsten, 1977; C.J. Chapman, pers. comm. to A.C. Taylor). Pollution-generated events in the Kattegat and Skagerrak, resulting in the water becoming anoxic have already led to a crash of a demersal fishery (Hagerman & Baden, 1988; S.P. Baden, pers. comm. to A.C. Taylor). Additionally, Truchot & Duhamel-Jouve (1980) and Taylor & Spicer (1987) have reported that exposed intertidal rock pools can become anoxic at night during the summer.

The broad aims of this study were therefore to investigate the anaerobic metabolism of decapods, exposed to environmental anoxia. The rock pool environment was chosen, because of easy access and also that it has been previously shown to become anoxic at night during the summer. The common shore crab, *Carcinus maenas*, was used since it is abundant in intertidal pools, easy to maintain in aquariums and there is already a great deal of information on many aspects of its respiratory physiology (Taylor, 1976; Cumberlidge & Uglow, 1977; Taylor & Wheatly, 1979; Truchot & Jouve-Duhamel, 1980).

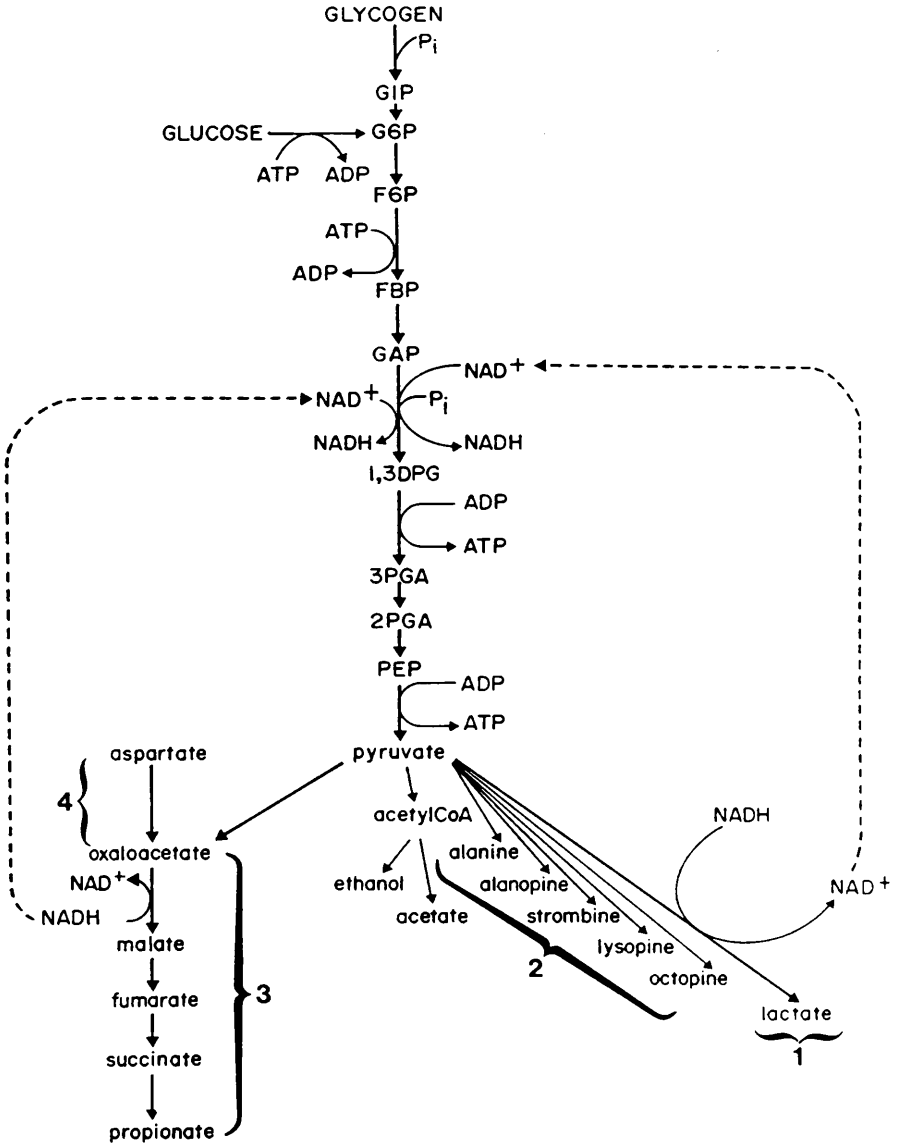
The primary aim of the study was to determine the extent of the exposure of *Carcinus maenas* to severe hypoxia and anoxia and the importance of anaerobic metabolism in the field. In addition, the possible effect of seasonal variations in

energy metabolites was investigated.

Although L-lactate undoubtedly accumulates during anaerobic metabolism, there has been no previous comprehensive study to investigate the occurrence of other anaerobic pathways in decapod crustaceans. A major aim of the present study was therefore to establish their relative importance. Currently, the most comprehensive review of the anaerobic pathways is that by Livingstone (1983). In this review he proposes four main groups of pathways, and by referring to Figure 1.1 it is possible to see how the various pathways fit into the overall glycolytic system. The first three groups all use glucosyl units (the constituent unit of glycogen and oligosaccharides) exclusively as their initial substrate, whilst the last pathway utilises amino acids as well. The best known of the pathways is that ending in L-lactate. Lactate dehydrogenase (EC 1.1.1.28) catalyses the reaction that oxidises NADH to NAD^+, whilst it reduces pyruvate to L-lactate. The second group of pathways result in the formation of opines. These are the condensation products of pyruvate with specific amino acids. Opines are weaker acids than L-lactate and therefore cause less disturbance to the acid-base balance of the organism concerned. The third group of pathways involve a reversal of part of the Tricarboxylic acid cycle (TCA cycle). Phosphoenolpyruvate (PEP) is converted to oxaloacetate by phosphoenolpyruvate carboxykinase (EC 4.1.1.49), whilst pyruvate can be converted to the same compound by pyruvate carboxylase (EC 6.4.1.1). The oxaloacetate is then reduced to malate, with the concurrent oxidation of NADH to NAD^+ which maintains the redox balance of the cell. The end products of these pathways are the organic acids: malate, succinate, fumarate and propionate.

The final pathway involves the amino acid aspartate which is transaminated by glutamate-oxaloacetate transaminase (EC 2.6.1.1) to oxaloacetate, in the presence of pyruvate and NADH. The pathways involving the production of the organic acids yield larger amounts of ATP molecules per mole of substrate, as compared with the

FIG. 1.1 Major anaerobic pathways present in the Animal Kingdom (from Hochachka & Somero, 1984). Numbers refer to the four main groups of end products (see text for further details).


GLYCOGEN
Pi
GIP
GLUCOSE
G6P
ATP
ADP
F6P
ATP
ADP
FBP
GAP
NAD+
NAD+
Pi
NADH
NADH
1,3DPG
ADP
ATP
3PGA
2PGA
PEP
ADP
ATP
aspartate
pyruvate
4
acetylCoA
NADH
oxaloacetate
alanine
ethanol
alanopine
NAD+
NAD+
NADH
acetate
strombine
malate
lysopine
2
octopine
fumarate
3
lactate
succinate
1
propionate

pathways that culminate in L-lactate and opines, but the rate at which they do so is somewhat lower. Therefore the organic acid pathways are found in those organisms that experience long term oxygen stress, whilst the lactate forming pathway tends to be favoured in organisms that require rapid bursts of energy over short periods of time.

The comparative study of the various anaerobic pathways present in the Animal Kingdom, was really initiated in the 1940's, when von Brand (1946), who working on endoparasitic worms, found that volatile fatty acids were the end products of glycogen metabolism. Further studies revealed that propionate and acetate were produced in gastropods (von Brand *et al.*, 1950) and in the liver fluke, *Fasciola hepatica* (Lahoud *et al.*, 1971). In the mid 1960's alanine and succinate were discovered to be the principle end products of the clam, *Rangia cuneata* (Simpson & Awapara, 1966). In more recent years the proliferation in the study of potential anaerobic end products, has led to the discovery of a wide range of metabolites in several groups of invertebrates. In the cestode and trematode Platyhelminthes, anaerobic metabolism culminates in the production of mainly L-lactate and succinate. In addition, sessile bivalves accumulate succinate, propionate and alanine (de Zwaan & Zandee, 1972a; Kluytmans *et al.*, 1975; Zebe, 1975), whilst, swimming bivalves and cephalopods produce predominately octopine as their anaerobic end product (Gäde, 1980; Livingstone *et al.*, 1981). Much of the present study is directed to determining whether or not any of these other anaerobic end products are present in decapod crustaceans.

Of fundamental importance to an investigation of the consequences of anaerobic metabolism in decapods, is an understanding of the fate of L-lactate, during the post-anoxic/hypoxic recovery period. In contrast to the extensive literature concerning the accumulation of L-lactate in crustaceans, there is little information on the elimination of this end product (Phillips *et al.*, 1977; Gäde *et al.*, 1986). Therefore the fate of L-lactate during recovery in *Carcinus maenas* was investigated

in the present study, using ion exchange chromatography and radiolabelling techniques.

A more complete understanding of the post-anoxic recovery period could only be obtained by comparing the metabolic data with certain respiratory measurements. Consequently, oxygen consumption ($\dot{M}o_2$), heart rates and certain acid-base parameters were determined, not only during the recovery period, but also throughout the preceding exposure to anoxia. In addition, calorimetric measurements of heat dissipation values were made, to investigate the possibility of a metabolic depression during anoxia and also to help provide further information on post-anoxic recovery.

CHAPTER 2 - FIELD BASED INVESTIGATIONS INTO THE ANAEROBIC METABOLISM OF *CARCINUS MAENAS.*

2.1 INTRODUCTION.

The primary aim of this project was to carry out a detailed investigation into the anaerobic metabolism of the shore crab, *Carcinus maenas.* Although the present study was confined to an investigation of this species, it was felt that the results would apply to other decapod crustaceans. Before any detailed biochemical and physiological investigation of the anaerobic metabolism of *C. maenas* was carried out, it was important to study certain aspects of the behaviour and ecology of the crab in its natural environment. In addition, it was important to establish when, and to what extent, the rock pools, in which *C. maenas* were known to be present, become hypoxic. Finally, under hypoxic conditions, did the crabs respire anaerobically or did they employ other adaptations to survive periods of hypoxia ? The results in this chapter are divided into 6 sections.

I) Measurement of seasonal and diel physico-chemical variations in the rock pool environment. Since Morris & Taylor (1983) had already carried out an extremely detailed investigation into this subject for the Isle of Cumbrae in 1982, this present study was essentially confirmatory.

II) A population study monitoring the distribution of *Carcinus maenas* in rock pools, over a 12 month period.

III) An investigation into the accumulation of L-lactate in response to various behavioural adaptations in *Carcinus maenas* during environmental hypoxia.

IV) A study determining the reliance on anaerobic metabolism of those crabs that remain totally immersed during hypoxia.

V) A seasonal study of variations in some energy metabolites of *Carcinus maenas* in an attempt to assess their role in determining the susceptibility of crabs to

hypoxic stress.

VI) Measurement of the effects of handling disturbance on the concentration of haemolymph sugars, in an effort to design a more stringent haemolymph sampling programme for later experiments.

The huge diel and seasonal variations in environmental conditions that rock pool animals experience, are areas of study that have attracted much attention. Powers (1920) and Humphrey & Macy (1930) first showed that the partial pressure of oxygen (Po_2) varied according to time of day. Gail (1919) reported that the pH in pools containing vegetation was 7.43 just before sunrise, but increased to 8.8 in late afternoon. Over the next 50 years there were regular studies, investigating variations in temperature, salinity, pH and Po_2 (Klugh, 1924; Johnson & Skutch, 1928; Stephenson *et al.*, 1934; Pyefinch, 1943; Naylor & Slinn, 1958; Daniel & Boyden, 1975; Taylor, 1988 for review). The more recent work of Truchot & Duhamel-Jouve, (1980) and Morris & Taylor, (1983), in addition to measuring the above parameters, have concentrated on measuring the partial pressure of CO_2 and other components of the carbonate system. Many authors have reported naturally occurring severe hypoxia, in middle to high shore rock pools (Truchot & Jouve-Duhamel, 1980; Morris & Taylor, 1983; Agnew & Taylor, 1986; Taylor & Spicer, 1987). Severe hypoxia occurs at night during the summer, when the pools are uncovered and there is a greater biomass of actively respiring plants and animals, as compared with the pools in winter and spring. Since the water is not being reoxygenated at night by photosynthesis, the pools can often become severely hypoxic.

Various authors have described seasonal and diel differences in the distribution of *Carcinus maenas* in the intertidal zone (Edwards, 1958; Naylor, 1962; Crothers, 1968; Atkinson & Parsons, 1973). It is clear from the work of these authors that there are considerable differences between different populations of these crabs. A

knowledge, specific to the Kames Bay population, of all these variations is therefore essential if one is to be able to predict the extent to which these crabs are likely to be exposed to severely hypoxic conditions.

Given that *Carcinus maenas* does occur in hypoxic rock pools, it has been shown to exhibit a variety of behavioural responses. These were first reported in 1897 by Bohn, who observed that the crab moved into the shallow regions of the pools and adopted a raised posture resulting in the anterior part of the body being held clear of the surface of the water (Fig. 2.1). This has become known as the partial emersion response and has previously been reported in the freshwater prawn *Macrobrachium rosenbergii* (Willis & Berrigan, 1977) and in the prawn *Palaemon elegans* (Taylor & Spicer, 1987). *C. maenas* has also been observed to leave the hypoxic water completely and to start respiring aerially (Taylor *et al.*, 1973). In this study, the crabs were observed to exhibit these responses in the field. An experiment was carried out to determine the extent of L-lactate accumulation, associated with each of these responses.

Before the above experiment could be carried out, the effects of handling disturbance needed to be assessed. This was best done by monitoring haemolymph sugar concentrations, since it has been widely reported that the action of handling can cause a hyperglycaemic response (Florkin & Duchateau, 1939; Abramowitz *et al.*, 1944; Riegel, 1960; Lynch & Webb, 1973; Telford, 1973). Since circulating haemolymph sugars are more generally available to all tissues for glycolysis than their storage compounds, which may be unevenly distributed (e.g. glycogen), hyperglycaemia is regarded as an adaptation to rapidly produce energy, in excess of the basal rate. If seasonal and oxygen stress-induced variations were going to be separated from those caused by handling, it was essential to determine the extent of this hyperglycaemic response in *Carcinus maenas*. The results from this experiment could then be used to design a controlled haemolymph sampling programme.

FIG. 2.1 Partial emersion of *Carcinus maenas* in response to severe hypoxia. The crab moves into the shallow regions of the pool and adopts a raised posture resulting in the anterior part of the body being held clear of the water. The scaphognathite beat is reversed and the air enters the branchial chamber via the anterior openings on either side of the mouth.

Under certain circumstances, rock pool decapods will be forced to remain fully immersed in an hypoxic pool. An *in situ* study by Taylor & Spicer (1987), showed that under such conditions L-lactate accumulated in the prawn, *Palaemon elegans* (Rathke). The aim of this study was to investigate whether *Carcinus maenas* similarly accumulated L-lactate when exposed to severe hypoxia. The study was carried out using the facilities of the University Marine Biological Station Millport (UMBSM) on the Isle of Cumbrae, Scotland.

Since glycogen is the major endogenous substrate for glycolysis in crustaceans (Livingstone, 1983) any tissue variations in its concentration, may have an important effect on the animal's ability to tolerate environmental stress. Although diel changes in various energy metabolites of crustaceans have been extensively studied, there has been relatively little work on seasonal changes (Barnes *et al.*, 1963; Heath & Barnes, 1970). Heath & Barnes studied *Carcinus maenas* collected from Millport, but fairly large crabs (carapace width = 60 $\pm$ 3.0 mm) were used and only the hepatopancreas and the ovary were monitored. A monitoring programme was therefore carried out to examine both pooled tissue samples and haemolymph samples from specimens collected from intertidal rock pools.

2.2 MATERIALS AND METHODS.

2.2.1 Seasonal changes in the distribution of *Carcinus maenas* and in the physico-chemical parameters of the rock pool environment.

A study of the *Carcinus maenas* population of the rock pools at Kames Bay was carried out every month for a period of 12 months. Once a month, 1 h was spent collecting *C. maenas* (L.) by hand from 2 adjacent intertidal rock pools, on a rocky promontory, to the West of Kames Bay on the Isle of Cumbrae, Firth of Clyde, Scotland. As it was impossible to remove and count all the crabs within a pool each month, this method was used, since it gave a good indication of the relative numbers of crabs present in the pools. This procedure assumed that the collecting ability of the sampler was constant throughout the year and that a similar proportion of the total number of crabs in the pools was being collected each month. Since this is unlikely, the results of the study can only be used to give an indication of the changes in the crab population during the year. This survey was always carried out immediately after the pools had become uncovered, by the falling tide. The rock pool water temperature was measured using a mercury thermometer. Salinity was determined using a hand held, portable refractometer (Atago, Japan). The crabs were then transferred to the UMBSM, where the following observations and measurements were made:

a) Fresh Weight: The fresh weight of individual crabs was measured, by blotting off as much sea water as possible using tissue paper, and then weighing on a top-pan balance, accurate to 0.01 g (Sartorius).

b) Carapace Width: The maximum width across the carapace was measured to the nearest mm using a pair of vernier callipers,

c) Sex Ratio: Crabs whose maximum carapace width was over 15 mm were sexed by examining the shape of their abdomen. Individuals

smaller than 15 mm were recorded as being 'juvenile' (Crothers, 1967).

Before any field investigation using *Carcinus maenas* could be carried out, it was important to determine whether the rock pools actually became naturally severely hypoxic. Truchot & Jouve-Duhamel (1980) and Taylor & Spicer (1987) reported that the Po_2 of rock pools became severely hypoxic during the night in summer. Therefore the Po_2 of a rock pool near the UMBSM was measured, using an oxygen electrode (E5046 Radiometer, Denmark) connected to a portable battery-powered oxygen meter (Strathkelvin Instruments, Glasgow). Nights were chosen when the pool had been exposed for long periods during the hours of darkness.

2.2.2 Effects of handling disturbance on the concentration of haemolymph sugars and L-lactate.

Carcinus maenas (L.), were collected by hand from intertidal rock pools as described in section 2.2.1. These animals were transported to the Department of Zoology, University of Glasgow, in plastic tanks containing sea water. The animals were then transferred to large tanks, in a recirculating sea water aquarium, maintained at 10 $\pm$ 1°C. They were fed on mussels, *Mytilus edulis* during the first week, and starved for the second week. Some half-tiles were introduced into the tanks, to provide shelter and to reduce the incidence of the aggressive interactions which are sometimes observed between individuals. Only intermoult male animals (fresh wt. range = 2 - 25 g) were used in the experiments.

The experiment was carried out in a constant temperature room (10 $\pm$ 1°C), in which the crabs were exposed to a 12:12 light:dark cycle. Artificial sea water (salinity = 30 ‰), made up from sea salt (Tropic Marin) and distilled water was used throughout, and always allowed to equilibrate to 10°C before use. Crabs were placed in plastic tanks (volume = 10 l) containing fully aerated sea water and left

undisturbed for 48 h prior to the start of the experiment.

30 crabs maintained in the aquarium were transferred to 6 plastic tanks, containing aerated sea water and left undisturbed for 48 h. At the end of this time haemolymph samples (100ul) were taken from batches of 5 animals, after they had been handled for 15, 30, 45, 60, and 120 seconds. Haemolymph samples were taken using a 1 ml syringe, the needle (26 g) of which was inserted into the arthrodial membrane of the 4th walking leg. Since removing the haemolymph took approximately 10 seconds, sampling started after 5, 20, 35, 50 and 110 seconds of handling respectively. Any haemolymph sample taking more than 10 seconds to obtain was discarded. Control samples were taken from previously undisturbed crabs, within 10 seconds of handling. The haemolymph was then prepared and analysed for D-glucose, total hexose/pentose sugars and L-lactate (Appendices 1,2,4 and 5).

2.2.3 L-lactate accumulation in response to behavioural adaptations during hypoxia in rock pools.

Observations made during the night indicated that when rock pools became severely hypoxic, some crabs remained fully immersed; others came up to the shallows (partial emersion), or if conditions become even more extreme, they became fully emersed and respired aerially. It was decided, therefore, to determine whether crabs accumulated L-lactate, when exhibiting any of these behavioural responses to hypoxia. It was hoped that the experiment could be carried out in the field, but after an initial attempt, this proved to be impractical owing to disturbance amongst the partially and fully emersed crabs, caused when haemolymph samples were being taken. Consequently, all subsequent experiments were carried out under laboratory conditions at Glasgow University.

Animals were collected from the Isle of Cumbrae and kept at Glasgow University as described in section 2.2.2. The experiment was carried out in a constant temperature room (10 ± 1°C), in which the crabs were exposed to a 12:12 light:dark cycle. Artificial sea water (salinity = 30 ‰) was used throughout as previously described. Only intermoult males (fresh wt. range = 10 - 25 g) were used.

40 crabs were distributed equally between 4 plastic tanks (volume = 20 l). Each tank had previously been filled with sea water (7 litres) and left for 24 h to ensure temperature equilibration. Rocks were put into 2 of the tanks, in such a manner that they protruded above the surface of the water. This gave the crabs the option of becoming partially emersed. Polystyrene sheets were cut to fit the two other tanks exactly and floated on the surface of the water, in order to limit the absorption of atmospheric oxygen. An oxygen electrode (E5046 Radiometer, Denmark) coupled to an oxygen meter (Strathkelvin Instruments, Glasgow) was used to monitor the oxygen tension (Po_2) throughout the experiment. The Po_2 of the water was regulated using a gas mixture, produced by a precision gas mixing system, which was pumped through air-stones into each of the tanks. The crabs were left undisturbed for 24 h, during which time the water was constantly aerated. At the end of the 24 h period, the Po_2 of the water was reduced to < 1 Torr by bubbling a mixture of nitrogen and carbon dioxide into it. Carbon dioxide was added to maintain the pH of the water at 7.8 throughout the experiment.

A further 20 animals were distributed equally between 2 tanks containing no water but which were lined with filter paper soaked in sea water and covered with moistened paper towelling, to ensure the maintenance of a high relative humidity within the tanks. This procedure was used to simulate conditions in the field, when crabs become fully emersed. Finally, 8 control animals were placed in a single 10 l tank which contained fully aerated sea water.

Haemolymph samples were taken from 4 crabs in each of the 3 groups at 0, 1, 2, 3 and 4 h after the start of the experiment. Haemolymph samples from control crabs were taken at 0 and 4 h. The haemolymph was sampled as described in section 2.2.2. An equal volume of perchloric acid (0.6 M) was immediately added to each haemolymph sample to ensure that all metabolic reactions had ceased. The samples were firstly neutralised using a $^{1}/_{10}$th volume of K_2CO_3 (2 M), centrifuged at 14,000 g to precipitate the $KClO_4$ and then the supernatant stored at -20°C. Analyses for L-lactate were carried out according to methods described in Appendices 1 and 5.

2.2.4 A field investigation into the change in concentrations of L-lactate and glycogen during hypoxia.

Since it was not possible to carry out the previous experiment in the field, it was felt important to perform an *in situ* study to determine whether L-lactate accumulated in those crabs that remained fully immersed in the rock pool under hypoxic conditions. The problems experienced in disturbance of crabs whilst taking haemolymph samples, were not a problem here, since all the crabs were contained within a submerged metal cage and less prone to disturbance than those crabs that had become partially emersed and fully emersed (section 2.2.3).

A high shore rock pool (3.2 m above chart datum) was chosen, since it was regularly inhabited by *Carcinus maenas* and, being exposed for long periods, was likely to experience environmental extremes. Since there were not enough crabs occurring naturally in the experimental rock pool, others were collected from the pools in Kames Bay as described above (section 2.2.1.). Instead of being transported to Glasgow, they were transferred to outdoor holding tanks, at the UMBSM where they were maintained in a recirculating system for 48 h prior to the start of the

experiment.

24 crabs, (fresh wt. range = 2 - 25 g) were placed in a metal cage (40 cm in diameter). A number of small rocks and pieces of seaweed were also introduced into the cage, in order to provide shelter and to reduce the contact between the crabs and hence minimise any aggressive interactions. The cage was then transferred to the rock pool 12 h prior to the start of the experiment. A further 4 animals were kept in the holding tanks to act as controls. The experiment was carried out 3 times, in April and August 1987 and finally in April 1988. The results are taken from the last 2 experiments, since April 1987 was largely a pilot experiment. The timing of the sampling was dictated by the tidal cycle. In August, the sampling times were: 02.00, 06.00, 09.00, 14.30 and 20.30 h, whilst the following April they were: 01.45, 05.30, 07.15, 15.00 and 19.40 h . This sampling regime was used to ensure that samples were taken just after the pool was uncovered and also just prior to it being inundated by the rising tide. At each sampling interval 4 animals were taken for pooled tissue analysis. The control animals were taken at 14.00 h in August and 14.30 h in April. These crabs were immediately frozen in liquid nitrogen and stored at -20 ^{o}C. Tissue preparation and biochemical analysis for pooled tissue L-lactate and glycogen was carried out, according to the method described in Appendices 1,3 and 5.

The Po_2 in the rock pool was monitored throughout the 24 h period, using an oxygen electrode (E5046 Radiometer, Denmark) connected to a portable battery-powered oxygen meter (Strathkelvin Instruments, Glasgow). The water temperature was measured using a mercury thermometer. The salinity of the water in the pool was determined using a portable refractometer (Atago, Japan). Values for the Cco_2 and pH of the rock pool water, were obtained with the assistance of Dr. J.I. Spicer. The total Cco_2 content of the rock pool water was determined on duplicate 10 ul samples using the method of Cameron (1971) (Chapter 5 for further details). The pH of the rock pool was determined to the nearest 0.01 units, using a Yellow

Springs portable pH meter.

2.2.5 Seasonal changes in the energy metabolites.

Seasonal variations in the concentrations of glycogen and L-lactate were estimated in pooled tissue samples, collected over a 12 month period. In addition, the concentrations of D-glucose, total hexose/pentose sugars and L-lactate in the haemolymph were also measured.

In addition to the crabs collected in Section 2.2.1., 5 male and 5 female crabs were collected by hand and immediately dropped into a Dewar vacuum flask of liquid nitrogen. Care was taken to catch the animal as quickly as possible, to reduce stress-induced changes in metabolite concentration, which have been reported previously (Telford, 1973; Burke, 1979). Finally, haemolymph samples were taken from a further 5 male and 5 female crabs, using the method described in 2.2.2. An equal volume of perchloric acid (0.6 M) was immediately added to each haemolymph sample to ensure that all metabolic reactions had stopped and that the protein had precipitated out. In the laboratory, the tissue and haemolymph samples were stored at -20 ^{o}C. Tissue preparation and biochemical analyses were carried out according to the methods described in Appendices 1, 2, 3, 4 and 5.

2.3 RESULTS.

2.3.1 Seasonal changes in the distribution of *Carcinus maenas* and in the physico-chemical parameters of the rock pool environment.

The mean monthly temperatures of the 2 rock pools, ranged from 17.5 ^{o}C in July, to 4.6 ^{o}C in January (Fig. 2.2). The salinity reached a mean low of 28 ‰ in January and a mean high of 33 ‰ in May.

Carcinus maenas were found to be most common in intertidal pools during the summer, with maximum numbers being recorded during July (Fig. 2.3). During August, unseasonably high winds meant that collection was more difficult and the numbers probably under estimate the actual population size. Numbers of crabs in the pools decreased in the winter, with a minimum occurring during December.

During the winter months the lowest monthly mean crab weight was 1.64 g, whilst in the summer the mean increased to a maximum of 14.1 g (Fig. 2.4). This offshore migration of the larger (and hence older individuals) has been widely reported by others and is presumably a strategy to avoid the environmental extremes associated with winter. It was interesting to observe that males were more common in the pools throughout the year (Fig 2.3). Observations by divers indicated, however, that there were large numbers of female crabs in the subtidal zone of Kames Bay, throughout the year. Females were most abundant in the pools in late summer and early autumn, when it has been reported that reproductive pairings are most common (Atkinson & Parsons, 1973).

The Po_2 of the rock pool water decreased to below 10 Torr at nights during June, July and August, when the pool was exposed for over 3 h. The pool Po_2 decreased to only 109 and 88 Torr respectively, at nights during December and March.

FIG. 2.2 Seasonal variation in the mean monthly temperatures of the rock pools (□) and of the surrounding air (■).

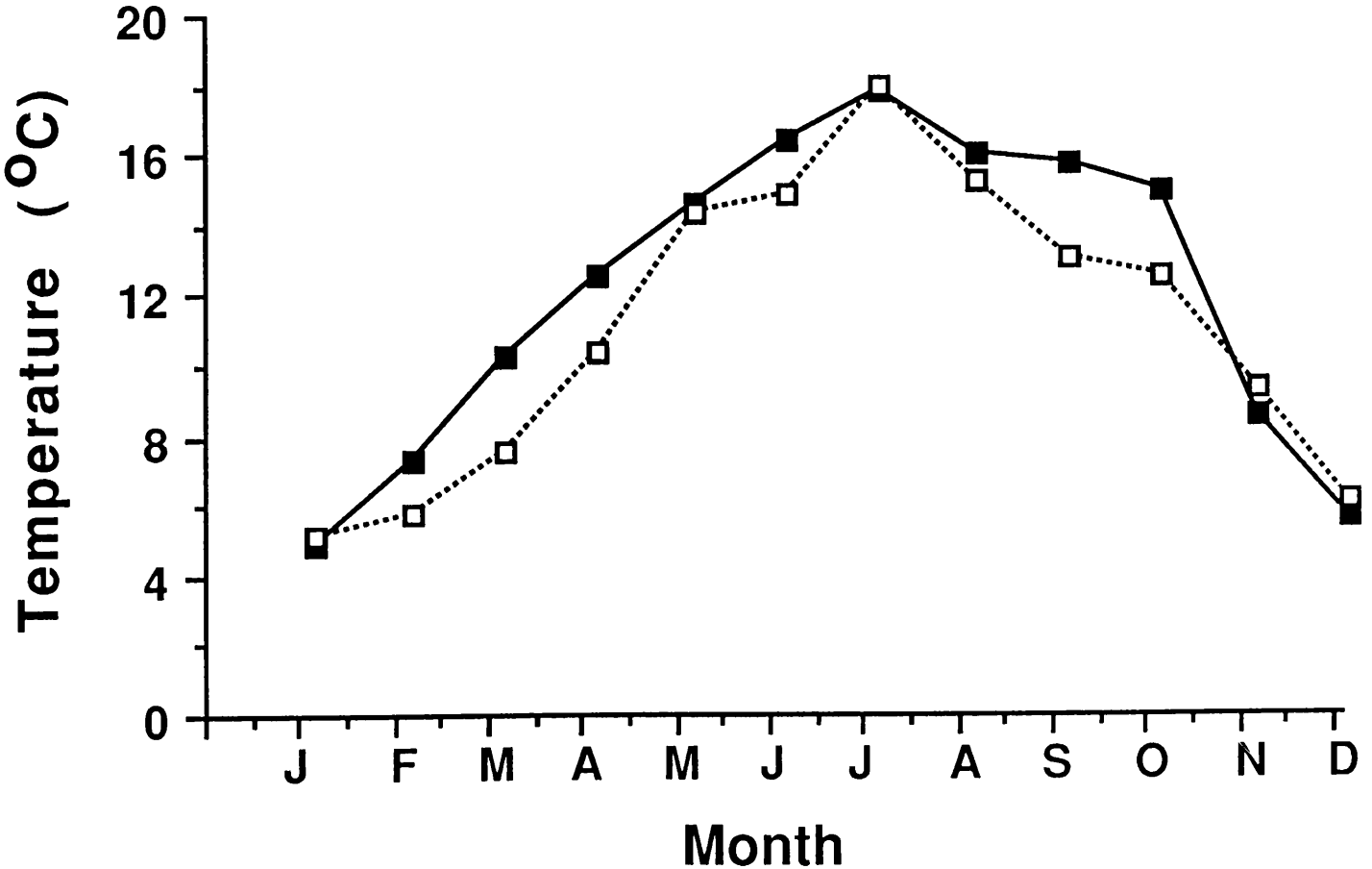

Temperature (°C)
20
16
12
8
4
0
J
F
M
A
M
J
J
A
S
O
N
D
Month

FIG. 2.3 Seasonal variation in the number of male (▨) and female (□) crabs present in the 2 rock pools sampled over a 12 month period.

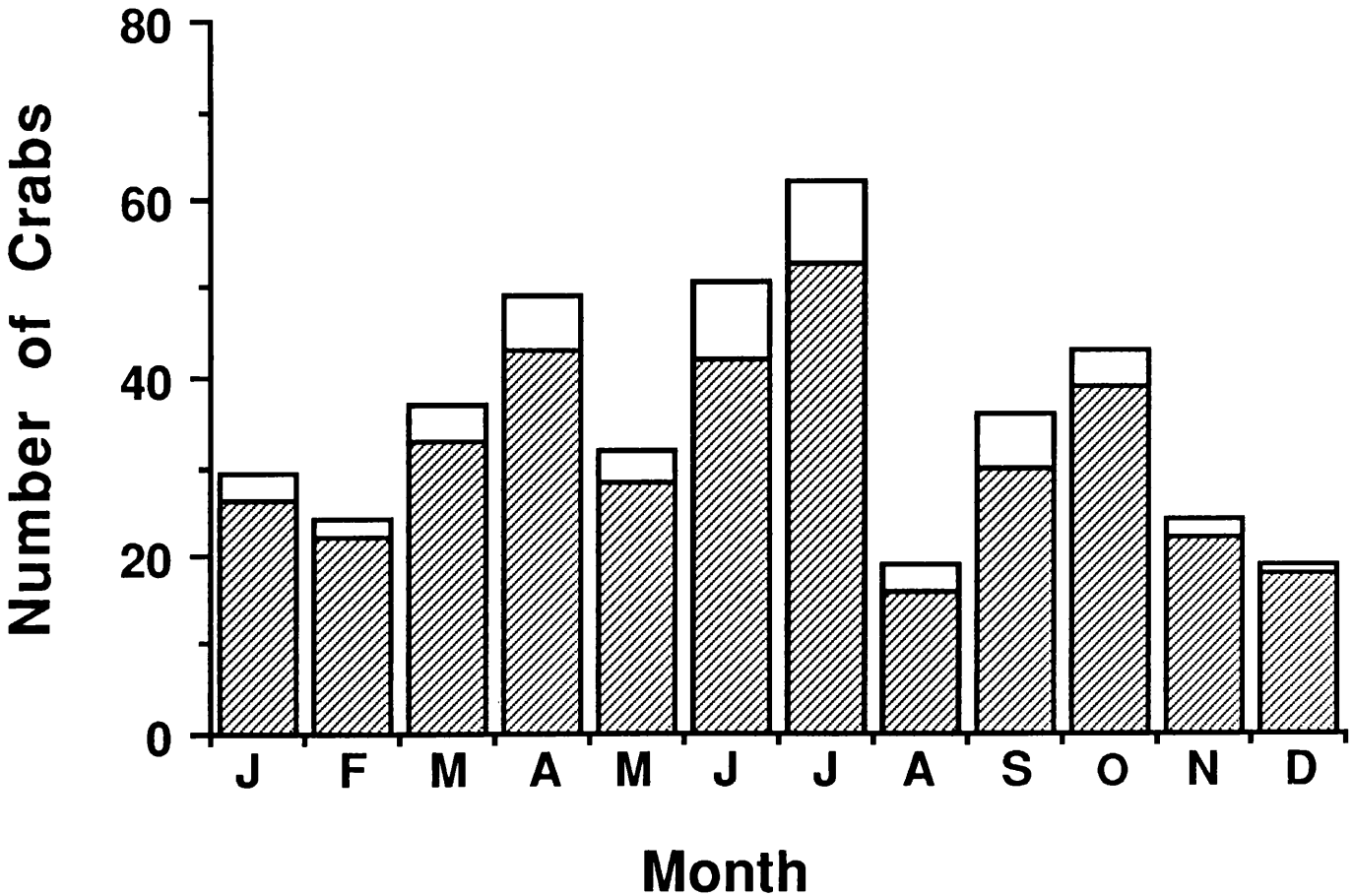
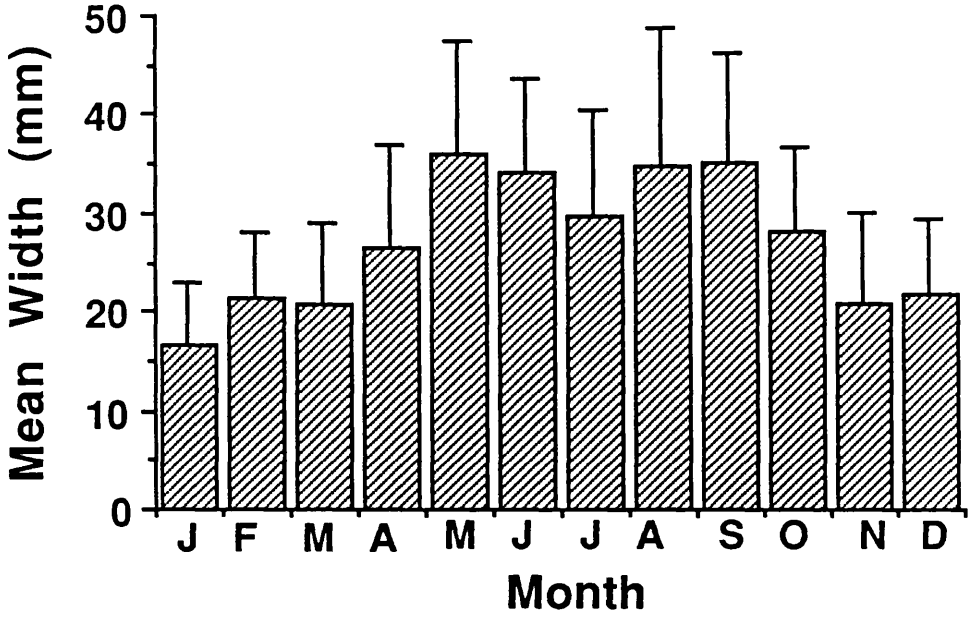
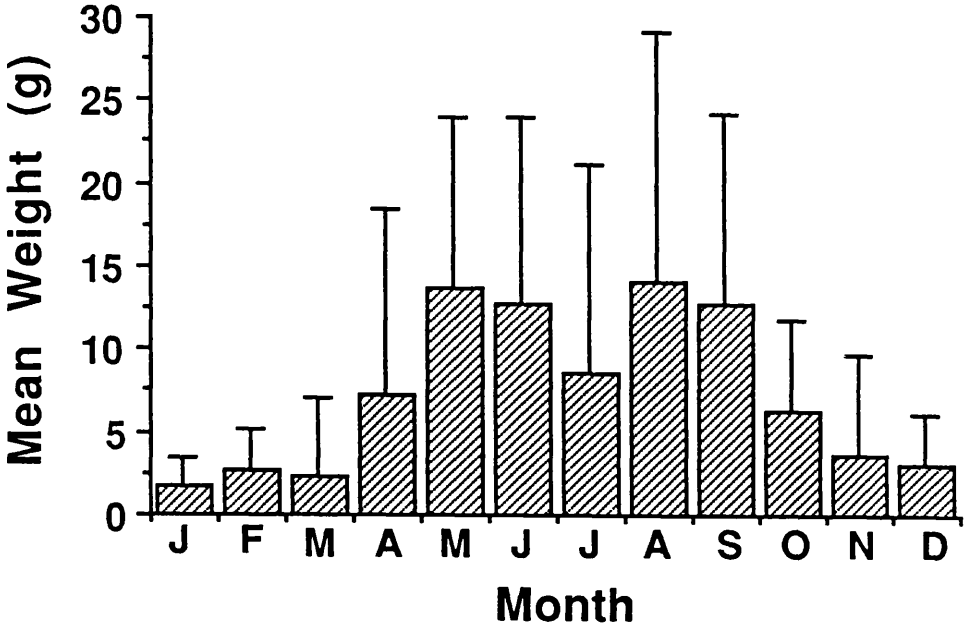

Number of Crabs
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
D
Month

FIG. 2.4 Seasonal variation in the a) width and b) weight of *Carcinus maenas* present in the 2 rock pools sampled over a 12 month period. Values are means ± S.D.

a


Mean Width (mm)
50
40
30
20
10
0
J F M A M J J A S O N D
Month

b


Mean Weight (g)
30
25
20
15
10
5
0
J F M A M J J A S O N D
Month

2.3.2 Effects of handling disturbance on the concentration of haemolymph sugars and L-lactate.

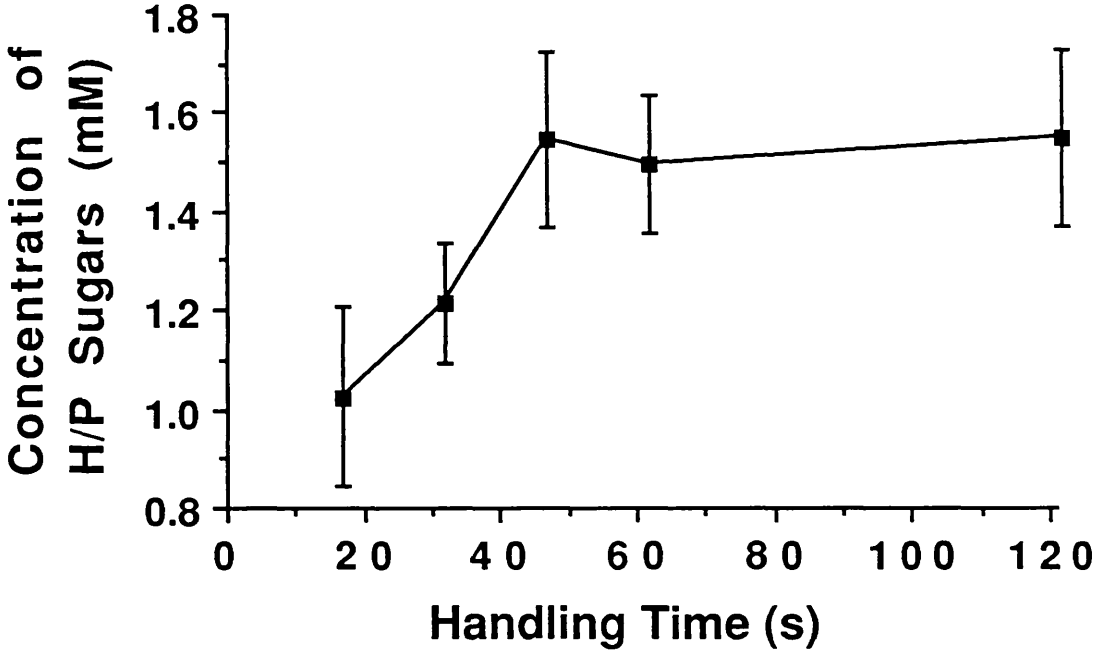
The concentration of total hexose/pentose sugars in the haemolymph of crabs exposed to the minimum of handling (i.e. control animals) was 0.98 ± 0.21 mM (Fig. 2.5). Handling for 30 seconds had no significant effect on the concentration of these sugars. After 45 seconds, however, there was a significant increase in the concentration of hexose/pentose sugars from 1.19 ± 0.12 to 1.52 ± 0.18 mM ($P < 0.05$). There was no further change in the concentration of the sugar over the next 75 seconds. As might be expected, a similar trend was observed for D-glucose; the concentration of which increased significantly from 0.125 ± 0.03 to 0.179 ± 0.04 mM, following 45 seconds of handling ($P < 0.05$). No significant ($P > 0.05$) change in the concentration of haemolymph L-lactate was detected, even after 2 minutes of handling (Fig. 2.6).

2.3.3 L-lactate accumulation in response to behavioural adaptations during hypoxia in rock pools.

The mean concentration of haemolymph L-lactate increased significantly ($P < 0.05$) in the constantly immersed group of animals, from an initial concentration of 0.57 ± 0.02 to 7.9 ± 0.75 mM after 4 h of hypoxia (Fig 2.7). The mean concentration of L-lactate in the partially and fully emersed animals, remained constant at 0.38 ± 0.10 and 0.31 ± 0.11 mM respectively. The concentration of L-lactate in the controls did not change significantly ($P > 0.05$).

FIG. 2.5 Changes in the concentration of a) total Hexose/Pentose (H/P) sugars and b) D-glucose in the haemolymph of *Carcinus maenas* during 2 minutes of handling disturbance. Values are means $\pm$ S.D.

a


Concentration of H/P Sugars (mM)
1.8
1.6
1.4
1.2
1.0
0.8
0
20
40
60
80
100
120
Handling Time (s)

b

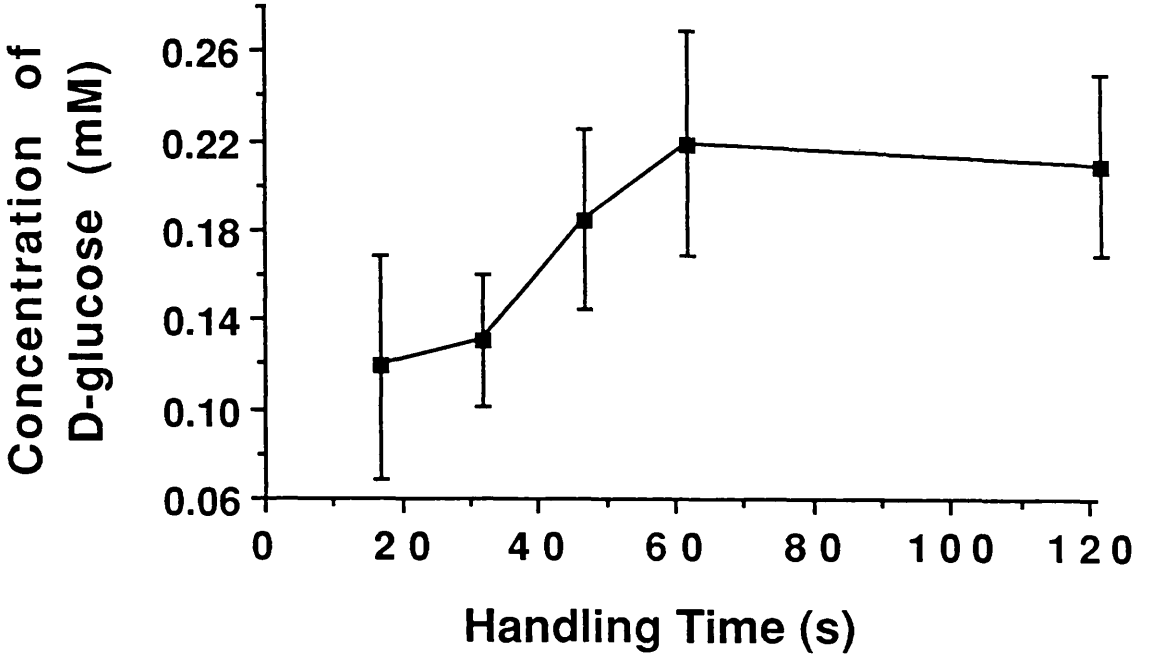

Concentration of D-glucose (mM)
0.26
0.22
0.18
0.14
0.10
0.06
0
20
40
60
80
100
120
Handling Time (s)

FIG. 2.6 Changes in the concentration of L-lactate in the haemolymph of *Carcinus maenas* during 2 minutes of handling disturbance. Values are means ± S.D.

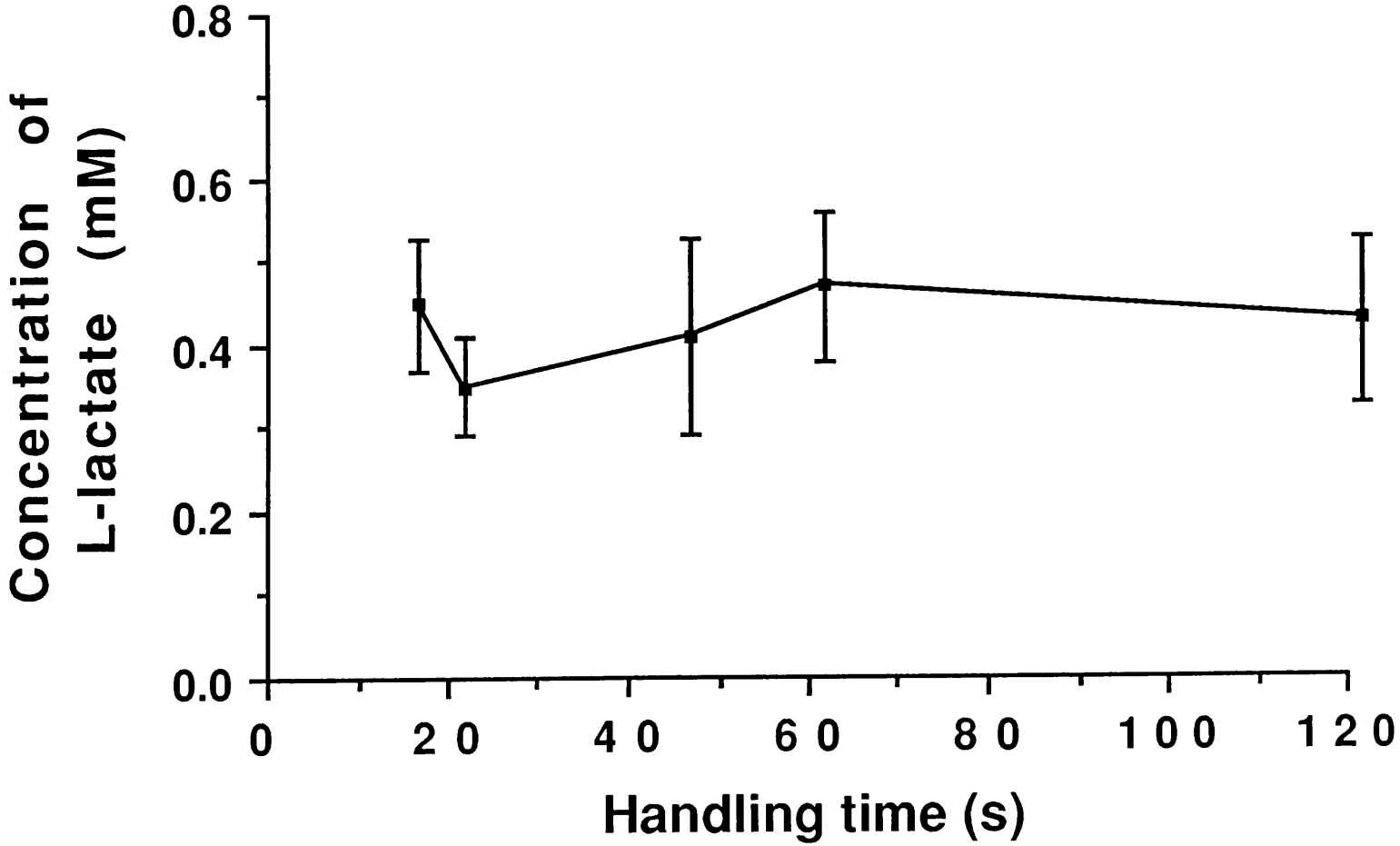
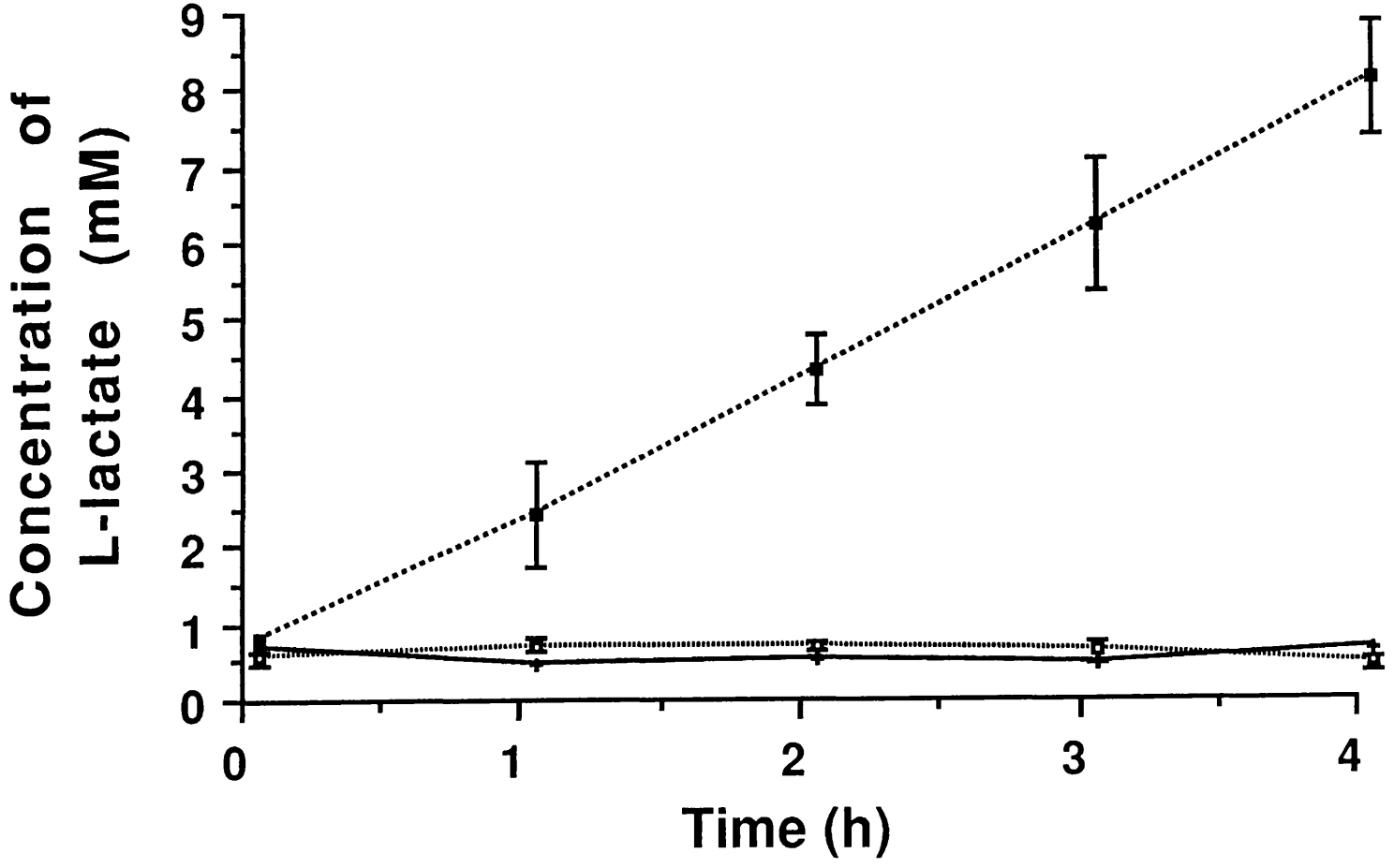

Concentration of L-lactate (mM)
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Handling time (s)

FIG. 2.7 Changes in the concentration of L-lactate in the haemolymph of *Carcinus maenas*, in response to immersion (■), partial emersion (□) and full emersion (+). Values are means ± S.D.


Concentration of L-lactate (mM)
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
Time (h)

2.3.4 A field investigation into the change in concentrations of L-lactate and glycogen during hypoxia.

Details of the physico-chemical conditions in the experimental rock pool during August (1987) and April (1988) are tabulated below in Table 2.1.

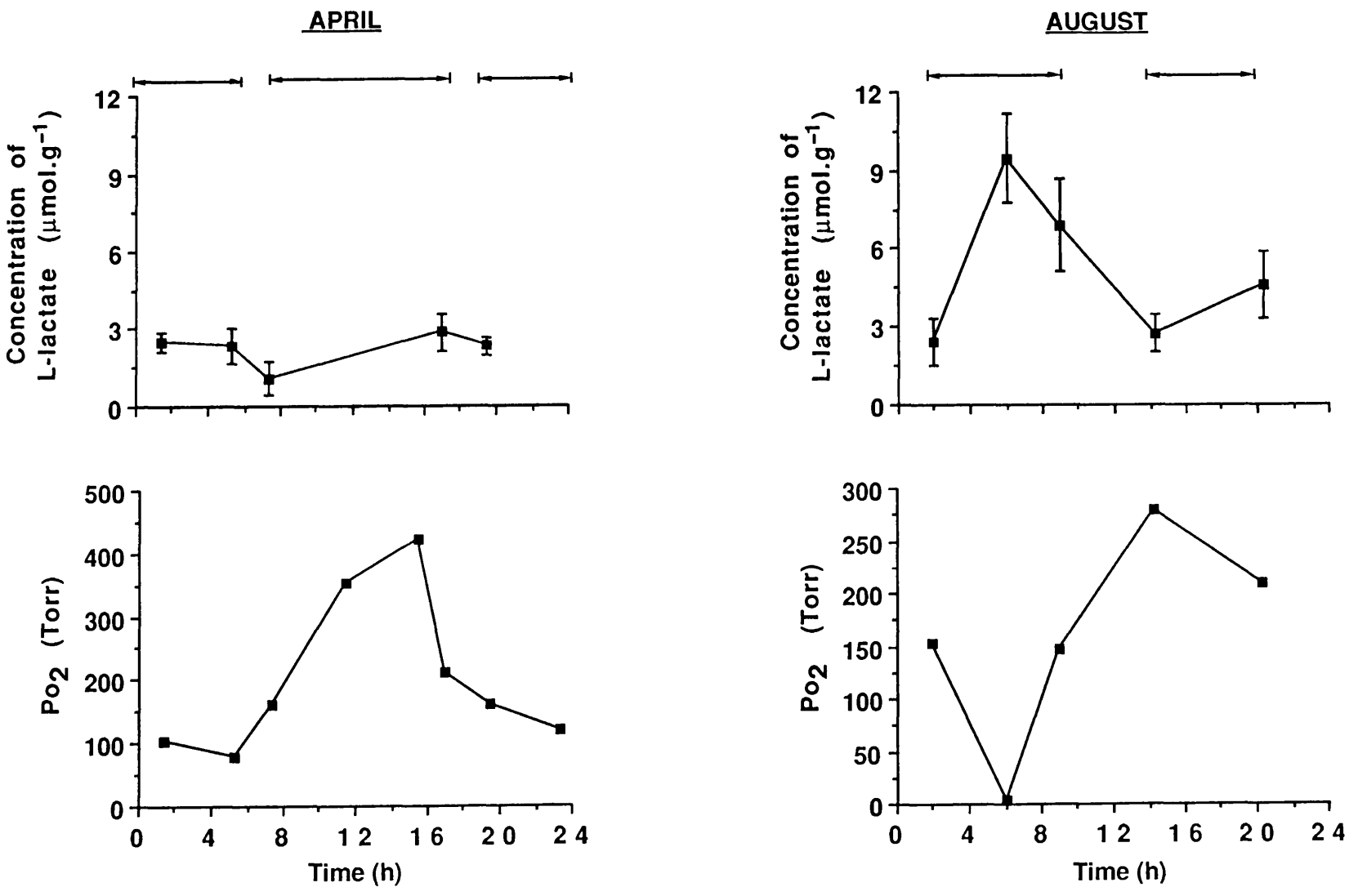
Table 2.1 Physico-chemical parameters of rock pool during field investigation.

Time of Sampling	pH	Cco_2 (mM)	Temp. (^{o}C)	Salinity (‰)
April 1988				
01.45	7.88	5.9	5.9	34
05.30	7.69	4.9	6.1	34
07.15	8.02	4.8	8.7	32
15.00	8.20	1.3	8.3	33
19.40	7.91	4.2	7.3	33
August 1987				
02.00	7.70	1.6	13.3	32
06.00	7.69	3.7	12.4	33
09.00	7.76	1.0	14.1	32
14.30	7.91	1.2	11.6	32
20.30	7.81	2.3	13.9	33

Maximum pH's of 8.2 and 7.91 were recorded during the afternoon in April and August respectively (Table 2.1). Maximum concentrations of Cco_2 were measured during the night in both April and August and the pool's salinity was found to be fairly constant in both months. As expected, the Po_2 in the rock pools was highest during the day and lowest during low tides at night (Fig. 2.8). When conditions were sunny during the day the pools became hyperoxic in August.

In April 1988 the Po_2 of the rock pool decreased at night only to 78 Torr, whereas in the previous August, it reached the much lower level of 4.6 Torr (Fig. 2.8). The

FIG. 2.8 Changes in the concentration of L-lactate in the pooled tissue of *Carcinus maenas* (upper graphs) in response to the diel fluctuations in the Po_2 of rock pool water (lower graphs), in April 1988 and August 1987. The L-lactate values are means $\pm$ S.D. The bars above the graphs indicate the times when the pool was uncovered.


APRIL
AUGUST
Concentration of L-lactate (µmol.g⁻¹)
12
9
6
3
0
Po2 (Torr)
500
400
300
200
100
0
300
250
200
150
100
50
0
4
8
12
16
20
24
Time (h)

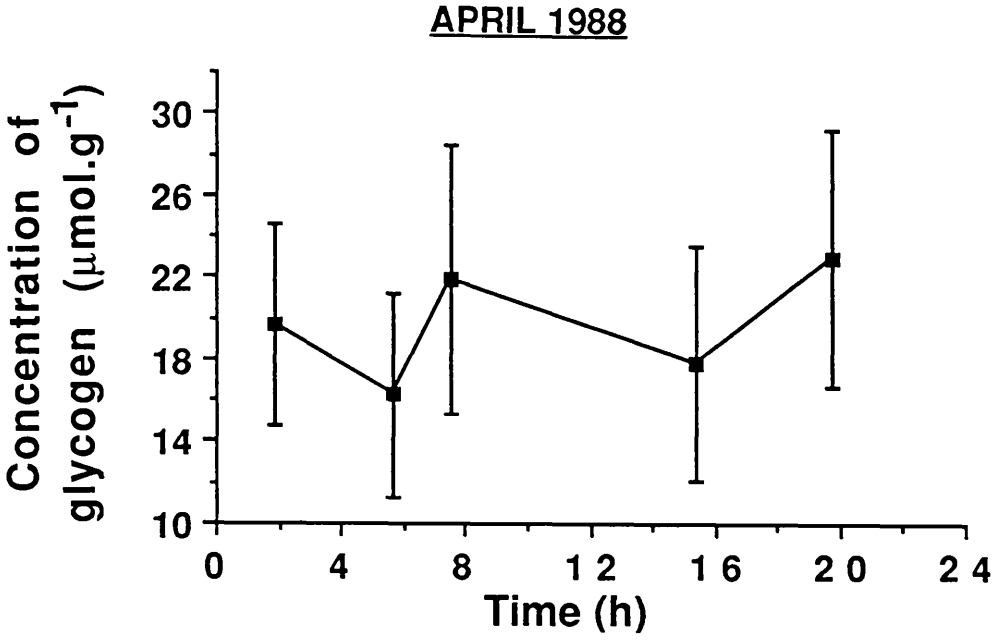
mean concentration of L-lactate, in the tissues of *C. maenas* collected, in August 1987, increased significantly ($P < 0.05$) from 2.4 ± 0.53 umol.g^{-1} during the day, to 9.46 ± 1.9 umol. g^{-1} at 06.00, in the early morning when it was still dark and the Po_2 was at its lowest. In April, the concentration of L-lactate in the tissues stayed fairly constant throughout the 24 h , with a mean concentration of 2.09 ± 0.62 umol.g^{-1} (Fig. 2.8). The concentration of total tissue glycogen was highly variable and no significant changes were discernible (Fig. 2.9) ($P > 0.05$).

2.3.5 Seasonal changes in the energy metabolites.

The monthly mean concentrations of total tissue L-lactate ranged from 3.62 ± 0.93 umol.g^{-1} in August to 1.31 ± 0.39 umol.g^{-1} in March (Fig 2.10). This decrease during the autumn and winter months was significant ($P < 0.05$). The mean monthly concentrations of total tissue glycogen were highly variable with maxima occurring during late summer and minima during the winter (Fig 2.11). These differences, however, were not significant, owing to the large standard deviations ($P > 0.05$). It had initially been intended that variations between the sexes could be investigated, but this was impossible owing to the paucity of females on the shore. Concentrations quoted here are therefore for pooled data (i.e. males and females).

The mean monthly concentrations of haemolymph L-lactate ranged from a maximum in September of 0.68 ± 0.35 mM to a minimum of 0.19 ± 0.05 mM in January (Fig. 2.12). This decrease was not significant ($P > 0.05$). The mean monthly haemolymph concentrations of total hexose\pentose sugars and D-glucose ranged from a maximum in June of 2.14 ± 0.83 and 0.41 ± 0.97 mM respectively, to a minimum in November of 1.06 ± 0.84 and 0.13 ± 0.04 mM respectively (Fig 2.13) ($P < 0.05$ in both cases). The haemolymph concentrations of L-lactate and total carbohydrates directly reflect the seasonal variations present in the respective pooled tissue samples, as described above.

FIG. 2.9 Changes in the concentration of glycogen in the pooled tissue of *Carcinus maenas* in response to the diel fluctuations in the Po_2 of rock pool water, in August 1987 and April 1988. The glycogen values are means $\pm$ S.D. Refer to Fig. 2.10 for the simultaneous water Po_2 measurements. (n = 4 for each point).


APRIL 1988
Concentration of glycogen (µmol.g-1)
30
26
22
18
14
10
0
4
8
12
16
20
24
Time (h)

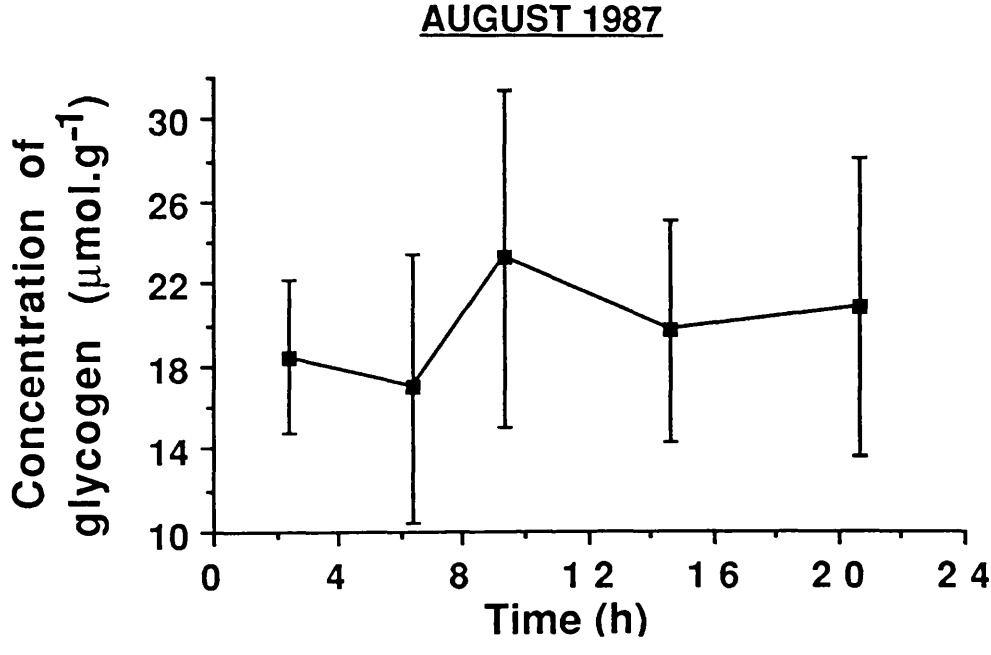

AUGUST 1987
Concentration of glycogen (µmol.g-1)
30
26
22
18
14
10
0
4
8
12
16
20
24
Time (h)

FIG. 2.10 Seasonal variation in the concentration of L-lactate in the pooled tissue of *Carcinus maenas* collected from the 2 rock pools sampled over a 12 month period. Values are monthly means $\pm$ S.D.

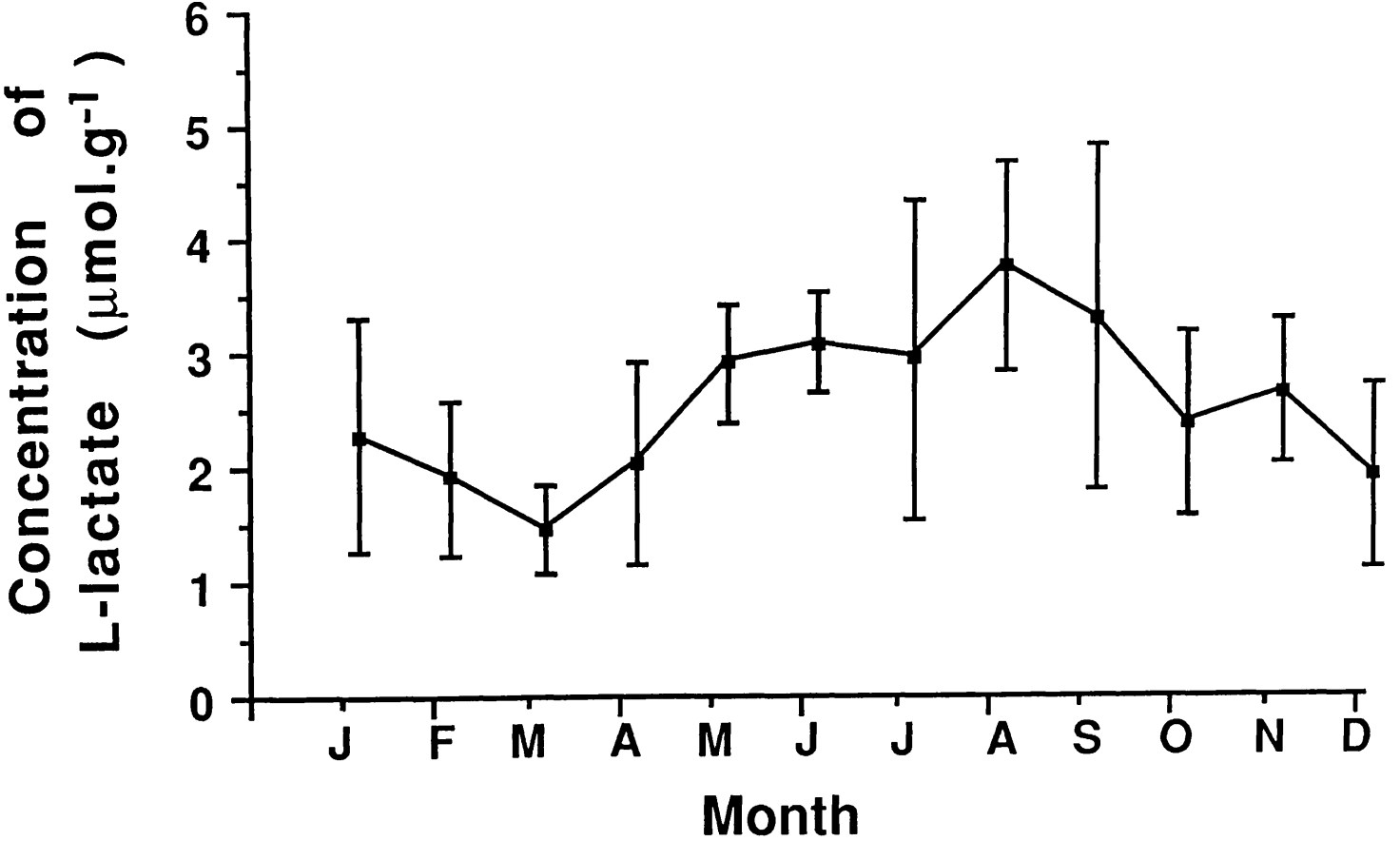

Concentration of L-lactate (μmol.g-1)
0
1
2
3
4
5
6
J
F
M
A
M
J
J
A
S
O
N
D
Month

FIG. 2.11 Seasonal variation in the concentration of glycogen in the pooled tissue of *Carcinus maenas* collected from the 2 rock pools sampled over a 12 month period. Values are monthly means $\pm$ S.D.

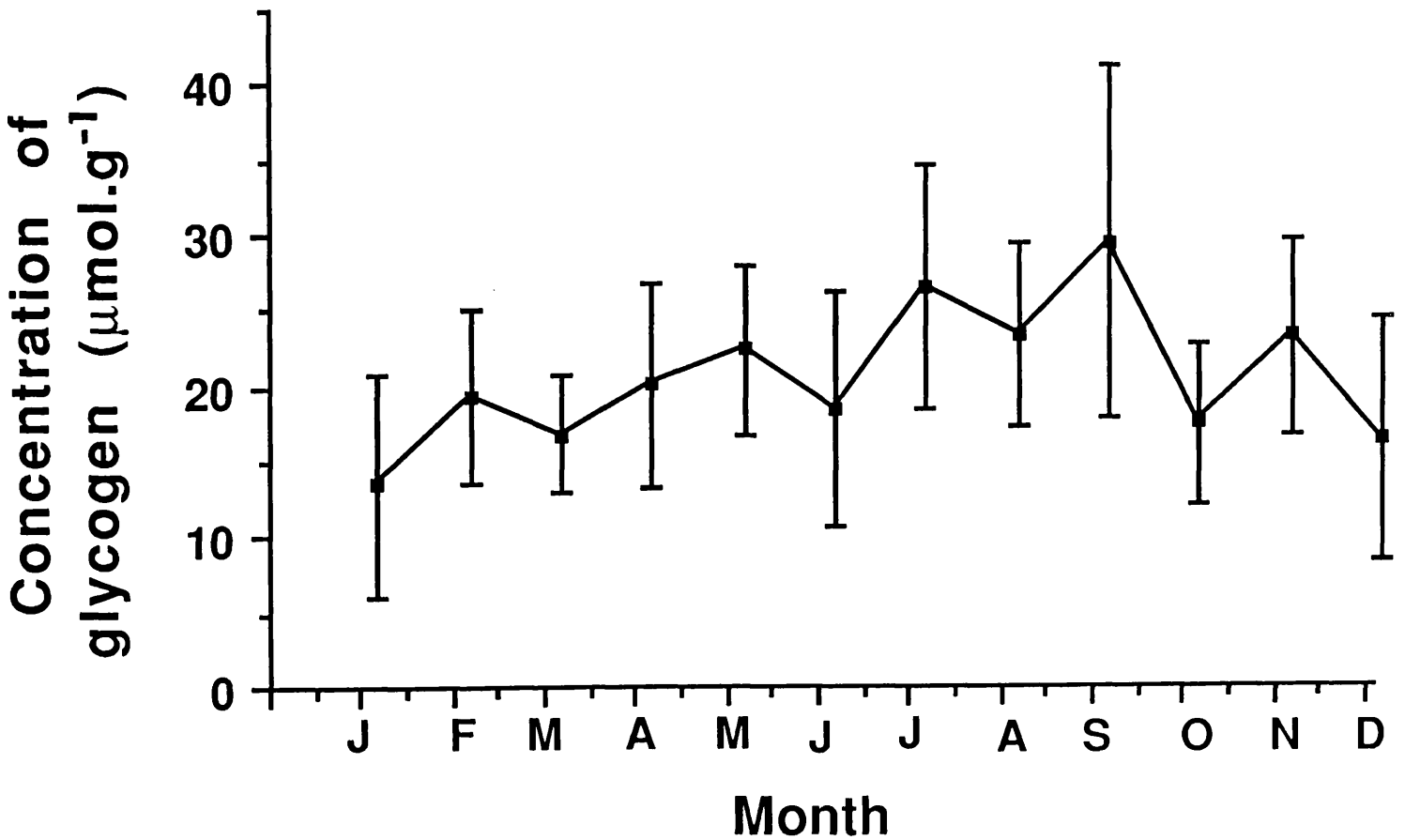

Concentration of glycogen (μmol.g-1)
0
10
20
30
40
J
F
M
A
M
J
J
A
S
O
N
D
Month

FIG. 2.12 Seasonal variation in the concentration of L-lactate in the haemolymph of *Carcinus maenas* collected from the 2 rock pools sampled over a 12 month period. Values are monthly means $\pm$ S.D.

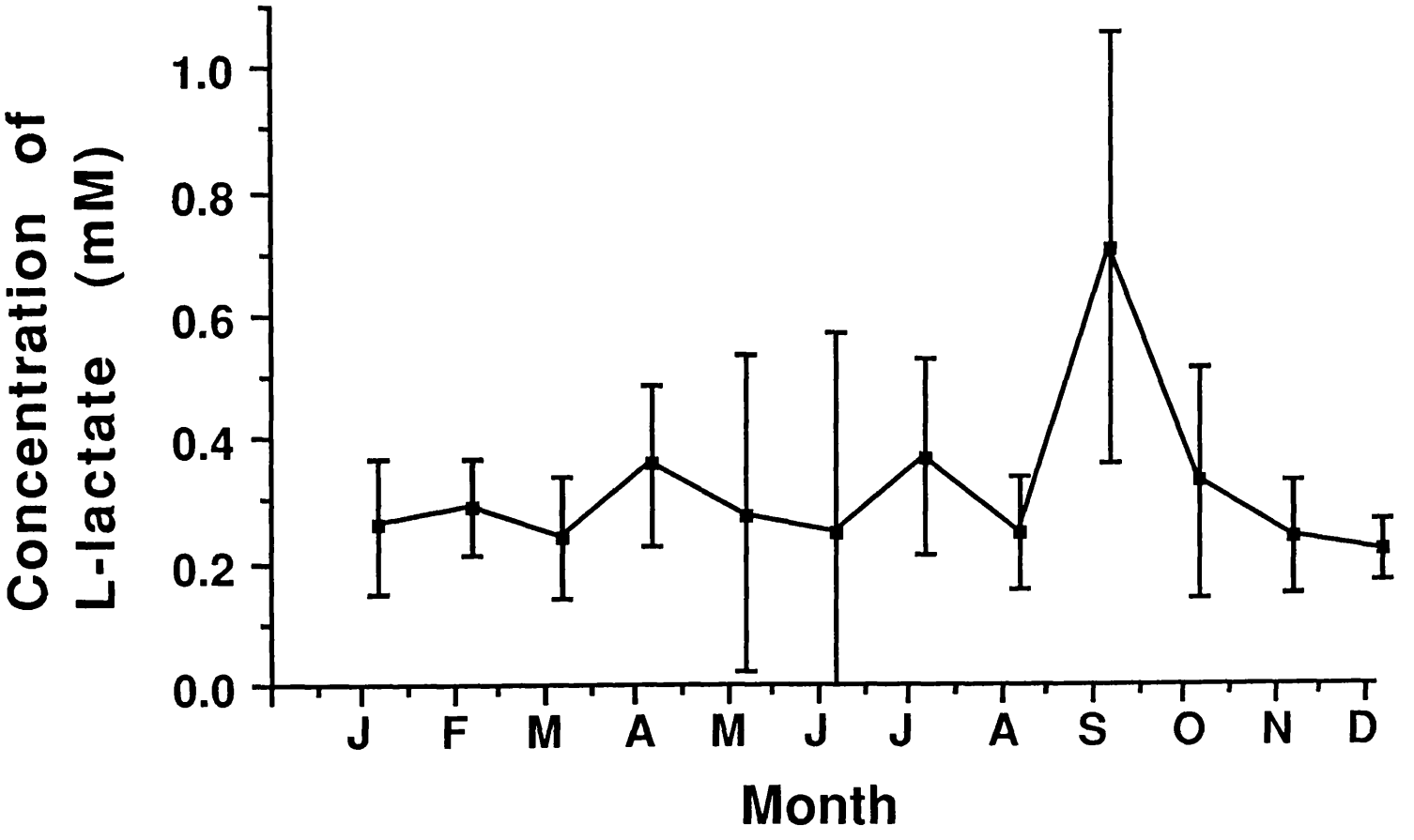
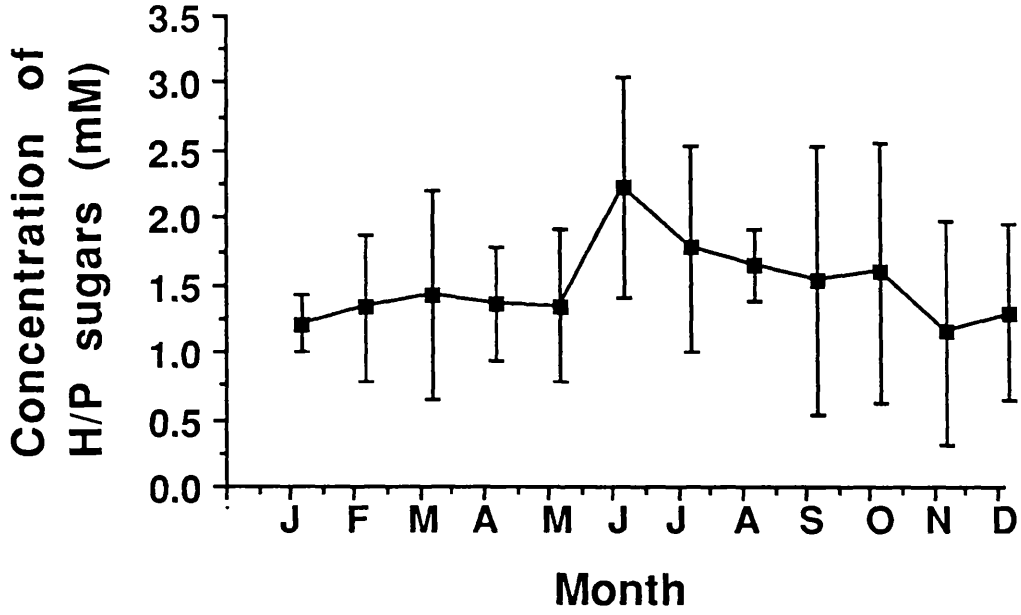
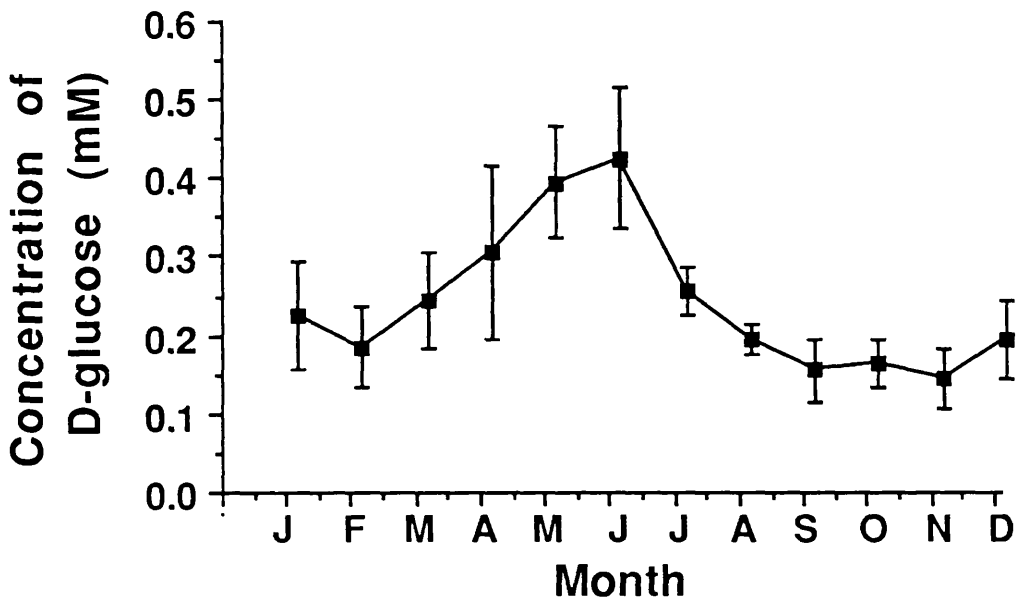

Concentration of L-lactate (mM)
1.0
0.8
0.6
0.4
0.2
0.0
J
F
M
A
M
J
J
A
S
O
N
D
Month

FIG. 2.13 Seasonal variation in the concentration of a) Hexose/Pentose (H/P) sugars and b) D-glucose in the haemolymph of *Carcinus maenas* collected from the 2 rock pools sampled over a 12 month period. Values are monthly means $\pm$ S.D.

a


Concentration of H/P sugars (mM)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
J F M A M J J A S O N D
Month

b


Concentration of D-glucose (mM)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
J F M A M J J A S O N D
Month

2.4 DISCUSSION.

2.4.1 The Rock Pool Environment.

2.4.1.1 Seasonal Variations.

Until the beginning of this decade environmental variations in rock pools had been studied only over short periods of time (Powers, 1920; Ambler & Chapman, 1950). Morris & Taylor (1983) were the first to investigate the seasonal variations of the physico-chemical conditions within intertidal rock pools, on the Isle of Cumbrae, Firth of Clyde. Although this study was carried out on different pools, the magnitude of diel variations in physico-chemical conditions were similar to those recorded by Morris & Taylor (1983).

The biota of the pools was observed to vary considerably throughout the year, depending on rates of algal growth, the settlement of animal larvae and the migration of species into and out of the pools. Factors including the length of day and water temperatures were of obvious importance in affecting metabolic rates of the pool inhabitants and also in providing natural stimulae for on-shore migrations. The temperatures of the mid-shore rock pools, not surprisingly, were highest during the summer months, with a mean maximum of 17.7 ^{o}C in July. Since the rock pools were fairly shallow (maximum depth of 25 cm) and without many fissures and crevices, the temperature profile of the pool was comparatively constant. Where spatial variations were found the mean temperature was taken. Temperature can also alter the solubilities of oxygen and carbon dioxide (both gases being more soluble at low temperatures). The maximal Po_2 ranges, occur in the summer and will be discussed in more detail in section 2.4.1.2.

The salinity of the pools varied seasonally, with the annual low of 28 ‰ occurring in January, the period of maximal rainfall. This trend was also reported by Morris

& Taylor (1983), who recorded an annual minimum of 23 ‰ in an upper shore pool during late winter. A sudden salinity change occurred in August, 1987, when, after a heavy summer thunderstorm, the salinity of the top 1.5 cm of the pool was reduced to 24 ‰, whereas the salinity of the water beneath this layer was 32 ‰ Unfortunately, it was impossible to follow the gradual mixing of the 2 layers owing to the incoming tide. Such salinity stratifications have been recorded in other studies (Ganning, 1971; Morris & Taylor, 1983).

2.4.1.2 Diel Variations.

During the course of the field hypoxia experiment (section 2.2.4), measurements of rock pool sea water temperature, salinity, Po_2, pH and Cco_2 were made during both studies carried out in August, 1987 and April, 1988 (Table 2.1 and Fig. 2.8). The trends observed agreed with those recorded by Ambler & Chapman (1950), Naylor & Slinn (1958) and more recently by Daniel & Boyden (1975) and Morris & Taylor (1983). Diel fluctuations in Po_2, Cco_2 and in pH are directly related to the photosynthetic activity of the pool flora and to the respiration of both flora and fauna. During the day oxygen production from algal photosynthesis exceeds the respiratory demands of the pool inhabitants and the water becomes increasingly hyperoxic and hypocapnic. At night, however, photosynthesis ceases and the respiration of the pools' flora and fauna gradually depletes the available oxygen whilst the Cco_2 increases. The maximum diel range of oxygen tension occurs during the summer, as a result of elevated temperatures and increased biomass. For example, in August the rock pool in this study became hyperoxic (Po_2 = 280 Torr) during the day and severely hypoxic (5 Torr) and hypercapnic during the night. In fact Truchot & Duhamel-Jouve (1980) reported that in their study of rock pools on the north-west coast of France, the Po_2 in one of the pools fell to almost zero on one occasion. In April, the Po_2 of the rock pools decreased only to 78 Torr, which is above the critical oxygen partial pressure at which *C. maenas* is unable to maintain its rate of oxygen consumption independent of the external Po_2

(Pc point) (Taylor, 1976).

During the summer, therefore, the rock pools can become severely hypoxic for up to 4 h at night and any animals remaining in them are likely to have to resort to some form of anaerobic metabolism.

2.4.2 Distribution of *Carcinus maenas* on the intertidal shore.

Carcinus maenas is common intertidally throughout the summer. Edwards (1958) suggested that there were seasonal migrations of *C. maenas* off-shore to deep water, in the autumn. Similar offshore migrations have previously been reported for the edible crab, *Cancer pagurus* (Williamson, 1900; Meek, 1913). Edwards found that in the summer at low tide there was a rapid migration of *C. maenas* up and down the shore following the tide, which involved the majority of individuals. Small crabs, with a carapace width of less than 35 mm tended, however, to remain on the shore permanently. Crabs having a mean width of 36 mm and 20-30 mm for males and females respectively have been reported to be about one year old (Broekhuysen, 1936). It therefore appears that the non-migratory part of the population are those that are less than one year old. This tidal rhythmicity did not occur, however, during the winter months. Naylor (1962), however, suggested that the situation was more complicated, and that there was no complete off-shore migration. His results showed that during the less cold months of December and March large crabs continued to migrate in-shore with each tide but very few were stranded between the tidemarks at low tide. In the particularly cold months of January and February there was no evidence of any in-shore migration, but neither was there any evidence for an off-shore migration to deeper water (Naylor, 1962). The findings of Atkinson & Parsons (1973) agreed with those of Naylor, but they did find that ovigerous females rarely displayed tidal rhymicity. They also suggested that there was a temperature threshold of $8^{o}C$ below which this

rhymicity was less common.

The results of the present study largely agree with the above. Number and sizes of crabs in the pools were greatest during the summer months. During the winter, juveniles (< 15 mm) were present in the pools at all times. On one occasion in January, 1987 the pools became frozen over, yet some small individuals were observed underneath the ice.

Observations by divers revealed that there were moderate numbers of crabs present in the intertidal pools at high tide during the winter, indicating the presence of tidal rhymicity in winter , as reported by Naylor (1962) (I.P. Smith pers. comm.). The predominance of males on the shore throughout the year, as shown here, has been reported by numerous authors including Edwards (1958) and Crothers (1968). Again, diving observations made during the summer revealed a predominance of females off-shore at this time (J. Sturtivant pers. comm.). There does seem to be local variations, however, since a sex ratio of 1:1 among crabs on the shore was observed in the Isle of Man population in the summer of 1970 (Atkinson & Parsons, 1973). In the present study, females were most common on the shore in mid to late summer, presumably just prior to mating.

To conclude, large numbers of crabs were present in the pools in summer when conditions at night become very hypoxic. These conditions can last for up to 4 h and require the crabs to either respire anaerobically or to respond with certain behavioural adaptations.

2.4.3 Effects of handling disturbance on the concentration of haemolymph sugars and L-lactate.

If seasonal and oxygen stress-induced variations were going to be realistically

examined, it was essential to take into account the effects of handling disturbance on the concentrations of haemolymph sugars and of L-lactate. The size of the hyperglycaemic response found to occur as a result of handling in this study, is of the same order of magnitude as reported for other decapod crustaceans:

Table 2.2 Size of the hyperglycaemic response in certain decapod crustaceans.

Species	Hyperglycaemic response (% increase in D-glucose after 2 minutes of handling)	Author
Homarus americanus	135 (107*) (after 5 minutes)	Telford, 1968
Orconectes propinquus	353	Telford, 1973
Cambarus robustus	513	Telford, 1973
Chasmagnathus granulata	132	Santos & Colares, 1986
Carcinus maenas	177 (155**)	Present study

* - % increase in haemolymph reducing sugars.
** - % increase in haemolymph total hexose/pentose sugars.

In previous studies, indicators of the presence or absence of hyperglycaemia have normally been restricted to increases in the concentration of haemolymph D-glucose. In addition, Telford (1968) measured reducing sugars but it is evident that the hyperglycaemic response observed could be explained simply by the increase in the concentration of D-glucose. In the present study, however, only 16.3 % of the hyperglycaemic response observed for the hexose/pentose sugars can be attributed to the increase in the concentration of D-glucose. The hyperglycaemic response, associated with handling disturbance, must therefore also involve haemolymph sugars other than D-glucose. The determination of the exact identity of these other sugars would be an interesting subject for future investigation.

Dendinger & Schatzlein (1973) reported a fourteenfold increase in the concentration of L-lactate in *Pachygrapsus crassipes* in response to handling disturbance. The experimental procedures that they used, however, involved 5 minutes of forced exercise. This is a far more extreme form of disturbance than merely handling, resulting in intense activity and causing the crabs to produce L-lactate. No similar increase in the concentration of haemolymph L-lactate, was observed in *Carcinus maenas* (present study) or in the thalassinid *Calocaris macandreae* (S.J. Anderson pers. comm.) as a result of simple handling disturbance.

Since the hyperglycaemic response in *Carcinus maenas* became evident after 30 seconds, it was decided that haemolymph samples should be taken from the crabs within 15 seconds of first handling.

2.4.4 L-lactate accumulation in response to behavioural adaptations during hypoxia in rock pools.

Observations made during the night, revealed that *Carcinus maenas* employ a variety of behavioural responses to hypoxic conditions within the pool. Many of the crabs moved to the surface of the water as the hypoxic conditions became more severe, with most occurring around the edge of the pools. As already mentioned in the introduction, this partial emersion response was first observed by Bohn in 1897, but has since been reported by many authors (Taylor *et al.*, 1973; Willis & Berrigan, 1977; Taylor & Spicer, 1987, 1988). In the laboratory, it can be shown that when *C. maenas* exhibits this partial emersion response, the scaphognathite beat is reversed and air enters the branchial chambers via the anterior openings on either side of the mouth.

When the pool conditions become most severe, crabs were seen to leave the water

completely and to remain on the surrounding rocks. Some of the other pools on the shore had steep walls which prevented the crabs becoming partially or fully emersed. Under such conditions they were observed to remain fully immersed during hypoxia.

In the laboratory study, L-lactate was shown to accumulate in the immersed hypoxic crabs but not in those that were partially or fully emersed. This is in contrast to *Palaemon elegans* in which L-lactate is seen to accumulate under all 3 conditions (Taylor & Spicer, 1988). The fact that *Carcinus maenas* is well adapted for aerial respiration is well established and has been the subject of much work (Arudpragasam & Naylor, 1964; Taylor *et al.*, 1977). Johnson & Uglow (1985) found that there was a significant increase in the concentration of L-lactate after 24 h of full emersion. The maximum length of time that *C. maenas* is likely to experience conditions of aerial exposure in the mid-shore is about 4 h., during which time the crab is unlikely to have recourse to anaerobic metabolism. When fully emersed, however, *C. maenas* experiences problems of carbon dioxide excretion and a respiratory acidosis has been observed to occur (Truchot, 1986). This acidosis is less marked in partially emersed crabs, presumably because the branchial chambers are kept partly full of sea water and CO_2 excretion is facilitated.

Since the degree of haemolymph acid-base disturbance is less during the partial emersion response, it would appear that it would be advantageous for *Carcinus maenas* to employ this behavioural response to hypoxia. In some pools this is not possible, for reasons already given. Employing some form of emersion response increases the risk to the crab of predation. In areas with large numbers of predatory birds (e.g. gulls and cormorants) or on moonlit nights, it might be advantageous for a crab to remain fully immersed and to respire anaerobically.

2.4.5 A field investigation into the change in concentrations of L-lactate and glycogen during hypoxia.

When crabs have no alternative but to remain fully immersed in a rock pool, they are forced to respire anaerobically. The previous laboratory based experiment revealed that L-lactate accumulated under such conditions. This has also been shown to occur by many other authors (Hawkins, 1970; Taylor *et al*, 1977; Pritchard & Eddy, 1979; Bridges & Brand, 1980; Spotts, 1983). The problem with these studies is that all of them were carried out in the laboratory. Admittedly it is far more difficult to maintain constant experimental conditions in the field, but such studies are necessary to ensure the validity of laboratory investigations. Jouve-Duhamel & Truchot (1983) investigated the role of ambient oxygen and carbon dioxide in controlling ventilation in *Carcinus maenas*, under both laboratory and field conditions. They found that their field study corroborated the results obtained in the laboratory. Taylor & Spicer (1987) investigated the metabolic responses of *Palaemon elegans* in rock pools, to naturally occurring hypoxia. This was done in conjunction with further laboratory studies on the subject and revealed that *P. elegans* produced L-lactate under hypoxic conditions.

In the present investigation, *Carcinus maenas* were subjected to the same environmental conditions as *Palaemon elegans* in Taylor & Spicer's investigation. In the August experiment, the Po_2 of the rock pool water decreased to about 5 Torr by 05.00 h . The crabs became very lethargic and the concentration of L-lactate in pooled tissue tripled, indicating that the crabs were respiring anaerobically. The lack of any discernible change in the concentration of pooled tissue glycogen, reflects the huge natural variations of this metabolite in *C. maenas* (Verne, 1924). Unfortunately, this natural variation necessitated the use of laboratory conditions to investigate the stoichiometric relationship between the accumulation of L-lactate and

depletion of glycogen during environmental hypoxia and anoxia (Chapter 3).

It is clear from these results that *Carcinus maenas* does normally experience hypoxic conditions within the rock pools in which it is present. At these times it may be forced to remain immersed within the pools and to respire anaerobically, with the resultant accumulation of L-lactate.

2.4.6 Seasonal changes in the energy metabolites.

The biochemical changes associated with the moulting cycle in crustaceans have received considerable attention, but relatively little work has been carried out to examine seasonal variations in body composition. As glycogen is the primary substrate for energy production, the extent to which a crab can withstand hypoxic stress must partly depend on the concentration of this substrate. Since the concentration of glycogen is governed by so many factors, ranging from the stage in the reproductive cycle to seasonal availability of food, one can expect huge fluctuations throughout the year. Large variations have been observed in decapod crustaceans for a long time (Morgulis, 1922: Hemmingsen, 1924; Kleinholz & Little, 1949). Some of this variation must surely be attributed to the fact that, in these early studies, little or no regard was given to seasonal changes. More recently, it has been shown that the concentration of tissue glycogen increases during the early summer when food availability is at its greatest, and decreases again in the autumn and winter (Ansell & Trevallion, 1967; Heath & Barnes, 1970; de Zwaan & Zandee, 1972; Ahmad & Chaplin, 1979). In this study there was a small, but not significant ($P > 0.05$), increase in the concentration of pooled tissue glycogen during the summer and then a decrease during the early autumn and winter. Heath & Barnes state that: '.... *Carcinus* were so beset by individual variations' (in glycogen) ' as to make it difficult to draw definite conclusions.' The concentration of haemolymph sugars (especially D-glucose) were shown to be highest during the early summer and

to decrease in late summer and early autumn. This agrees with the results obtained by Lynch & Webb, (1973) who found the concentration of haemolymph D-glucose reached a maximum in July, in the blue crab, *Callinectes sapidus*. In this species, there was then a rapid decrease in the concentration of D-glucose associated with the increased reproductive activity occurring in August and September.

Very little work has previously been carried out on seasonal influences on the concentration of either pooled tissue or haemolymph L-lactate. Morris *et al.* (1985) found that the concentration of L-lactate in the haemolymph increased from 0.13 $\pm$ 0.06 mM in the winter to 2.30 $\pm$ 0.44 mM in the summer in the prawn *Palaemon elegans*. It was found in the present study that the concentration of pooled tissue L-lactate was highest during the summer months and lowest during winter. The difference in concentration of L-lactate between winter and summer animals was far smaller than that reported by Morris *et al.* (1985). This summer peak might be associated either with the period of maximum locomotor activity and highest metabolic rates or with the environmental stresses that have been discussed previously.

The concentration of energy metabolites in animals is dependent on so many inter-relating factors, that to attribute trends to a single cause is entirely unrealistic. It is perfectly feasible that some of the trends reported above could be as easily attributed to moulting cycles as to the effects of the varying seasons. Whatever the cause of this variation in the biochemical composition, it is likely to have profound effects on an animal's ability to react to environmental stresses and requires further investigation.

2.4.7. Conclusions:

I) The seasonal and diel physico-chemical variations, are similar to those recorded by Morris & Taylor (1983). In the present study it was found that

during the summer, the rock pools at night became severely hypoxic.

II) Large numbers of crabs were present in the rock pools in summer, when conditions at night become very hypoxic.

III) It was found that during rock pool hypoxia, some crabs became fully or partially emersed, whilst some continued to remain fully immersed.

IV) In a laboratory study, L-lactate was shown to accumulate in the immersed crabs and not in those that were partially or fully emersed.

V) The investigation into the seasonal variations in the concentration of energy metabolites revealed clear trends, but it was found to be very difficult to ascribe too much functional significance to them, since so many other factors were inevitably involved.

VI) There was a pronounced hyperglycaemic response with concentrations of both D-glucose and total hexose/pentose sugars, increasing markedly after 30 seconds of handling disturbance.

CHAPTER 3 - THE METABOLIC RESPONSE OF *CARCINUS MAENAS* TO PERIODS OF ANOXIA AND SUBSEQUENT RECOVERY.

3.1 INTRODUCTION.

In the last chapter it was concluded that *Carcinus maenas* naturally experiences conditions of severe hypoxia in intertidal rock pools and that under such conditions it resorts to anaerobic metabolism and accumulates L-lactate as an end product. In this chapter this metabolic response of *C. maenas* to anoxia was investigated in detail, by means of a series of laboratory experiments.

The two functions of anaerobic respiration are: firstly, to produce energy, and secondly to maintain the redox balance within a cell. In the normal glycolytic pathway, glyceraldehyde 3-phosphate (GAP) is oxidised with the reduction of the cofactor NAD^+ and then phosphorylated to 1,3-diphosphoglycerate (1,3-DPG). If glycolysis is to be maintained, the resulting NADH must be constantly reoxidised. Under aerobic conditions the reoxidation of the NADH by the mitochondrial electron transport chain is not a problem, but where oxygen is limited, alternative mechanisms are needed. Therefore a central feature of all anaerobic pathways is their capacity to reoxidise the NADH, thereby maintaining a redox balance.

In decapod crustaceans, it has long been known that L-lactate accumulates during anaerobiosis, and some authors have implied that it is likely to be the only major end product of anaerobic metabolism in this group (Teal & Carey, 1967; Bridges & Brand, 1980; Gäde, 1984; Albert & Ellington, 1985). Until now, however, there has been no comprehensive study to determine the importance of other anaerobic end products. Therefore, a large part of the work in this chapter concentrated on investigating the possible occurrence of these other anaerobic pathways. This investigation involved examining the responses of *Carcinus maenas* to environmental anoxia, followed by normoxic recovery. Pooled tissue samples were then analysed

using three separate techniques. Firstly, enzymic estimation of the concentrations of L-lactate and glycogen allowed a stoichiometric comparison to be made. Secondly, the tissue samples were analysed for organic acids, using High Performance Liquid Chromatography (HPLC) and finally the concentrations of amino acids were determined using an ion exchange automated amino acid analyser.

As already mentioned, the primary function of anaerobic respiration is to produce energy in the form of ATP. In addition, in invertebrates ATP can also be produced from the breakdown of phospho-l-arginine (Livingstone *et al.*, 1981; Gäde, 1984; Bestwick, 1988). This is a highly labile organic phosphoryl compound which is able to phosphorylate ADP under the control of arginine kinase (EC 2.7.3.3). Although of secondary importance compared with anaerobic glycolysis, the breakdown of phospho-l-arginine does help to supplement the animal's overall energy production under conditions of oxygen stress. The samples obtained for the stoichiometric comparison (as described above) were therefore also analysed for their concentrations of phospho-l-arginine and adenylate nucleotides.

In order to provide information on the tolerance of *Carcinus maenas* to anoxia, an experiment was carried out to investigate the rate of mortality under such conditions. Amongst the thalassinids, *Callianassa* species have been shown to survive periods of up to 60 h of anoxia at 10 ^{o}C (Felder, 1979; Zebe, 1982). The LT_{50} of *C. maenas*, however, is likely to be considerably lower than that of thalassinids, since under natural conditions it is exposed to far shorter periods of hypoxic conditions. For instance *Munida rugosa* (a species of squat lobster that would not naturally be exposed to field hypoxia) has been shown to have an LT_{50} of only 4 h (K. Zainal, pers. comm.).

In the last chapter it was observed that there were large variations in the concentrations of sugars in the tissues of *Carcinus maenas* collected from the field. This great variability in the concentrations of tissue glycogen in crustaceans has also

been reported by other authors (Verne, 1924; Heath & Barnes, 1970). Since the above experiments required the use of pooled groups of animals, it was important to try to reduce this variation. As nutrition is likely to have a great influence on the concentration of glycogen and other sugars, 2 preliminary experiments were carried out. These investigated the effects of a single meal on the concentration of haemolymph sugars and the influence of a period of starvation on the concentration and variation of whole body glycogen.

3.2 MATERIALS AND METHODS.

3.2.1 Collection and maintenance of animals.

Carcinus maenas, were collected by hand from intertidal rock pools, on a rocky promontory, to the West of Kames Bay, on the Isle of Cumbrae, Firth of Clyde, Scotland. These animals were transported to the Department of Zoology, University of Glasgow, in plastic tanks containing sea water. The animals were then transferred to large tanks, in a recirculating seawater aquarium, maintained at 10 ± 1 ^{o}C. They were fed on mussels, (*Mytilus edulis*) during the first week, and starved in the second week, in order to reduce the natural individual variation in the concentration of glycogen in the tissue of *C. maenas*, that was observed to be present in freshly collected crabs (Chapter 2).

General experimental conditions.

All experiments described in this chapter were carried out in a constant temperature room (10 ± 1 ^{o}C), with a photoperiod of 12 h light and 12h dark. Artificial sea water made up from sea salt (Tropic Marin) with distilled water (salinity = 30 ‰) was used throughout and was always allowed to equilibrate to 10 ^{o}C before use.

3.2.2 Preliminary experiments.

3.2.2.1 The effects of feeding on the concentration of haemolymph sugars.

The effects of feeding on the concentrations of D-glucose and total hexose/pentose sugars in the haemolymph were investigated as follows: 15 crabs were placed in individual beakers (each containing 1 litre of sea water at a salinity of 30 ‰) and left undisturbed for 24 hours before the start of the experiment. On the following

day, 10 animals were fed on the adductor muscles (200 mg) of *Mytilus edulis*. The other 5 remained unfed and were used as controls. Haemolymph samples (100 ul) were taken on day 1, before introducing the food, and at the same time on each of the following 5 days. These were taken using a 1ml syringe, the needle (26 g) of which was inserted into the arthrodial membrane of the 4th walking leg. The samples were then prepared and analysed for D-glucose and total hexose/pentose sugars (Appendices 1, 2 and 4).

3.2.2.2 The effects of starvation on the concentration of tissue glycogen.

Immediately after capture, *Carcinus maenas* was starved for the following 14 days. 5 crabs were sacrificed every day for the duration of the experiment, by plunging them into liquid nitrogen and storing them at -20 ^{o}C. Pooled tissue samples were then prepared and the concentration of glycogen determined (Appendix 3).

3.2.3 Survival under anoxic conditions.

A total of 64 animals were placed in individual beakers (volume = 250 ml), in 8 clear perspex tanks (volume = 10 l). This procedure was used to reduce interactions between animals, which might have complicated the interpretation of the results. Each beaker was submerged to a minimum depth of 6 cm, in order to ensure thorough aeration. A heavy plastic mesh was placed over all the beakers and secured using weights. The tanks were covered with polystyrene sheets (5 mm thick), which were cut to fit the insides of the tanks and which, by floating on the water surface, substantially reduced the water/air interface and helped to prevent the diffusion of oxygen back into the water. An oxygen electrode (E5046 Radiometer, Denmark) was fitted through the polystyrene, to monitor the oxygen tension (Po_2) of the water throughout the experiment. The oxygen electrode was coupled to an oxygen meter (Strathkelvin Instruments, Glasgow). The Po_2 of the

water was regulated using a gas mixture, produced by a precision gas mixing system, which was pumped through air-stones into each of the tanks.

At the start of the experiment the crabs were left undisturbed for 24 h, during which time the water was constantly aerated. At the end of 24 h period the Po_2 of the water in 6 of the tanks was reduced to < 2 Torr by bubbling a mixture of nitrogen and carbon dioxide into the water. The carbon dioxide in the gas mixture was to maintain the pH constant throughout the experiment. The animals in the remaining 2 tanks were used as controls and were maintained under normoxic conditions. The number of mortalities occurring in each tank over a 20 hour period was recorded. During anoxia the crabs became very quiescent and at times it was difficult to assess whether or not an animal was dead or alive, but animals were confirmed dead, when they failed to respond to touching the eyestalk. Observations were made every hour, and any dead animals were immediately removed and transferred to a tank of normoxic water. This was to ensure that there was no contamination of the experimental water with metabolites of decomposition.

3.2.4 Anaerobic metabolism during environmental anoxia and subsequent recovery.

The general experimental design was as described in section 3.2.3. In this experiment 132 animals were placed in individual beakers which were in turn apportioned equally between 5 large tanks (volume = 40 l). They were left undisturbed for 24 hours, while the water was constantly aerated.

At the end of this period, the Po_2 of the water of 4 of the tanks was reduced to < 2 Torr by bubbling nitrogen through the water. Animals in the fifth tank were maintained under normoxic conditions and were used as controls. The Po_2 of the water in the experimental tanks reached anoxic levels (< 2 Torr) in approximately

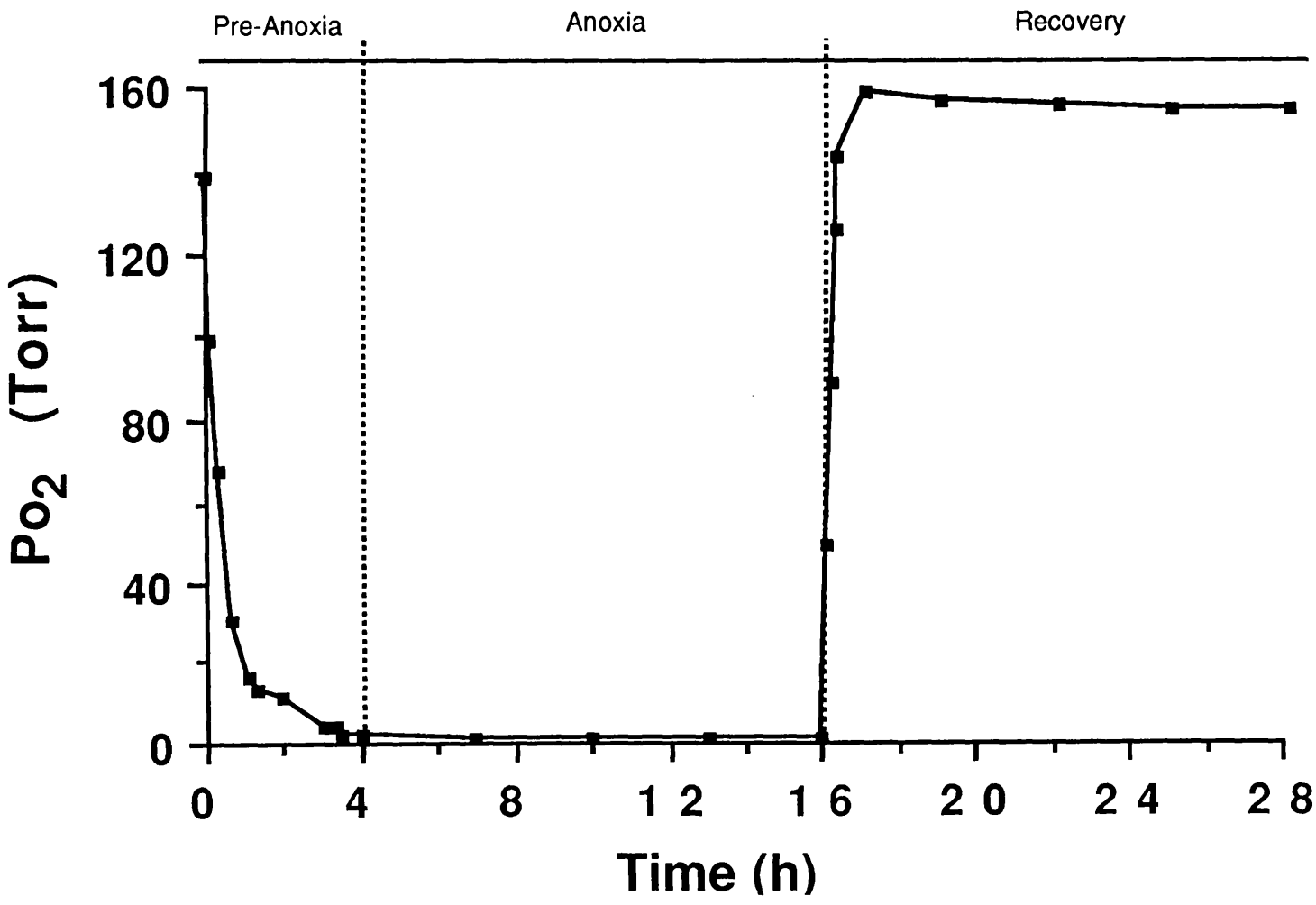
4 hours (Fig. 3.1). This time, (0 - 4 h) was called the 'pre-anoxic' period. After 0 and 2 h, 2 batches of 5 experimental and 2 control animals were removed and immediately plunged into liquid nitrogen, using long-nosed tongs. The frozen animals were then stored in a deep freeze (-70 ^{o}C) until required for biochemical analysis. During the 'anoxic period' (4 - 16 h), batches of 5 experimental and 2 control animals were taken at 4, 7, 10, 13 and 16 hours. After 12 hours of anoxia, the Po_2 of the water was returned to near saturation (approximately 160 Torr) within 20 minutes, by bubbling air through the tanks (Fig. 3.1.). During the 'recovery period' (16 - 28 h) a further 5 experimental and 2 control crabs were taken at 17, 19, 22, 25 and 28 hours. In addition, haemolymph samples (100 ul) were extracted from 2 experimental and 2 control animals at each of the 12 time intervals. The haemolymph was extracted as outlined above and then stored and processed as described in Appendix 1.

3.2.4.1 Biochemical analyses.

i) Enzymatic analysis.

Pooled tissue samples were prepared using the standard perchloric acid extraction technique, as used by Gäde *et al.* (1978) for frozen tissue (Appendix 1). Haemolymph samples were treated in a similar manner. The metabolites were assayed enzymically by the following methods: D- glucose and Glucose 6-phosphate (Kunst *et al.*, 1981); ATP (Trautschold *et al.*, 1981); Pyruvate, ADP and AMP (Jaworek & Welsch, 1981); Phospho-l-arginine (Heinz & Weiber, 1981); PEP and Succinate (Lamprecht & Heinz, 1981; Beutler, 1981). The concentration of L- lactate, in both haemolymph and pooled tissue samples was estimated using the method of Gutmann & Wahlefeld (1974) with modifications suggested by Engel & Jones (1978). Glycogen and oligosaccharides were measured enzymically using 1,4 -1,6-amyloglucosidase (EC 3.2.1.33) after boiling the tissue in potassium hydroxide (30% w/v) and subsequent alcohol precipitation

FIG. 3.1 Changes in the Po_2 of the experimental water during 4 hours of 'pre-anoxia' (see text for further explanation), 12 hours of anoxia and finally 12 hours of normoxic recovery. A Po_2 of less than 2 Torr, was taken to represent anoxia, since the oxygen electrode was unable to accurately measure a Po_2 of less than this value.


Pre-Anoxia
Anoxia
Recovery
160
120
80
40
0
Po_2 (Torr)
0
4
8
12
16
20
24
28
Time (h)

as described by Keppler & Decker (1974). Glycogen was also estimated using the anthrone method as reported by Carroll *et al.*, (1956). The results from both of these methods were compared. Full details of all these methods can be found in the Appendices. Concentrations of metabolites in pooled tissue samples were expressed as $umol.g^{-1}$ fresh weight. An index that compares the relative concentrations of the 3 adenylate nucleotides (ATP, ADP and AMP) is the adenylate energy charge (AEC), as proposed by Atkinson & Walton (1967) and was calculated using the following equation:

$$AEC = \frac{[ATP] + {}^{1}/_{2}\,[ADP]}{[AMP] + [ADP] + [ATP]}$$

ii) High Performance Liquid Chromatography (HPLC).

Samples were prepared as described in Appendix 1, with the additional step of centrifuging the final mixture for 15 minutes (at 14000 g). This ensured that the samples were free of any particulate matter that might block the HPLC column.

a) Nucleotides

The estimation of adenylate nucleotides was also carried out by an HPLC system (Gilson, France) coupled to a Partisil 10 SAX Column - 250 by 4.6 mm (HPLC Macclesfield). Adenylate mononucleotides were better resolved by using a mobile phase with a low pH. However, ADP and ATP resolve better when the pH of the mobile phase is higher. Therefore their analyses were carried out separately.

Phosphoric acid (pH 3.5), acting as a buffer, was used to separate AMP, with a mobile phase gradient (flow rate 1.4 ml. min^{-1}). The concentration of buffers A and B were 5 mM and 300 mM respectively. Since the pH of the buffer was so low, it was essential that the column was flushed thoroughly after a batch of samples had been passed through.

ADP and ATP were estimated using the buffer ammonium sulphate, at a pH of 7.0, with a mobile phase gradient (flow rate 1.4 ml. min^{-1}). The concentration of buffers A and B were 20 mM and 300 mM respectively.

All samples (20 ul) were introduced into the column using a 25 ul Hamilton micro-syringe. For both procedures all nucleotides were eluted within a 30 minute period and were detected by a spectrophotometer at a wavelength of 254 nm. The results were recorded on a chart recorder (Tekman TE200/1, Tekman Ltd, England). Standards of all three nucleotides were run to establish their respective retention times.

b) Organic acids

The concentrations of the organic acids were estimated using a similar HPLC system to the one described above, but, in this case, a Bio Rad Aminex HPX-87H organic acid analysis column (300 by 7.8 mm) was used. Samples (25 ul) were introduced into the column using a 25 ul Hamilton micro-syringe. The column was eluted with H_2SO_4 (25 mM) at a rate of 1 ml. min^{-1} . All the organic acids of interest were eluted within a 16 minute period and detected spectrophotometrically at a wavelength of 210 nm. The following standards (1 mM) were run in order to establish the identity of the endogenous organic acids: oxaloacetate, malate, pyruvate, succinate, lactate, acetate, fumarate and propionate.

iii) Amino Acid Analysis.

The estimation of the amino acids was carried out courtesy of Mr. J. Jardine of the Department of Biochemistry, using an automated amino acid analyser. The analyser consisted of an ion exchange column, from which the various amino acids were eluted by a pH gradient, using a sodium citrate buffer. The samples were

prepared in the same way as described for the HPLC.

3.3 RESULTS.

3.3.1 Preliminary experiments.

3.3.1.1 The effects of feeding on the concentrations of haemolymph sugars.

The concentrations of total hexose/pentose sugars and D-glucose in the haemolymph of animals prior to feeding were 0.95 ± 0.22 mM and 0.106 ± 0.06 mM respectively (Fig. 3.2). On the following day after feeding there had been a significant increase in the concentrations of these compounds, to 2.48 ± 0.66 mM and 0.781 ± 0.27 mM respectively ($P < 0.05$). Two days later, in the absence of food, the concentration of haemolymph sugars had decreased significantly ($P < 0.05$) to 1.95 ± 0.14 mM and 0.151 ± 0.05 mM for total hexose/pentose sugars and D-glucose respectively.

3.3.1.2 The effects of starvation on the concentration of tissue glycogen.

A large variation was observed in the concentration of pooled tissue glycogen, from individual animals killed immediately after collection in the field (mean = 17.1 ± 6.15 $umol.g^{-1}$) (Fig. 3.3). At the end of the 14th day there had been no significant ($P > 0.05$) decrease in the concentration of glycogen. When expressed as a percentage of the concentration of glycogen, however, the standard deviation decreased from ± 35 % immediately after capture, to ± 9 % after 2 weeks of captivity. A Spearman Rank correlation, between the number of days of captivity and the coefficient of variation of the concentration of the glycogen, resulted in a coefficient of -0.693, indicating a significant negative relationship.

3.3.2 Survival under anoxic conditions.

In order to devise an appropriate protocol, it was necessary to know the length of

FIG 3.2 Changes in the concentration of Hexose/Pentose (H/P) sugars (■) and D-glucose (□) in the haemolymph of *Carcinus maenas*, following a single meal (on Day '0'). Values are means ± S.D. (n = 5).

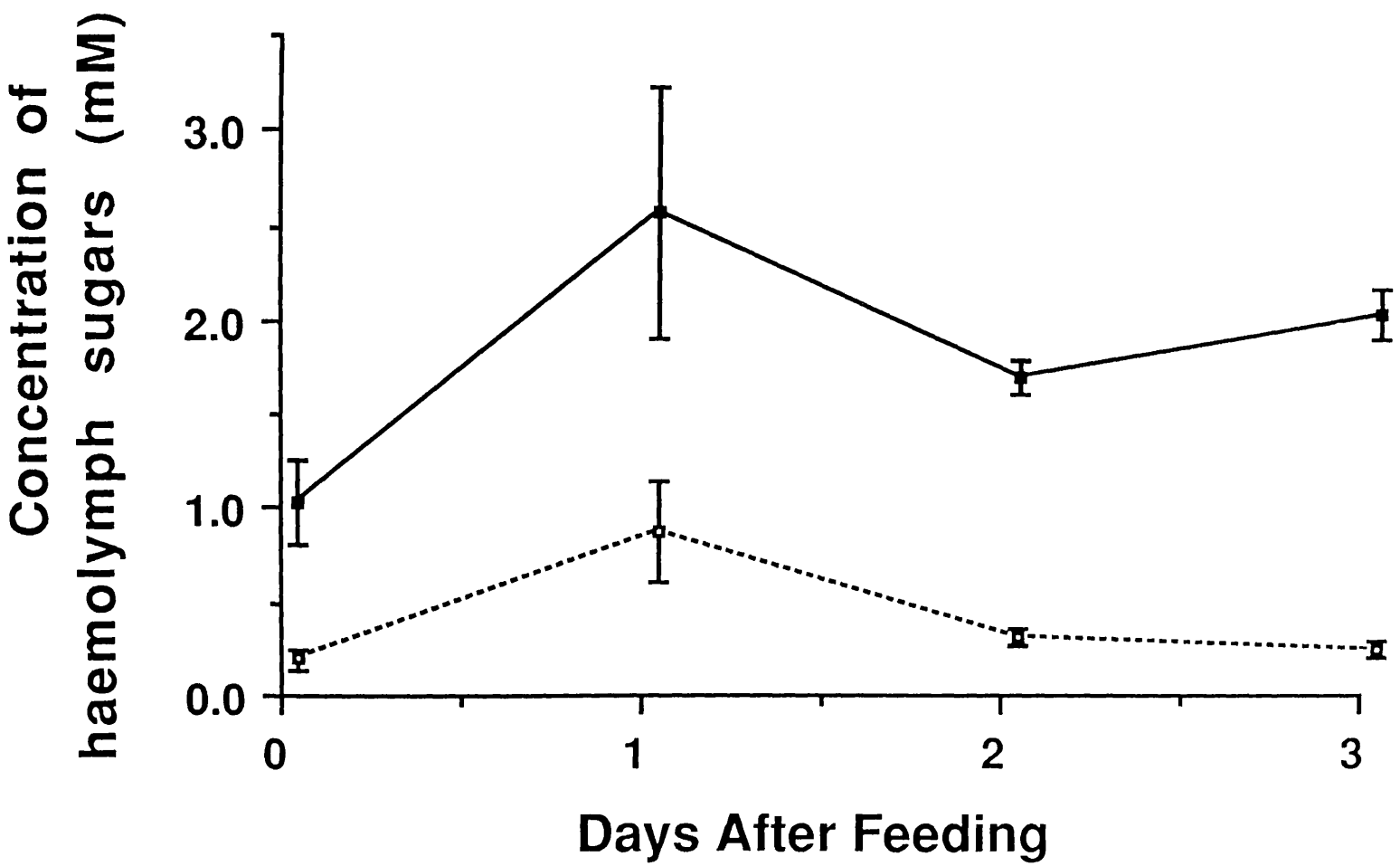
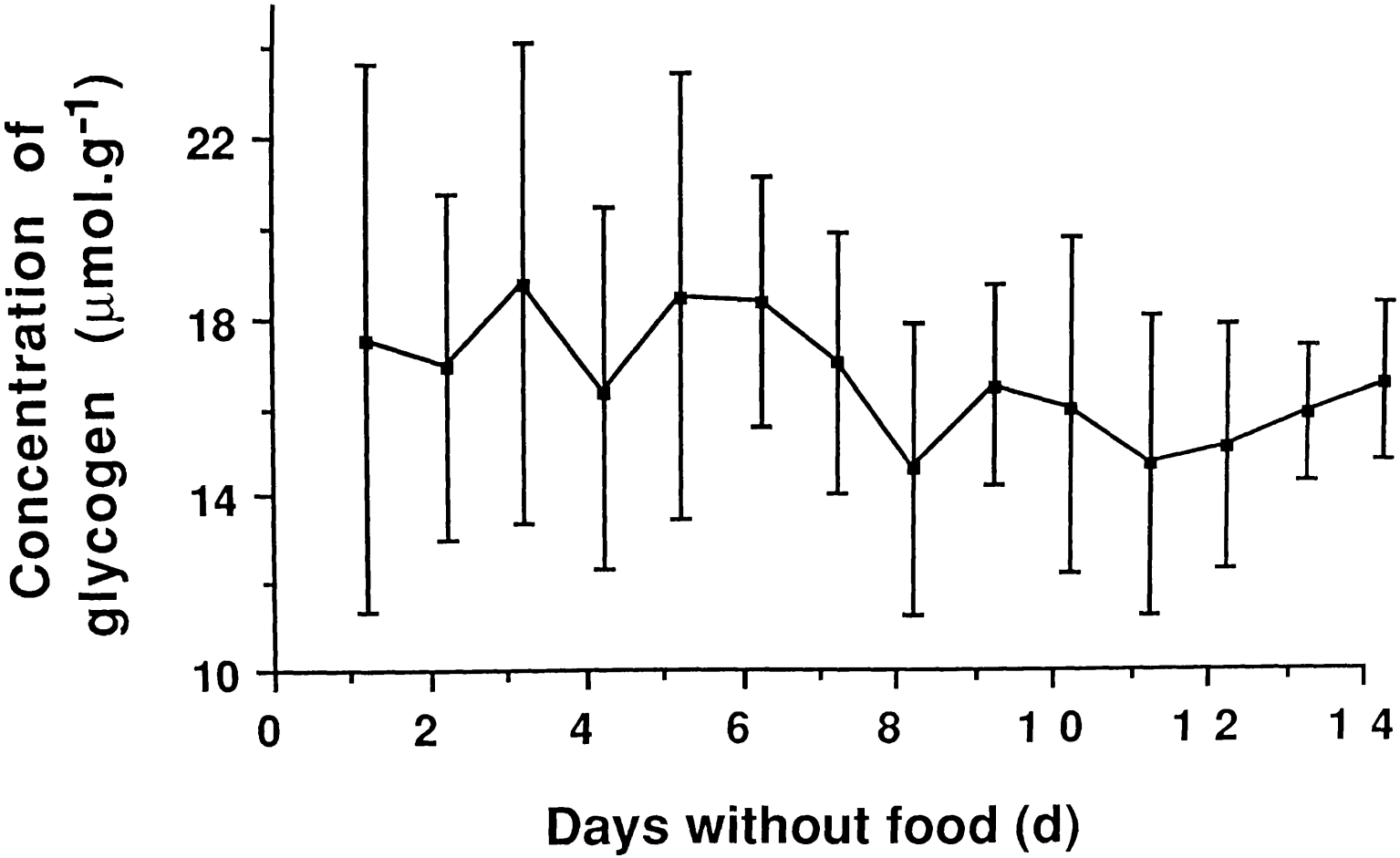

Concentration of haemolymph sugars (mM)
3.0
2.0
1.0
0.0
0
1
2
3
Days After Feeding

FIG. 3.3 Changes in the concentration of glycogen in the pooled tissue of *Carcinus maenas* over a starvation period of 14 days, following initial collection from the field. Values are means $\pm$ S.D. (n = 5 for each point).


Concentration of glycogen (µmol.g-1)
22
18
14
10
0
2
4
6
8
10
12
14
Days without food (d)

time that the animals could survive under conditions of total anoxia. The results of this preliminary experiment indicated that survival rate was high for the first 14 h of anoxia (Fig. 3.4). After this, however, there was a rapid increase in mortality and all the crabs were dead by 18 h . In contrast, only 1 out of 16 of the control crabs died during the course of the experiment. None of the crabs removed from the experimental tanks, having 'deemed' to have died, recovered after being placed in normoxic water.

3.3.3 Anaerobic metabolism during environmental anoxia and subsequent recovery.

3.3.3.1 L-lactate and carbohydrate stores.

The mean concentration of L-lactate in the pooled tissue samples of animals sampled 4 h prior to the onset of anoxia was 4.49 $\pm$ 3.28 umol.g^{-1} fresh wt. but increased significantly ($P < 0.05$) to 20.3 $\pm$ 4.5 umol.g^{-1} by the end of the 12 h period of anoxia (Fig. 3.5). Of particular interest was the fact that the concentration of L-lactate continued to increase during the first hour of recovery to a concentration of 41.3 $\pm$ 6.6 umol.g^{-1} . The rate of L-lactate accumulation over the first 17 h of the experiment thus averaged 2.16 umol.h^{-1}.g^{-1} . During the remainder of recovery, the concentration of L-lactate decreased to 8.1 $\pm$ 1.33 umol.g^{-1} . The concentration of L-lactate in the control animals did not change significantly (mean = 3.2 $\pm$ 1.95 umol.g^{-1}) throughout the 28 hour period. The concentration of L-lactate in the haemolymph increased significantly ($P < 0.05$) from 3.19 $\pm$ 0.71 to 16.7 $\pm$ 3.2 mM by the 4th and 10th hour of the experiment respectively (Fig. 3.6). This appeared to be the maximum concentration, since no further increase was observed until 6 h into the recovery period when the concentration of L-lactate decreased significantly ($P < 0.05$) from 16.1 $\pm$ 1.3 to 5.1 $\pm$ 0.6 mM after 22 and 28 h respectively. The concept of haemolymph L-lactate reaching a maximum concentration, even though tissue L-lactate was still

FIG. 3.4 Cumulative mortality (%) recorded during exposure of *Carcinus maenas* to anoxia. The experiment was carried out at 10 ^{o}C. (n = 64).

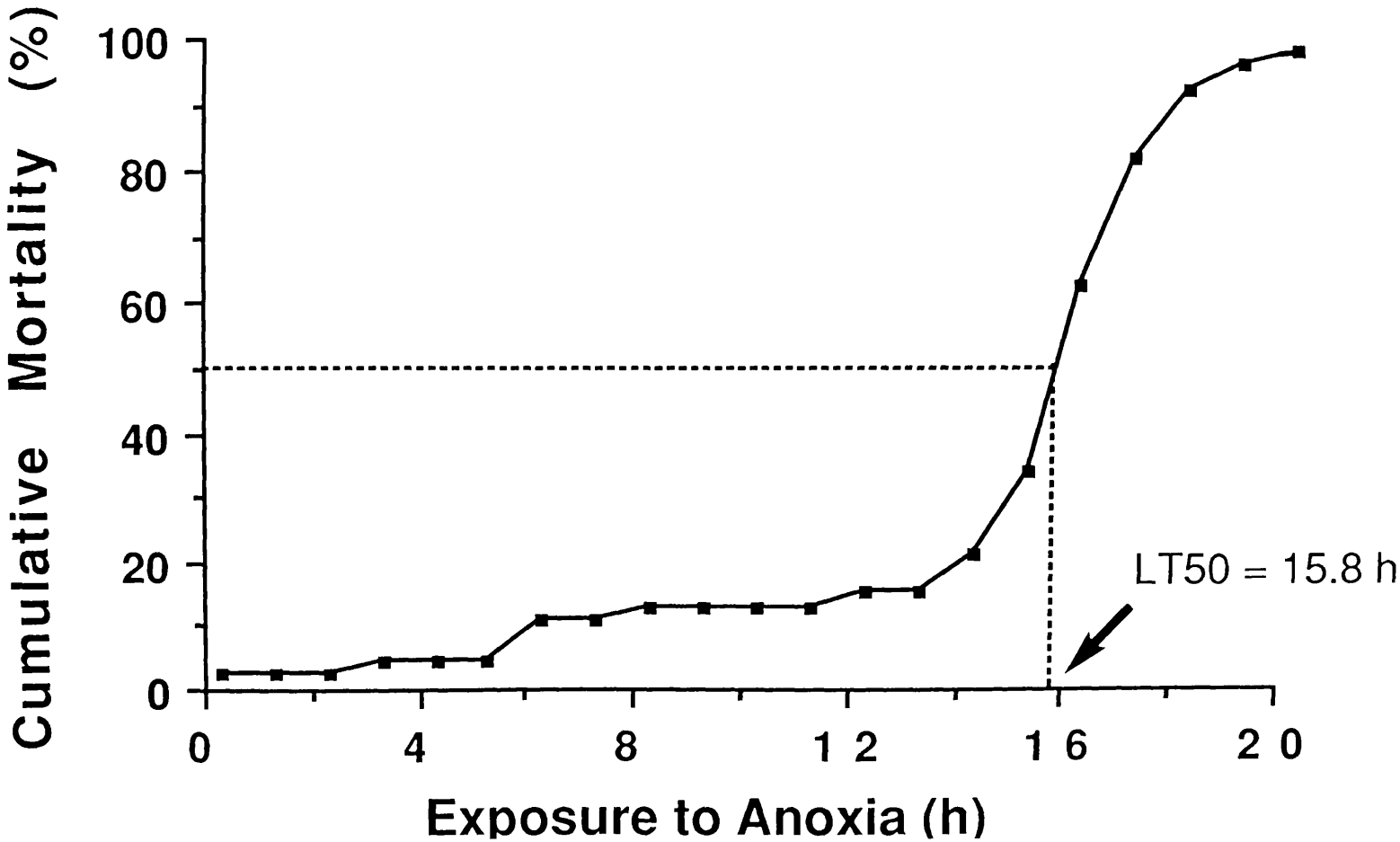

Cumulative Mortality (%)
100
80
60
40
20
0
0
4
8
12
16
20
LT50 = 15.8 h
Exposure to Anoxia (h)

FIG. 3.5 Changes in the concentration of L-lactate in the pooled tissue of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D. (n = 5 for each point).

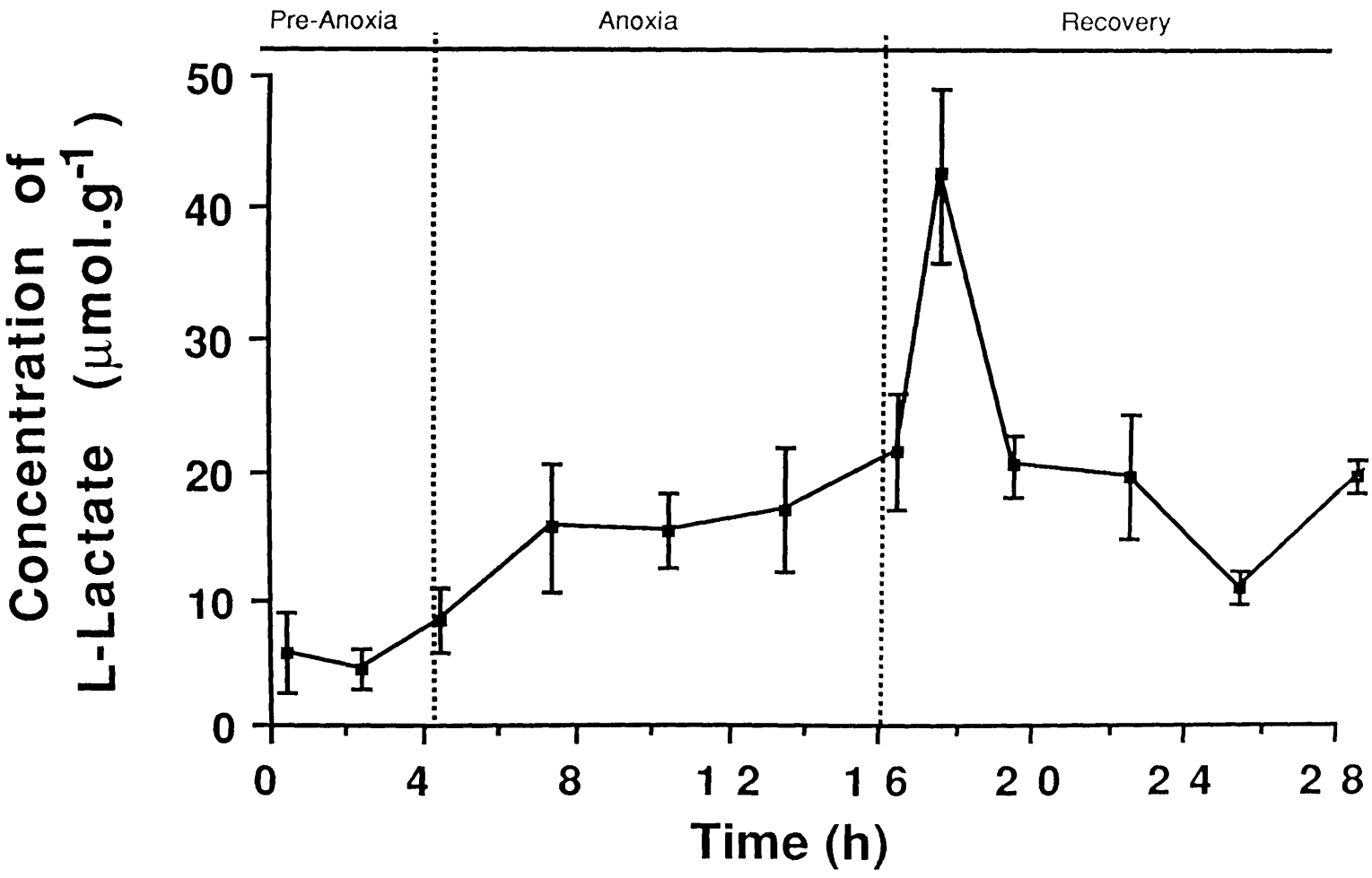
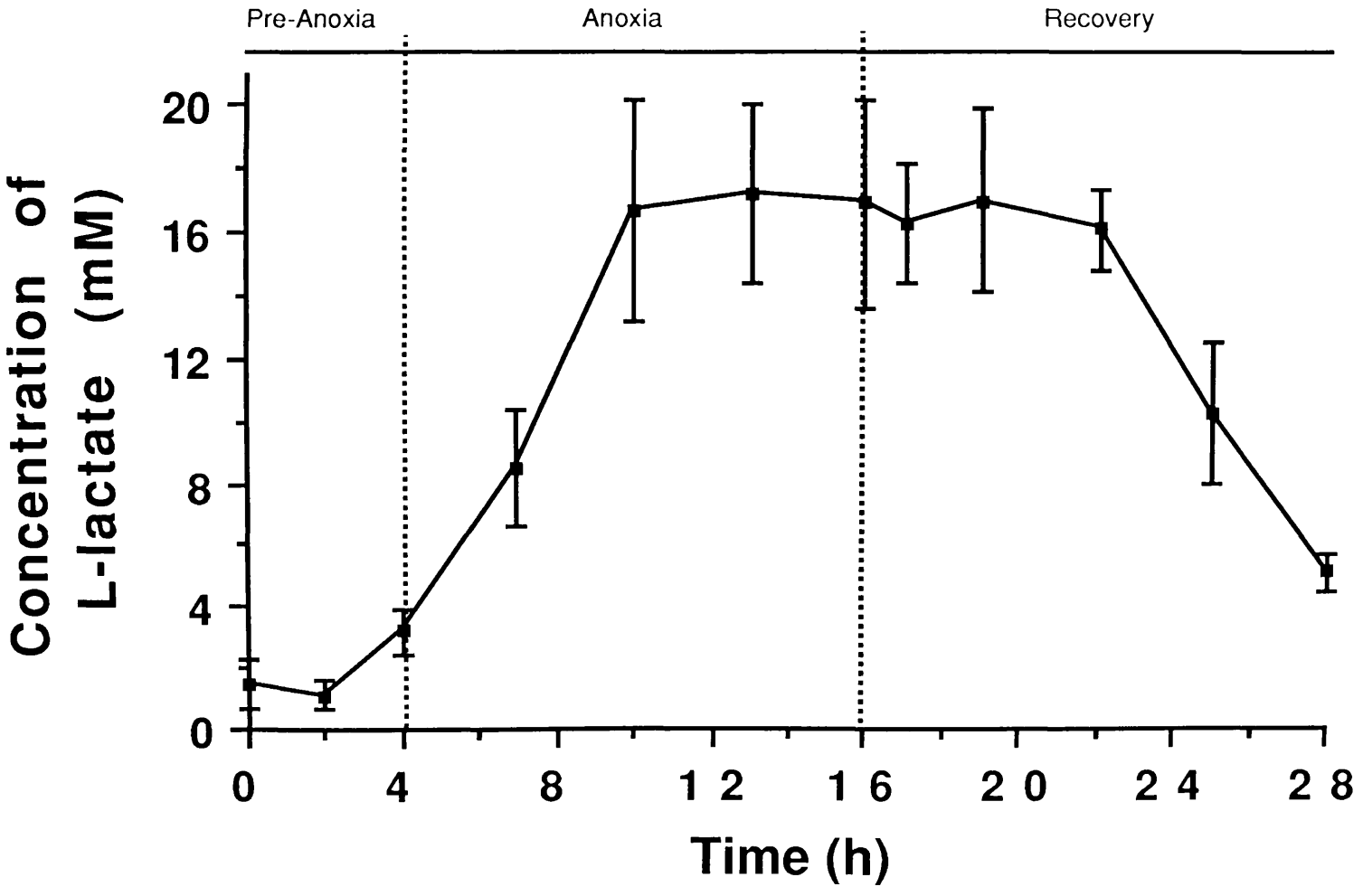

Pre-Anoxia
Anoxia
Recovery
Concentration of L-Lactate (µmol.g-1)
50
40
30
20
10
0
0
4
8
12
16
20
24
28
Time (h)

FIG. 3.6 Changes in the concentration of L-lactate in the haemolymph of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means ± S.D.

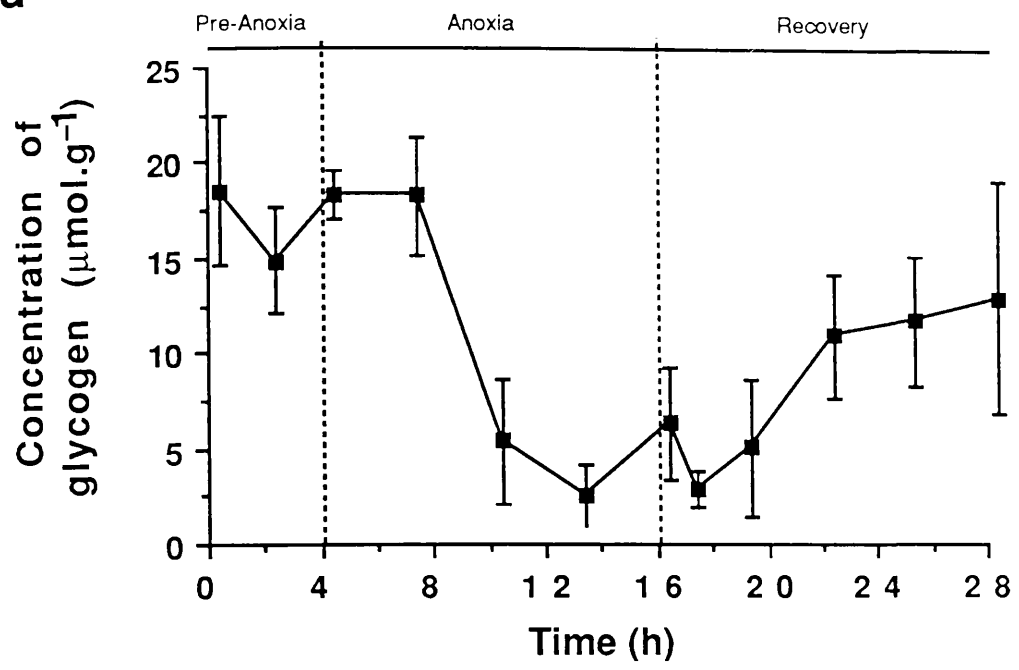

Pre-Anoxia
Anoxia
Recovery
Concentration of L-lactate (mM)
0
4
8
12
16
20
0
4
8
12
16
20
24
28
Time (h)

accumulating during anoxia, has previously been observed by other authors (Bridges & Brand, 1980; Taylor & Spicer, 1987).

During the 'pre-anoxic' and 'anoxic' periods, the concentrations of glycogen and of total circulating soluble carbohydrates (including D-glucose) decreased from 17.8 $\pm$ 3.9 to 2.22 $\pm$ 1.02 umol.g^{-1} and from 6.98 $\pm$ 1.3 to 2.57 $\pm$ 0.76 umol.g^{-1} respectively (Fig. 3.7). The concentration of glycogen of pooled tissue samples was estimated both enzymically and chemically. No significant differences ($P > 0.05$) were observed between the results of the two methods. It was interesting to note that there appeared to be lag of about 2 hours between the depletion of glycogen and free circulating soluble carbohydrates and the main increase in the concentration of pooled tissue L-lactate. By adding the mean concentrations of glycogen and free circulating sugars together, it was possible to estimate the size of the total glucosyl unit store that was available to the animal. This glucosyl unit store was depleted at a mean rate of 1.17 umol.$g^{-1}.h^{-1}$ during the anoxic period. Given that 2 molecules of L-lactate may be produced from 1 molecule of glucosyl units, it would appear from these results that approximately 92% of the glucosyl units catabolised have gone into the production of L-lactate (Fig. 3.8). During recovery, the concentrations of pooled tissue glycogen and soluble carbohydrates, increased from 2.22 $\pm$ 1.02 to 12.3 $\pm$ 2.7 umol.g^{-1} and from 2.57 $\pm$ 0.76 to 7.32 $\pm$ 2.2 umol.g^{-1} respectively. The same stoichiometric comparison as used previously during anoxia, can be applied to the recovery period, between L-lactate depletion and glucosyl unit accumulation. This result suggests that most of the L-lactate is being converted to glucosyl units via gluconeogenic pathways, and is therefore not being lost to the surrounding medium or fully oxidised to CO_2.

FIG. 3.7 Changes in the concentration of pooled tissue a) glycogen and b) water soluble carbohydrates in *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means ± S.D. (Water soluble carbohydrates consist of oligosaccharides and monosaccharides).

a


Pre-Anoxia
Anoxia
Recovery
Concentration of glycogen (μmol.g⁻¹)
25
20
15
10
5
0
0
4
8
12
16
20
24
28
Time (h)

b

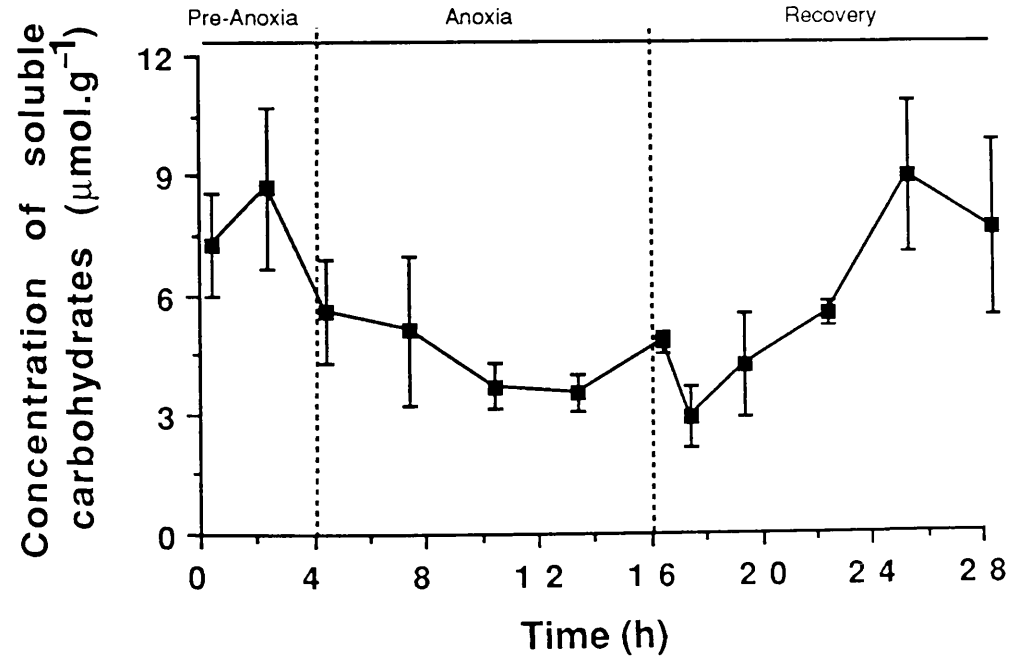
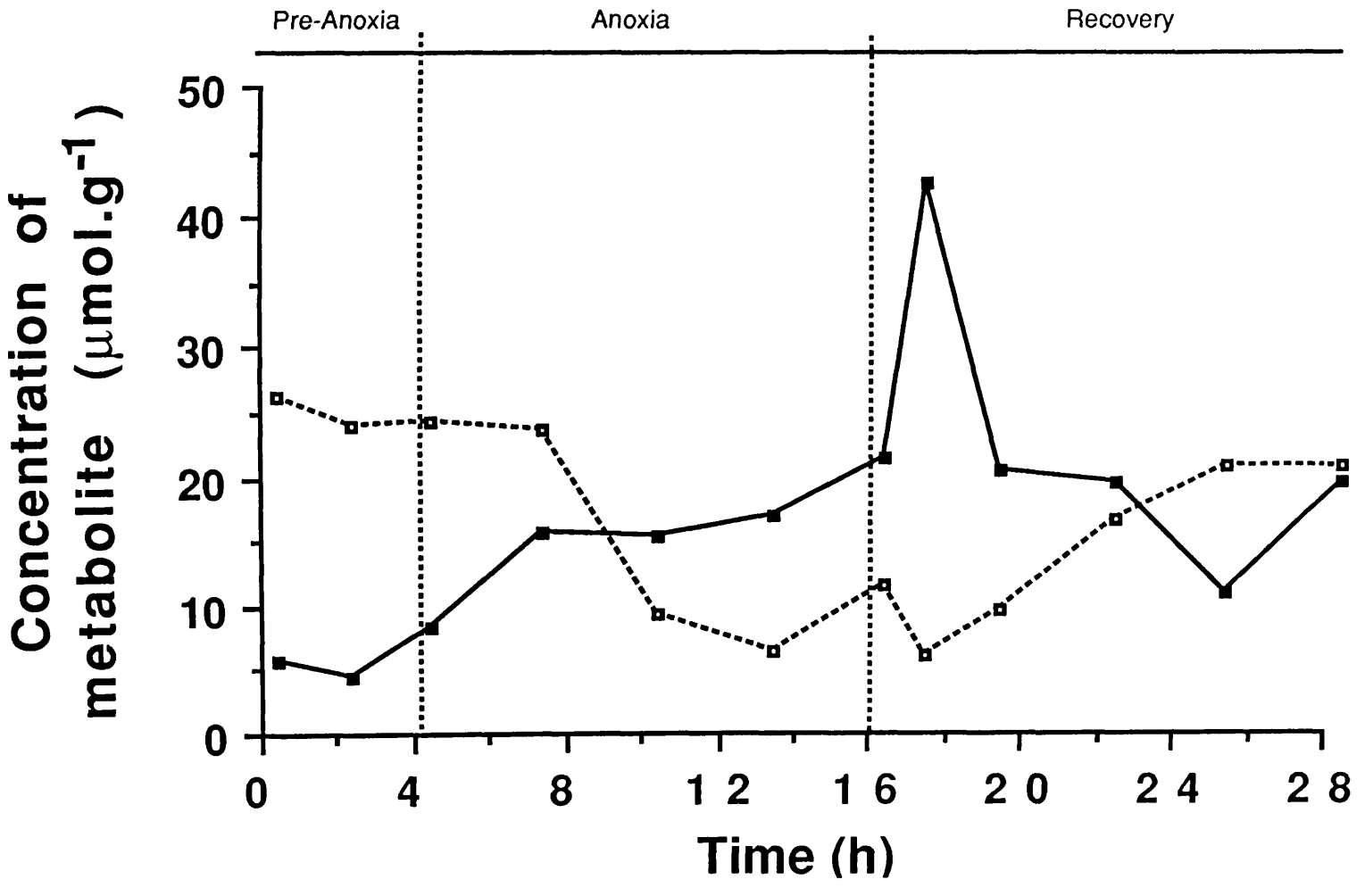

Pre-Anoxia
Anoxia
Recovery
Concentration of soluble carbohydrates (μmol.g⁻¹)
12
9
6
3
0
0
4
8
12
16
20
24
28
Time (h)

FIG. 3.8 Changes in the concentration of L-lactate (■) and glucosyl units (□) in the pooled tissue of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. A stoichiometric comparison revealed that during anoxia, 92 % of the glucosyl units catabolised could be explained by the production of L-lactate. A similar comparison made during normoxic recovery, indicated that approximately 88 % of the depletion of L-lactate could be explained by the increase in the concentration of the carbohydrate pool. (See text for further details).


Pre-Anoxia
Anoxia
Recovery
Concentration of metabolite (µmol.g-1)
50
40
30
20
10
0
0
4
8
12
16
20
24
28
Time (h)

3.3.3.2 Adenylate nucleotides and phospho-l-arginine.

The mean concentration of pooled tissue phospho-l-arginine was 7.5 ± 0.74 umol.g^{-1} in quiescent animals under normoxic conditions, but this decreased significantly ($P < 0.05$) to 2.53 ± 0.53 umol.g^{-1} after 12 hours of anoxia. After 9 hours of recovery the concentration of the phospho-l-arginine had increased again to 6.4 ± 0.3 umol.g^{-1} (Fig. 3.9).

The concentrations of adenylate nucleotides were estimated using both HPLC and enzymic assays. There was no significant difference between the two methods for any of the 3 nucleotides ($P > 0.05$ in all three cases).

The utilisation of phospho-l-arginine during the first 6 hours of anoxia, helped to ensure that there was no significant ($P > 0.05$) decrease in the concentration of ATP (Fig. 3.10a). There was, however, a significant decrease in the concentration of ATP to 1.07 ± 0.16 umol.g^{-1}, during the first hour of recovery. During the later stages of recovery, the concentration of ATP increased again to 4.14 ± 0.48 umol.g^{-1} before returning to original levels of 2.17 ± 0.156 umol.g^{-1} at the end of the experimental period.

The concentration of ADP did not change significantly throughout the experiment ($P > 0.05$) (Fig. 3.10b). When exposed to anoxia, the concentration of AMP remained constant at 0.23 ± 0.02 umol.g^{-1}, but during the first 3 hours of recovery there was a significant transient increase from 0.25 ± 0.03 to 0.37 ± 0.09 umol.g^{-1} (Fig. 3.10c).

The adenylate energy charge (AEC) was estimated from the nucleotide concentrations as obtained above (Fig. 3.11). The only significant change occurred during the first hour of recovery, when the energy charge decreased temporarily from 0.71 ± 0.005 to 0.65 ± 0.02 ($P < 0.05$). It soon recovered, however, increasing

FIG. 3.9 Changes in the concentration of phospho-l-arginine in the pooled tissue of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means ± S.D.

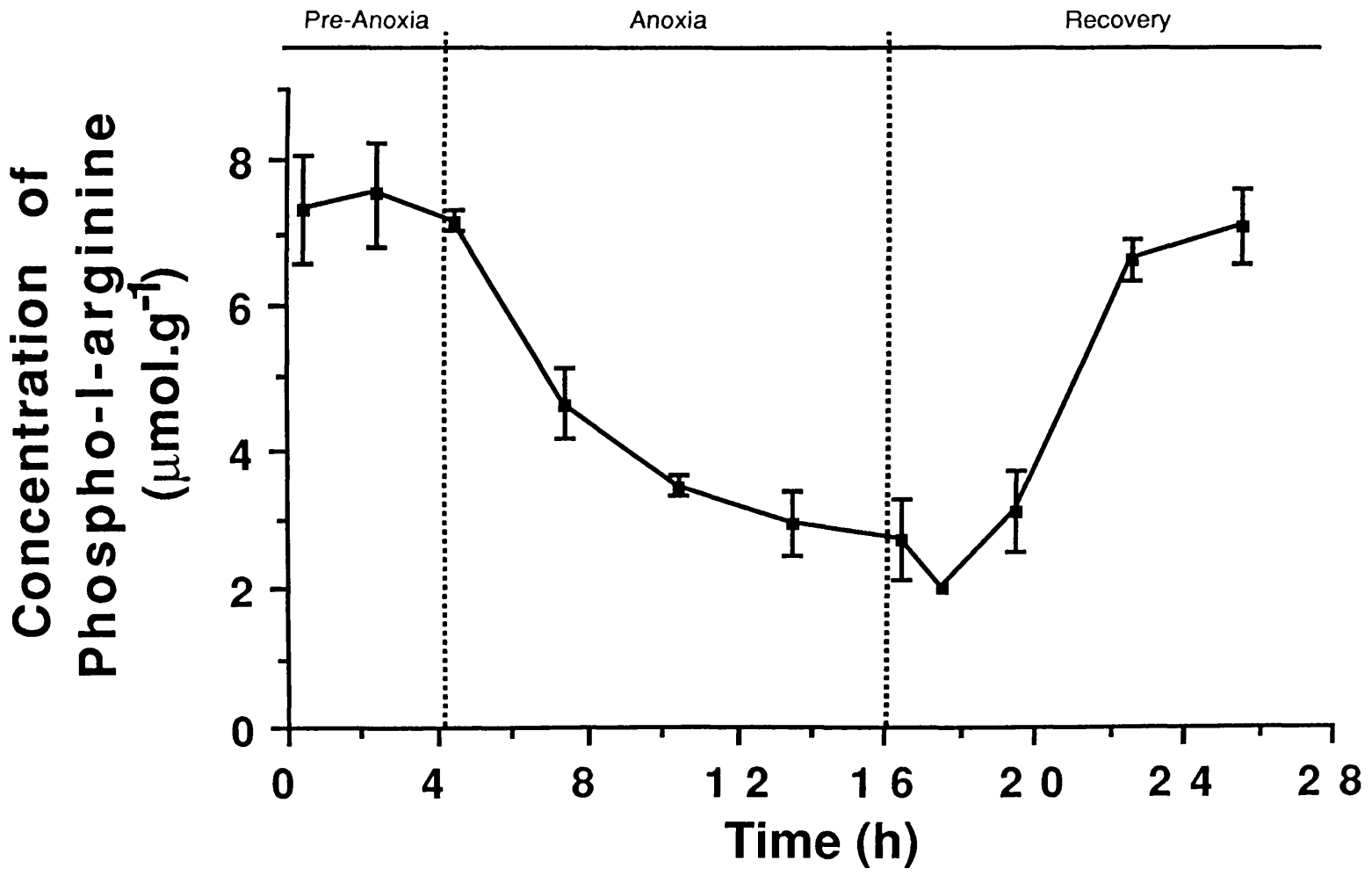

Pre-Anoxia
Anoxia
Recovery
Concentration of Phospho-l-arginine (µmol.g-1)
0
2
4
6
8
0
4
8
12
16
20
24
28
Time (h)

FIG. 3.10 Changes in the concentration of a) ATP; b) ADP and c) AMP in the pooled tissue of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D.

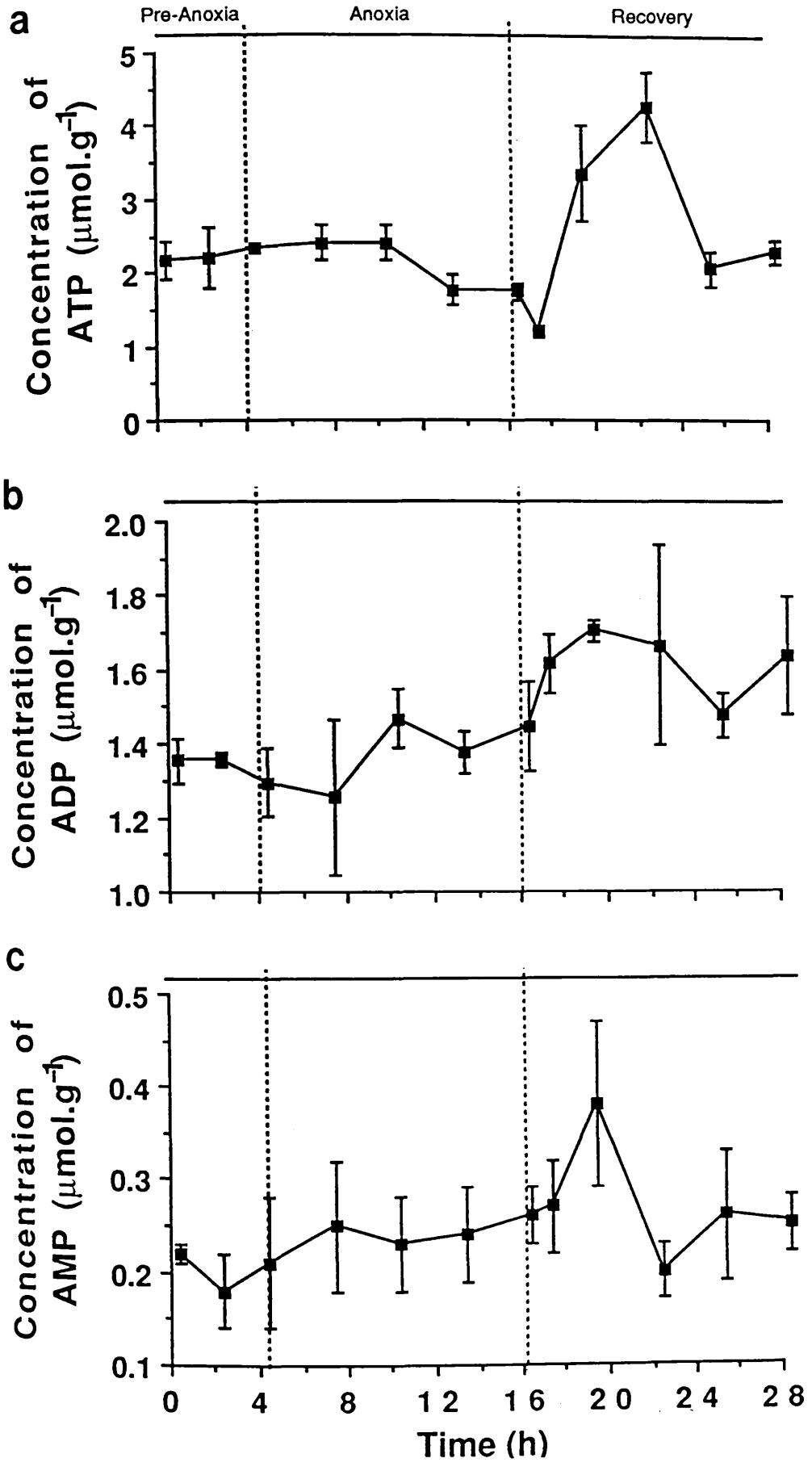
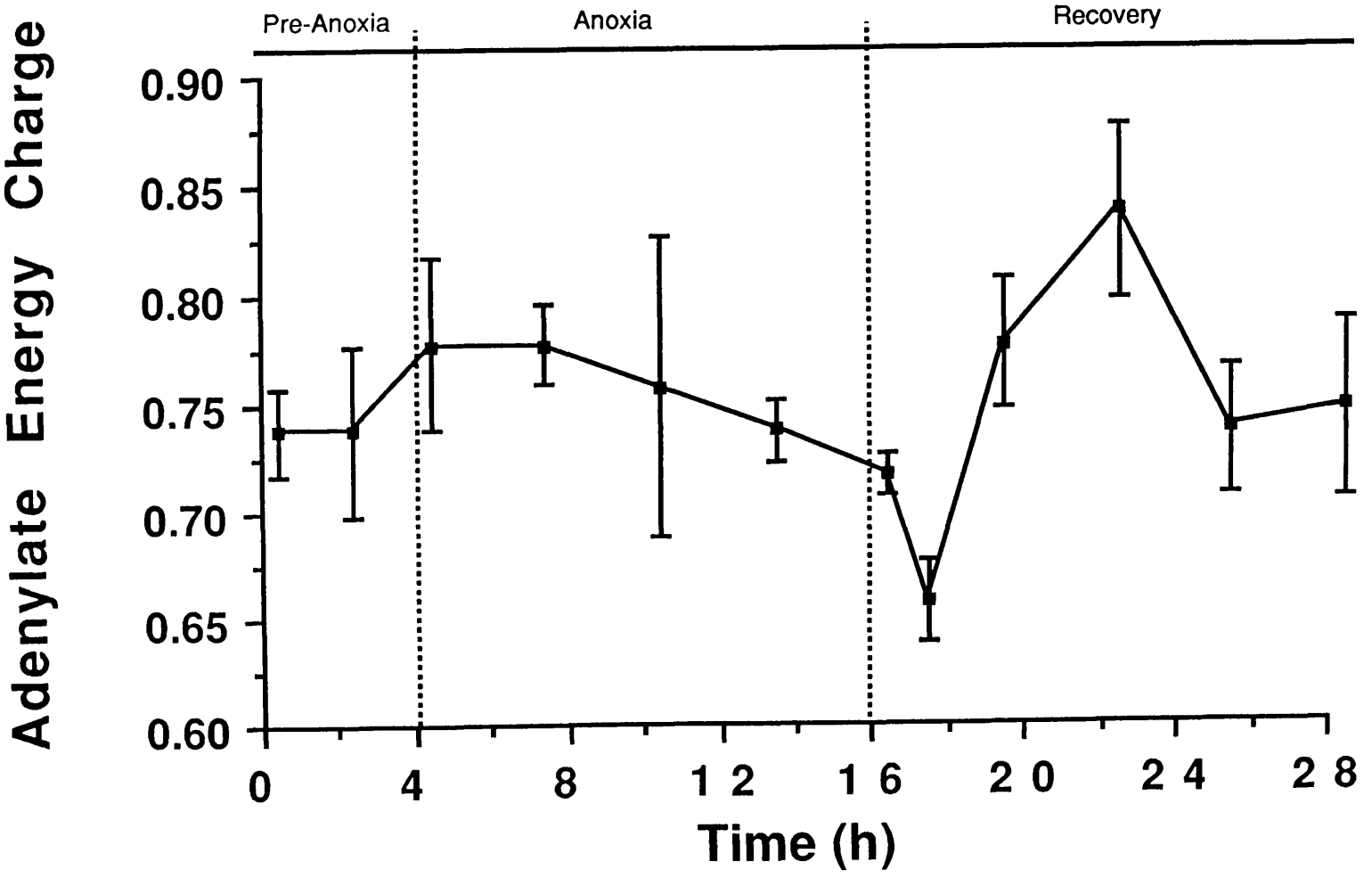

a
Pre-Anoxia
Anoxia
Recovery
Concentration of ATP (μmol.g-1)
5
4
3
2
1
0
b
Concentration of ADP (μmol.g-1)
2.0
1.8
1.6
1.4
1.2
1.0
c
Concentration of AMP (μmol.g-1)
0.5
0.4
0.3
0.2
0.1
0
4
8
12
16
20
24
28
Time (h)

FIG. 3.11 Changes in the adenylate energy charge (AEC) of the pooled tissue of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D. (See text for further details).


Pre-Anoxia
Anoxia
Recovery
Adenylate Energy Charge
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0
4
8
12
16
20
24
28
Time (h)

to 0.77 ± 0.04 after the 19th hour of the experiment.

3.3.3.3 Organic Acids.

The retention times for the organic acid standards used, can be seen in the following table:

Table 3.1 - HPLC retention times (RT) for the organic acid standards.

Organic acid standard	RT (min)
Oxaloacetate	5.74
Malate	6.90
Pyruvate	7.06
Succinate	8.68
L-lactate	8.85
Acetate	10.8
Fumarate	12.6
Propionate	13.0

All the acids could be resolved, with the exception of succinate and L-lactate, which had almost identical retention times of 8.68 and 8.85 minutes respectively (Fig. 3.12). Succinate was therefore estimated enzymically, following the method of Beutler (1981) (Appendix 6). The concentrations of succinate are tabulated overleaf:

FIG. 3.12 HPLC traces of resolved organic acids from *Carcinus maenas* exposed to A) normoxic conditions; B) 4 hours of declining Po_2; C) 12 hours of anoxia and D) 1 hour of recovery.

SF - Solvent front.
Peak 'a' - pyruvate.
Peak 'b' - L-lactate/succinate.
Peak 'c' - acetate.
Peak 'd' - fumarate.

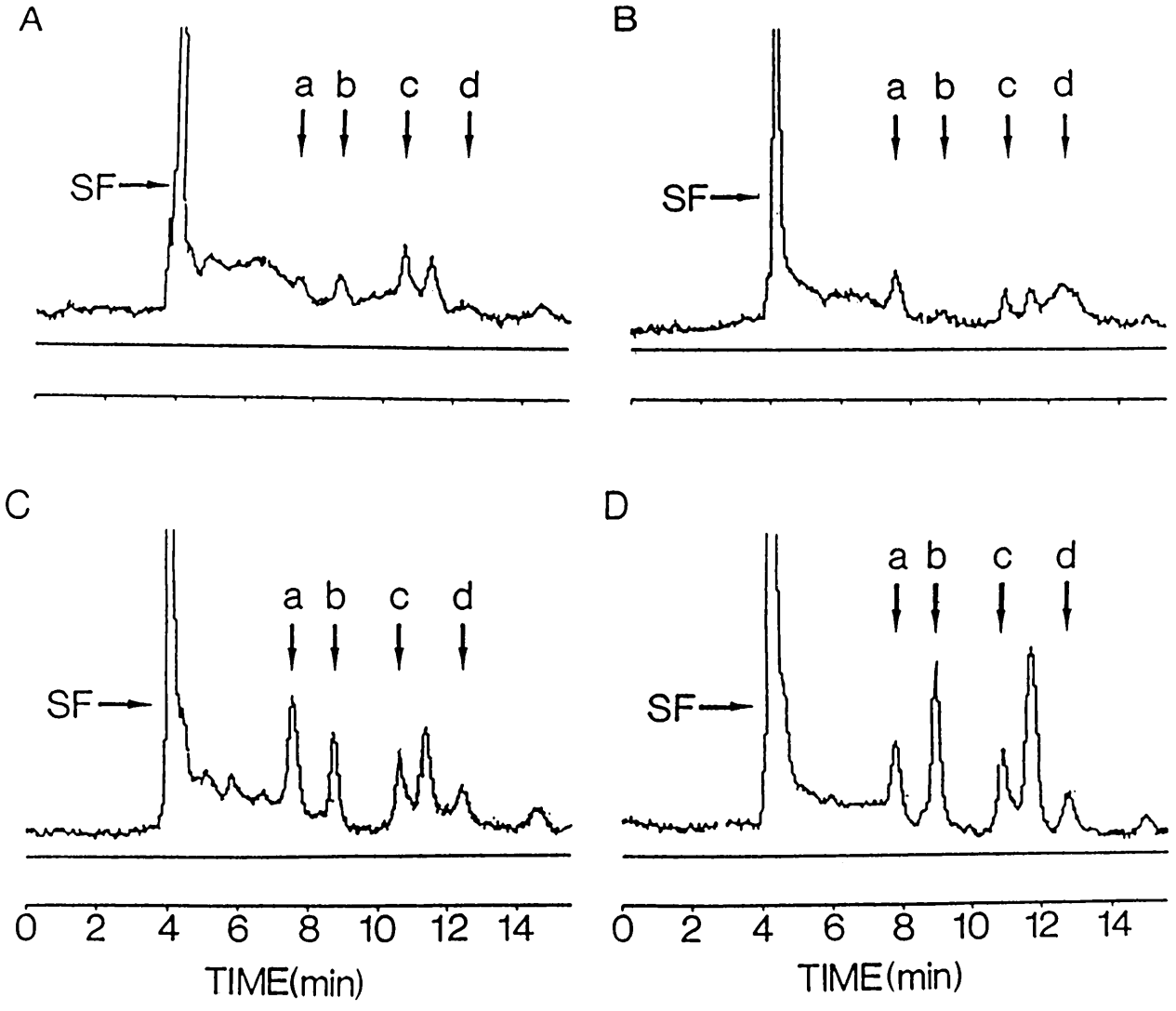

A
B
C
D
a b c d
SF
0 2 4 6 8 10 12 14
TIME(min)

Table 3.2 - Changes in the concentration of succinate during exposure to prolonged anoxia and subsequent recovery.

Time after start of experiment (h)	Condition	Succinate ($umol.g^{-1}$ fresh wt.)
Control	Normoxic	1.2 ± 0.03
4	Anoxic	0.90 ± 0.02
10	Anoxic	0.81 ± 0.15
16	Anoxic	0.76 ± 0.21
17	Recovery	0.89 ± 0.09
22	Recovery	0.79 -
28	Recovery	0.94 ± 0.17

The concentration of succinate in the tissues of *Carcinus maenas* was very low and remained constant throughout the experiment. Therefore most of the peaks labelled 'b' in Figure 3.12 can be attributed to L-lactate. This was confirmed using standards, since it was calculated that the concentrations of L-lactate that these peaks (b) represented, were not significantly ($P > 0.05$) different from the results obtained for L-lactate via enzymic methods.

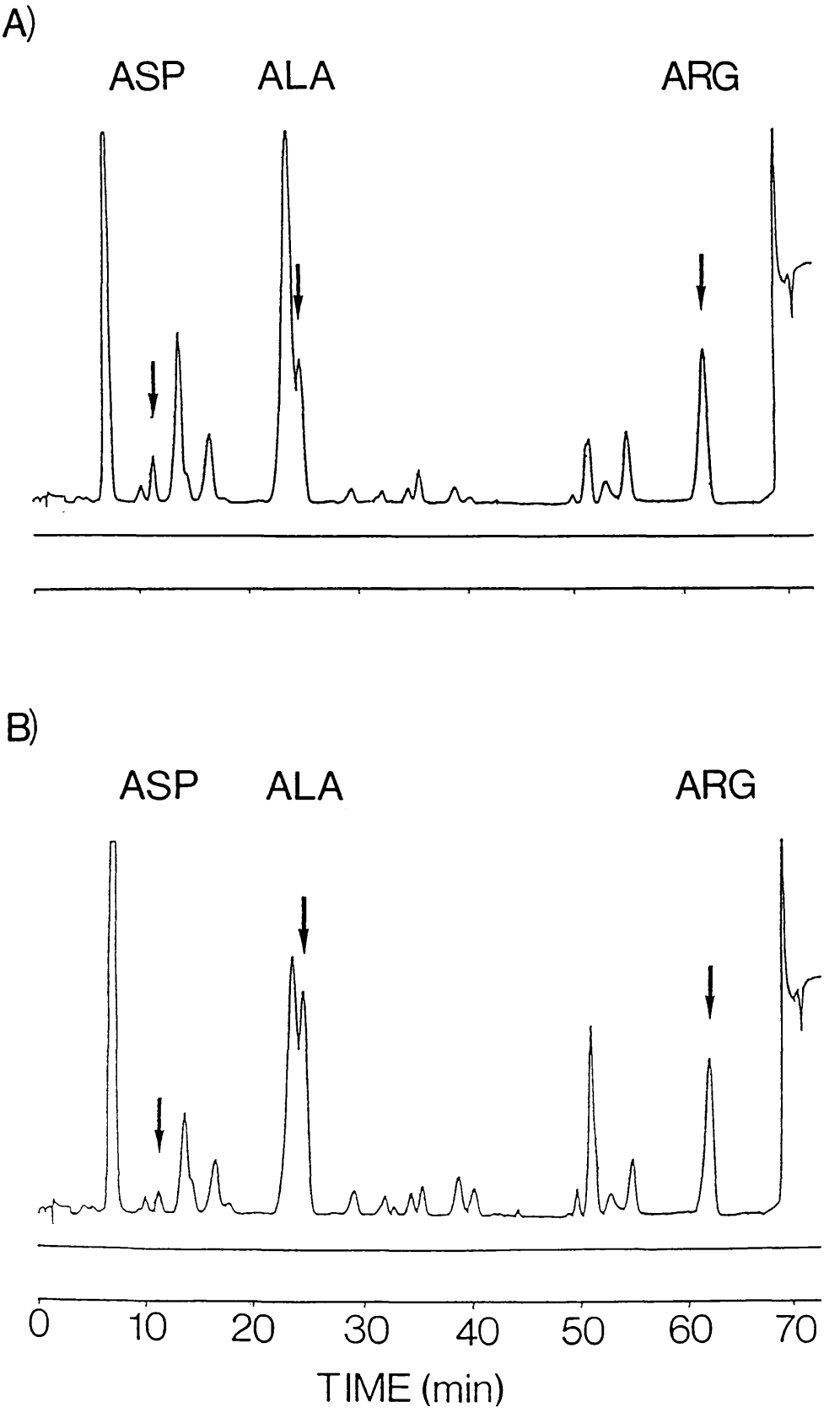
The concentration of acetate (peak c) did not increase significantly, throughout the course of the experiment. The concentrations of pyruvate (peak a) and fumarate (peak d) increased significantly during anoxia, from 1.65 ± 0.11 to 2.95 ± 0.15 $umol.g^{-1}$ and from 0.64 ± 0.06 to 0.91 ± 0.04 $umol.g^{-1}$ respectively ($P < 0.05$ in both cases). The magnitude of change in the concentration of fumarate was insignificant, however, when compared with the enormous increase in the concentration of L-lactate during exposure to anoxia. The other organic acids (malate, oxaloacetate, and propionate) were present only in very low concentrations, which could not be accurately determined by the HPLC system.

3.3.3.4 Amino Acids.

Figure 3.13 shows 2 automated amino acid analysis traces (representing extracts from crabs taken at O h (A) and 12 h (B) in the time course). Of all the amino acids estimated, 3 were of particular interest, owing to the fact that they are known to be important in anaerobic glycolysis. The concentration of aspartate (ASP) decreased from 1.25 $\pm$ 0.34 to 0.50 $\pm$ 0.13 umol.g^{-1} during the anoxic period and then increased again to 0.93 $\pm$ 0.14 umol.g^{-1} during recovery ($P < 0.05$ in both cases). The concentration of alanine (ALA) increased from 5.77 $\pm$ 0.7 to 8.90 $\pm$ 1.13 umol.g^{-1} during anoxia, but decreased to 7.72 $\pm$ 0.6 umol.g^{-1}, after 12 hours of recovery ($P < 0.05$ in both cases). Finally, the mean concentration of arginine decreased significantly ($P < 0.05$) from 9.67 $\pm$ 0.41 to 7.49 $\pm$ 1.4 umol.g^{-1} after 0 and 12 h of anoxia. For the remainder of the experiment the mean concentration of arginine did not change significantly ($P > 0.05$) from 7.08 $\pm$ 0.52 umol.g^{-1}. The concentration of the remaining amino acids can be found in Appendix 9.

FIG. 3.13 Automated amino acid analysis traces of resolved amino acids from *Carcinus maenas* exposed to A) normoxic conditions; B) 12 hours of anoxia.

ASP - aspartate.
ALA - alanine.
ARG - arginine.


A)
ASP
ALA
ARG
B)
ASP
ALA
ARG
0
10
20
30
40
50
60
70
TIME (min)

3.4 DISCUSSION.

3.4.1 Preliminary experiments.

Although there have been a considerable number of studies on the influence of starvation on the metabolic reserves of crustaceans, interpretation of the results of these studies is difficult since some authors have analysed the pooled tissue samples while others have considered only specific tissues and organs. Whyte *et al.* (1986) state that there is a conflict in the literature on the relative abundance and order of utilisation of major energy reserves in decapod crustaceans during starvation. Heath & Barnes (1970) described a large decrease in the concentration of lipid reserves during starvation in *Carcinus maenas*. Lipids have also been shown to be the main energy source in the crayfish *Orconectes nair* (Armitage *et al.*, 1972). This contrasts with the preferential use of protein reserves in *Hemigrapsus nudus* (Neiland & Scheer, 1953), *O. virilis* (Hazlett *et al.*, 1975), *Panulirus longipes* and *Nephrops norvegicus* (Dall, 1974, 1981). Even within the same species there seems to be a certain amount of confusion. Thus Cuzon & Ceccaldi (1972) found that in the shrimp *Crangon crangon*, carbohydrates were primarily used during a starvation period of 4 weeks. Regnault (1981) stated that, based on the oxygen to nitrogen ratios, lipids and proteins were the principal sources of energy in this species.

There is also considerable confusion concerning the utilisation of energy reserves in *Carcinus maenas*. Schönborn (1911) found that, after 25 days of starvation, the glycogen store in *Carcinus maenas* had been largely consumed, whereas Neiland & Scheer (1953) failed to find any diminution in either the concentration of glycogen or lipids after a period of starvation. Munn (1963) found a steady decrease in the concentration of hepatopancreas glycogen, over a starvation period of 20 days. Heath & Barnes (1970) recorded a decrease in glycogen after 40 days of starvation, but 5 times as much lipid was consumed over the same period. Finally, Marsden

et al. (1973) stated that there was no significant decrease in the concentration of carbohydrates, triglycerides or phospholipids in either the hepatopancreas or gill tissue of *Carcinus maenas* following starvation for 2 weeks. Differences in the experimental procedures used by different authors make it more difficult to draw firm conclusions but it would appear that over a period of 14 days, the effects of starvation on the concentrations of carbohydrates, lipids and proteins are likely to be very small. In order to reduce these effects still further, it was decided that, in all experiments carried out during the present study, the crabs would be fed once during the 2 weeks that they were kept in the aquariums prior to use in experiments. Since feeding immediately prior to an experiment would interfere with the concentration of certain metabolites, a preliminary experiment was carried out to determine the length of time taken for the concentration of haemolymph sugars to return to normal after a single meal. The results from this experiment indicated that it took as little as 2 days for this to happen. It was therefore decided that the crabs would be fed on day 5 of the 14 day period and then starved for the remaining 9 days. This 9 day starvation did not decrease the concentration of pooled tissue glycogen, but the overall variation between individuals did decline. This was advantageous, since in later experiments, it would facilitate the interpretation of the significance of any differences, observed between experimental groups.

3.4.2 Survival under anoxic conditions.

Carcinus maenas was shown to have a mean tolerance of anoxia of 15.8 h (LT_{50}) at 10 ^{o}C, with no individuals surviving beyond 18 h . By way of comparison, it has been demonstrated that thalassinid crustaceans have much higher LT_{50} values. Thompson & Pritchard (1969) and Zebe (1982) reported maximum survival times in *Callianassa californiensis*, under conditions of anoxia, of 138 and 60 h respectively. In *Calocaris macandreae* the maximum survival time in anoxia was about 50 h, with

an LT_{50} of 43 h (Anderson, 1989). Another thalassinid *Upogebia pugettensis* was found to be less tolerant, but still managed to survive up to 30 h of anoxia (Zebe, 1982). There are very few comparative studies from other decapod groups. In 1941, however, Vallin (cited in Lindroth, 1950) stated that crayfish can survive for 20 h under experimental conditions when the oxygen concentrations of the surrounding medium was only 0.2 $mg.l^{-1}$. Gäde (1984) found that the crayfish *Orconectes virilis* could survive at least 16 h of anoxia at 14 ^{o}C. Interspecific comparisons of survival rates during exposure to anoxia were made more difficult, however, since there is growing evidence that tolerance of anoxic conditions is reduced at higher temperatures, owing to higher metabolic rates. The great tolerance that the thalassinids have for anoxic exposure, is possibly an adaptation to their life in burrows, in which they regularly experience severely hypoxic or even anoxic conditions (Atkinson & Taylor, 1988). Based on the results of this experiment, an anoxic period of 12 h was chosen for future experiments, since this was felt to be sufficient time to investigate the maximum capacity of *C. maenas* for anaerobic metabolism.

3.4.3 The accumulation of end products of anaerobic energy metabolism in crustaceans.

Since one molecule of glucosyl units may theoretically be converted into 2 molecules of L-lactate, it should be possible to establish whether glucosyl units and L-lactate are the major substrate and end product respectively of anaerobic metabolism in *Carcinus maenas*. Teal & Carey (1967) compared the depletion rate of glucosyl units with the accumulation rate of L-lactate in the pooled tissue samples of the marsh crab *Uca pugnax* and found that there was an approximate ratio of 1:2 (glucosyl units : L-lactate respectively). They found, however, that there were huge variations in the concentration of glucosyl units and that they could not attach too much statistical significance to their estimation of the rate of glucosyl unit depletion

during anoxia. Zebe (1982) reported ratios of 1:2 and 1:1.93 for *Upogebia pugettensis* and *Callianassa californiensis* respectively after 24 h of exposure to anoxia. Taylor & Spicer (1987) found a ratio of approximately 1:2 in the prawns *Palaemon elegans* and *P. serratus* and concluded that alternative metabolic pathways are unlikely to be of significance during anaerobic metabolism. Results from this present study confirm the findings of the above authors. The ratio of glucosyl depletion and L-lactate accumulation for *Carcinus maenas* was 1:1.92, indicating the almost total reliance on the pathway culminating in the production of L-lactate.

Only a few studies have previously measured the concentrations of organic acids in decapod crustaceans that have been exposed to anoxia. Van Aardt & Wolmarans (1987) used gas chromatography to measure changes in the concentration of certain organic acids in the freshwater crab *Potamon warreni* during exposure to anoxia. They showed that the concentration of L-lactate increased eighteenfold during a 6 h exposure to anoxia, and that there was a small increase in the concentration of succinate. Similar increases in the concentration of succinate in decapods have also been reported by Zebe, 1982; Gäde, 1984; Albert & Ellington, 1985. Zebe (1982) suggested that the succinate might have originated from the activity of micro-organisms in the alimentary tract. Whether this is true or not is doubtful, but all the authors agree that succinate is at the very most of trivial importance in providing energy for decapod crustaceans during anoxia. Van Aardt & Wolmarans (1987) found that other organic acids and the more volatile of the fatty acids could not be determined using gas chromatography. A recent study by Anderson (1989) using HPLC, confirmed that L-lactate was the only organic acid to increase significantly during anoxia in the mud burrowing shrimp *Calocaris macandreae*.

Results from the present investigation agree with previous studies, except for the concentration of the succinate which remained constant throughout the exposure to anoxia. Amongst the other organic acids measured, a small increase in both the

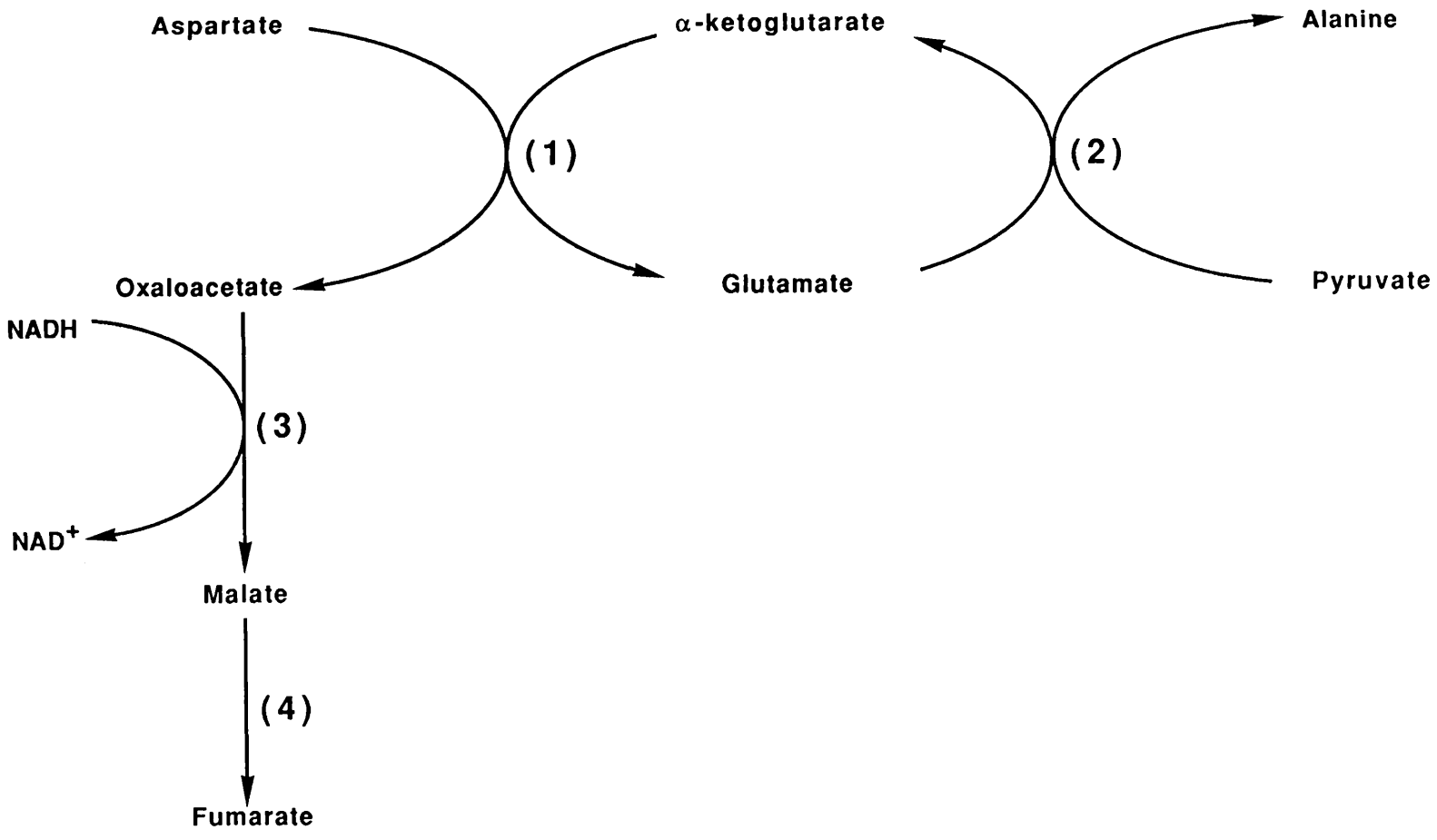
concentrations of pyruvate and fumarate was observed. The concentration of malate, oxaloacetate and propionate could not be accurately determined on the HPLC system. Oxaloacetate is readily decarboxylated to pyruvate, and it is possible that the small increase in the concentration of pyruvate could be due in part to the decarboxylation of oxaloacetate. Compared to the much greater increase in the concentration of the L-lactate, however, these changes are insignificant.

It has been known for many years that amino acids can be intimately involved in anaerobic metabolism, e.g. as a substrate (aspartate); an intermediary in opine formation (taurine, lysine); and also as an anaerobic end product (alanine). In previous work on decapod crustaceans, alanine and aspartate are the 2 amino acids most often measured. Aspartate was first established as a substrate for anaerobic metabolism by Collicutt & Hochachka (1977) working on oyster heart muscle. Livingstone (1983) describes an anaerobic pathway in molluscs, in which aspartate is converted to oxaloacetate via a series of transamination reactions in the presence of pyruvate and glutamate-oxaloacetate transaminase (EC 2.6.1.1) (Fig. 3.14). Zebe (1982) reports that the concentration of aspartate decreases from 0.87 $\pm$ 0.19 to 0.38 $\pm$ 0.16 umol.g^{-1} fresh wt. after 12 h of anoxia in the thalassinid *Callianassa californiensis*. At the same time, there is a concomitant increase in succinate from 0.21 $\pm$ 0.03 to 0.52 $\pm$ 0.14 umol.g^{-1} fresh wt. Van Aardt & Wolmarans (1987) reported a similar situation in the freshwater crab *Potamon warreni*. In the present study, the concentration of aspartate was shown to decrease during anoxia, but fumarate rather than succinate was the end product that accumulated. The concentrations of aspartate and succinate/fumarate in all these studies are extremely low and are insignificant in comparison to the quantities of L-lactate that accumulated. This pathway, utilising aspartate as a substrate, becomes more important in other groups of invertebrates in which the stores of free aspartate are far greater (Gäde, 1983).

Both D- and L-alanine have been found to increase in the tissues of many decapod

FIG. 3.14 Pathway involving the transamination of aspartate to oxaloacetate.

(1) - glutamate-oxaloacetate transaminase (EC 2.6.1.1).
(2) - alanine transaminase (EC 2.6.1.2).
(3) - malate dehydrogenase (EC 1.1.1.37).
(4) - fumarase (EC 4.2.1.2).


Aspartate
α-ketoglutarate
Alanine
(1)
(2)
Oxaloacetate
Glutamate
Pyruvate
NADH
(3)
NAD+
Malate
(4)
Fumarate

crustaceans during exposure to anoxia (Zebe, 1982; Gäde, 1984; Albert & Ellington, 1985; Van Aardt & Wolmarans, 1987). The alanine is produced as a by-product of the transamination of aspartate to oxaloacetate, as described above. In the present study, this series of reactions, could only account for about 25 % of the total amount of alanine produced. The mean of 3.13 umol.g^{-1} of alanine produced during anoxia, accounts for only 7.2 % of the total end product accumulated, and is therefore of relatively minor importance in comparison to L-lactate, to the anaerobic energy metabolism of *Carcinus maenas*.

The results from this present study indicate that L-lactate is the major end product of anaerobic metabolism in decapod crustaceans, with a small amount of alanine also being produced. In addition, there was evidence to suggest that the amino acid aspartate is utilised as a substrate, with fumarate the accumulating end product. In comparison with the accumulation of L-lactate, however, both alanine and fumarate are of only very minor importance in crustacean anaerobic metabolism.

3.4.4 Comparative aspects of energy production during environmental anaerobiosis.

3.4.4.1 Anaerobic glycolysis in decapod crustaceans.

The primary method of energy production in decapod crustaceans involves the utilisation of the carbohydrate pool and the eventual production of L-lactate (section 3.4.3). In this study, glycogen was found to constitute about 75 % of the total carbohydrate pool and the remaining 25 % being soluble carbohydrate (mainly monosaccharides and oligosaccharides). This compares favourably with the situation in *Calocaris macandreae* in which glycogen accounts for about 73 % of the carbohydrate pool (Anderson, 1989). In many studies, the concentration of glycogen has been estimated but that of the oligosaccharides ignored. Therefore the

calculated rates are likely to underestimate the total carbohydrate pool utilisation. The following table presents the carbohydrate pool utilisation rates in decapod crustaceans, during periods of exposure to environmental anoxia.

Table 3.3 The rates of carbohydrate (glucosyl unit) utilisation in the tissues of decapod crustaceans during exposure to anoxia.

Species	Rate of glucosyl unit utilisation (umol. $h^{-1}.g^{-1}$ fresh wt.)	Reference
Uca pugnax	0.30	Teal & Carey, 1967
Upogebia pugettensis	0.79	Zebe, 1982
Callianassa californiensis	0.35	Zebe, 1982
Orconectes limosus	2.38	Gäde, 1984*
Palaemon elegans	1.06	Taylor & Spicer, 1987
Palaemon serratus	1.55	Taylor & Spicer, 1987
Calocaris macandreae	0.42	Anderson, 1989**
Carcinus maenas	1.08	Present study**

* glucosyl unit utilisation rate of tail muscle only.
** rate includes oligosaccharide consumption as well as glycogen.

The lowest rates of glucosyl unit utilisation have been observed in those species which experience severe hypoxia most frequently (i.e. the mud burrowing shrimps of the family Thalassinidea). The prawn, *Palaemon serratus* is primarily a subtidal species and is not normally exposed to severely hypoxic conditions in the field (Taylor & Spicer, 1987). This is reflected in the relatively high rate of glucosyl unit utilisation observed in this species. The rate of 1.08 $umol.g^{-1}.h^{-1}$ observed in *Carcinus maenas* reflects the fact that its anoxia tolerance is intermediate between that of the thalassinids and *P. serratus*.

The results presented in Table 3.3 suggest that there is a direct relationship between rate of glucosyl unit utilisation and duration of the period of exposure to anoxia. In

Carcinus maenas there was a rapid decrease in the concentration of the carbohydrate pool after 5 or 6 h of anoxia. No immediate increase in the concentration of L-lactate was observed (Fig. 3.5). This implies that there may be some glycolytic intermediates present perhaps in the form of dihydroxy-acetone phosphate. Similar accumulations of glycolytic intermediates are well documented in insects, in which glycerol 1-phosphate is produced (Hochachka & Somero, 1973; Jabbar & Strang, 1987). In decapod crustaceans, glycolytic intermediates have been suggested to occur as a result of post-mortem degradative changes (Hiltz & Bishop, 1975). Further discussion of this subject is given in Chapter 4 (section 4.4.2), in which radio-isotopes were used to try to establish the presence of pathways resulting in the accumulation of any glycolytic intermediates.

The accumulation of L-lactate in both the tissue and haemolymph of decapod crustaceans has been reported by a number of authors in a variety of species. The following table summarises the accumulation rates ($umol.h^{-1}.g^{-1}$ fresh wt. and $mmol.l^{-1}.h^{-1}$ for tissue and haemolymph respectively) and the mean maximum concentration of L-lactate measured ($umol.g^{-1}$ fresh wt. and $mmol.l^{-1}$ for tissue and haemolymph respectively). Metabolites were estimated in pooled tissue sample and deproteinised haemolymph.

Table 3.4 Accumulation rates and maximum concentrations of L-lactate in the tissue and haemolymph of decapod crustaceans, following exposure to environmental anoxia (10 °C).

Species	Accumulation rate[1]	Maximum L-lactate concentration[2]	Reference
Tissue			
Uca pugnax (20°C)	2.20	40.0	Teal & Carey, 1967
Callianassa californiensis(12°C)	0.70	16.8	Zebe, 1982
Upogebia pugettensis (12°C)	1.52	6.40	Zebe, 1982
Orconectes limosus (13°C)	1.50	19.3	Gade, 1984
Menippe mercenaria (25°C)	1.50	16-20	Albert & Ellington, 1985**
Palaemon elegans	3.97	16.7	Taylor & Spicer, 1987
Palaemon serratus	5.60	9.60	Taylor & Spicer, 1987
Calocaris macandreae	0.90	30.2	Anderson, PhD Thesis, 1989
Carcinus maenas	1.10	20.3	Present study
Haemolymph			
Upogebia pugettensis	1.96	39.2	Pritchard & Eddy, 1979*
Atelecyclus rotundatus	1.26	6.30	Bridges & Brand, 1980*
Carcinus maenas	0.91	4.54	Bridges & Brand, 1980*
Corystes cassivelaunus	1.73	8.63	Bridges & Brand, 1980*
Galathea strigosa	1.46	7.13	Bridges & Brand, 1980*
Homarus gammarus	0.95	7.63	Bridges & Brand, 1980*
Nephrops norvegicus	1.75	8.74	Bridges & Brand, 1980*
Macrobrachium rosenbergii (22°C)	5.85	12.0	Mauro & Malecha, 1984
Orconectes limosus (13°C)	3.75	60.0	Gade, 1984
Menippe mercenaria (25°C)	3.33	46.7	Albert & Ellington, 1985
Callinectes sapidus (25°C)	9.70	41.7	Lowery & Tate, 1986
Callinectes sapidus (32°C)	22.2	44.4	Lowery & Tate, 1986
Palaemon elegans	2.98	13.0	Taylor & Spicer, 1987
Palaemon serratus	6.35	6.93	Taylor & Spicer, 1987
Potamon warreni (25°C)	6.40	40.3	van Aardt, 1988
Calocaris macandreae	0.85	40.2	Anderson, PhD Thesis, 1989
Carcinus maenas	1.16	17.1	Present study

1 - The accumulation rate of L-lactate during anoxia (umol.h^{-1}.g^{-1} fresh wt. and mmol.l^{-1}.h^{-1} for tissue and haemolymph samples respectively).

2 - Maximum mean concentration of L-lactate measured (umol.g^{-1} fresh wt. and mmol.l^{-1}).

* - Original data re-calculated using the above units.

** - Mean concentration taken of heart, leg socket and cheliped muscles.

An inverse relationship can be observed between the accumulation rate of L-lactate and the anoxia tolerance of each of the species cited in Table 3.4. The thalassinids, with the greatest tolerance to anoxia, have the lowest rate of L-lactate accumulation (Zebe, 1982). *Palaemon serratus* which has previously been shown to have a high glucosyl unit utilisation rate, also has a very high rate of L-lactate accumulation (Taylor & Spicer, 1987). It is interesting to note that increasing the ambient

temperature, increases the rate of haemolymph L-lactate accumulation. The accumulation rate of L-lactate measured for *Carcinus maenas* in this study, is consistent with an animal which is moderately well adapted to survive exposure to anoxic conditions.

3.4.4.2 The role of phospho-l-arginine and adenylate nucleotides.

The majority of studies investigating the adenylate nucleotide metabolism in marine invertebrates have concentrated on functional (exercise) anaerobiosis (Onnen & Zebe, 1983; Thébault *et al.*, 1987). Wijsman (1976) investigated the effects of aerial exposure on the adenylate nucleotides of *Mytilus edulis*, but no mention was made of the phosphagen, phospho-l-arginine. Phospho-l-arginine was first detected and isolated in crustaceans by Meyerhof and Lohmann in 1928 (cited in Onnen & Zebe, 1983). It is an extremely labile organic phosphoryl compound which is able to phosphorylate ADP under the control of arginine kinase to produce arginine and, more importantly, ATP. During conditions of stress, the breakdown of phospho-l-arginine helps to maintain the concentration of ATP (and hence the adenylate energy charge) at or near its normoxic level (Ebberink *et al.*, 1979, Onnen & Zebe, 1983; Gäde, 1983). Results from this study agree with the above, with the concentration of ATP remaining constant throughout the first 6 h of anoxia as a result of the transphosphorylation of ADP by phospho-l-arginine. In recent years, phosphagens have also been shown to be potential regulators of glycolysis by direct inhibition of such key enzymes as phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) (Guderley *et al.*, 1976; Storey, 1981; Gäde, 1983, 1984). This subject will be discussed in greater detail in Chapter 7.

The adenylate energy charge (AEC) remained constant throughout the anoxic period and only decreased significantly after 12 h of anoxia. This ratio was devised by Atkinson & Walton (1967) as a measure of the relative proportions of the high

energy phosphate groups. It has since been used as a means of assessing the physiological condition of an organism under a variety of stressful situations (Bestwick, 1988). In this study, the AEC was shown to be a rather insensitive index of metabolic condition, since a significant decrease was only observed after the crabs had been exposed to 12 h of anoxia. Wegener (1988) reported, however, that in the frog, a decrease in the AEC during 2 h of anoxia was measurable only in certain tissues (e.g. brain, liver and kidney tissue). Since pooled tissue samples were taken in the present study, it is possible that subtle changes in the AEC of specific tissues were missed.

3.4.4.3 Energy production under aerobic and anaerobic conditions.

One of the main problems with anaerobic respiration is the incomplete oxidation of the initial substrate. Under aerobic conditions a theoretical total of 39 molecules of ATP are produced from 1 glucosyl unit molecule (from glycogen). When the anaerobic pathway, culminating in the production of L-lactate, is utilised only 3 molecules of ATP are produced for 1 glucosyl unit molecule consumed. Therefore under anaerobic conditions, if aerobic rates of ATP production are to be maintained, the substrate store (carbohydrate pool) is soon going to be consumed. From measurements made in this study and the rates of oxygen consumption of quiescent crabs (Chapter 5), it is possible to calculate the aerobic and anaerobic rates of ATP production. In addition to the carbohydrate catabolism, the contribution of the transphosphorylation of ADP by phospho-l-arginine, to ATP production has also been calculated.

Under aerobic conditions the oxygen consumption rate of quiescent crabs was 1.27 umol $O_2.h^{-1}.g^{-1}$ fresh wt. (section 5.3.2). Assuming that for every 1 molecule of glucosyl units, 6 molecules of O_2 are consumed and 39 molecules of ATP are produced, the rate of ATP production can be calculated to be 8.26 $umol.h^{-1}.g^{-1}$ fresh wt.

Under anaerobic conditions, the rate of L-lactate production in *Carcinus maenas* was 1.22 umol.h^{-1}.g^{-1} fresh wt. (Table 3.4). The glycolytic flux was therefore 0.61 umol.h^{-1}.g^{-1} fresh wt., which resulted in a rate of ATP production of 1.83 umol.h^{-1}.g^{-1} fresh wt. The contribution of phospho-l-arginine to the production of ATP was directly proportional to its breakdown and amounted to 0.37 umol.h^{-1}.g^{-1} fresh wt. Therefore the overall rate of ATP production during anaerobiosis was 2.2 umol.h^{-1}.g^{-1} fresh wt., as compared with 8.26 umol.h^{-1}.g^{-1} fresh wt. under aerobic conditions. There had obviously been a large reduction in energy demand during anoxia, in an effort to conserve the carbohydrate pool and reduce the accumulation of L-lactate. During anoxia, *C. maenas* was observed to cease all locomotor activity, which fits in with this reduction in energy demand. The concepts of metabolic regulation will be discussed further in Chapter 7.

If glycolysis continued at the aerobic rate during anoxia, one would have expected an ATP production rate of only 0.64 umol.h^{-1}.g^{-1} fresh wt. If the concentration of ATP does not decrease, it follows that there has been an increase in the rate of glycolysis during anoxia. This phenomenon is known as the 'Pasteur effect' and was defined by Storey (1985a) as 'the effect of oxygen deprivation in increasing the rate of carbohydrate uptake and catabolism'. In this study the glycolytic flux under aerobic conditions was 0.21 umol.h^{-1}.g^{-1} fresh wt., whilst during anoxia it increased to 0.61 umol.h^{-1}.g^{-1} fresh wt. This represents an increase in the rate of glycolysis by a factor of 2.89. The Pasteur effect can only really be utilised by organisms that experience anoxia for short periods of time since, by definition, it increases the rate at which carbohydrates are consumed. Since *Carcinus maenas* is exposed to anoxia for relatively short periods of time (Chapter 2, section 2.4.1.2), the Pasteur effect is extremely useful in increasing the rate of glycolysis to meet the crabs' energy requirements. In many organisms, that are exposed to longer periods of anoxia, the Pasteur effect has not been demonstrated (de Zwaan & Wijsman, 1976; Gäde, 1983).

3.4.5 Comparative aspects of the recovery of decapod crustaceans from anaerobiosis.

Ellington (1983) stated that 2 basic processes occur during recovery from a period of exposure to anoxia. Firstly, there is the recharging of the phosphagen and ATP stores, and secondly, the disposal of the end products of anaerobic metabolism.

3.4.5.1 The recharging of the phosphagen and ATP stores.

Numerous authors have reported that the concentrations of the phosphagens and ATP recover extremely rapidly (Ellington, 1981; Gäde & Meinardus, 1981; Onnen & Zebe, 1983; Ellington, 1983). The results from this study indicated that the crabs are still partially respiring anaerobically during the first hour of recovery. This meant that the recovery of ATP and phospho-l-arginine was delayed for about 1 h . A full recovery to pre-anoxic concentrations, required a further 2 and 8 h for ATP and phospho-l-arginine respectively. These recovery times are longer than have been reported in the literature for most molluscs (Ellington, 1983), but are consistent with the situation in *Orconectes limosus* (Gäde, 1984), in which the concentration of phospho-l-arginine took 6 h to recover to normal levels.

3.4.5.2 The disposal of the anaerobic end products.

The reduction in the concentration of L-lactate in the haemolymph and tissue of decapod crustaceans has been widely reported. The following table summarises the available literature:

Table 3.5 L-lactate elimination rates in the tissue and haemolymph of decapod crustaceans.

Species	Elimination rate[1]	Duration of anoxia/ and elimination[2]	Reference
Tissue			
Orconectes limosus (13°C)	1.09	12/12+	Gade, 1984
Menippe mercenaria (25°C)	0.68	12/24	Albert & Ellington, 1985**
Palaemon elegans	1.55	6/6	Taylor & Spicer, 1987
Carcinus maenas	2.68	12/12	Present study
Haemolymph			
Atelecyclus rotundatus	1.05	5/6	Bridges & Brand, 1980*
Carcinus maenas	0.87	5/5	Bridges & Brand, 1980*
Corystes cassivelaunus	1.99	5/4	Bridges & Brand, 1980*
Galathea strigosa	0.25	5/20	Bridges & Brand, 1980*
Homarus gammarus	0.26	8/24	Bridges & Brand, 1980*
Nephrops norvegicus	1.20	5/6	Bridges & Brand, 1980*
Macrobrachium rosenbergii (22°C)	2.95	6/3	Mauro & Malecha, 1984
Menippe mercenaria (25°C)	1.71	12/24	Albert & Ellington, 1985
Callinectes sapidus (25°C)	5.20	5/7	Lowery & Tate, 1986
Callinectes sapidus (32°C)	5.00	2/11	Lowery & Tate, 1986
Carcinus maenas	1.00	12/12	Present study

1 - The elimination rate of L-lactate during recovery ($umol.h^{-1}.g^{-1}$ fresh wt. and $mmol.l^{-1}.h^{-1}$ for tissue and haemolymph samples respectively).
2 - Duration of initial exposure to anoxia (h) / duration of L-lactate elimination (h).
* - Original data re-calculated using the above units.
** - Mean concentration taken of heart, leg socket and cheliped muscles.

It can be seen from the above that the rate of L-lactate elimination is relatively slow compared with the initial rate of accumulation. It is also clear that the more anoxia-tolerant species have the fastest L-lactate elimination rates. The concentration of L-lactate in *Carcinus maenas* decreases quite rapidly, again reflecting the fact that this species is relatively well adapted to tolerate exposure to anoxia.

An interesting feature of the recovery from exposure to anoxia in *Carcinus maenas* was the extremely large increase in the concentration of L-lactate, observed during the first hour after the return to normoxia (Fig. 3.5). This feature has previously been reported in respect of functional anaerobiosis in *Cherax destructor* in which 'anaerobic glycolysis makes a significant contribution to the metabolic recovery process' (Head & Baldwin, 1986). Observations made during this study indicated

that during the later stages of anoxia, *C. maenas* became extremely inactive, but resumed activity again as soon as the oxygen was reintroduced. In addition to this locomotor activity, the crab had to reinstate the ventilatory system and generally restore the tissue oxygen concentration. The energy requirements of this period would have been extremely large and it is highly likely that *C. maenas* was suffering from functional fatigue in certain tissues at this time and was having to utilise anaerobic metabolism to supplement energy requirements.

The exact mechanism of end product disposal is the subject of much debate but one of the proposed alternatives is that the L-lactate could be converted to its original substrate, via a gluconeogenic pathway. The stoichiometric comparison that was applied to L-lactate and the carbohydrate pool during anoxia (section 3.4.3) can be equally well applied to the recovery period and results in the ratio of glucosyl unit : L-lactate of 1:2.25. Expressed as a percentage, 88.5 % of the L-lactate reduction could be explained by the increase in concentration of the carbohydrate pool. This seems to indicate that most of the L-lactate is in fact being converted back to its initial substrate. Since a more direct means of investigating this subject would be to use radio-isotopes to trace the fate of L-lactate, further discussion of this subject will continue in Chapter 4.

CHAPTER 4 - THE FATE OF L-LACTATE DURING RECOVERY FROM ENVIRONMENTAL ANOXIA.

4.1 INTRODUCTION.

The metabolic responses of organisms to environmental anoxia have been fairly well studied in decapod Crustacea (Teal & Carey, 1967; Bridges & Brand, 1980; Gäde, 1983; Albert & Ellington, 1985; Taylor & Spicer, 1987; van Aardt & Wolmarans, 1987) (Chapter 3). Until recently, however, investigations into the metabolic responses of an organism during recovery from environmental anoxia have been mainly limited to determining the rate at which L-lactate disappears from the animal in question (Chapter 3, section 3.4.5.2). Over the last decade, a few studies have looked at this subject in more detail. As described in the last chapter, Ellington (1983) stated that recovery firstly involved the restoration of the phosphagen and ATP store, and secondly the elimination of the anaerobic end products. The mechanism(s) by which crustaceans eliminate L-lactate during recovery is the subject of this chapter.

There are potentially three methods of clearance of anaerobic end products available to marine invertebrates (Ellington, 1983). Firstly, the organism can excrete the end product into the surrounding water. This has been observed to be common amongst the annelids (Ruby & Fox, 1976; Pionetti & Toulmond, 1980). Secondly, the end product could be metabolised back to its precursor, via a biochemical pathway (e.g. gluconeogenesis). Gluconeogenesis has been demonstrated in crustaceans by Phillips *et al.* (1977), Gäde *et al.* (1986) and van Aardt (1988). Resynthesis of the anaerobic substrate, aspartate, has also been shown to occur in the polychaete *Arenicola marina* (Pörtner *et al.*, 1979) and in the sea anemone *Bunodosoma cavernata* (Ellington, 1982). Finally, the end product can be fully oxidised to carbon dioxide. This particular mechanism of end product clearance has been observed in bivalves (Zurburg *et al.*, 1982), cephalopods (Monneuse-Doublet *et al.*, 1980) and crustaceans

(Gäde *et al.*, 1986)

In this chapter the results of a series of experiments carried out to establish which of the methods of L-lactate clearance were employed in *Carcinus maenas* are presented. All the experiments involved using radio-isotopes, in the forms of D-[U-^{14}C]-glucose and L-[U-^{14}C]-lactate (Amersham International, England). Ion exchange chromatography was used in order to fractionate particular categories of labelled metabolites. The radioactivity in the resultant categories was then counted using a liquid scintillation counter. Similar techniques have been used previously to investigate recovery from exposure to anoxia in decapod crustaceans (Phillips *et al.*, 1977; Gäde *et al.*, 1986; van Aardt, 1988).

Prior to directly addressing the question of L-lactate clearance, it was necessary to investigate the fate of D-[U-^{14}C]-glucose under aerobic conditions, when disturbance of the crabs had been kept to a minimum. Since some of the D-[U-^{14}C]-glucose would inevitably be metabolised and released as $^{14}CO_2$, a second preliminary experiment was required to investigate the loss of radioactivity as a result of the excretion of carbon dioxide. The results of these experiments were to be used as controls and compared directly with the data from subsequent experiments, in which periods of normoxic recovery (12 h) had been preceded by anoxia (12 h).

4.2 MATERIALS AND METHODS.

4.2.1 Techniques.

4.2.1.1 Ion Exchange Chromatography.

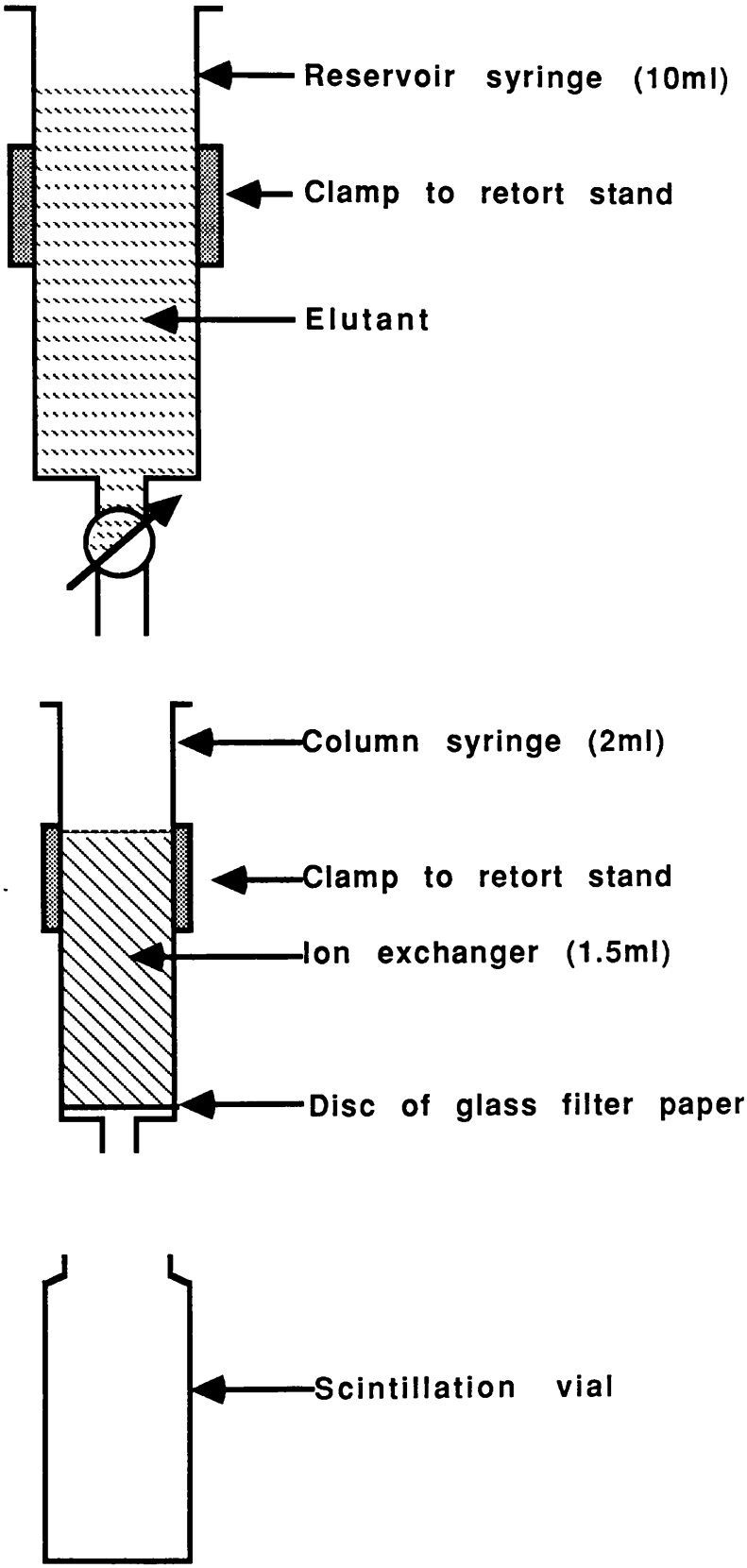
All ion exchange columns consist of an insoluble porous matrix, usually in the form of polystyrene beads, with attached ionisable functional groups. In addition to the matrix and functional group, resins also carry an exchangeable counterion opposite in charge to the functional group (e.g. SO_3^- : Na^+).

In ion exchange chromatography, the counterions are replaced by sample ions of the same charge. Neutral molecules and those having the same charge as the functional group, pass through the column, and are separated from the adsorbed ions. Another ion may then be used to displace and elute the adsorbed ions, or they may be fractionated by various elution methods which release them preferentially, starting with the most weakly bound. Using these techniques, various groups of radiolabelled metabolites were separated and the amount of radioactivity in each measured by scintillation counting.

Both cation (Dowex AG 50W-X8 (200-400 mesh)) and anion (Dowex 1-X8 (200-400 mesh)) columns were employed (both Sigma, England). In order to set up a column, a disc (13 mm in diameter) of glass fibre filter paper was placed in the base of a 2ml plastic syringe (Fig.4.1). 1.5 ml of the suspension of exchanger in water was poured into the syringe and allowed to settle. A larger plastic syringe (10 ml) was attached above the column, and acted as a reservoir for subsequent elutants. A 2-way tap was fitted to the base of the reservoir syringe, to control the elutant flow rate through the column.

Since the exchanger was used more than once, after each experiment it was

FIG. 4.1 Diagram of an ion exchange column used to separate particular categories of labelled metabolites. See text for explanation.


Reservoir syringe (10ml)
Clamp to retort stand
Elutant
Column syringe (2ml)
Clamp to retort stand
Ion exchanger (1.5ml)
Disc of glass filter paper
Scintillation vial

necessary to regenerate it by restoring it to a low affinity ionic form. This required that the cation and anion columns be reconverted to the chloride and acetate forms respectively. The cation exchanger was regenerated by washing with 20 bed volumes of 1 M HCl. The anion exchanger was regenerated by washing with 20 bed volumes of 1 M NaOH, followed by an equal volume of 1 M acetic acid. The column was then washed with distilled water, until the washings were shown to be neutral using pH indicator paper.

The metabolic groupings of glycogen (F1), amino acids (F2), neutral metabolites (F3), weak and strong acids (F4 and F5 respectively) were separated as shown in Figure 4.2. In order to check the efficiency of separation and recovery, selected pure commercial standard metabolites were passed through the columns. As shown in Appendix 14 recovery of the pure standards was in excess of 90 % .

4.2.1.2 Liquid scintillation counting.

Radioactivity causes excitation of certain compounds (fluors) which then fluoresce (scintillate). A photocathode detects the light emitted and converts the photons into an electric pulse. This is then amplified by using a photomultiplier. In liquid scintillation the sample is mixed with a solvent, which contains the appropriate scintillator and then placed in the counter. This allows B-emitters to be detected with great sensitivity.

The light emitted from scintillation is normally of a very short wavelength and is not efficiently detected by most photocathodes. In addition, various factors cause interference with this energy transfer, known as 'quenching'. It is important, therefore, that the degree of this quenching is determined. In the present study, ^{14}C-toluene was used as a standard to determine the efficiency of counting, based on the channel ratio method. Standards of known disintegrations per minute (dpm) were set up in a fluor, under exactly the same conditions as the subsequent samples

FIG. 4.2 The elution scheme used to fractionate particular categories of labelled metabolites. Sample preparation was carried out as described in Appendix 10.

Homogenise tissue in liquid nitrogen and add to 75 % ethanol. Leave on ice for 2 hours to precipitate glycogen. Centrifuge at 12,000 g for 10 minutes. (Appendix 10).

Remove supernatant - ethanol soluble fraction.

Dissolve the glycogen within the pellet in 1 ml of water and centrifuge at 12,000 g for 5 minutes. Remove supernatant - ethanol insoluble fraction (Appendix 10)

Glycogen (F1).

Add to short columns of cation exchange resin (Dowex 50W X8 in the hydrogen form).

Elute neutral and anionic metabolites with 6 ml of water.

Elute cations with 5 ml of ammonium hydroxide (3 M).

Amino acids (F2).

Add to short column of anion exchange resin (Dowex 1W X8 in the acetate form).

Elute with 4 ml of water.

Elute with 7 ml of acetic acid (5 M)

Elute with 5 ml hydrochloric acid (1 M).

Neutral compounds (F3).

Weak acids (F4).

Strong acids (F5).

were set up in a fluor, under exactly the same conditions as the subsequent samples and the resultant counts per minute (cpm) recorded. The counting efficiency could then be expressed as a percentage by dividing the cpm by dpm and then multiplying by 100.

4.2.2 General experimental conditions.

4.2.2.1 Injection of radioactivity.

Teflon gas-chromatography septa (3 mm in diameter) were attached to the carapace of the crabs (fresh wt. = 3 - 7 g), immediately above the heart, using cyanocrylate adhesive (Permabond, England). This was to ensure that there was no leakage of haemolymph or radio-labelled compound from the heart, once the injection had been made. There was no need to drill a hole through the carapace of the crabs, because with crabs of this size the carapaces are relatively thin. It was quite simple to insert a hypodermic needle through the septum and the carapace immediately prior to the radiolabelled injection. Following the attachment of the septa , the animals were left undisturbed in fully aerated sea water for 48 hours, to allow them to recover from any handling stress. The stock solutions of radio-isotopes were diluted with *Carcinus* Ringer (Appendix 12) to obtain a final radioactivity concentration of 0.1 uCi. ul^{-1} and 0.05 uCi. ul^{-1} for D-[U-^{14}C]-glucose and L-[U-^{14}C]-lactate respectively. The solutions containing the radioactivity were then injected (50 ul) directly into the hearts of the crabs, through the septa and carapace, using a Hamilton syringe (50 ul).

4.2.2.2 Tissue preparation and metabolite separation.

The frozen crabs were weighed and then the tissue was prepared according to the method described in Appendix 10. The elution scheme shown in Figure 4.2 was followed, using 1 ml of the 75 % ethanol supernatant. Each fraction was collected

in a separate plastic scintillation vial. The strong acids (F5) were further fractionated by precipitation of acid-soluble phosphates in the presence of barium salts (Kaplan and Greenberg, 1944; Sacks, 1949). The procedure for this precipitation can be found in Appendix 13. The strong acid fraction was thus divided into glycolytic phosphates (F5g) and tricarboxylic acid cycle acids (F5t).

4.2.2.3 Scintillation counting.

Scintillation counting involved mixing 0.5 ml of each fraction, with 5 ml of Ecoscint (Nuclear Medical Electronic Systems & Services Ltd, U.K.) in plastic scintillation vials . After vigorous mixing, to ensure homogeneity, these vials were transferred to the scintillation counter. Background radioactivity was determined by substituting distilled water for the sample fraction. The results were expressed in cpm and were then converted to dpm using the following equation:

$$\text{dpm} = \frac{100}{\text{Counting Efficiency}} \times \text{cpm}$$

After subtracting the background counts from the dpm values, the percentages of the total amount of radioactivity recovered were then calculated. Since the results were all presented as percentages, it was necessary to transform the data to ensure that they were normally distributed before statistical analysis could be carried out. Therefore all statistical analysis was performed on arcsine transformed data (Sokal and Rohlf, 1981). Unless otherwise stated, all figures are expressed as a percentage of radioactivity injected.

4.2.3 Preliminary experiments.

4.2.3.1 Metabolism of D-[U-^{14}C]-glucose during normoxia.

Septa were attached to the carapaces of 8 crabs (fresh wt. = 3 - 7 g) which were left undisturbed for 48 h as described in section 4.2.2.1 . Crabs were equally distributed between 2 plastic tanks (volume = 10 l) containing fully aerated artificial sea water (Chapter 2, section 2.2.2) and left undisturbed for a further 24 h .

At the end of the 24 h period, each crab was carefully removed from the tank and was injected with 50 ul of the solution containing D-[U-^{14}C]-glucose (5 uCi). Special care was taken to minimise the disturbance to the crabs. The crabs were immediately returned to the tanks, and were maintained under aerobic conditions for the duration of the experiment (12 h).

Groups of 4 crabs were individually sampled at 6 h and 12 h post-injection and killed by plunging them into liquid nitrogen. A sample of the water (10 ml) was also taken at each time interval and stored in a glass vial. Both the tissue and water samples were then stored at -20 ^{o}C until needed. The samples were prepared and fractionated according to the methods described in Appendix 10 and in section 4.2.2.2 . The radioactivity in each of the fractions could then be measured using the scintillation counter (section 4.2.2.3). To determine the approximate amount of radioactivity incorporated into acid labile and volatile metabolites within the water, 0.5 ml of hydrochloric acid (0.1 M) was added to 0.5 ml of the water sample. The acidified samples were then shaken thoroughly and left for 2 h , since previous experiments had indicated that acidified samples that had been left for this time, contained very low counts of radioactivity. 0.5 ml of the samples were then mixed with 5 ml of the Ecoscint and counted as described previously.

4.2.3.2 Incorporation of radioactivity from D-[U-^{14}C]-glucose into carbon dioxide during periods of normoxia.

Septa were attached to the carapaces of 4 crabs and the animals were left for 48 h as described in section 4.2.2.1 . Each individual crab was injected with 5 uCi of D-[U-^{14}C]-glucose (50 ul) and placed in a perspex chamber, containing 300 ml of artificial sea water (Fig. 4.3).

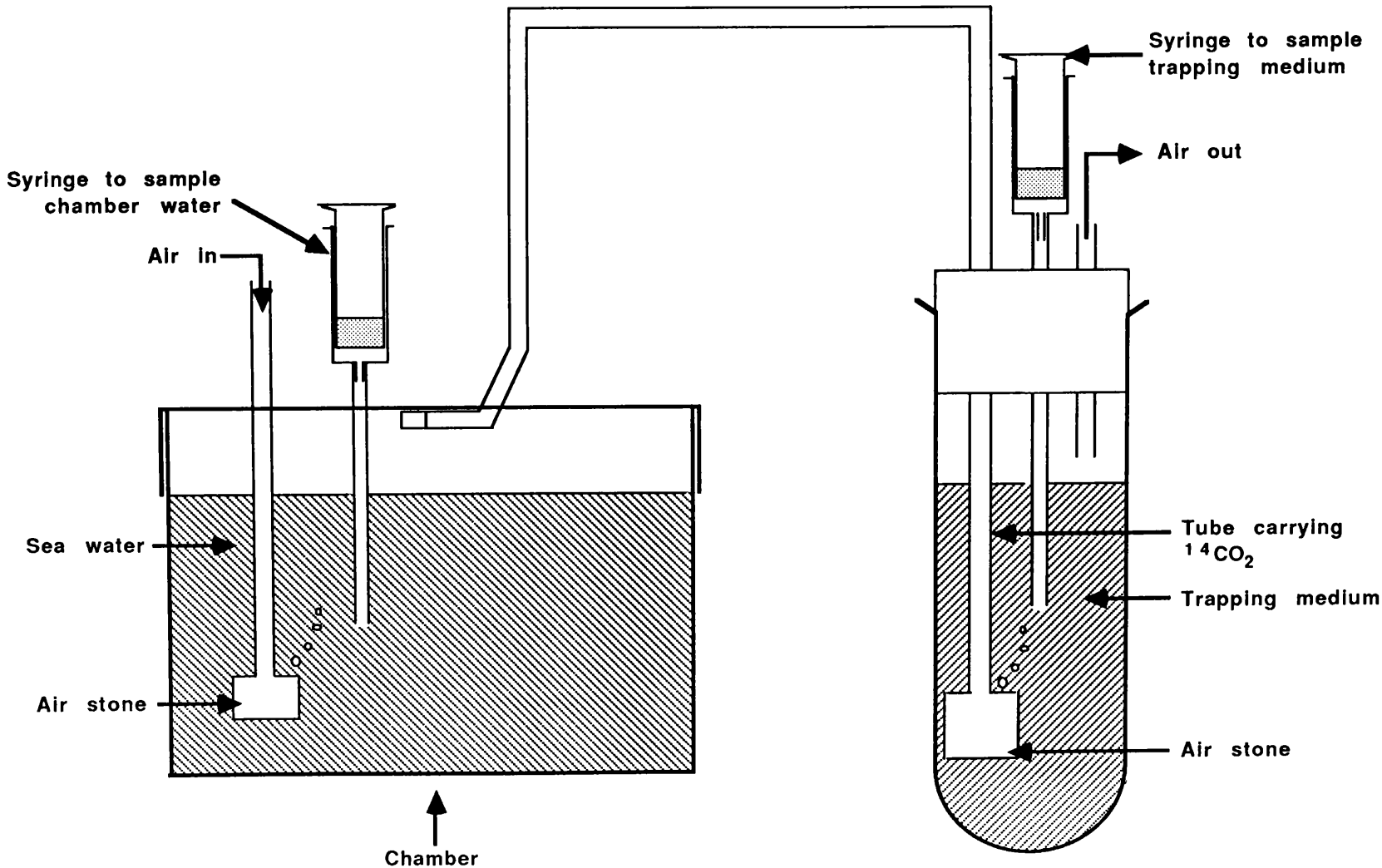
The experiment was carried out in a constant temperature room, maintained at 10 ± 1^{o}C. The water was left for 24 hours prior to each set of measurements, to allow it to equilibrate to the ambient temperature. The boiling tube contained 25 ml of 'Hyamine hydroxide'(10 % solution of methylbenzethonium hydroxide in methanol). Air was bubbled through the chamber containing the crab, in order to drive off any $^{14}CO_2$, which was then trapped in the 'Hyamine hydroxide' within the boiling tube. Any radiolabelled bicarbonate would remain in solution in the chamber. 2 duplicate samples (0.5 ml) were taken from the chamber (S1 & S2) and a duplicate sample from the boiling tube (S3) at 10 minute intervals during the first h, then at 20 minute intervals during the next 3 h and at 1 h intervals during the final 2 h of the experiment. After sampling, appropriate amounts of sea water and 'Hyamine hydroxide' were added to the chamber and the boiling tube in order to maintain constant volumes.

0.5 ml of hydrochloric acid (0.1 M) was added to the duplicate water samples (S2). The acidified samples were thoroughly shaken and left for 2 h . 0.5 ml of each of the samples were then mixed with 5 ml of Ecoscint, in plastic scintillation vials and counted as described in section 4.2.2.3 . The interpretation of the radioactivity in each of the samples, was as follows:

S1: Total radioactivity in chamber water.

S2: Radioactivity in chamber water, incorporated into non acid labile and

FIG. 4.3 Diagram of the apparatus used for measuring the incorporation of D-[U-^{14}C]-glucose and L-[U-^{14}C]- lactate into carbon dioxide by *Carcinus maenas*. See text for explanation.


Syringe to sample trapping medium
Air out
Syringe to sample chamber water
Air in
Sea water
Air stone
Chamber
Tube carrying $^{14}CO_2$
Trapping medium
Air stone

S2: Radioactivity in chamber water, incorporated into non acid labile and volatile compounds.

S3: Total radioactivity in boiling tube.The differences between S1 and S2 were the amounts of radioactivity incorporated into bicarbonate/carbonate (acid labile and volatile fractions).

4.2.4 Investigation to determine the fate of L-lactate during recovery from anoxia.

The aim of the following experiments was to determine which of the 3 methods of L-lactate clearance described by Ellington (1983), are utilised in *Carcinus maenas*.

4.2.4.1 Investigation into the conversion of L-lactate back to its initial precursor, using i) D-[U-^{14}C]-glucose and ii) L-[U-^{14}C]-lactate.

i) D-[U-^{14}C]-glucose.

Septa were attached to the carapaces of 48 crabs which were left for 48 h . 36 crabs were then equally distributed between 4 plastic tanks (tanks 1 - 4) and a further 12 were placed in tank 5 and left undisturbed for 24 h . The experimental design and procedure for attaining 12 h of anoxia, followed by 12 h of recovery was exactly as described in Chapter 3, section 3.2.3.

The Po_2 of tanks 1 - 4 was reduced to < 1 Torr, by bubbling nitrogen through the water. The water in tank 5 was maintained fully aerated for the duration of the experiment and the animals it contained used as controls. Once the water in tanks 1 - 4 had become anoxic, all 48 crabs were injected with 5 uCi of D-[U-^{14}C]-glucose (50 ul), after which they were immediately returned to their respective tanks.

During the 'anoxic period' (0 - 12 h), batches of 4 experimental crabs were removed

from the tanks at 1, 4, 8, and 12 h after the start of the experiment. The crabs were then rapidly killed by plunging them into liquid nitrogen. The tissue samples were then stored in a deep freeze (-20 ^{o}C) until required for extraction and the subsequent counting of radioactivity (Appendix 10 and section 4.2.2). After 12 h of anoxia, the Po_2 of the water was returned to near saturation (Po_2 approx. 160 Torr), by bubbling air through the tanks. During the 'recovery period' (12 - 24 h) further batches of 4 crabs were taken at 12.5, 13, 16, 20, and 24 h . Controls consisted of sampling batches of 4 crabs from the aerated tank at 0, 1 and 24 h after the start of the experiment.

The crabs were prepared as described in Appendix 10. The resultant extracts were then passed through the ion exchange columns (section 4.2.1.1) and finally the radioactivity in each fraction measured on the scintillation counter (section 4.2.2.3). After the counting efficiency had been taken into account, the recoveries of radioactivity were calculated, as a percentage of the total radioactivity recovered.

ii) L-[U-^{14}C]-lactate:

Septa were attached to the carapace of 16 crabs which were left for 48 h as described in section 4.2.2.1 . 10 and 6 of the crabs were then transferred to tanks 1 and 2 respectively. All crabs were left undisturbed for a further 24 h . Apart from the above modifications, the experimental procedures for attaining 12 h of anoxia, followed by 12 h of recovery were exactly as described in Chapter 3, section 3.2.3.

The Po_2 of both tanks was reduced to < 2 Torr and maintained at this partial pressure for 12 h , by bubbling nitrogen through the sea water. In this experiment, unlike that in section 4.2.4.1, the radioactivity was injected only after 12 h of anoxia. The injection (50 ul) was administered in exactly the same way as previously described, and consisted of 2.5 uCi of radioactivity injected in the form

of L-[U-^{14}C]-lactate. After 12 h of anoxia, the Po_2 of the water in tank 1 was returned to near saturation (Po_2 approx. 160 Torr). During the 'recovery period' (12 to 24 h) 2 sample batches, each of 5 crabs were taken at 16 and 20 h. The crabs were rapidly killed by plunging them into liquid nitrogen and then stored at -20^{o}C. A control for this experiment necessitated the continued exposure of crabs to anoxia. Controls therefore consisted of 3 sample batches of 2 crabs taken at 12, 16 and 20 h after the start of the experiment.

All the crabs were prepared as described in Appendix 10. The resultant extracts were then passed through the ion exchange columns (section 4.2.1.1) and finally the radioactivity in each fraction was measured on the scintillation counter (section 4.2.2.3). After the correcting for the counting efficiency, the percentage of recovered radioactivity in metabolic fractions (F1-F5) was calculated.

4.2.4.2 Investigation to determine whether L-lactate is oxidised to CO_2.

The procedure for this experiment was exactly the same as that described in section 4.2.3.2. apart from the following modifications. Prior to being injected, a crab with a septum attached to its carapace, was placed in a beaker containing 500 ml of artificial sea water (see above), maintained at 10 $\pm$ 1^{o}C. Anoxic conditions were obtained by bubbling nitrogen through the water, as described above. The beaker was covered with a polystyrene sheet (5 mm thick), which was cut to fit the inside of the beaker and which, by floating on the water surface, substantially reduced the water/air interface and helped to prevent the diffusion of oxygen back into the water. The water was sampled frequently, and the Po_2 determined using an oxygen electrode (E5046, Radiometer, Denmark), coupled to an oxygen meter (Strathkelvin Instruments, Glasgow). At the end of the 12 h of anoxia, the crab was removed and injected (50 ul) with 2.5 uCi of radioactivity in the form of L-[U-^{14}C]-lactate. The crab was then immediately transferred to the chamber (Fig. 4.3) and the experiment was continued exactly as described in section 4.2.3.2. Four control crabs

which had not been exposed to the 12 h anoxic period, were injected with the L-[U-^{14}C]-lactate as above and their $^{14}CO_2$ production measured over the following 6 h .

4.2.4.3 Investigation to determine whether L-lactate is excreted.

Septa were attached to the carapaces of 5 crabs which were left for 48 h as described in section 4.2.2.1 . These were then placed in 5 plastic beakers (volume = 500 ml), containing 300 ml of fully aerated artificial sea water. The crabs were left undisturbed for a further 24 h. Polystyrene discs were cut to fit inside the beakers and float on the water surface, in order to reduce the possibility of any oxygen from the air dissolving in the water. The mechanism for regulating the Po_2 of the water was as described in Chapter 3, section 3.2.3.

The Po_2 of the water in all 5 beakers was reduced to < 2 Torr. Crabs were then injected with 5 uCi of D-[U-^{14}C]-glucose (50 ul), in the manner described previously. After 12 h of anoxia the Po_2 of the water was returned to saturation, and the crabs left to recover for a period of 12 h under normoxic conditions, after which all 5 crabs were removed from the tanks and rapidly killed using liquid nitrogen. At the same time, the sea water in each beaker was collected and immediately passed through the ion exchange columns. The radioactivity in each of the resultant fractions was then measured on the scintillation counter. The tissue samples were prepared according to the methods given in Appendix 10, but the resulting extracts were not fractionated using ion exchange chromatography, since it was sufficient to know only the total amount of radioactivity retained within the tissue of the whole crab.

4.3 RESULTS.

4.3.1 The efficiency of scintillation counting.

^{14}C-Toluene was used as an internal standard, to determine the degree of quenching in each sample. The mean cpm expressed as a percentage of dpm was 74.5 ± 1.37. Since the channel ratios were constant throughout the experiments, counts per minute (cpm) were multiplied by 1.34 to convert to disintegrations per minute (dpm).

4.3.2 Preliminary experiments.

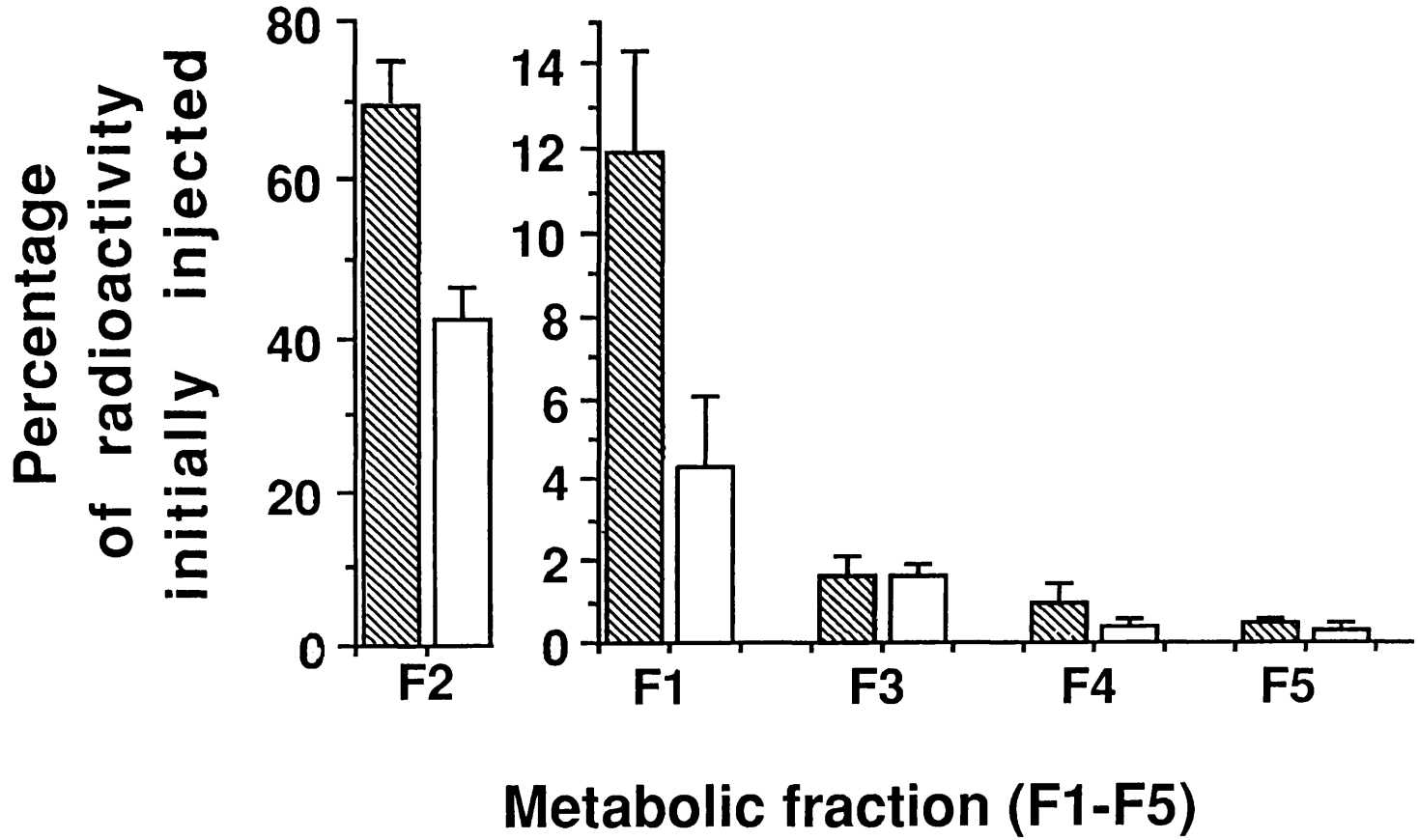
4.3.2.1 Metabolism of D-[U-^{14}C]-glucose during normoxia.

After 6 h post-injection, only 1.6 ± 0.51 % of the radioactivity initially injected, remained in the neutral fraction (mainly D-glucose and oligosaccharides) (Fig. 4.4). By this time, 69.5 ± 5.6 % of the radioactivity had been incorporated into the amino acid fraction. The other main metabolite to incorporate radioactivity was glycogen, which averaged 11.9 ± 2.4 %, 6 h post-injection. Radioactivity in both the weak and strong acid fractions was less than 1 % of the total amount recovered, and was therefore very low.

It was found that 12 h post-injection there was evidence of turnover in both amino acids and glycogen, because the amount of radioactivity in these fractions had decreased significantly ($P < 0.05$) to 42.4 ± 4.1 % and 4.3 ± 1.8 % respectively. There were no significant ($P > 0.05$) changes in the amount of radioactivity in any of the 3 other fractions (Fig. 4.4).

FIG. 4.4 The percentage of D-[U-^{14}C]-glucose initially injected into *Carcinus maenas*, that was incorporated into specific metabolic fractions (F1 - F5) under normoxic conditions. Measurements were taken after 6 (▨) and 12 (□) hours. Values are means ± S.D.

F1 - glycogen.
F2 - amino acids.
F3 - neutral compounds (including D-glucose).
F4 - 'weak acids' (mainly L-lactate).
F5 - 'strong acids' (glycolytic phosphates and TCA acids).


Percentage of radioactivity initially injected
80
60
40
20
0
14
12
10
8
6
4
2
0
F2
F1
F3
F4
F5
Metabolic fraction (F1-F5)

The following table summarises the actual recoveries, expressed as a percentage of the total radioactivity initially injected.

Table 4.1 Actual recoveries, expressed as a percentage of the total radioactivity initially injected.

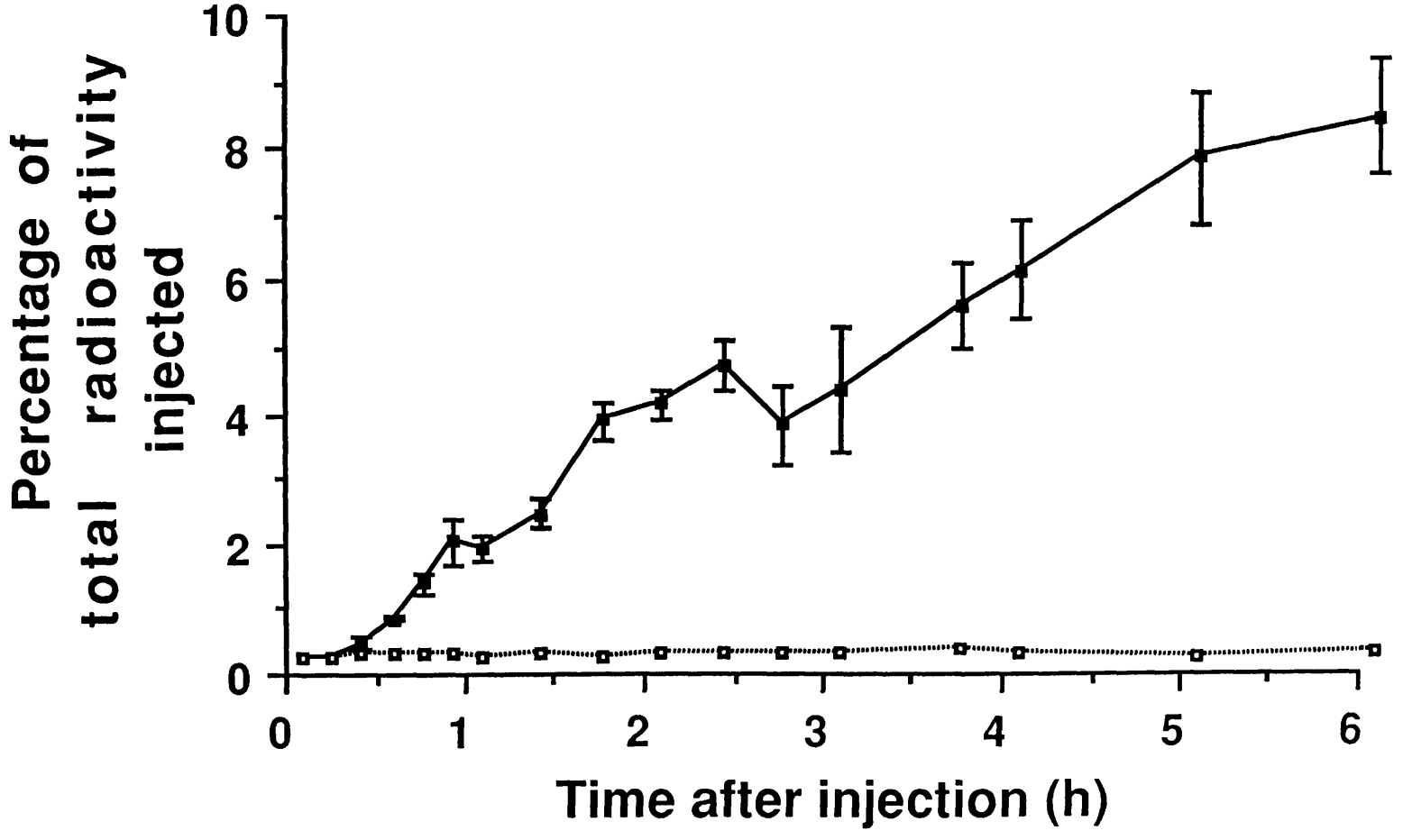
Location of radioactivity	Radioactivity recovered (%)	
	6 h	12 h
Tissue	84.1	48.9
Water		
- Acid labile and volatile compounds	14.7	36.8
- Acid stable compounds	0.9	1.3
Total radioactivity recovered	99.7	87.0

The addition of the hydrochloric acid to a water sample resulted in 94 and 97 % of the radioactivity contained within the water being lost to the surrounding air, after 6 and 12 h respectively. This acid labile and volatile fraction was likely to have consisted almost exclusively of labelled carbon dioxide and bicarbonate/carbonate.

4.3.2.2 Incorporation of radioactivity from D-[U-^{14}C]-glucose into carbon dioxide during periods of normoxia.

The first indication of any radioactivity in the water of the chamber occurred at 20 minutes post-injection, when a trace amount of radioactivity was measured (Fig. 4.5). The presence of radioactivity in the water was less than 1 % of the radioactivity initially injected, but was still well above the background count and well within the limits of sensitivity of this experiment. There was then a gradual but significant ($P < 0.05$) increase during the next 6 h until a maximum of 5.74 $\pm$ 0.94 % of the total radioactivity injected was reached. By comparing the counts S1 and S2, it was found that at least 98 % of the radioactivity within the chamber

FIG. 4.5 The percentage of D-[U-^{14}C]-glucose initially injected into *Carcinus maenas*, that was released from the crab into the surrounding water (■) under normoxic conditions. The radioactivity incorporated into acid stable compounds, is represented by the open squares (□). Values are means ± S.D.


Percentage of total radioactivity injected
10
8
6
4
2
0
0
1
2
3
4
5
6
Time after injection (h)

water was acid labile and volatile in nature, and that it was most likely to have been incorporated into either bicarbonate or carbonate.

The presence of radioactivity trapped in the 'Hyamine hydroxide' (S3) was detected approximately 30 minutes post-injection, when trace amounts of the total radioactivity injected were detected. This percentage increased significantly ($P < 0.05$) during the course of the experiment to reach a maximum of 2.49 ± 0.20 % 6 h post-injection.

4.3.3 Investigation to determine the fate of L-lactate during recovery from anoxia.

4.3.3.1 Investigation into the conversion of L-lactate back to its initial substrate, using i) D-[U-^{14}C]-glucose and ii) L-[U-^{14}C]-lactate.

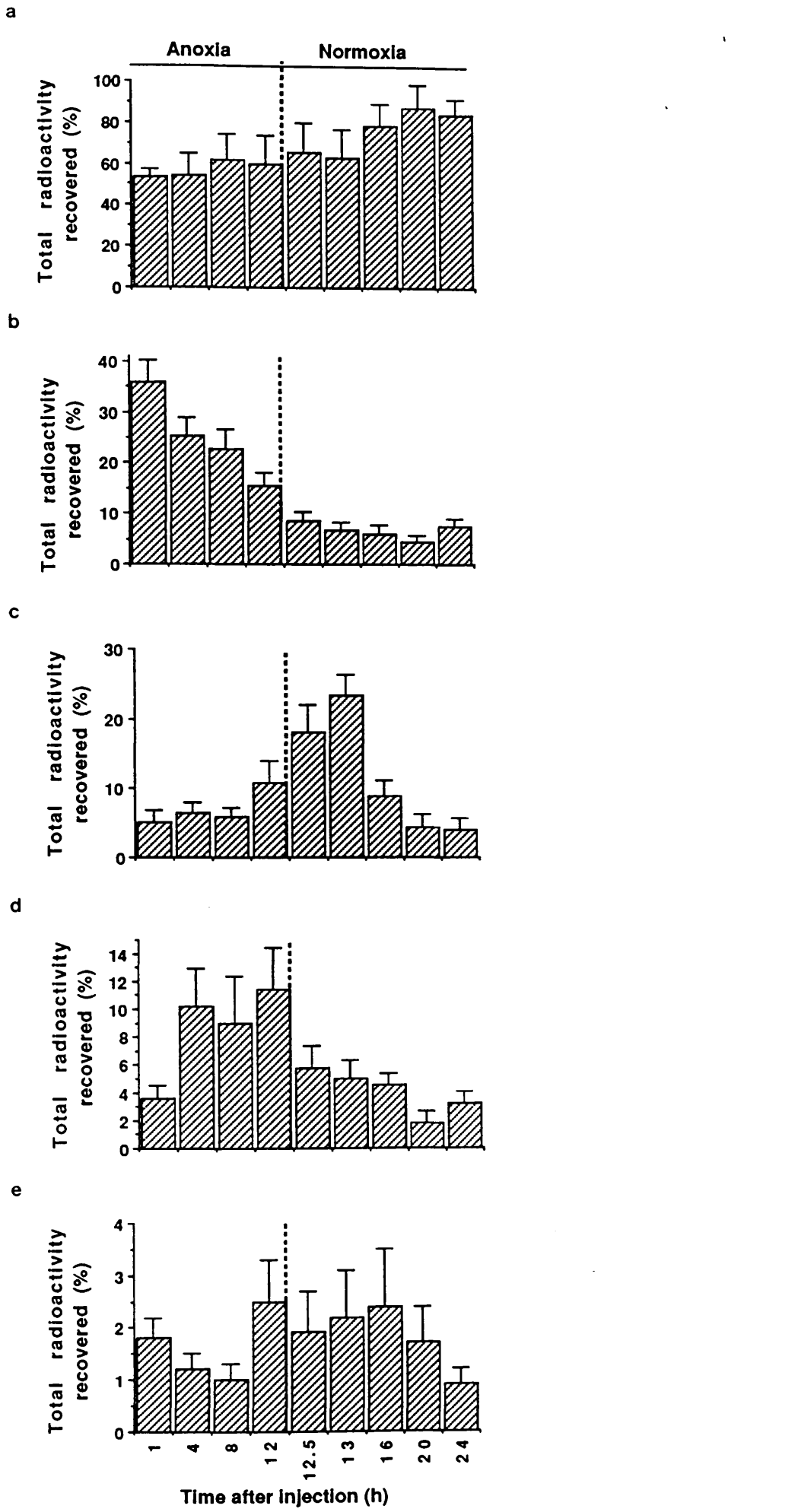
i) D-[U-^{14}C]-glucose.

At 1 h post-injection 73.4 ± 0.52 % of the total radioactivity recovered in the tissue samples had been incorporated into the amino acid fraction. This figure did not change significantly over the remainder of the 24 h experimental period. The fact that the amino acids had incorporated such a high percentage of the radioactivity, meant that many of the trends amongst the other metabolic fractions were masked. Therefore, in Figure 4.6, the amino acid fraction has been omitted and the percentages for the other metabolic groups (F1, F3, F4, F5) recalculated in relation to radioactivity in non-amino acid fractions.

There was a significant ($P < 0.05$) incorporation of radioactivity into the weak acid fraction (mainly L-lactate - Appendix 14) during anoxia, increasing from 1.9 ± 0.9 % to 10.7 ± 3.3 % after 1 and 12 h of anoxia respectively. During the same period

FIG. 4.6 The incorporation of D-[U-^{14}C]-glucose into specific metabolic fractions during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. The radioactivity is expressed as the percentage recovered in the tissues of *Carcinus maenas*. For reasons outlined in the text, the amino acid fraction has been omitted from these calculations. Values are means $\pm$ S.D.

a) glycogen.
b) neutral compounds.
c) 'weak acids'.
d) glycolytic phosphates.
e) TCA acids.


a
Anoxia
Normoxia
Total radioactivity recovered (%)
100
80
60
40
20
0
b
Total radioactivity recovered (%)
40
30
20
10
0
c
Total radioactivity recovered (%)
30
20
10
0
d
Total radioactivity recovered (%)
14
12
10
8
6
4
2
0
e
Total radioactivity recovered (%)
4
3
2
1
0
1
4
8
12
12.5
13
16
20
24
Time after injection (h)

there was a corresponding decrease in the radioactivity in the neutral fraction from 35.9 ± 4.3 % to 15.6 ± 2.3 % after 1 and 12 h of anoxia respectively. Although there was an increase in the radioactivity in glycogen during this period, this change was found to be not significant ($P > 0.05$). The radioactivity in the glycolytic phosphate fraction increased from 3.6 ± 0.9 % to 11.4 ± 3.0 % during the anoxic period, indicating the possibility of an accumulation of glycolytic intermediates. There was no significant ($P > 0.05$) change in the radioactivity in the tricarboxylic acid fraction.

During the first hour of recovery, the percentage of radioactivity in the weak acid fraction increased significantly ($P < 0.05$) from 10.7 ± 3.3 % to 23.4 ± 3.1 %, reflecting the heavy reliance on anaerobic metabolism that was observed in Chapter 3, section 3.3.3.1 . Thereafter the percentage decreased significantly ($P < 0.05$) to 3.9 ± 1.7 %, whilst the radioactivity within the glycogen fraction increased significantly ($P < 0.05$) from 59.5 ± 13.9 to 84.5 ± 7.4 % . During the recovery period the radioactivity in the neutral compounds and the tricarboxylic acid fractions remained constant (mean values = 6.6 ± 1.3 % and 1.83 ± 0.51 % respectively). There was, however, a significant ($P < 0.05$) decrease in the radioactivity within the glycolytic phosphate fraction, particularly during the first 30 minutes of recovery.

In those crabs exposed to anoxia there was significantly ($P < 0.05$) less radioactivity incorporated into the glycogen fraction during the first hour of the experiment, than was incorporated by control crabs maintained under normoxic conditions.

ii) L-[U-^{14}C]-lactate.

At 4 h post-injection (16 h after the start of the experiment) 68.8 ± 6.7 % of the radioactivity, recovered in the tissue, was still located in the weak acid fraction, 13.9 ± 4.9 %, 11.2 ± 2.7 % and 5.63 ± 2.2 % was now found in the glycogen, amino

acid, and undifferentiated strong acid fractions respectively.

At 8 h post-injection (20 h after the start of the experiment) there had been no significant ($P > 0.05$) change in the incorporation of radioactivity into the glycogen fraction. But the radioactivity in the weak acid fraction had decreased to 19.9 ± 3.8 %, whilst the proportion incorporated into the amino acid fraction had increased significantly ($P < 0.05$) to 66.7 ± 5.5 % (Table 4.2)

Table 4.2 - Incorporation of L-[U-^{14}C]-lactate during recovery period following environmental anoxia.

Time post-injection (h)	n	Radioactivity recovered in specific fractions (%)[1] F1*	F2*	F3*	F4*	F5*
4 (Control)**	1	3.37	< 1	< 1	95.0	-
4	4	13.9 (4.9)	11.2 (2.7)	< 1	68.8 (6.7)	5.63 (2.2)
8	4	10.4 (3.1)	66.7 (5.4)	< 1	19.9 (3.8)	2.09 (0.8)

1 Expressed as a percentage of the radioactivity initially injected.

* F1 = Glycogen; F2 = Amino Acids; F3 = Neutral Compounds; F4 = Weak Acids and F5 = Strong Acids (See text for further information).

** Animals maintained under continued anoxia.

- Values given in parentheses are standard deviations of the means.

The LT_{50} for *Carcinus maenas* under anoxia was found to be 15.8 h (Chapter 3, section 3.3.2), so it was not surprising that 3 out of the 4 control crabs kept under continued anoxia died in this experiment. In the single crab that survived, 95 % of the radioactivity remained in the weak acid fraction as lactate, 4 h after the initial injection. This compares with the situation in the experimental crabs in which only

68.8 ± 6.7 % of the radioactivity remained in the weak acid fraction, after the same length of time. Since only a single control crab survived, no statistical significance could be attached to this difference.

4.3.3.2 Investigation to determine whether L-lactate is oxidised to CO_2.

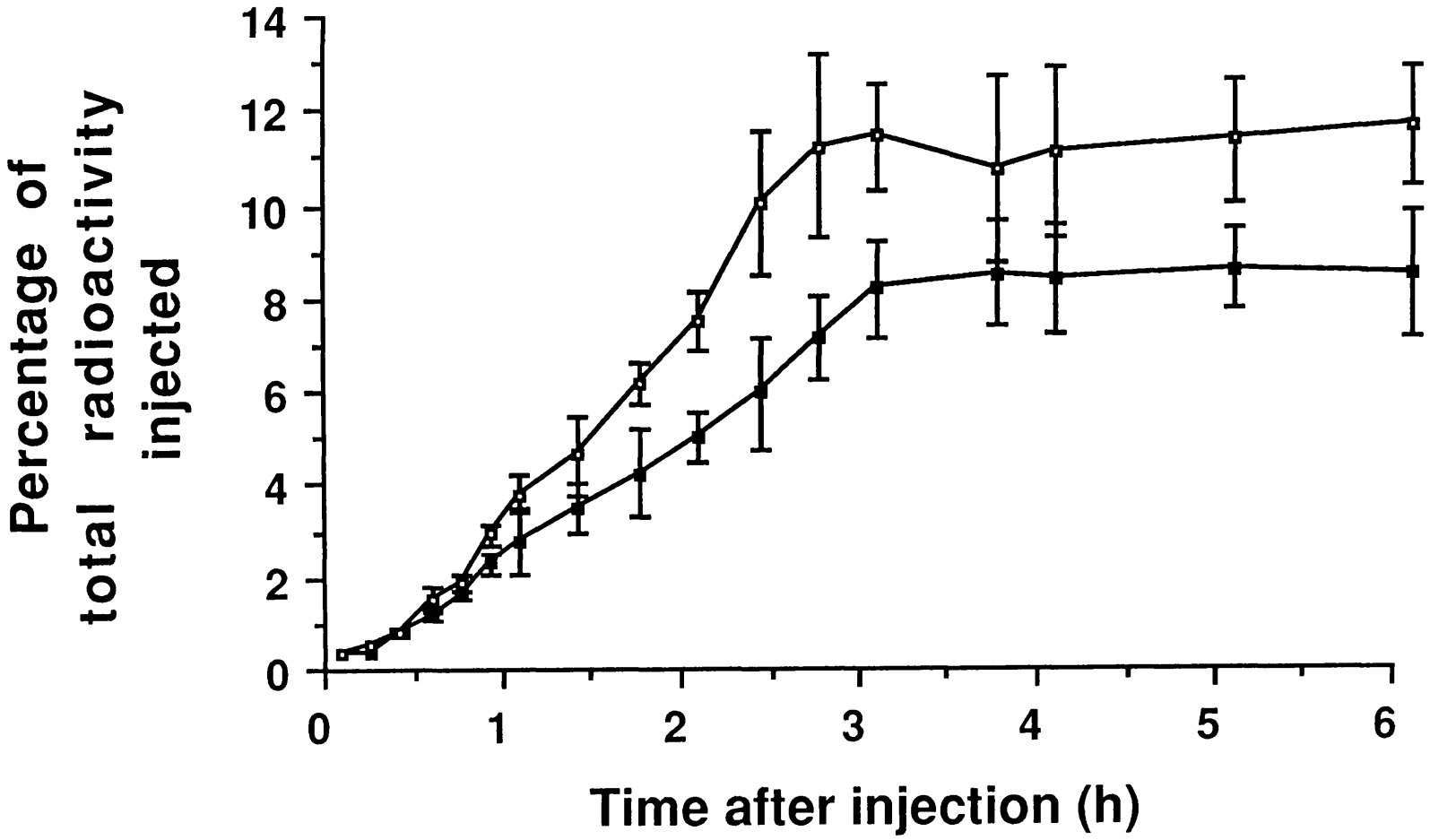
Radioactivity was first detected in the chamber water 20 minutes post-injection, when trace amounts were measured (Fig 4.7). The rate of incorporation of radioactivity into $^{14}CO_2/HCO_3^-$ was initially very rapid, but slowed down after 3 h . Incorporation of radioactivity into $^{14}CO_2/HCO_3^-$ levelled-off at about 8 % of the total radioactivity injected. In the control crabs the temporal trends were the same as in the experimental animals, but the maximum percentage incorporation of radioactivity into the $^{14}CO_2/HCO_3^-$ was greater with a mean of 11.3 ± 1.26 % .

Acidifying the chamber water samples revealed, as in section 4.3.2.2., that at least 98 % of the radioactivity observed in the water was acid labile, and likely to be attributable to bicarbonate, carbonate and carbon dioxide.

4.3.3.3 Investigation to determine whether L-lactate is excreted.

At the end of the experiment 74.5 ± 2.06 % of the radioactivity recovered, remained within the crab tissue and 25.5 ± 1.87 % was detected in the incubation water (Table 4.3). To answer the question relating to the possible excretion of metabolites, it was unnecessary to fractionate the tissue extracts into the various metabolite groups. The sea water in each beaker contained on average 25.5 % of the total amount of radioactivity recovered, of which 24 ± 2.4 % was located in the carbon dioxide/bicarbonate fraction. The amino acid, neutral, weak and strong

FIG. 4.7 The percentage of L-[U-^{14}C]-lactate initially injected into *Carcinus maenas*, that was released from the crab into the surrounding water during recovery under normoxia. Data for control crabs previously maintained under normoxia for 12 hours are represented by open squares (□), whilst those previously maintained under anoxia for 12 hours are represented by the closed squares (■). Values are means ± S.D.


Percentage of total radioactivity injected
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
Time after injection (h)

acid fractions all contained very low counts of radioactivity (< 1 %). There was therefore no evidence to suggest that L-lactate or any other of the metabolites, apart from carbon dioxide and bicarbonate, were excreted during either anoxia or the normoxic recovery period.

Table 4.3 - Distribution of radioactivity within the incubation water, following recovery from environmental anoxia.

Metabolite fraction	Radioactivity recovered in incubation water (%)[1]
Carbon dioxide /bicarbonate	24.0 ± 2.40
Amino Acids	0.32 ± 0.08
Neutral Compounds	0.38 ± 0.23
'Weak' Acids	0.27 ± 0.19
'Strong' Acids	0.05 ± 0.03

* - Expressed as a percentage of the total radioactivity recovered.
n.b. - The remaining 76 % of the radioactivity was recovered in the tissue of the experimental animals.

4.4 DISCUSSION.

4.4.1 Incorporation of D-[U-^{14}C]-glucose in *Carcinus maenas* during normoxia.

D-[U-^{14}C]-glucose has been used to investigate carbohydrate metabolism in crustaceans for over 30 years. When Scheer & Scheer (1951) failed to find any labelled carbon dioxide being produced in *Panulirus penicillatus* or *P. japonicus*, after an injection of D-[U-^{14}C]-glucose, it was thought that glucose metabolism in crustaceans was radically different to that in other invertebrate groups. Hu (1958) showed conclusively, however, that an injection of uniformly labelled D-[U-^{14}C]-glucose into intermoult crabs, did result in the production of labelled respiratory carbon dioxide. Hu ascribed the inability of Scheer & Scheer to demonstrate the production of $^{14}CO_2$, to differences in the moulting cycles of the 2 types of crustaceans, but others have felt that the problems have more to do with the techniques used. Similar findings to those of Hu (1958) have been reported by Bergreen *et al.* (1961) confirming the existence of the glycolytic pathway in decapod crustaceans.

The results of the present study are in agreement with those reported above, with labelled carbon dioxide being detected after an injection of labelled D-[U-^{14}C]-glucose. The exact percentage of incorporation of total radioactivity into carbon dioxide, varies considerably, ranging from 41 % in the xiphosuran *Limulus polyphemus* after 3 h (Stetten, 1982) to 9 % in *Hemigrapsus nudus* after 13 h (Hu, 1958). Bergreen *et al.* (1961) tried to relate the influences of sex and stage of moult cycle to the extent of glucose oxidation (carbon dioxide excretion), but concluded that these factors could not explain the variations in CO_2 production observed between individuals. In this present, study 8.23 $\pm$ 0.94 % (Fig. 4.5) of the total radioactivity injected was incorporated into $^{14}CO_2$ and/or HCO_3^- under normoxic

conditions. It is difficult to distinguish between $^{14}HCO_3^-$ and $^{14}CO_2$, because a great deal of the former readily dissociates into the latter in sea water.

Incorporation of D-[U-^{14}C]-glucose into other metabolites, have been measured previously in *Hemigrapsus nudus* (Hu, 1958) in which radioactivity was detected in glycogen and oligosaccharides and glycolytic sugar phosphates. Huggins (1966) found that D-[U-^{14}C]-glucose was incorporated into L-lactate, sugar phosphates and alanine, in the gill, hepatopancreas and muscle tissue of *Carcinus maenas in vitro*. Since little activity was detected in tricarboxylic acid cycle intermediates, Huggins was of the opinion that pyruvate was probably being transaminated or reduced rather than oxidised via the TCA cycle. More recently, Zaba & Davies (1980) found that in mantle tissue preparations of the bivalve *Mytilus edulis*, D-[U-^{14}C]-glucose was metabolised *in vitro* into glycogen (46 %), amino acids (35 %), organic acids (13 %), CO_2 (5 %) and lipids (1 %).

In the present study, a far greater incorporation of D-[U-^{14}C]-glucose into amino acids was found, at the expense of glycogen and the organic acids (Fig. 4.4). This incorporation of D-[U-^{14}C]-glucose into the other metabolic fractions is extremely rapid, since only 1.6 % of the injected radioactivity remained in the form of D-glucose after 6 h . There was also evidence for glycogen and amino acid turnover, since percentage incorporation in crabs taken after 12 h post-injection was significantly ($P < 0.05$) lower than those taken after 6 h .

4.4.2 Investigation into the conversion of L-lactate back to its initial substrate during recovery.

Although the primary aim of this chapter was to investigate the energy metabolism involved in the recovery from anaerobic metabolism of decapod crustaceans, studies involving D-[U-^{14}C]-glucose have also revealed a great deal about the metabolic

responses of *Carcinus maenas* to the anaerobic conditions themselves. The following observations serve largely to reinforce what has already been described in Chapter 3.

The trends observed in the incorporation of radioactivity into the weak acid fraction, (mainly L-lactate), are very similar to those reported previously for the changes in concentration of L-lactate (Chapter 3) (Fig. 4.8).

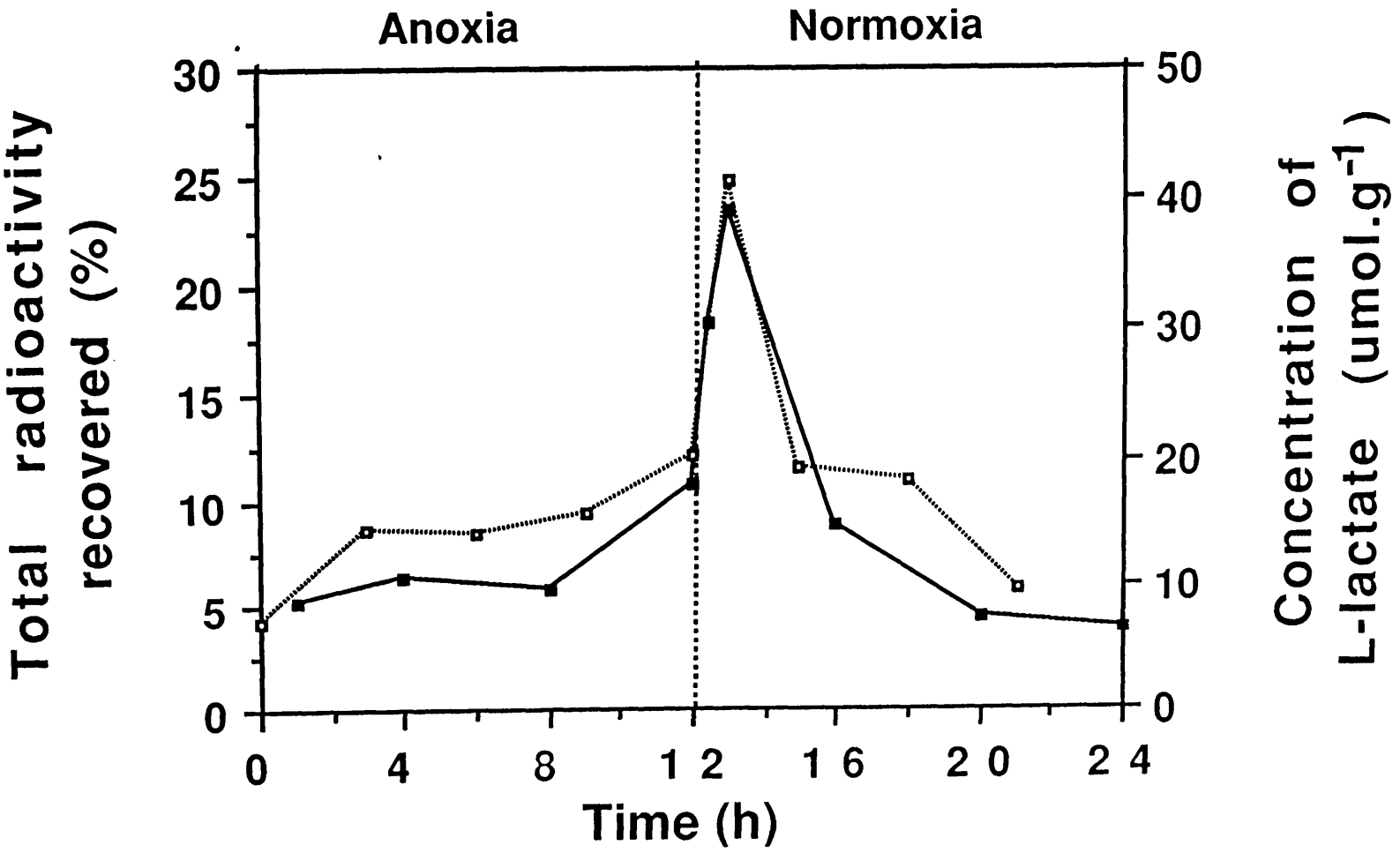
The lack of significant change in the incorporation of radioactivity into the TCA fraction, lends support to the statement made in section 3.3.3.3, that anaerobic pathways culminating in the production of organic acids are of minor importance in decapod crustaceans.

Finally, the existence of an accumulation of glycolytic intermediates was postulated, since a lag was observed between the catabolism of glycogen and the production of L-lactate (section 3.4.4.1.). Since this accumulation would take the form of glycolytic phosphates, one would expect an increase in the incorporation of radioactivity into this fraction (F5g). A significant ($P < 0.05$) increase was observed during this study, but the exact identity of the metabolites labelled in this fraction were not determined and so the existence of this intermediary accumulation remains in question.

After the first hour of the recovery period, the percentage incorporation of radioactivity in the weak acid fraction (F4) decreased dramatically, whilst there was an increase in the radioactivity found in glycogen. This indicated the occurrence of gluconeogenesis, but there was no direct evidence to suggest that the radioactivity involved in the increased incorporation into the glycogen was in fact from the weak acid fraction.

To answer this question unambiguously, L-[U-^{14}C]-lactate was used as it was considered to be a better labelled precursor with which to investigate the presence

FIG. 4.8 A comparison of 2 methods of assessing the trends in pooled tissue L-lactate in *Carcinus maenas* during 12 hours of exposure to anoxia, followed by 12 hours of recovery under normoxia. The percentage of radioactivity recovered from the pooled tissues of *Carcinus maenas*, that was incorporated into L-lactate (■)(values from Fig. 4.6). The change in the concentration of L-lactate determined by enzymic methods (□) (values from Fig. 3.5). See text for further details.


Anoxia
Normoxia
Total radioactivity recovered (%)
Concentration of L-lactate (umol.g-1)
Time (h)
0
4
8
12
16
20
24
0
5
10
15
20
25
30
0
10
20
30
40
50

or absence of gluconeogenesis. Phillips *et al.* (1977) suggested that decapod crustaceans had a poor capacity for lactate metabolism, but still showed a slow incorporation of L-[U-^{14}C]-lactate into glucose in the haemolymph of *Cherax destructor*. Stetten (1982) failed to show gluconeogenesis in the xiphosuran *Limulus polyphemus*, following an injection of L-[U-^{14}C]-lactate. This was not surprising, however, since *L. polyphemus* contains a lactate dehydrogenase which is specific for the D-lactate stereoisomer only (Long & Kaplan, 1973). Gäde *et al.* (1986) repeated the experiment by injecting *L. polyphemus* with D-[U-^{14}C]-lactate and *Menippe mercenaria* with L-[U-^{14}C]-lactate and clearly demonstrated gluconeogenesis, as well as a large incorporation of radioactivity into amino acids (mainly alanine). Recently, van Aardt (1988) showed that gluconeogenesis occurred in the river crab *Potamonautes warreni*. In all these studies the authors stressed that the rates of gluconeogenesis were extremely slow when compared with the process in vertebrates. Coulson (1987) reports for example that a 2 g shrew after 3 minutes of forced exercise, will restore their glycogen levels in just 1.6 minutes.

Results from the present study show conclusively that L-[U-^{14}C]-lactate is incorporated into glycogen and then into amino acids, in the shore crab *Carcinus maenas*. Percentage recoveries in the particular fractions are very similar to those reported by Gäde *et al.* (1986) in *Menippe mercenaria*.

The sites of lactate metabolism and gluconeogenesis in decapod crustaceans remain a matter of controversy. Many studies on this subject have used *Carcinus maenas* as the experimental animal. Thabrew *et al.*, (1971) demonstrated that the gill tissue of this crab was capable of gluconeogenesis. It was also reported in that investigation, that there were seasonal variations in the glucose production rate and also that fatty acids stimulated gluconeogenesis by inhibiting pyruvate oxidation. At about the same time there were a series of studies demonstrating that the haemocytes in *Libinia emarginata* and *Carcinus maenas* contained glycogen and glucose-6-phosphatase (Johnston & Fisher, 1968; Johnston *et al.*, 1971; Johnston & Davies,

1972; Johnston *et al.*, 1973). These studies demonstrated the presence of small amounts of phosphoenolpyruvate-carboxykinase and pyruvate carboxylase in the haemocytes, indicating a possible site of gluconeogenesis. Finally, Munday & Poat (1971) have suggested that the hepatopancreas is the major site for gluconeogenesis in crustaceans. Phillips *et al.* (1977) found, however, that the conversion of lactate to glucose could not be demonstrated with *in vitro* preparations of the hepatopancreas, gill or haemolymph of *Cherax destructor*.

4.4.3 Investigation to determine whether L-lactate is oxidised to CO_2.

In addition to the present study, the production of $^{14}CO_2$ during recovery, following an injection of L-[U-^{14}C]-lactate has been determined in 2 other investigations, involving 2 separate species. A similar experiment was performed using the xiphosuran *Limulus polyphemus* and is included in the following table by way of comparison:

Table 4.4 Percentage of injected L-[U-^{14}C]-lactate incorporated into carbon dioxide during recovery from environmental anoxia.

Species	Incorporation of L-[U-^{14}C]-lactate as carbon dioxide (%)	Time after injection (h)	Reference
Crustacea			
Menippe mercenaria	9-21*	4	Gäde *et al.*, (1986)
Potamonautes warreni	10	5	van Aardt, (1988)
Carcinus maenas	7-9	6	Present study.
Xiphosuran			
Limulus polyphemus	14-45*	4	Gäde *et al.*, (1986)

* Incorporation expressed as a percentage of radioactivity recovered.

Gäde *et al.* (1986) found that the incorporation of radioactivity into carbon dioxide varied considerably, depending on the activity level and previous physiological history of the animal. It is inadvisable to attach too much significance to the differences in Table 4.4, since each investigation had different methods and was carried out using different species of animals. The oxidation of L-lactate in the decapod crustaceans appear to represent a very minor method of anaerobic end product clearance. This is in contrast to *Limulus polyphemus*, which is far more heavily reliant on complete oxidation for the clearance of D-lactate.

4.4.4. Investigation to determine whether L-lactate is excreted.

As reported in the introduction, excretion of anaerobic end products is common in annelids and has been shown to occur in bivalves. In these groups it is more advantageous to lose the energy-rich carbon skeleton of the end product, than to expend further energy in re-metabolising it into a more useful and less toxic compound. Amongst the decapod crustaceans, excretion of L-lactate has been reported only rarely. de Zwaan & Skjoldl (1979) found that between 27 and 52 % of the total L-lactate produced by the isopod *Cirolana borealis* was excreted into the surrounding water.

In an investigation by van Aardt (1988) of the anaerobic metabolism in the crab, *Potamonautes warreni*, a significant difference was observed in acid-stable radioactivity within the incubation water, between experimental and control crabs. An explanation was put forward by W.R. Ellington (quoted in van Aardt, 1988), tentatively postulating the existence of a possible lactate:H symport system in the gills. In the present study and that of Gäde *et al.* (1986), very little or no acid-stable radioactivity was observed, indicating that lactate was not in fact excreted. This is consistent with the findings of Bridges & Brand (1980) who failed to

demonstrate any L-lactate excretion in a range of marine crustaceans.

Results from the present study, give the clearest evidence so far that L-lactate is not excreted during the recovery period. 24 $\pm$ 2.4 out of 25.5 $\pm$ 1.87 % of the radioactivity found in the incubation water can be attributed to carbon dioxide/bicarbonate. Only trace amounts of radioactivity were found in the other metabolic fractions. These results are in close agreement with those of Gäde *et al.* (1986).

This chapter has shown quite clearly that, in *Carcinus maenas*, end product clearance primarily involves the conversion of L-lactate back to its initial substrate. There is also evidence of complete oxidation of end products to carbon dioxide, but it is impossible to state whether this is direct oxidation of pyruvate from L-lactate or oxidation of pyruvate from gluconeogenically produced radiolabelled D-glucose.

CHAPTER 5 - THE RESPIRATORY RESPONSES OF *CARCINUS MAENAS* TO CONDITIONS OF ANOXIA AND SUBSEQUENT RECOVERY.

5.1 INTRODUCTION.

Previous chapters have concentrated on the metabolic responses of *Carcinus maenas* to conditions of anoxia and recovery, with little attention being given to the respiratory consequences of such conditions. Although the respiratory aspects of decapod crustaceans to declining oxygen tension have been previously studied (McMahon & Wilkens, 1983 - for review), it was felt that an investigation into these respiratory responses in the present study was necessary for two reasons. Firstly, to complement the extensive metabolic data already collected, it would be useful to have the comparable respiratory data measured under identical conditions on the same population of animals. Secondly, it was hoped that some insight into the respiratory responses of *C. maenas* to anoxia, might help to explain the heavy reliance on anaerobic metabolism observed during the initial stages of recovery from the period of anoxic conditions.

The experiments and measurements described in this chapter were confirmatory in nature and were not intended to be a comprehensive study of the respiratory physiology of *Carcinus maenas*. The experiments were divided into 2 main subject areas:

i) An investigation of oxygen regulation during exposure to conditions of declining Po_2 and subsequent recovery.

ii) An investigation of some of the parameters effecting the acid-base balance during anoxia and subsequent recovery.

Throughout all the previous experiments involving anoxia, it has been determined that the Po_2 of the sea water decreased to < 2 Torr, when a gaseous mixture of nitrogen /carbon dioxide was bubbled through it, and it has been assumed that at

the same time the crab's haemolymph became oxygen depleted. Similarly, when air was reintroduced and the sea water became re-aerated at the end of the anoxic period, it was assumed that the haemolymph of the crabs would also rapidly again become saturated with oxygen. In order to substantiate these assumptions, an experiment was carried out to measure the Po_2 of the postbranchial haemolymph, both under conditions of declining Po_2 and when the air was reintroduced again at the beginning of the recovery period.

The rate of weight specific oxygen consumption ($\dot{M}o_2$) was measured throughout the experiment, to obtain a further measure of metabolic rate. In addition, the heart rate was determined, since, amongst decapod crustaceans, it has previously been shown to be sensitive to environmental variations (McMahon *et al.*, 1974; Taylor, 1981; Wheatly & Taylor, 1981; Bradford & Taylor, 1982; Taylor, 1984).

The acid-base balance of *Carcinus maenas* under conditions of anoxia and subsequent recovery was studied by measuring the total carbon dioxide concentration (Cco_2) and the pH of the haemolymph. The concentration of bicarbonate (HCO_3^-) and the partial pressure of carbon dioxide (Pco_2) were then calculated. To assess the possible role of exoskeletal carbonate buffers (mainly calcium carbonate), the concentration of calcium was also determined.

5.2 MATERIALS AND METHODS.

5.2.1 Po_2 of postbranchial haemolymph.

A small hole was drilled through the carapace above the pericardial cavity of 42 crabs (fresh wt. range = 5 - 15 g). The holes were drilled using a dental drill (Baxter Dental Supplies), with care being taken to avoid penetrating the hypodermis. The holes were drilled to facilitate the insertion of the hypodermic needle, during haemolymph sampling. The crabs were then left to recover for 24 h in fully aerated artificial sea water (Tropic Marin - Chapter 2, section 2.2.2) maintained at $10 \pm 1^{o}C$.

The crabs were then distributed equally between 10 plastic tanks (volume = 10 l) and left undisturbed for a further 24 h . The Po_2 of water in the tanks was reduced to < 2 Torr, by bubbling a nitrogen/carbon dioxide mixture (Chapter 2, section 2.2.3) through the sea water. Before the haemolymph samples were taken, the syringes and needles were filled with nitrogen, to try to prevent oxygen in the air contained within the dead space of the needle from augmenting the oxygen in the haemolymph. Postbranchial haemolymph samples (500 ul) were taken from 3 crabs at the following times: 0, 0.5, 1, 2, 3 and 4 h after the start of the experiment. This was done by removing the crabs from the water and inserting the hypodermic needle (26 g) directly into the heart through the hole in the carapace. Care was taken to take the sample within 15 seconds and to avoid the entry of air bubbles into the syringe. Attempts were made to obtain haemolymph samples from crabs still under the water, but this technique was not successful since it appeared to cause more disturbance to the crab and also to the surrounding crabs, than simply removing the animal from the tank.

After the crabs had been exposed to anoxia for a period of 12 h, the Po_2 of the water was returned to normoxic levels by bubbling air through it. Further haemolymph samples from 3 crabs were taken at the following times after they had

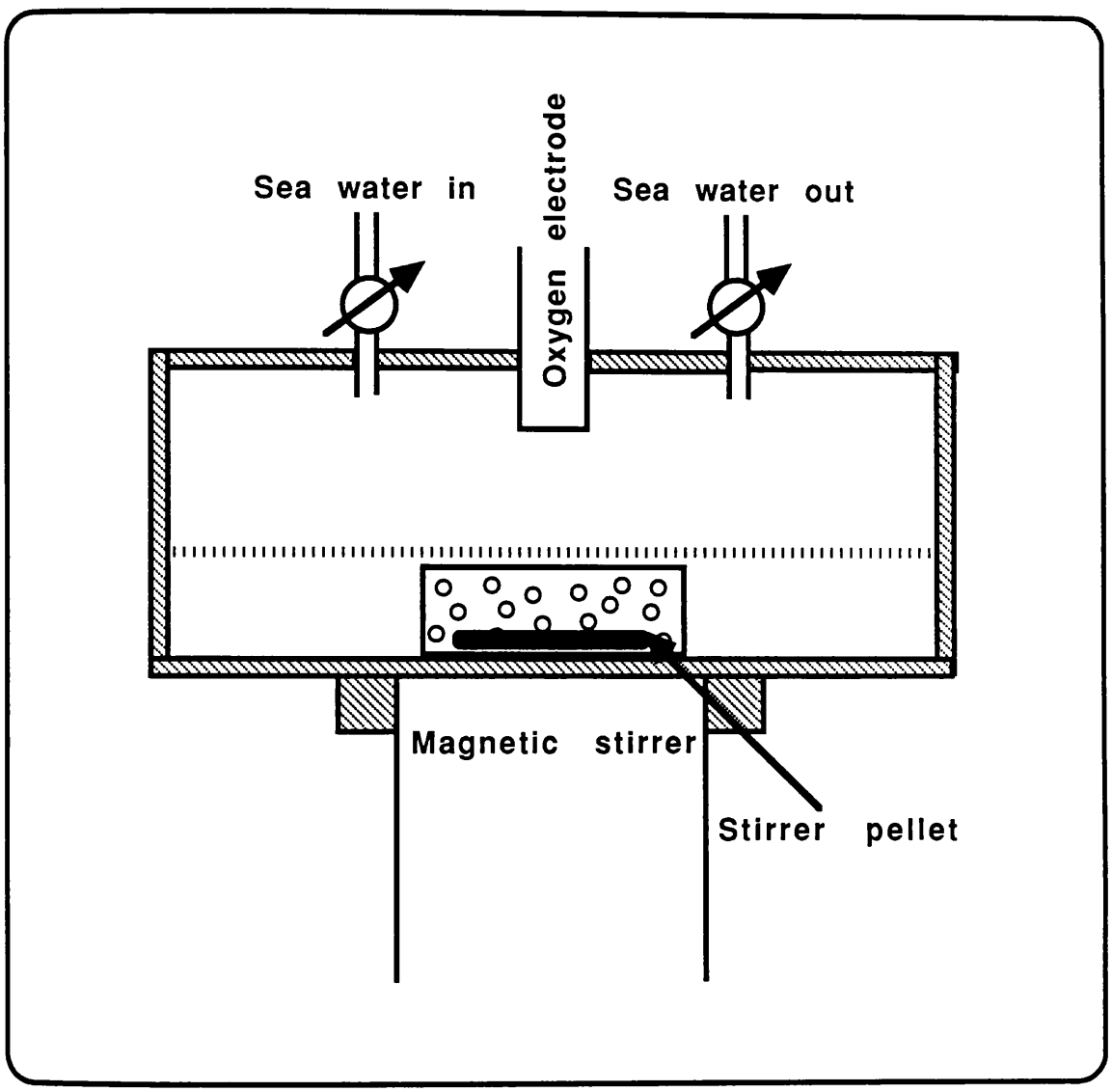
been returned to normoxic conditions: 0, 5, 10, 20, 30, 40, 50, 60 minutes. The Po_2 of the haemolymph sample was determined using an oxygen electrode (E5046, Radiometer, Denmark) contained within a thermostatted water jacket, which was maintained at $10 \pm 1^{o}C$. The oxygen electrode was coupled to an oxygen meter (Strathkelvin Instruments, Glasgow) and a pen recorder (Tekman). The Po_2 electrode was calibrated using a solution of sodium tetraborate (0.01M) containing a few milligrams of sodium sulphite to produce a Po_2 of zero. Saturated sea water was also used to calibrate the oxygen electrode. Haemolymph samples were injected into the chamber surrounding the electrode and the Po_2 measured. After each measurement the system was flushed with distilled water.

Sea water samples (500 ul) were also taken at the same time as the haemolymph samples, and their Po_2 measured as above. These were then compared directly with the Po_2 measurements of the postbranchial haemolymph (P_ao_2).

5.2.2 Oxygen Consumption.

A closed respirometer was used to determine the rate of oxygen consumption of crabs during the recovery period. The respirometer consisted of a perspex container (volume 175 ml) with an enclosed magnetic stirring bar, which ensured that the sea water was constantly mixed (Fig. 5.1). Changes in the Po_2 of the water in the respirometer were monitored using an oxygen electrode (E5046, Radiometer, Denmark), connected to an oxygen meter (Strathkelvin Instruments, Glasgow) and a pen recorder (Tekman). The electrode was inserted into the lid of the chamber, so that the Po_2 could be continuously monitored. The electrode was calibrated as described previously (section 5.2.1). A storage column was placed above the respirometer and connected to it via a 2-way tap. This allowed the rate of flow of water through the respirometer to be carefully controlled when the water was changed. A header tank was placed above the storage column, to reduce bubbles

FIG. 5.1 Diagram of the closed respirometer used for measuring oxygen consumption in *Carcinus maenas*. See text for explanation.


Sea water in
Oxygen electrode
Sea water out
Magnetic stirrer
Stirrer pellet

caused through aeration of the water from entering the respirometer. The experiment was carried out in a constant temperature room, at 10 ± 1°C.

Preliminary experiments using sea water from the aquarium system showed that background respiration rates were high due to microflora living within the system. Artificial sea water was therefore used and made up freshly before each experimental run. In addition, the respirometer, reservoir column and the header tank were all sterilised using a 10 % solution of sodium hypochlorite solution between each experiment.

A nitrogen/carbon dioxide mixture (Chapter 2, section 2.2.3.), was bubbled through the sea water in the header tank to reduce the Po_2 to < 2 Torr. The header tank was covered with a polystyrene sheet (5 mm thick), which was cut to fit the inside of the tank and which, by floating on the water surface, substantially reduced the water/air interface and helped to prevent the diffusion of oxygen back into the water. The water from the header tank then drained into the storage column, which in turn flowed into the respirometer. When normoxic conditions were required, air was substituted for the nitrogen/carbon dioxide mixture.

5 crabs (fresh wt. range = 1 - 5 g) were placed in a plastic tank (volume = 10 l) of continually aerated artificial sea water and were left undisturbed for 24 h . One crab was transferred to the respirometer, which was then filled with aerated sea water from the reservoir and the inflow and outflow taps were then closed. It was left for 12 h under normoxic conditions, to allow the crab sufficient time to recover from handling stress. The rate of oxygen consumption was measured throughout this period. It was necessary to flush the chamber with fresh water after every 4 h to ensure that the water did not become too hypoxic and to prevent the accumulation of metabolic waste products. This was done by opening both the inflow and outflow taps and allowing fully aerated sea water to flow through the

respirometer and then closing both taps again.

After this 12 h period, the Po_2 of the water in the header tank was reduced, as described earlier. The inflow and outflow taps were again opened and the anoxic water was flushed through. When the respirometer water was fully anoxic the taps were closed. Anoxia was maintained for 12 h during which time the water was changed after 4 and 8 h to ensure that metabolic waste products did not accumulate.

After the crabs had been exposed to anoxia for 12 h, the Po_2 of the water was returned to normoxic levels by bubbling air through the header tank and then passing this aerated water through the storage column and into the respirometer. The oxygen consumption was then measured during the subsequent recovery period (12 h). Fresh, aerated sea water was flushed through the respirometer every 4 h , to prevent the water becoming hypoxic and the accumulation of metabolic waste products. After the recovery period, the crab was removed and the background oxygen consumption of the chamber microflora was then measured.

The rates of weight specific oxygen consumption ($\dot{M}o_2$) were then calculated using the following equation:

$$\dot{M}o_2 = \frac{(((Po_{2i} - Po_{2f}) \times \alpha O_2) \times V)}{W} \times 2$$

$\dot{M}o_2$ = Oxygen consumption (umol $O_2.h^{-1}.g^{-1}$ fresh wt.).

Po_{2i} = Initial partial pressure of oxygen in respirometer.

Po_{2f} = Final partial pressure of oxygen in respirometer (i.e. after 30 minutes).

αO_2 = Solubility coefficient of oxygen at 10 ^{o}C.

V = Volume of the respirometer (litres).

W = Wet weight of crab (g).

The background rate of oxygen consumption was then subtracted from the rates measured for individual crabs.

5.2.3 Heart rate.

The heart rate was recorded using the impedance technique as originally described by Hoggarth and Trueman (1967), with modifications suggested by Spaargaren (1973) and by Dyer and Uglow (1977). The electrodes consisted of shellac-coated copper wire (0.28 mm in diameter), from which the last 1 millimetre of shellac had been removed. This wire electrode was inserted through the carapace immediately above the heart and held in place by cyanoacrylate adhesive (Permabond, England). The hardening of the adhesive was accelerated using methyl methacrylate. Care was taken not to add too much methyl methacrylate since it was highly toxic to the crabs. 5 crabs (fresh wt. range = 5 - 15 g) had electrodes inserted in this manner and were then left to recover from handling stress for a least 48 h in fully aerated sea water, in a constant temperature room at 10 $\pm$ 1^{o}C. The electrodes were attached to a Washington impedance pneumograph and pen recorder (Tekman). A small oscillating current (2 uA, 25kHz) was induced between a fine wire electrode inserted into the crab and a larger reference electrode consisting of an aluminium plate placed in the tank. Normal beating of the heart, caused a change in the impedance, between the electrodes and this was converted to a voltage which was amplified and recorded on a chart recorder.

A crab with an electrode attached to it was transferred to a beaker (volume = 1 l) containing 800 ml of aerated artificial sea water. Following a 24 h period, during which time the crab was left undisturbed, the Po_2 of the water was reduced to < 2 Torr (over a 4 h period), using a gas mixture of nitrogen and carbon dioxide, regulated by a precision gas mixing system, which was pumped through an air-stone into the container. The Po_2 of the water was monitored continuously using an oxygen electrode coupled to an oxygen meter as described previously. The beaker

was covered with a polystyrene sheet for reasons previously described. Following a 12 h anoxic period, the sea water in the beaker was re-aerated. The heart rate was monitored throughout the experimental period, along with the corresponding Po_2 of the water.

5.2.4 Acid-base balance during anoxia and recovery

5.2.4.1 General experimental procedure.

A small hole was drilled through the carapace above the pericardial cavity of 26 crabs (fresh wt. range = 5 - 15 g), as described previously (5.2.1). The crabs were then left undisturbed for 24 h, in fully aerated artificial sea water maintained at 10 $\pm$ 1^{o}C.

26 of the crabs were then distributed amongst 6 plastic tanks (volume = 10 l) (4 crabs in tank 6) containing aerated sea water and left for a further 24 h. In all 6 tanks the sea water was continuously aerated and maintained at 10 $\pm$ 1^{o}C.

The Po_2 of the sea water in the first 5 tanks was reduced to $<$ 2 Torr by bubbling a nitrogen/carbon dioxide mixture through the water (Chapter2, section 2.2.3 for more details). The water in the 6th tank was maintained fully aerated throughout the experiment. After the crabs had been exposed to anoxia for a period of 12 h, the Po_2 of the water in the 5 tanks was returned to normoxic levels by bubbling air through it. Haemolymph samples (1 ml) were taken from a further 2 crabs at each of the following times 0, 3, 6, 9, 12, 12.5, 13, 15, 18, 21 and 24 h after the start of the experiment (5.2.1). Haemolymph samples from control crabs, maintained under normoxic conditions were obtained at 0 and 24 h .

5.2.4.2 Measurement of the Cco_2 and the pH of the haemolymph.

The total carbon dioxide content of the haemolymph (Cco_2) (i.e. CO_2 + HCO_3^- + CO_3^{2-}) was determined on duplicate samples (10 ul) following the method of Cameron (1971). This method utilises the fact that the carbon dioxide/bicarbonate system is pH dependent. Therefore in the presence of HCl (0.01 M), any bicarbonate and carbonate in a haemolymph sample immediately forms carbon dioxide. In the present study, a Pco_2 electrode (Radiometer E5037) was connected to a Radiometer PHM73 meter. The electrode was inserted into a thermostatted, stirred cell (volume 345 ul), filled with HCl (0.01 M) and thermostatted to 30 °C to speed the reaction. The haemolymph sample (10 ul) was injected into this cell and the bicarbonate or carbonate displaced as carbon dioxide. The increase in the Pco_2 of the solution was detected by the electrode and recorded on a chart recorder. By using potassium bicarbonate (10 mM) as a standard, it was possible to calculate the concentration of total carbon dioxide in the original haemolymph sample.

The pH of the haemolymph was determined by drawing haemolymph samples into the microcapillary pH electrode (thermostatted to 10 °C) of a Radiometer BMS II which was connected to a PHM 73 pH meter (Radiometer, Copenhagen). Calibration involved using precision buffers (Radiometer S1500 and S1510).

5.2.4.3 Calculation of Pco_2 and HCO_3^-.

The Pco_2 of the haemolymph was calculated from the values of pH and Cco_2, using the modified Henderson-Hasselbalch equation (McMahon et al., 1978).

$$pH = pK'_1 + \log\frac{Cco_2 - \alpha.Pco_2}{\alpha.Pco_2}$$

Values for the constants pK'_1 and αCO_2 (solubility coefficient of carbon dioxide) at the appropriate temperature (10 °C) and salinity (32 ‰), were taken from Truchot (1976).

The concentration of haemolymph bicarbonate was calculated from the following equation:

$$[HCO_3^-] = Cco_2 - (\alpha.Pco_2)$$

This assumes that, at physiological pH values, the concentrations of carbonates and carbamates are negligible.

The remaining haemolymph samples were then centrifuged (10,000 g) for 10 minutes to remove any cellular debris and then stored at -20 ^{o}C until required for ionic analysis. Calcium was determined, following dilution with deionized water, using an atomic absorption spectrophotometer (Philips PU 9200). In order to release any bound Ca^{2+}, lanthanum chloride was added to each diluted haemolymph sample (final concentration of $LaCl_3.7H_2O$ = 1 %). Calibration curves were constructed using standard solutions of calcium nitrate containing $LaCl_3$.

5.3 RESULTS.

5.3.1 Po_2 of postbranchial haemolymph.

Anoxic conditions were obtained (< 2 Torr) after bubbling the nitrogen/carbon dioxide mixture through the water for 4 h . This was to simulate as nearly as possible the conditions that an animal would experience in the field (Chapter 2). The observed decrease in Po_2 of the postbranchial haemolymph (P_aO_2) initially lagged approximately 15 minutes behind the Po_2 decrease in the sea water (Fig. 5.2). After 4 h, however, both the Po_2 of the sea water and the postbranchial haemolymph had decreased to below 2 Torr.

When air was reintroduced into the anoxic tanks, the Po_2 of the water increased to normoxic levels (approximately 160 Torr) within 5 minutes. This rapid return to normoxic conditions simulates the situation in the field when the incoming tide floods a previously exposed rock pool (Chapter 2). After the reintroduction of air, the postbranchial haemolymph remained oxygen depleted for 5 minutes. The Po_2 then increased quite rapidly to become approximately 80 Torr again after about 30 minutes (Fig. 5.2).

5.3.2 Oxygen Consumption.

Since the weight of the crabs used in these experiments was very similar, size related differences in oxygen consumption ($\dot{M}o_2$) could be ignored and the rates of oxygen consumption from all 5 crabs could be pooled (Fig. 5.3). The background $\dot{M}o_2$ amounted to a mean of 0.37 ± 0.11 umol $O_2.h^{-1}$, representing between 6.75 and 22.5 % of the total $\dot{M}o_2$.

The mean $\dot{M}o_2$ of undisturbed crabs under normoxic conditions was 1.27 ± 0.17 umol $O_2.h^{-1}.g^{-1}$ fresh wt. Since the closed respirometric method used in this

FIG. 5.2 Changes in the Po_2 of the experimental water (+) and postbranchial haemolymph (P_ao_2) (■) during 4 hours of 'pre-anoxia' (see text for further explanation), 12 hours of anoxia and finally during a 12 hour period of recovery under normoxic conditions. Values are means ± S.D.

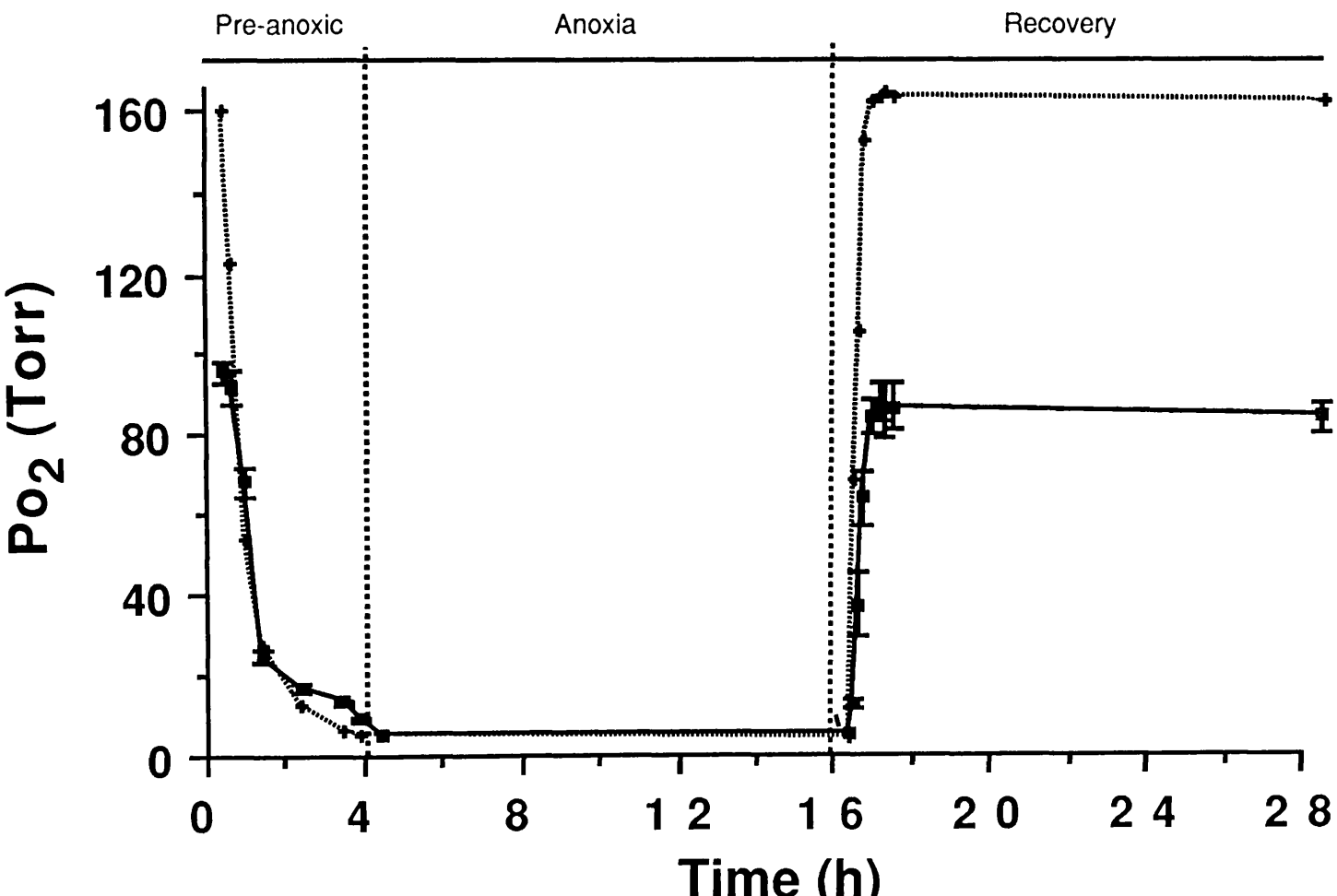
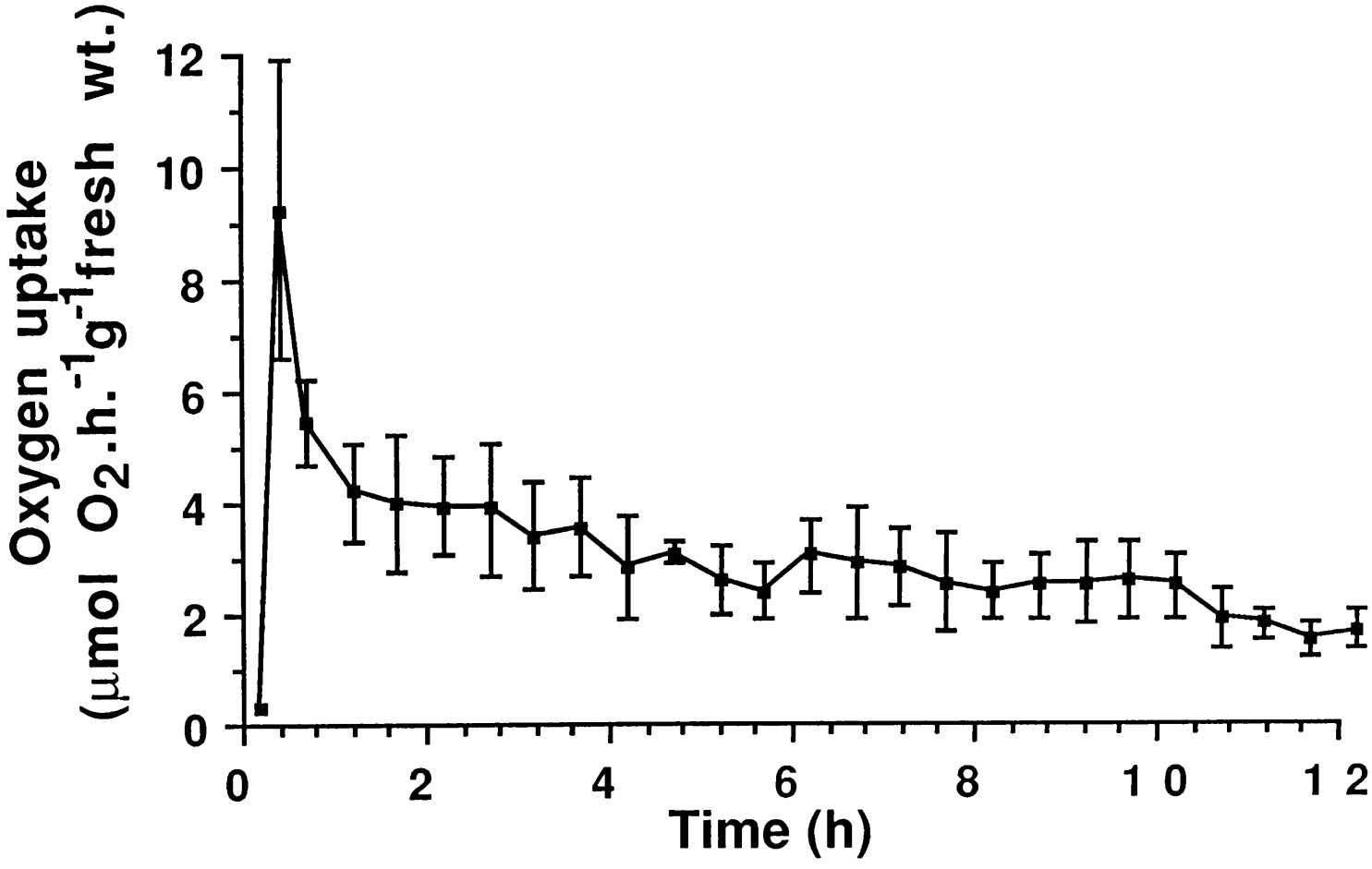

Pre-anoxic
Anoxia
Recovery
160
120
80
40
0
Po_2 (Torr)
0
4
8
12
16
20
24
28
Time (h)

FIG. 5.3 Changes in the rate of weight specific oxygen consumption ($\dot{M}o_2$) in *Carcinus maenas*, recorded during a 12 hour recovery period, following exposure to 12 hours of anoxia. Values are means $\pm$ S.D. (n = 5).


Oxygen uptake
(μmol O_2.h.$^{-1}$g^{-1}fresh wt.)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
Time (h)

experiment involved the direct replacement of normoxic with anoxic sea water, it was not possible to determine the Po_2 at which respiratory independence was lost. No discernible $\dot{M}o_2$ was measured during the anoxic period, but during the first 30 minutes of recovery, however, there was a rapid and significant ($P < 0.05$) increase in $\dot{M}o_2$ to 5.11 ± 0.71 umol $O_2.h^{-1}.\ g^{-1}$ fresh wt. The $\dot{M}o_2$ then gradually decreased during the remainder of the recovery period, returning to the control rate of 1.40 ± 0.37 umol $O_2.h^{-1}.g^{-1}$ fresh wt. after 12 h .

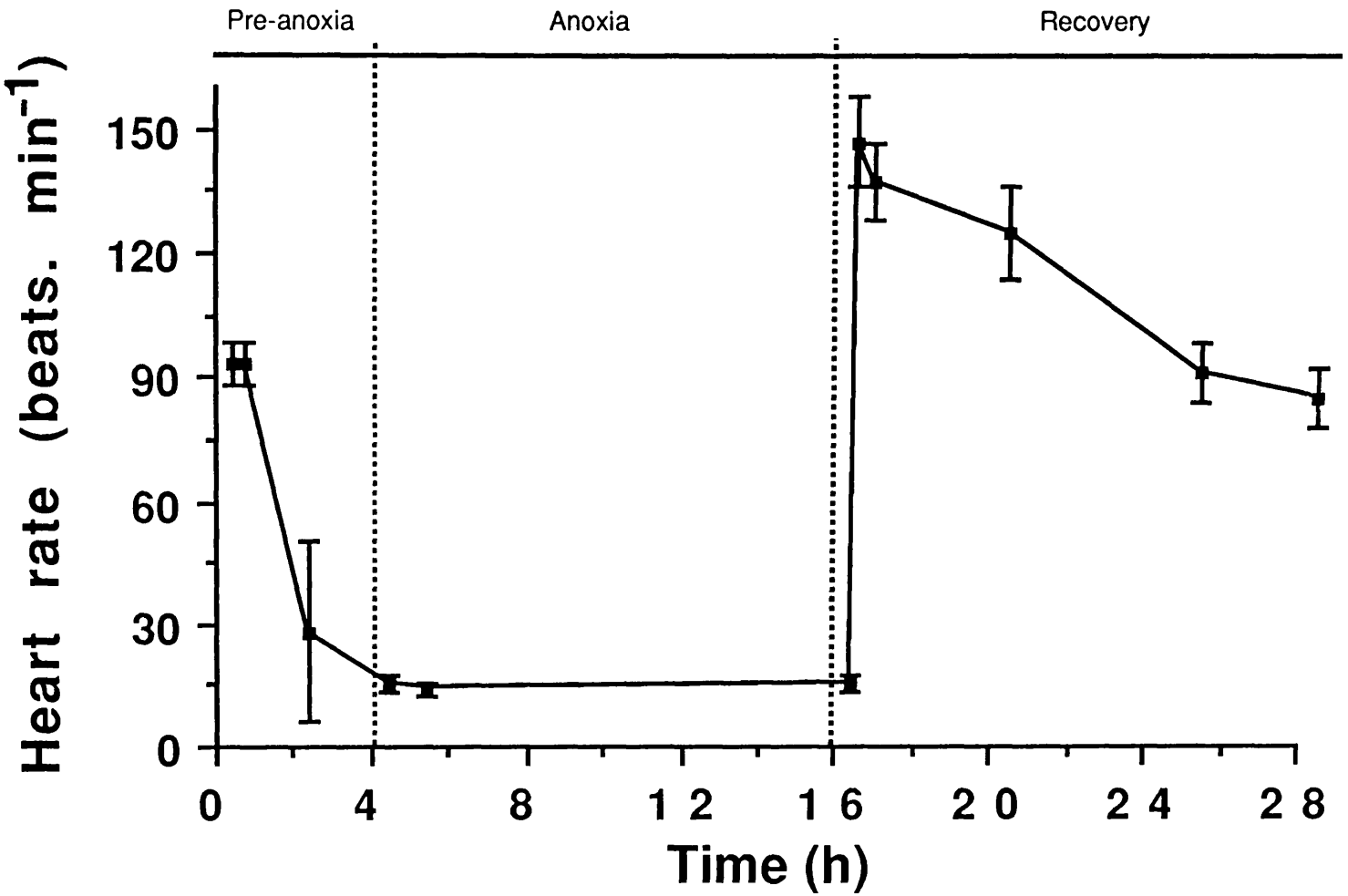
5.3.3 Heart rate.

Very high heart rates were measured in 11 crabs (mean = 119 ± 12 beats. min^{-1}) immediately following transfer to the experimental beakers, which appeared to be due to handling stress since during the next 3 h a gradual reduction in heart rate was recorded. The mean heart rate of quiescent crabs of this size was 92 ± 5 beats. min^{-1}.

Whilst the Po_2 of the sea water was being reduced by the nitrogen/carbon dioxide mixture, the heart rate remained approximately constant until the Po_2 of the water reached approximately 60 Torr. Below this Po_2 the heart rate decreased rapidly, until at the onset of anoxia the heart rate had declined to only 14 ± 2 beats. min^{-1} (Fig. 5.4). During the period of exposure to anoxia the heart continued to beat but at a very low rate (mean = 15 ± 1.7 beats. min^{-1}).

When the water in the beaker was aerated and the Po_2 returned to normoxic levels again, the heart rate increased significantly ($P < 0.05$) to 143 ± 11.2 beats. min^{-1} within 10 minutes. The mean heart rate during the first 30 minutes of the recovery period was 133 ± 9.1 beats. min^{-1}, but returned to control rates after about another 9 h .

FIG. 5.4 Changes in the heart rate of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D. (n = 5).


Pre-anoxia
Anoxia
Recovery
Heart rate (beats. min-1)
150
120
90
60
30
0
0
4
8
12
16
20
24
28
Time (h)

5.3.4 Haemolymph acid-base balance during anoxia and recovery.

The mean *in vivo* pH of the postbranchial haemolymph of control crabs maintained under normoxic conditions was 7.86 ± 0.05. A reduction in the Po_2 of the sea water within the experimental tanks resulted in a significant increase ($P < 0.05$) in the pH to 8.06 ± 0.04 (Fig. 5.5), but the mean *in vivo* pH then decreased significantly ($P < 0.05$) to 7.60 ± 0.03 after 12 h of anoxia. During the recovery period, the *in vivo* pH increased to return to control values after 12 h .

The total concentration of carbon dioxide decreased significantly ($P < 0.05$) from an initial value of 7.68 ± 0.17 to 3.24 ± 0.17 mM after 12 h of exposure to anoxia (Fig. 5.6). Cco_2 increased significantly ($P < 0.05$) again from 3.75 ± 0.34 to 8.02 ± 0.51 mM after 0.5 and 12 h of recovery.

There was no significant change ($P > 0.05$) in the calculated values for Pco_2 throughout the anoxic period (Fig. 5.7). During recovery, however, there was a significant ($P < 0.05$) increase from 1.57 ± 0.22 to 2.42 ± 0.19 Torr at the end of the first hour, before returning to 1.89 ± 0.05 Torr after 6 h of recovery.

The calculated values for the concentration of HCO_3^- ions decreased significantly ($P < 0.05$) during the 12 h period of anoxia, from an initial value of 6.52 ± 0.22 to 3.17 ± 0.17 mM after 12 h (Fig. 5.8). The concentration of HCO_3^- ions returned to control levels after 9 h of recovery and then did not change significantly during the remaining 3 h of the experiment.

There appeared to be no mobilisation of calcium from the carapace, in response to the acidosis, since the mean concentration of Ca^{2+} in the haemolymph remained constant throughout the anoxic period, (24.7 ± 1.88 $umol.g^{-1}$) (Fig. 5.9). During

FIG. 5.5 Changes in the pH of the postbranchial haemolymph of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D.

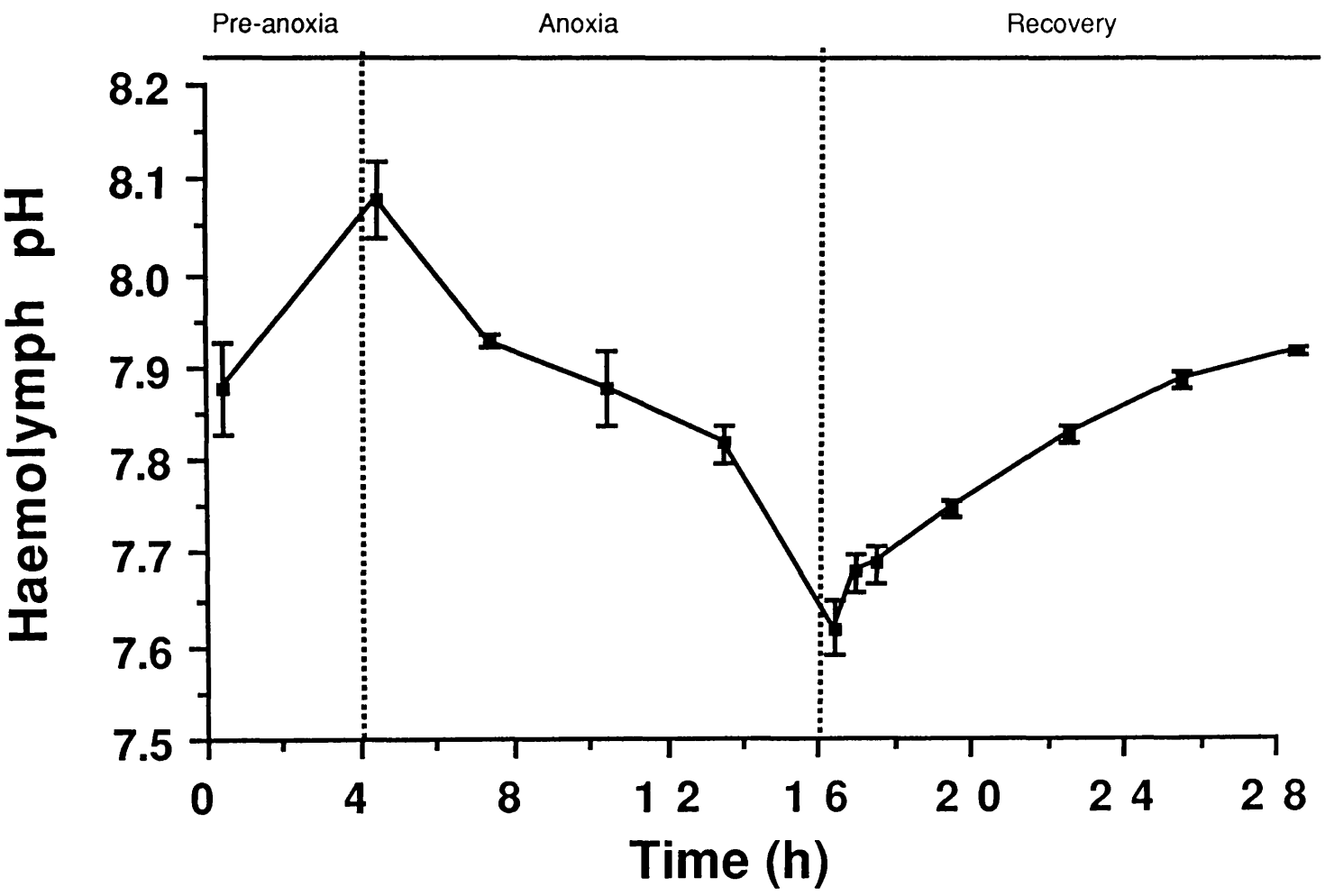

Pre-anoxia
Anoxia
Recovery
Haemolymph pH
8.2
8.1
8.0
7.9
7.8
7.7
7.6
7.5
0
4
8
12
16
20
24
28
Time (h)

FIG. 5.6 Changes in the concentration of total carbon dioxide (Cco_2) in the postbranchial haemolymph of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D.

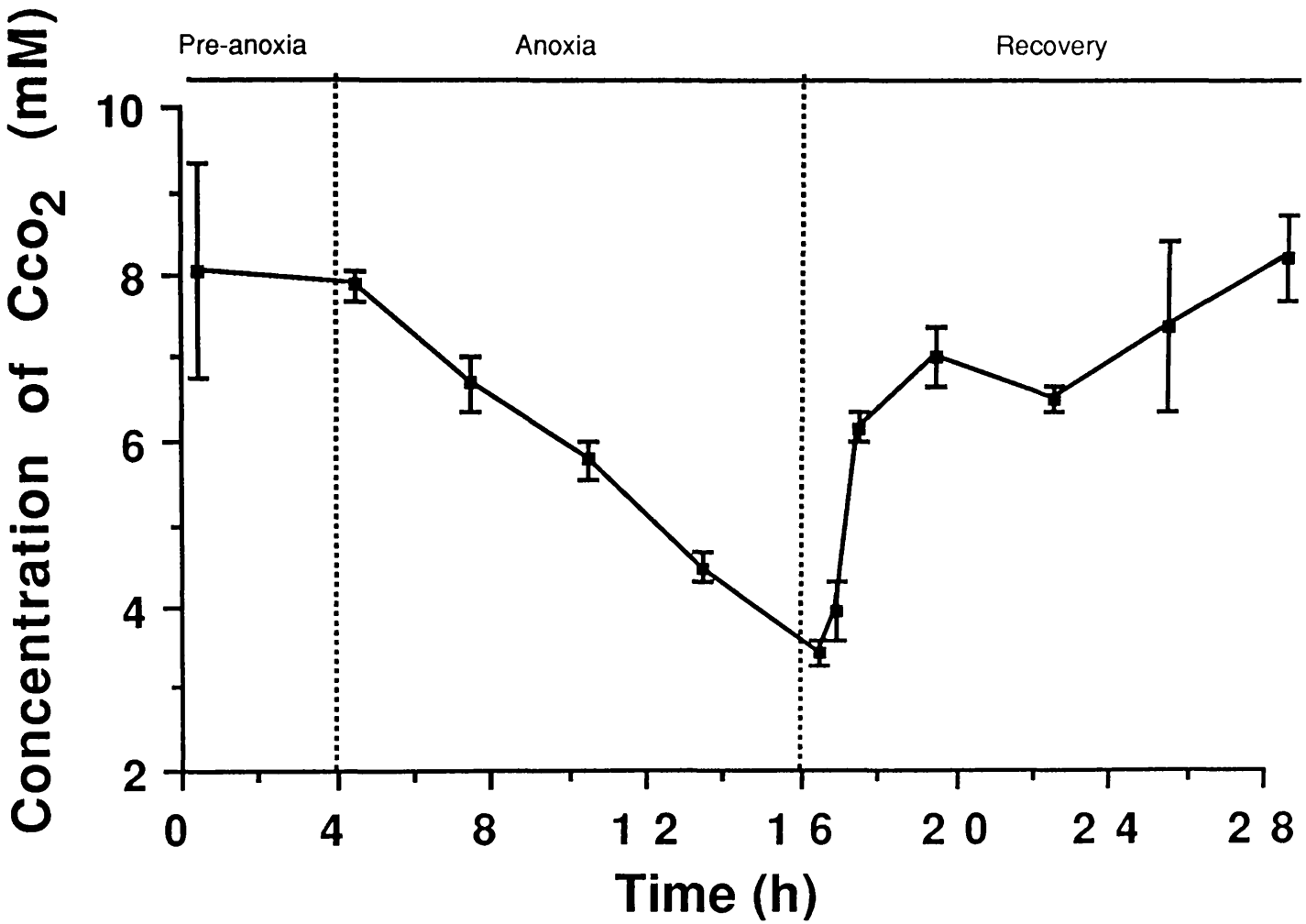

Pre-anoxia
Anoxia
Recovery
Concentration of Cco_2 (mM)
10
8
6
4
2
0
4
8
12
16
20
24
28
Time (h)

FIG. 5.7 Changes in the partial pressure of carbon dioxide (Pco_2) in the postbranchial haemolymph of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D.

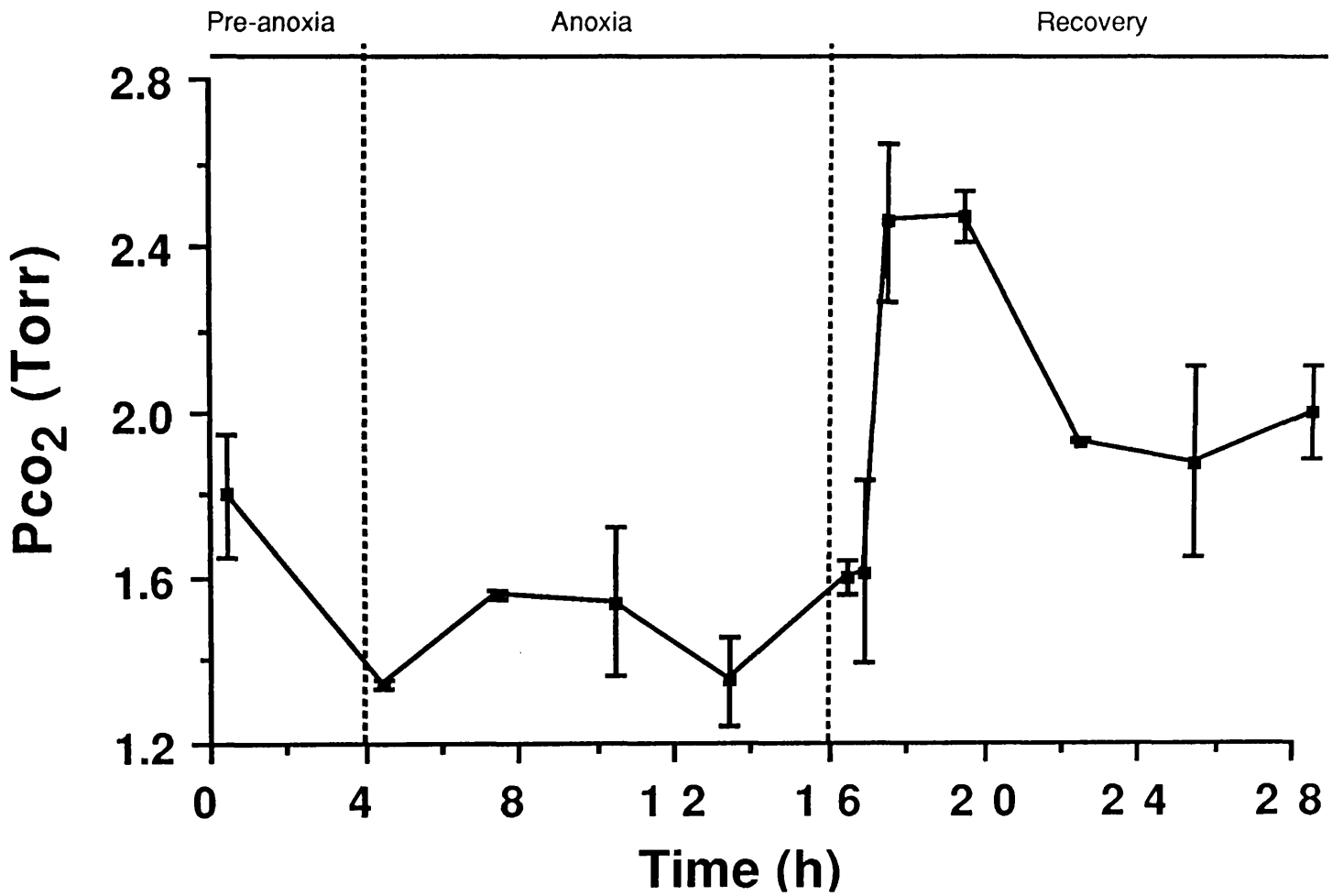

Pre-anoxia
Anoxia
Recovery
2.8
2.4
2.0
1.6
1.2
P_{CO_2} (Torr)
0
4
8
12
16
20
24
28
Time (h)

FIG. 5.8 Changes in the concentration of bicarbonate (HCO_3^-) in the postbranchial haemolymph of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D.

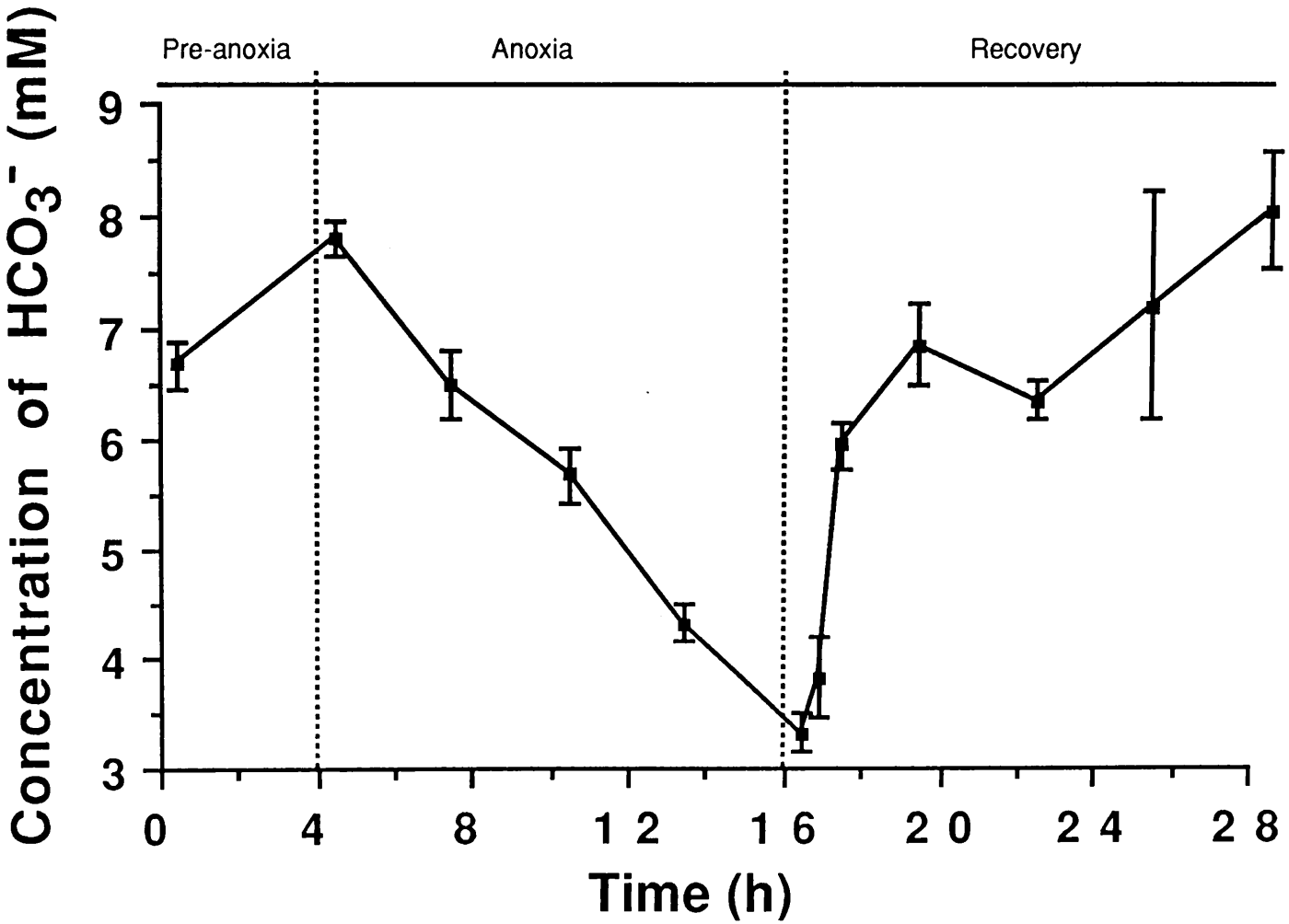
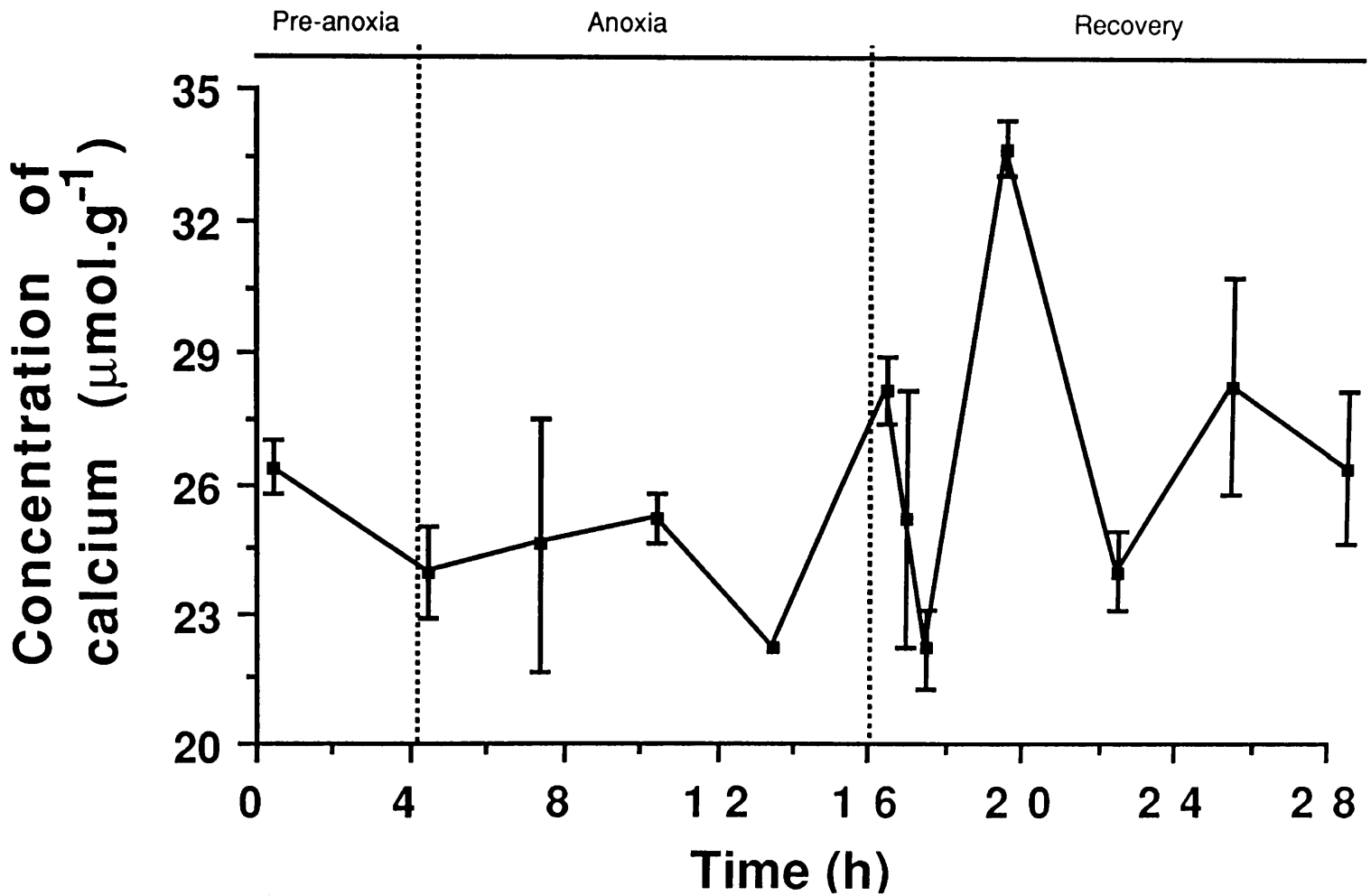

Pre-anoxia
Anoxia
Recovery
Concentration of HCO_3^- (mM)
9
8
7
6
5
4
3
0
4
8
12
16
20
24
28
Time (h)

FIG. 5.9 Changes in the concentration of calcium (Ca^{2+}) in the postbranchial haemolymph of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Values are means $\pm$ S.D.


Pre-anoxia
Anoxia
Recovery
Concentration of calcium (µmol.g-1)
35
32
29
26
23
20
0
4
8
12
16
20
24
28
Time (h)

the recovery period, there was a transient but significant increase ($P < 0.05$) in the Ca^{2+} concentration to 33.3 $\pm$ 2.3 umol.g^{-1} after 3 h, before decreasing again to a constant 25.8 $\pm$ 1.76 umol.g^{-1} for the remainder of the experiment.

5.4 DISCUSSION.

5.4.1 Oxygen regulation in *Carcinus maenas*.

5.4.1.1 Oxygen uptake.

McMahon & Wilkens (1983) described oxygen uptake as '... the most frequently used (and perhaps misused) parameter in crustacean respiratory physiology'. Although the oxygen consumption of a large number of crustacean species has been determined (Wolvekamp & Waterman, 1960; McMahon & Wilkens, 1983 for reviews), direct comparisons are difficult owing to large inter- and intra-specific variations, as well as differences in experimental procedures.

In the present study, the $\dot{M}o_2$ of *Carcinus maenas* was determined using a form of closed respirometry. This form of $\dot{M}o_2$ measurement has been criticised by many authors, including Tang (1933), Kamler (1969) and more recently by von Oertzen (1984), who summarised the 4 main groups of criticisms:

1) Disturbance of the animals whilst transferring them to the respirometer at the beginning of the experiment.

2) Fluctuation of the pH of the water within the respirometer, as a result of CO_2 evolution.

3) Variations in the accumulation of metabolic waste products in the respirometer water, owing to variations in the biomass-to-volume ratio, duration of the experiment and also the type of metabolism of the animal concerned.

4) Absence of current and turbulence and the consequent sub-optimal supply and removal of the gases associated with metabolism.

Care was taken in the present study to minimise the effects of the above criticisms.

Following the transference to the respirometer, the crabs were left undisturbed for 12 h . During this time the $\dot{M}o_2$ was measured and although it was found to be initially high, it decreased to a lower, more constant rate after 6 h . This constant rate was taken to represent the crabs quiescent rate under conditions of normoxia. The water in the respirometer was changed every 4 h, but no increase in the $\dot{M}o_2$ was observed when this was carried out. The pH of the water was measured and found to remain constant throughout the experiment, indicating no major accumulation of CO_2. Since the respirometer water was changed at regular intervals and continuously stirred, the problems associated with criticisms 3 and 4 were almost completely eliminated.

5.4.1.2 The mechanism of oxygen regulation during hypoxia, anoxia and subsequent recovery.

It is now well established that many decapod crustaceans are capable of exhibiting a high degree of respiratory independence during hypoxia, i.e. they are able to maintain their oxygen consumption independent of the ambient oxygen tension over a wide range of Po_2 (McMahon & Wilkens, 1975; Taylor, 1976; Bradford & Taylor, 1982). This is in contrast to early investigations, in which it was reported that the rate of oxygen consumption in some species was dependent on the ambient Po_2. It appears that the animals used in these early studies were either active or disturbed.

Under conditions of hypoxia, increasing ventilatory volume helps to maintain the supply of oxygen to the respiratory surfaces despite the reduced oxygen content of the medium. However, an increase in ventilatory activity will itself increase the oxygen demand of the animal until eventually the oxygen supplied is sufficient only to meet the energy requirements of the ventilatory pump. It is at this point, which corresponds to the critical oxygen tension (Pc), that respiratory independence can no longer be maintained. Taylor (1976) reported that the Pc in *Carcinus maenas* was

about 60 - 80 Torr and this was confirmed by the present study, in which it was observed that the heart rate of *C. maenas* started to decrease after the Po_2 of the surrounding water had decreased to about 60 Torr. This reduction of the heart rate, (bradycardia) associated with the Po_2 of the surrounding water decreasing to below the Pc point, has been observed in a variety of decapod crustacean species (Taylor *et al.*, 1973; McMahon & Wilkens, 1975; Taylor, 1976; Wheatly & Taylor, 1981; Bradford & Taylor, 1982). A reduction in the heart rate does not necessarily indicate reduced haemolymph flow, however, since, in some fish, gill perfusion is maintained, despite the bradycardia, by an increase in stroke volume. Little information on changes in stroke volume, however, is available for decapod crustaceans.

An increase in the frequency of beating of the scaphognathites (hyperventilation) is a very common response to environmental hypoxia amongst decapod crustaceans, since it is one of the mechanisms which help to maintain the rate of oxygen consumption independent of the ambient oxygen tension over a wide range of Po_2. An increase in ventilatory rate has been reported by many authors (Arudpragasam & Naylor, 1964; Taylor, 1976; McMahon & Wilkens, 1977; Butler *et al.*, 1978; Burnett, 1979; Bradford & Taylor, 1982; Morris & Taylor, 1985; Johnson & Uglow, 1987). Indirect evidence from postbranchial haemolymph pH and a decrease in Pco_2 values, suggested that hyperventilation occurred during the pre-anoxic period, implying an increase in the frequency of scaphognathite pumping. The role of hyperventilation in haemolymph acid-base balance will be discussed in greater detail in relation to the acid-base balance of *Carcinus maenas* in section 5.4.2.

In order to determine the combined effects of these hypoxia-induced regulatory responses, the Po_2 of the postbranchial haemolymph (P_aO_2) was measured throughout this period. The P_aO_2 of *Carcinus maenas*, maintained under normoxic conditions was 83 $\pm$ 6.4 Torr, which is similar to the values of 97 and 74.9 $\pm$ 7.3

Torr obtained by Taylor (1976) and by Taylor & Butler (1978) respectively. It was found that when the Po_2 of the sea water was reduced below the Pc of 60 Torr (for heart rate), a lag of about 15 minutes was observed before the P_aO_2 started to decrease. After 4 h, however the P_aO_2 had declined to < 2 Torr.

The vast majority of studies reported in the literature, have concentrated on measuring the respiratory responses, associated with exposure to hypoxia, so there are very few comparative studies for animals under anoxic conditions. In the present investigation, there was no discernible measurement of $\dot{M}O_2$ during anoxia, since both the Po_2 and the P_aO_2 were < 2 Torr. The heart, however, continued to beat at a very low rate throughout the anoxic period, despite the fact that the postbranchial haemolymph was shown to be almost devoid of oxygen (section 5.3.1). Anderson (1989) also observed a continued heart beat during prolonged anoxia, in a study on the thalassinid *Calocaris macandreae* and suggested 2 possible explanations for this phenomenon. Firstly, it was argued that, in the absence of haemolymph circulation, localised L-lactate accumulation in the tissues and haemolymph might limit the anaerobic capability of the animal. Therefore, a circulating, haemolymph might represent a larger 'sink' for anaerobic end-products, which would minimise localised acid-base disturbances. Secondly, since the neural Po_2 detector in decapods appears to be located in the vascular system, a maintained circulation might allow a rapid response to improved environmental conditions during recovery from hypoxia (A.C. Taylor, pers. comm.).

During the early stages of recovery, both heart rate and the $\dot{M}O_2$ were extremely high, presumably representing the 'repayment' of an oxygen debt. This 'overshoot' in oxygen consumption has also been demonstrated by Bridges & Brand in both *Corystes cassivelaunus* and *Galathea strigosa* (1980). During the first 15 minutes of recovery, the $\dot{M}O_2$ was 9.2 umol $O_2.h^{-1}.g^{-1}$ fresh wt., which represents an increase by a factor of 7 over the $\dot{M}O_2$ of quiescent crabs under normoxic conditions.

Similar calculations, where the maximal $\dot{M}o_2$ has been divided by the quiescent $\dot{M}o_2$, have been performed by a variety of authors and termed the 'aerobic metabolic scope' by Bennet (1978). McMahon & Wilkens (1983) demonstrated that the aerobic scope is typically 4 - 5 in decapod crustaceans, although these calculations were based on only a few studies. The 'aerobic metabolic scope' measured in *Carcinus maenas* in the present study is therefore very high, but this probably reflects the extreme conditions to which the crabs had been exposed.

The increase in the Po_2 of the haemolymph observed during the first 30 minutes of recovery of this study has been also reported by Butler *et al.* (1978) in the lobster *Homarus vulgaris*, which had been exposed to a period of hypoxia. The 5 minute lag between the reoxygenation of the sea water and the increase in the Po_2 of the postbranchial haemolymph has not been reported before and is likely to be a response to the extreme conditions present during anoxia. This does mean, however, that during the first few minutes of recovery, the crabs were forced to respire almost totally anaerobically. This observation is somewhat confusing since Figure 5.4 shows that the frequency of heart beat was very high even after only 10 minutes, but presumably the initial demand for oxygen was far in excess of the amount that could delivered. Since the crabs were observed to be extremely active during this time, the energy demand was high, resulting in a rapid accumulation of L-lactate. This might provide an explanation for the observation described in Chapter 3, section 3.3.3.1, in which it was reported that the concentration of L-lactate doubled during the first hour of recovery.

5.4.2 Acid-base balance disturbance during anoxia and during recovery.

Studies of the effects of anoxia on the acid-base balance of invertebrates have tended to concentrate on bivalve and gastropod molluscs (see reviews by Campbell and Boyan, 1974; Zandee *et al.*, 1980; Grieshaber, 1982). In decapod crustaceans,

acid-base studies have concentrated on the effects of environmental hypoxia (Dejours & Armand, 1980; Wheatly & Taylor, 1981; Truchot, 1986; Johnson & Uglow, 1987), aerial respiration (Truchot, 1975); Innes *et al.*, 1986) and strenuous exercise (McDonald *et al.*, 1979; Smatresk *et al.*, 1979; Wood & Randall, 1981; Booth *et al.*, 1984).

As mentioned in section 5.2.4.3, Pco_2 and $[HCO_3^-]$ were calculated using data measured directly and also taken from *in vitro* work by Truchot (1976). Direct measurements of *in vivo* Pco_2 of decapods are rare because of low Pco_2 values which are difficult to measure directly and also the difficulties of obtaining sufficient haemolymph samples. The use of the Henderson-Hasselbalch equation to calculate Pco_2 and HCO_3^-, assumes that the bicarbonate system in the haemolymph is in equilibrium. Carbonic anhydrase (EC 4.2.1.1), however, is absent from the haemolymph of decapods (Randall and Wood, 1981; Henry & Cameron, 1982; McMahon *et al.*, 1984) and the non-catalysed hydration/dehydration rate constants for $CO_2/HCO_3^-/CO_3^{2-}$ are low (Edsall, 1969). Together with the work of deFur *et al.* (1980), this indicates that the bicarbonate system may not be in equilibrium for much of the time. It is therefore important to view the calculated values of Pco_2 and HCO_3^-, with a certain amount of caution.

As mentioned in section 5.4.1.2., there was evidence to suggest that the crabs were hyperventilating during the initial stages of the 'pre-anoxic' period. In the context of the haemolymph acid-base balance, hyperventilation, causes carbon dioxide to be excreted across the gills, resulting in a respiratory alkalosis. This alkalosis can be clearly seen in the present study, with the pH of postbranchial haemolymph increasing during the 'pre-anoxic' period and a concurrent decrease in Pco_2 (Figs. 5.5 & 5.7). Burnett & Johansen (1981) stated that, in *Carcinus maenas*, hyperventilation could not totally explain the observed alkalosis and that a small metabolic component was present, in the form of bicarbonate and/or carbonate.

There was some evidence to support this in the present study, since an increase in the concentration of haemolymph bicarbonate was observed during the 'pre-anoxic' period. The functional explanation of this increase, however, is unclear.

In the present study, a pronounced acidosis developed during the anoxic period, that had both respiratory and metabolic components; the respiratory component arising from the depletion of bicarbonate, and the metabolic component from the accumulation of L-lactate. It has been previously reported that the respiratory acidosis associated with anoxia, was normally the result of the accumulation of carbon dioxide in the absence of gas exchange (Cameron, 1986). In the present study, however, the Pco_2 remained relatively constant throughout the anoxic period, which probably reflects the lack of carbon dioxide production. It is well established that HCO_3^- accounts for about 95 % of the total carbon dioxide pool (Cco_2) in the haemolymph of decapods. The marked depletion of Cco_2 can therefore be largely attributed to a decline in the concentration of HCO_3^-. The reason for this depletion is unclear, but it would certainly account for a large proportion of the observed acidosis. It is likely that the H^+ ions produced in association with L-lactate result in the breakdown of the HCO_3^- ions (R. Burton pers. comm.)

During the early stages of recovery, the Pco_2 rapidly increased to a value in excess of the 'pre-anoxic' values, reflecting the increased rate of energy metabolism associated with repaying the 'oxygen debt' or a change in the excretion rate. The alkalosis observed during recovery, resulted from both the replenishment of the haemolymph bicarbonate and also the elimination of L-lactate.

Stewart (1978) stated that the regulation of haemolymph pH is largely brought about by the total weak acid activity, Pco_2 and the strong ion difference (SID). In decapod crustaceans, the weak acid buffering is mainly due to haemolymph proteins (Truchot, 1983) and usually remains fairly constant over short term fluctuations in the acid-base balance. Bicarbonate buffering (respiratory compensation) depends on

the haemolymph Pco_2 and pK^1 values, since it involves the relative concentrations of CO_2, HCO_3^- and CO_3^{2-}. The buffering ability of the SID (metabolic compensation) relies on the difference in activity between anions and cations, which do not change their dissociation in the physiological pH range. Factors which contribute to changes in SID include the major haemolymph ions (Na^+, K^+, Ca^{2+}, Mg^+, Cl^- and SO_4^{2-}) and metabolic acids such as L-lactic acid.

Currently, it is generally accepted that, in water breathing animals, ion exchange between haemolymph and water (Cl^- for HCO_3^- and/or Na^+ for H^+), usually across the gills, are responsible for the maintenance of pH in aquatic animals (Truchot, 1975, 1979; Cameron, 1978; Henry *et al.*, 1981). In terrestrial decapods, pH compensation is achieved partly by the mobilisation of a source of $CaCO_3$, possibly from the exoskeleton (Henry *et al.*, 1981) or from $CaCO_3$ granules from the digestive gland (Becker *et al.*, 1974) The concentration of calcium was measured in this present study, but no evidence of mobilisation was observed. This is consistent with the findings of Cameron (1985) who states that in the aquatic crab *Callinectes sapidus* the contribution of the carapace carbonates was calculated to be only 7.5 % of the total compensatory H^+ associated with a hypercapnic acidosis.

CHAPTER 6 - A CALORIMETRIC INVESTIGATION OF ANAEROBIC METABOLISM.

6.1 INTRODUCTION.

When exposed to anoxic conditions *Carcinus maenas* exhibited a large reduction in the metabolic rate (Chapter 3, section 3.4.4.3.). This helped to reduce the accumulation of L-lactate and also to conserve the carbohydrate pool. During anoxia *C. maenas* was also observed to cease all locomotor activity, which was at least partially responsible for the metabolic depression. It was felt that, to demonstrate this energy reduction more clearly, calorimetry should be used, since this technique measures the enthalpy changes of all the metabolic reactions within an organism, even if the nature of these reactions is not fully understood. Interpretation of heat dissipation results (direct calorimetry), is best carried out when considered together with basic biochemical data or with oxygen consumption rates (indirect calorimetry). Under certain circumstances the use of direct calorimetry, in conjunction with indirect calorimetry, enables the relative contribution of aerobic and anaerobic metabolism to be determined.

The work was carried out at the Plymouth Marine Laboratory, with the assistance of Dr. J. Widdows. The study was limited to measurements of only 5 individual crabs, since direct calorimetry is very time consuming and only one week was available for these experiments. This study was not intended to be comprehensive, but was carried out to provide an indication of the extent of the observed reduction in energy demand during anoxia and also to determine potential areas of future investigation.

The use of calorimetry to explore heat changes was integral to the origins of bioenergetics, 200 years ago: ' Respiration is therefore a combustion, a very slow one to be precise' (Lavoisier and LaPlace, 1783 - cited in Gnaiger, 1983). Direct calorimetry and gas exchange respirometry have been compared in man (Atwater &

Benedict, 1905), geese (Benedict & Lee, 1937), ruminants and other domestic animals (Kleiber, 1962; Blaxter, 1969) and *Tenebrio molitor* L. (Peakin, 1973). These studies involved the measurement of heat dissipation in animals only under normoxic conditions and it was not until the mid 1970's that the potential contribution of calorimetry, as a means of direct comparison of anaerobic and aerobic metabolism, was first fully appreciated (Hammen, 1976; Gnaiger, 1977). The majority of work on the comparison of anaerobic and aerobic metabolism in invertebrates, has been carried out on molluscs (Hammen, 1979, 1980; Pamatmat, 1979, Famme *et al.*, 1981; Widdows & Shick, 1985; Shick *et al.*, 1986, 1988). In crustaceans, such studies have been confined to those of Gnaiger (1981) and Hammen (1983).

The experiment described in this chapter, involved the use of microcalorimetry in conjunction with respirometry, to investigate the energy metabolism of *Carcinus maenas* under conditions of anoxia and subsequent recovery. The concentrations of L-lactate, as determined in Chapter 3, were used to help interpret heat dissipation during anoxia. A heat-flow calorimeter with perfusion (open-flow) animal chambers was used, since it enabled the environmental conditions in the chamber to be manipulated.

6.2 MATERIALS AND METHODS.

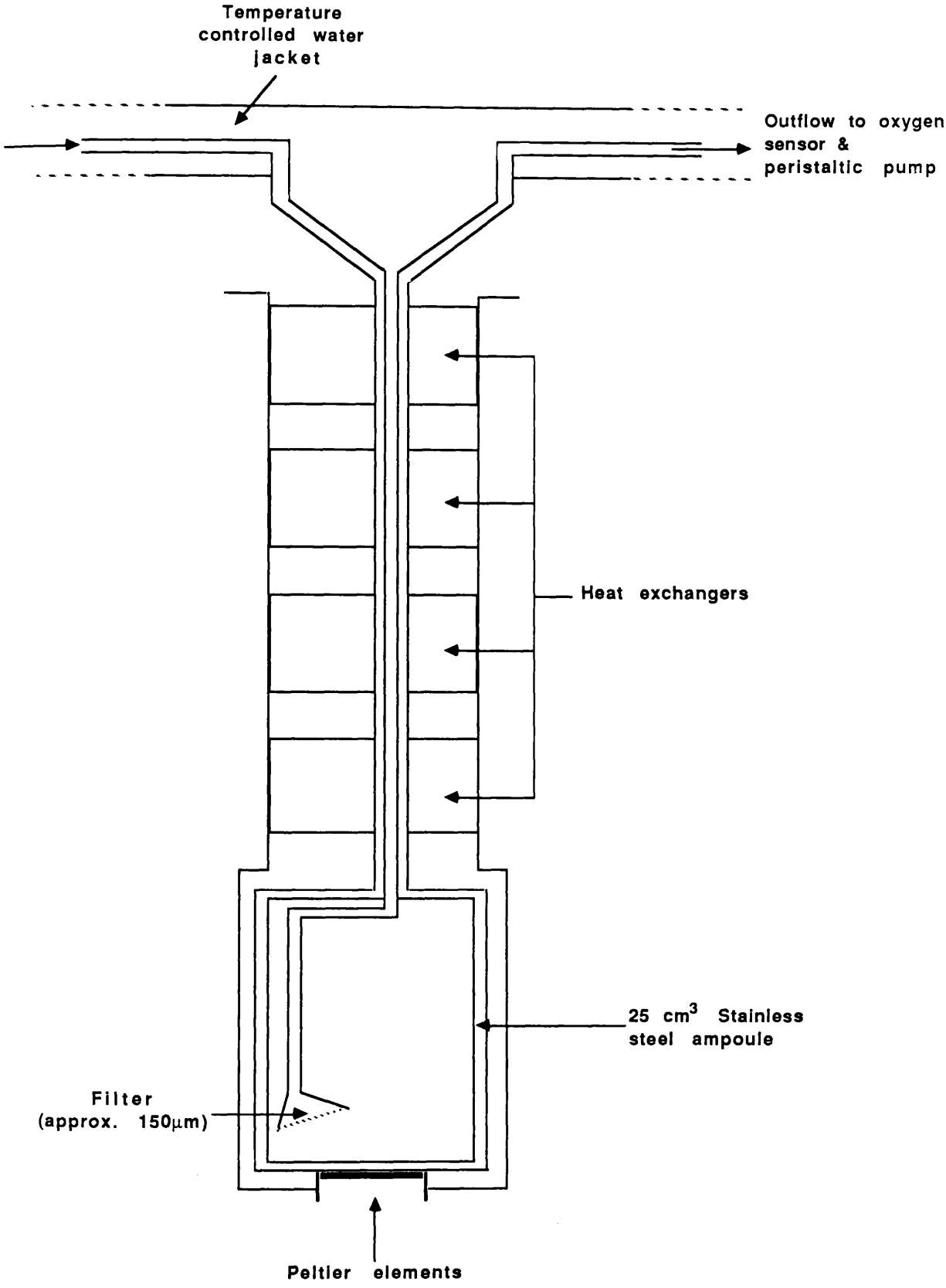
6.2.1 Calorimeter Design and Maintenance.

The microcalorimeter used was a LKB 2277 BioActivity Monitor. The animal was placed in a sample perfusion chamber, which was enclosed in a measuring vessel. This was in turn surrounded by metal heat sinks and immersed in a large temperature-controlled water bath (25 l). The large capacity heat sink arrangement allows heat to be exchanged with the measuring vessel. In between the measuring vessel and the heat sink is a pair of Peltier elements which respond to a temperature gradient between the measuring vessel and the heat sink of less than 1×10^{-6} of 1 degree Celsius. The voltage produced by the Peltier elements is proportional to the temperature difference and can be recorded on a chart recorder.

The perfusion chamber used was developed within the Plymouth Marine Laboratory by J. Widdows and P. Salkeld. Previously, microcalorimetry had been limited to using animals of biomass less than about 20 mg dry mass (Widdows, 1987). The new system consisted of a perfusion chamber (volume = 25 ml), which could accommodate animals of up to about 800 mg dry mass (Fig. 6.1). This also had the advantage that much higher rates of water flow could be used, without incurring unacceptable baseline noise.

Since a control was required, a blank was included which consisted of a chamber without an animal, under identical conditions to those of the experimental chamber. The sea water used had been obtained offshore, from around the Eddystone Lighthouse and transported to the Plymouth Marine Laboratory, where it was filtered, continuously circulated and aerated at a temperature of $10 \pm 1^{o}C$. After flowing through the perfusion chambers, the Po_2 of this sea water was measured with 2 thermostatted water-jacketed oxygen electrodes (E5046, Radiometer,

FIG. 6.1 Diagram of the stainless steel ampoule used in the calorimetric measurements (from Widdows, 1987). (Diameter of the ampoule is approximately 25 mm). See text for explanation.


Temperature controlled water jacket
Outflow to oxygen sensor & peristaltic pump
Heat exchangers
25 cm3 Stainless steel ampoule
Filter (approx. 150μm)
Peltier elements

Denmark) coupled to an oxygen meter (Strathkelvin Instruments, Glasgow).

The system was calibrated by means of an internal calibration capsule, which switched on and off a known power output at selected time intervals. The recorder deflection due to this thermal power gives a calibration level which may then be used to determine quantitative experimental results.

After each experimental run, the system was cleaned by flushing it through with a 10 % sodium hypochlorite solution, to remove organic matter and microbial growth from the walls. This procedure was essential to avoid discrepancies between heat dissipation and oxygen uptake.

6.2.2 Maintenance of Animals.

Carcinus maenas (L.) (fresh wt. range = 0.5 - 1.5 g), was collected by hand from intertidal rock pools on a rocky promontory, to the West of Kames Bay, on the Isle of Cumbrae, Firth of Clyde, Scotland. The crabs were then transported to the Plymouth Marine Laboratory in moist sea weed, contained within a plastic tank. On arrival, the animals were transferred to large tanks, in a recirculating sea water aquarium, maintained at 15 $\pm$ 1^{o}C. They were fed on *Mytilus edulis* until 72 h prior to the start of the experiments.

6.2.3 Experimental Procedure.

After cleaning, the perfusion chamber was filled with sea water and the system left for 2 h , during which time a steady baseline of the heat dissipation was obtained. A crab was then transferred from the aquarium to the perfusion chamber. It was essential to ensure that the lid of the perfusion chamber was screwed on tightly and that the outside of the chamber was both clean and dry, before lowering it into the

calorimeter.

Fully aerated sea water was then pumped through the chamber at a rate of 1 ml. min^{-1} for about 4 h, during which time a steady baseline of the heat dissipation was obtained. The oxygen consumption rate of the crab during the last hour of this period was taken to be the quiescent rate. Traces were obtained on the chart recorder, for the heat dissipation and the Po_2 of both the experimental and blank perfusion chambers.

Once a reasonably constant value of heat dissipation had been obtained (there was a some variation in the heat dissipation of the experimental chamber which reflected the varying activity patterns of the crab) the aerated sea water was completely replaced with sea water, made anoxic by bubbling nitrogen gas through a 25 l reservoir for about 3 hours. Anoxic conditions were maintained for 9 h , during which time the heat dissipation and the Po_2 of both chambers were continually monitored. At the end of this period, fully aerated sea water was pumped through the system and the crab allowed to recover. Since both the heat dissipation and oxygen consumption rates changed extremely rapidly during recovery, it was necessary to increase the speed of the chart recorder, to obtain a more detailed record of these changes.

6.2.4 Interpretation of Results.

A computer programme (J. Widdows) was used to calculate the heat dissipation rate ($_tQ$) ($J.h^{-1}$) and the oxygen consumption rate ($umol.h^{-1}$) from the chart recorder traces. The weight specific oxygen consumption rates ($\dot{M}o_2$) (umol O_2. $h^{-1}.g^{-1}$ fresh wt.) were calculated, so that they could be compared directly with $\dot{M}o_2$ values obtained from closed respirometry in Chapter 5, section 5.3.2). The values for $_tQ$ and $\dot{M}o_2$ were then used to calculate oxycaloric equivalents ($_kHO_2$), which are defined as the energy dissipated per mole of oxygen consumed. In aquatic

animals, this ranges from -440 to -480 kJ.mol$^{-1}O_2$ (Gnaiger 1983). If oxycaloric equivalents exceed this range, then partial anaerobiosis is indicated. Conversely, should the total heat equivalents fall below this empirical range, then endothermic anabolic processes are suggested (Widdows, 1987). This has been reported as being the case during the early stages of recovery from anoxia when there is an elevated rate of oxygen uptake, termed the 'oxygen debt payment' (Shick & Widdows, 1981).

6.3 RESULTS.

Owing to the limited time available, recordings from only 5 crabs could be measured in the calorimeter and, of these, 2 died during the anoxic period. The results obtained for the 3 remaining crabs showed similar trends throughout the experiment, although the absolute values varied between the individuals. This was to be expected (J. Widdows pers. comm.), however, and for this reason the results presented below are those obtained for only one of the three surviving individuals. The standard deviations are derived from the mean of several measurements, taken over a given time period, but for the same crab. The mean $\dot{M}o_2$ of the crab during normoxia was 4.07 $\pm$ 0.39 umol $O_2.h^{-1}.g^{-1}$ fresh wt. (Fig. 6.2). During the recovery period the $\dot{M}o_2$ was 16.6 umol $O_2.h^{-1}.g^{-1}$ fresh wt. after 20 minutes, decreasing to 4.84 umol $O_2.h^{-1}.g^{-1}$ fresh wt. after 4 h .

The values for $\dot{M}o_2$ obtained in these experiments are compared with those measured using closed respirometry (Chapter 5) (Fig. 6.2). It is widely accepted that smaller animals have a higher rate of weight specific oxygen uptake than do animals with a greater mass. Therefore, the slightly higher $\dot{M}o_2$'s obtained using open respirometry were not unexpected, since the animals used in the open respirometry experiments, had a fresh wt. of less than 1 gram, whilst the mean fresh wt. of the crabs in Chapter 5 was 4.4 g. The differences, however, between the rates determined using the two forms of respirometry were not significant ($P > 0.05$).

The mean weight specific rate of heat dissipation during normoxia was -1.89 $\pm$ 0.20 $J.h^{-1}.g^{-1}$ fresh wt., but this decreased significantly ($P < 0.05$) to a mean of -0.295 $\pm$ 0.035 $J.h^{-1}.g^{-1}$ fresh wt. during anoxia (Fig. 6.3). This represents a decrease of 84 % . During recovery an 'overshoot' was observed, with the rate of heat dissipation increasing significantly ($P < 0.05$) to -2.94 $J.h^{-1}.g^{-1}$ fresh wt. after 10 minutes and then decreasing again to become relatively constant at -2.15 $\pm$

FIG. 6.2 The rate of weight specific oxygen consumption ($\dot{M}o_2$) of *Carcinus maenas* during 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions. Results from open respirometry (■) are compared with those measured using closed respirometry (+).

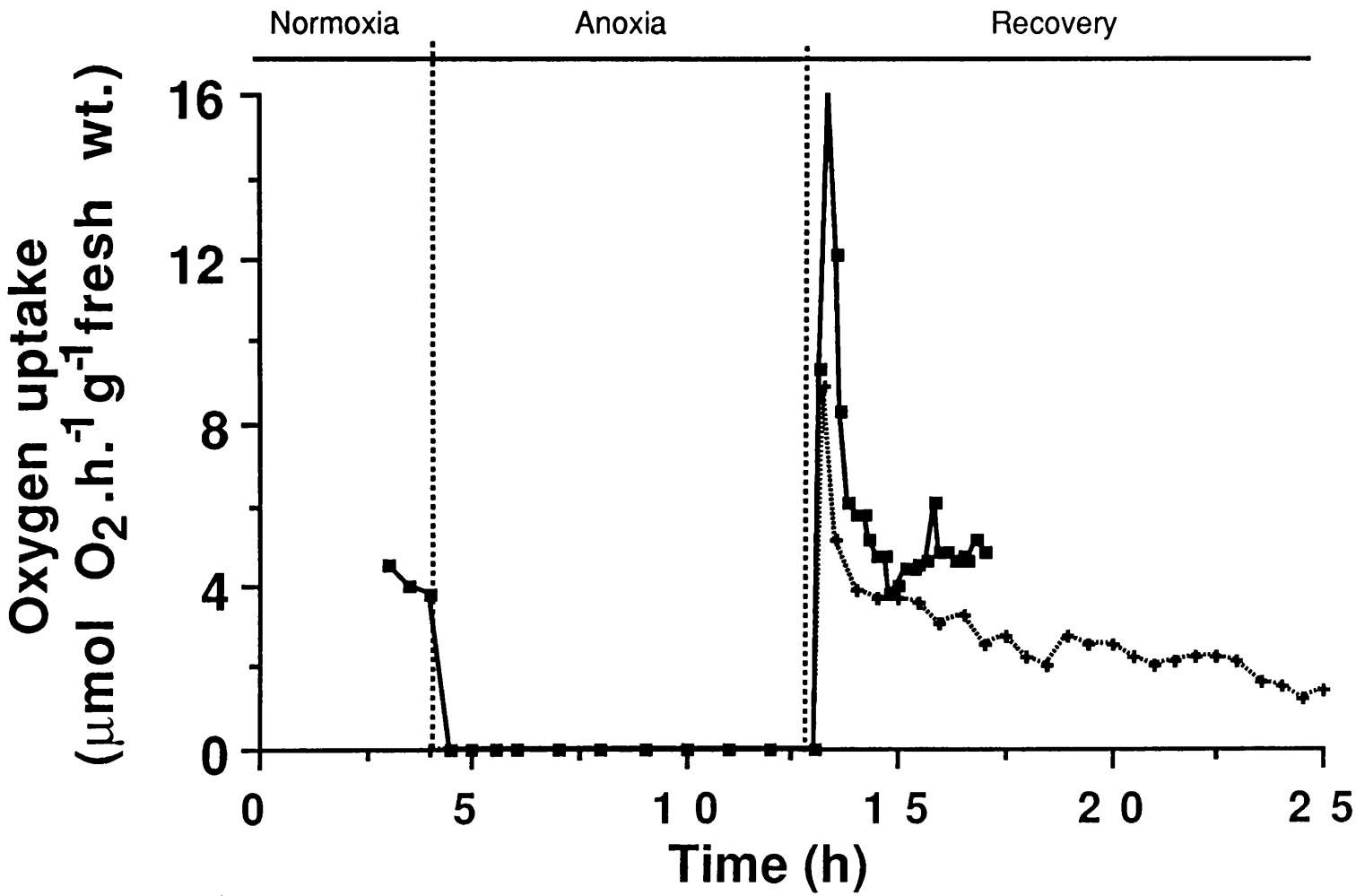
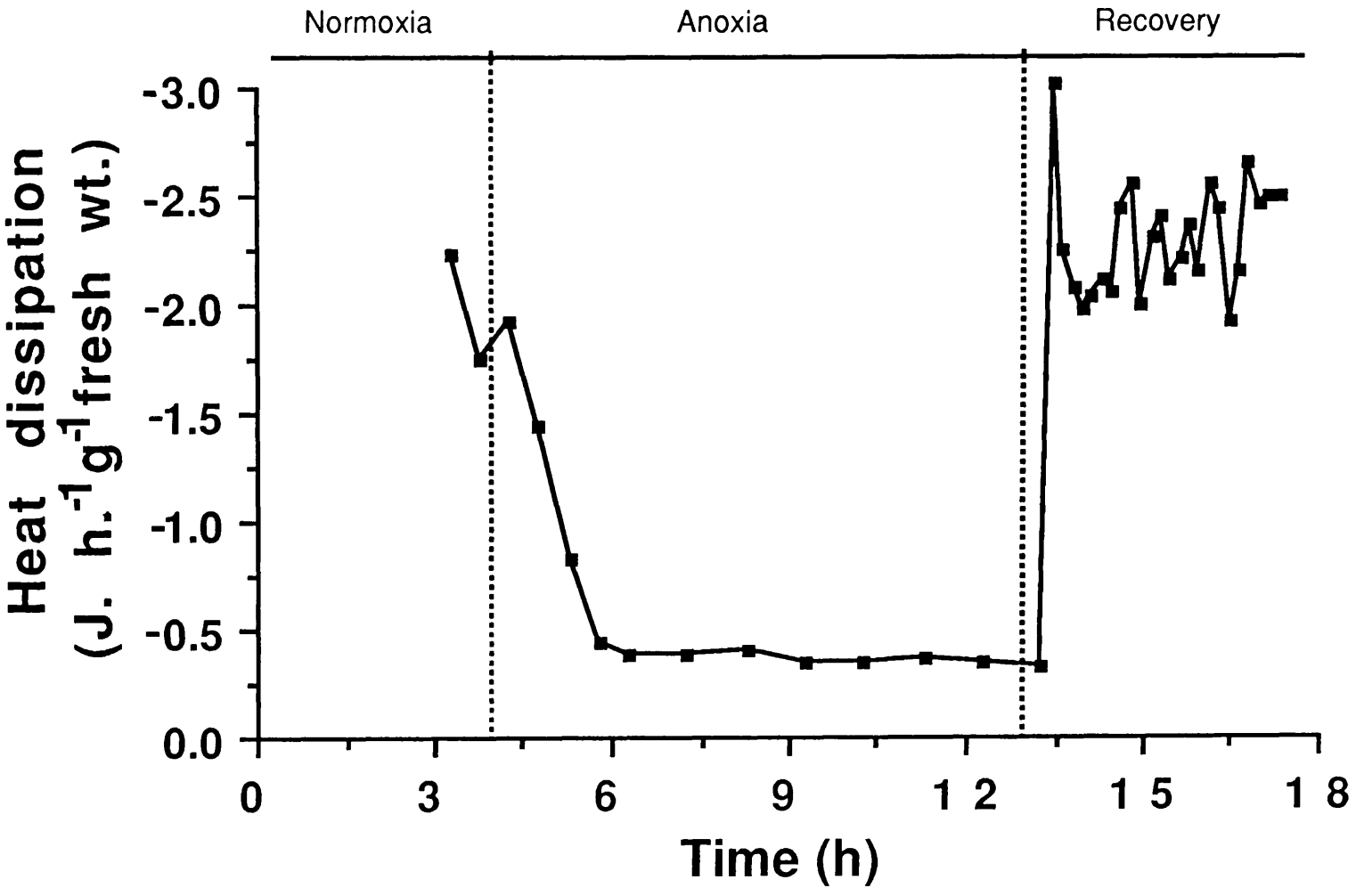

Normoxia
Anoxia
Recovery
Oxygen uptake
(µmol O2.h.-1 g-1 fresh wt.)
16
12
8
4
0
0
5
10
15
20
25
Time (h)

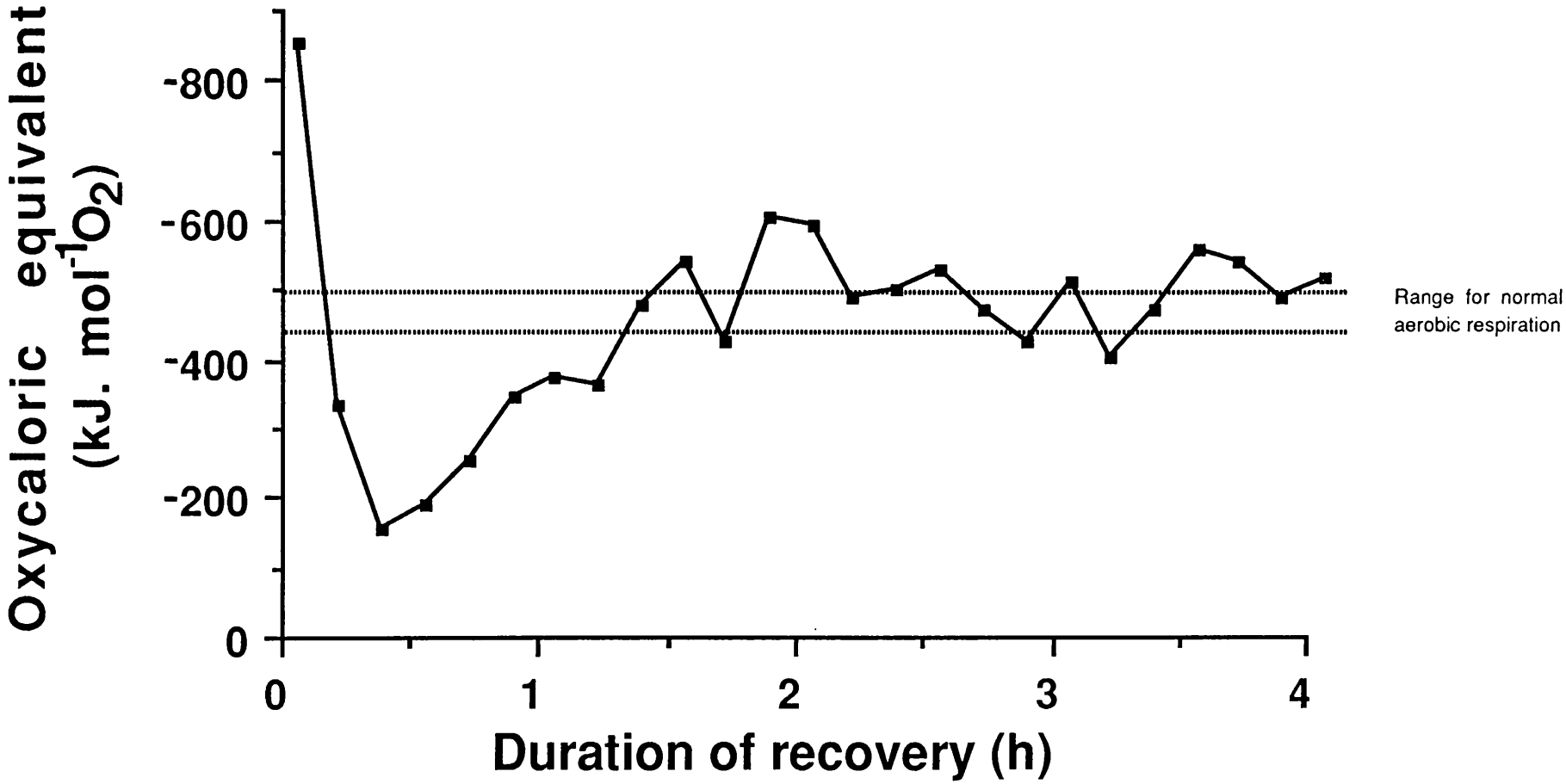
FIG. 6.3 Changes in the rate of heat dissipation ($J.h.^{-1}g^{-1}$ fresh wt.) in *Carcinus maenas* exposed to 12 hours of anoxia and during a 12 hour period of recovery under normoxic conditions.


Normoxia
Anoxia
Recovery
Heat dissipation
(J. h.-1g-1fresh wt.)
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0
3
6
9
12
15
18
Time (h)

0.184 $J.h^{-1}.g^{-1}$ fresh wt. for the remainder of the experiment.

The mean oxycaloric equivalent ($_kHO_2$) during the normoxic period was -459.7 ± 15.2 $kJ.mol^{-1}O_2$. Theoretically, it should not be possible to calculate an oxycaloric equivalent when animals are kept under anoxic conditions, since there should be no oxygen present in the water. In this study, therefore, the observed oxycaloric equivalent of -987.9 ± 123.8 $kJ.mol^{-1}O_2$ during anoxia, was possibly an experimental artefact, reflecting the fact that the oxygen electrodes were unable to accurately measure the Po_2 of the water below approximately 2 Torr. During the first hour of recovery, the $_kHO_2$ decreased significantly ($P < 0.05$) to a mean of -255.8 ± 82 $kJ.mol^{-1}O_2$, thereafter it increased again to a mean of -481 ± 54.4 $kJ.mol^{-1}O_2$ (Fig. 6.4). During the later stages of recovery the calculated values of $_kHO_2$ occasionally increased to over -500 $kJ.mol^{-1}O_2$, indicating the possibility of an anaerobic component to respiratory metabolism.

FIG. 6.4 Changes in the oxycaloric equivalent (kJ. mol^{-1} O_2), recorded during a 4 hour recovery period, following exposure of *Carcinus maenas* to anoxia for 9 hours.


Oxycaloric equivalent
(kJ. mol-1O2)
-800
-600
-400
-200
0
0
1
2
3
4
Duration of recovery (h)
Range for normal
aerobic respiration

6.4 DISCUSSION.

6.4.1 Metabolic depression during anoxia

ATP production by anaerobic metabolism is known to be inefficient, due to the incomplete oxidation of the glycolytic substrate. Therefore, if an animal is to maintain its normoxic energy demand during anoxia, the rate of glycolysis must be enhanced (i.e. 'Pasteur effect'). This is potentially maladaptive for animals that are exposed to prolonged anoxic periods, since it results in a very rapid depletion of the endogenous carbohydrate pool. Animals adapted to surviving long-term anaerobiosis, exhibit responses that conserve energy expenditure, rather than resorting to the Pasteur effect. This conservation of energy expenditure is brought about by a depression of the metabolic rate, as well as a selective inactivation of non-essential physiological and biochemical processes. This reduces the animal's energy demands and means that an increase in the glycolytic flux, on becoming exposed to anoxic conditions, may not be necessary. In addition to metabolic depression, bivalve molluscs also utilise alternative anaerobic pathways, which are more efficient in terms of ATP production than the pathway culminating L-lactate (de Zwaan, 1977).

In the present study, the magnitude of the metabolic depression has been calculated using the rate of ATP production (Chapter 3, section 3.4.4.3.) and also by direct calorimetry. The calculated depression of metabolic rate amounted to 74 and 84 % during anoxia (expressed as a percentage of the normoxic metabolic rate), for the biochemical and calorimetric methods respectively. The following table summarises the current literature concerning metabolic depression in adult invertebrates during anoxia (from Widdows, 1987).

Table 6.1 The extent of the metabolic depression in invertebrates during anoxia (from Widdows, 1987).

Species	Metabolic depression* (%)	Temp. (°C)	Reference
Coelenterates:			
Actinia equina	7	15	Shick, 1981
Bivalves:			
Modiolus demissus	7	20	Pamatmat, 1980
Cardium edule	41	20	Pamatmat, 1980.
Arctica islandica	23	15	Pamatmat, 1980.
Mytilus edulis	11	20	Famme *et al.*, 1981.
Mulinia lateralis	97	15	Shumway *et al.*, 1983.
Crustaceans:			
Cyclops abyssorum	17	6	Gnaiger, 1981
Carcinus maenas	16	15	Present study.

* Anoxic metabolic rate as a percentage of normoxic rate.

There is an indication that the more anoxia-tolerant species exhibit the greatest metabolic depression. For example, *Mytilus edulis* often experiences anaerobic stress, since during aerial exposure at low tide, it closes the shell valves and creates its own hypoxic/anoxic microenvironment (Widdows, 1987). In the study of Famme *et al.* (1981) the anoxic heat dissipation of *M. edulis* was shown to be only 11 % of the normoxic rate. In contrast, the coot clam *Mulinia lateralis*, which is less anoxia-tolerant and a much more active bivalve than *M. edulis*, exhibits no appreciable metabolic depression (Shumway *et al.*, 1983). Presumably the high anoxic energy demand of this species was met by the Pasteur effect, which is consistent with the fact that *M. lateralis* is only exposed to anoxic conditions for short periods of time. A similar comparison in Crustacea is difficult, owing to the lack of relevant studies. Comparisons between widely differing groups is always difficult, but one might tentatively say that *Carcinus maenas* appears to be well adapted to survive anoxic exposure, since it exhibits a large metabolic depression, which conserves the carbohydrate pool and reduces the rate of L-lactate

accumulation.

6.4.2 Thermodynamic and biochemical interpretation of anaerobiosis and recovery.

Gnaiger (1983) reported that, in experiments employing heat-flow calorimetry and simultaneous respirometry, the experimental (total) heat equivalents of oxygen consumption approximated to -450 $kJ.mol^{-1}O_2$ for freshwater and marine animals including gastropods, bivalves, crustaceans and fish (Famme *et al.*, 1981; Gnaiger, 1983; Pamatmat, 1978, 1983). The mean oxycaloric equivalent for the normoxic period in the present study was -459.7 $\pm$ 15.2 kJ. $mol^{-1}O_2$ and therefore agrees very closely with the above figure. The fact that the heat output values, derived from oxygen consumption measurements agree closely with those from direct calorimetry, explains why oxygen uptake has regularly been used as a measurement of an animal's metabolic rate.

There are, however, conditions in which the use of the generalised oxycaloric equivalent is totally inappropriate (Widdows, 1987). During exposure to severe hypoxia, the oxycaloric equivalents increase to above the value of -450 $kJ.mol^{-1}O_2$, owing to the inevitable reduction in oxygen consumption. In the present study, the oxycaloric equivalent was calculated to have increased to a mean of -988 $\pm$ 123 $kJ.mol^{-1}O_2$ during anoxia, indicating the occurrence of anaerobic metabolism. For reasons already described in section 6.3, this figure is probably an experimental artefact. It is preferable, therefore, to compare heat dissipation during anoxia, by direct calorimetry and also by indirect biochemical estimation of heat output based on the stoichiometric analysis of accumulated anaerobic end products and utilisation of the transphosphorylation of ADP by phospho-l-arginine. This was carried out using the caloric equivalents described by Shick *et al.* (1983) and Gnaiger (1983) (caloric equivalents for L-lactate and phospho-l-arginine are -60 and -55.2 $mJ.umol^{-1}$ respectively) and the changes in the concentrations of L-lactate (L-lac)

and phospho-l-arginine (PA) measured in Chapter 3, (sections 3.3.3.1. and 3.3.3.2. respectively).

Indirect calorimetry:

L-lac: 1.025 umol.h^{-1}.g^{-1} fresh wt. x -60 mJ = -61.5 mJ.h^{-1}.g^{-1} fresh wt.

PA : 0.431 umol.h^{-1}.g^{-1} fresh wt. x -55.2 mJ = -23.8 mJ.h^{-1}.g^{-1} fresh wt.

Total anoxic heat dissipation = -85.3 mJ.h^{-1}.g^{-1} fresh wt.

The total anoxic heat dissipation for direct and indirect calorimetry was therefore -295 and -85.3 mJ.h^{-1}.g^{-1} respectively. It can be seen that only 29 % of the total anoxic heat dissipation can be explained by end product accumulation and transphosphorylation of ADP by phospho-l-arginine. This large discrepancy between direct and indirect calorimetric methods has been reported by other authors and has become known as the 'exothermic gap', the size of which is partly governed by the duration of anoxia (Shick *et al.*, 1983). The size of the exothermic gap in this study is larger than reported for other investigations, but it should be remembered that these results are from only one individual.

Widdows (1987) divided the recovery period, following exposure to anoxia, into two distinct phases. Early recovery is characterised by a low oxycaloric equivalent (i.e. below -450 kJ.$mol^{-1}O_2$), because of the high rate of oxygen consumption associated with the 'repayment' of the oxygen debt. Figure 6.4 clearly shows this phenomenon, with low oxycaloric equivalents being observed for the first 1.5 h of recovery. It can be calculated that, based on the generalised oxycaloric equivalent (i.e. -450 kJ.$mol^{-1}O_2$) and a mean $\dot{M}o_2$ of 9.69 umol O_2.h^{-1}.g^{-1} fresh wt., the total expected heat dissipation over the first hour of recovery should be -4.36 J.h^{-1}.g^{-1} fresh wt. The observed rate of total heat dissipation measured only -2.90 J.h^{-1}.g^{-1} fresh wt., indicating a predominance of endothermic (anabolic) processes. This has been previously reported by de Zwaan (1977) and Widdows *et al.* (1979). The present study is further complicated by the fact that results from Chapters 3 and 4

demonstrate a pronounced anaerobic component of metabolism, during the first hour of recovery. This would call into question the justification of using the generalised oxycaloric equivalent, since it was stated by Widdows (1987) that it should only be used when environmental conditions were in a steady state. The calculations made previously in this section, should therefore be viewed with caution, but the discrepancy ('endothermic gap') was so large, that the conclusions concerning the predominance of endothermic processes must surely still apply.

The second phase of recovery (Widdows, 1987) is typified by an oxycaloric equivalent close to -450 $kJ.mol^{-1}O_2$. Similar values were found in the present study, with a mean oxycaloric equivalent of -473 $\pm$ 61.6 $kJ.mol^{-1}O_2$ for the later stages of recovery (1 to 4 h of recovery) (Fig. 6.4). Periodically during the recovery phase, the oxycaloric equivalent increased to above -480 $kJ.mol^{-1}O_2$ indicating anaerobic metabolism. This has been described previously and attributed to transient increases in locomotor activity (de Zwaan *et al.*, 1983).

Even though the scale of the present study was very limited, it has illustrated the importance and the potential of combining thermodynamic, biochemical and physiological (respirometry) approaches in the interpretation of aspects of anaerobic metabolism in *Carcinus maenas*. In particular, further research is required to investigate the regulation of the metabolic depression observed during anoxia and to obtain a more comprehensive explanation of the exothermic and endothermic gaps.

CHAPTER 7 - SUMMARY DISCUSSION AND CONCLUSIONS.

The aims of this study were to investigate the importance of anaerobic metabolism in the decapod crustacean, *Carcinus maenas*. These aims can be divided into 3 sections:

i) To determine the extent to which *Carcinus maenas* is exposed to severe hypoxia or anoxia in the field and to assess the importance of anaerobic metabolism under these conditions.

ii) To investigate the possible biochemical pathways involved in anaerobic metabolism.

iii) To establish the fate of anaerobic end products, once the animal has been returned to normoxic conditions.

In addition, two further areas of study, arising from the above, were investigated:

iv) To determine the role of anaerobic metabolism, during the early stages of recovery from exposure to anoxia.

v) To assess the metabolic adaptations that *Carcinus maenas* possesses to survive prolonged exposure to anoxic conditions.

The study was initiated by an investigation into the diel and seasonal environmental extremes that occur within intertidal rock pools. It was found that the diel ranges shown by the various physico-chemical factors become larger during the summer months. The fluctuations in the partial pressure of dissolved gases within the rock pools were the result of algal photosynthesis elevating the oxygen levels during the day and respiration of the pools' fauna and flora, in the absence of photosynthesis, causing a depletion at night. During the summer, the observed range of Po_2 was larger, owing to the greater pool biomass and also the higher metabolic rates associated with the increased summer temperatures (Chapter 2). Consequently, it was observed that in August the rock pool used for the field experiments became

hyperoxic (Po_2 = 280 Torr) during the day but severely hypoxic (Po_2 = 5 Torr) and hypercapnic during the night.

Studies investigating the distribution of *Carcinus maenas* throughout the year, revealed that far greater numbers of crabs (mainly male), were present in exposed intertidal rock pools during the summer than during the winter. This peak in the number of crabs present on the littoral zone coincided with the time of year that the pools became most hypoxic. Severe hypoxia (Po_2 < 10 Torr) lasted up to 4 h and required the crabs to either employ a behavioural response to avoid these extreme conditions or to resort to anaerobic metabolism.

Field observations showed that, in common with several other intertidal species of decapod crustaceans, *Carcinus maenas* exhibited partial and full emersion responses, when exposed to hypoxic conditions in rock pools (Fig. 2.1). As the hypoxic conditions became more severe, larger numbers of crabs became fully emersed and emerged onto the surrounding rocks. These crabs avoided the rock pool hypoxia and were able to take advantage of the air's rich oxygen supply. In addition, laboratory experiments demonstrated that under conditions of severe hypoxia, the concentration of L-lactate was shown to increase in fully immersed crabs, but no accumulation was observed in those crabs that had become partially or fully emersed. In terms of respiratory metabolism therefore, it appears that it would be advantageous for the hypoxic crabs to become at least partially emersed. Previous studies, however, have shown that aquatic animals have problems with excreting carbon dioxide. The CO_2 can accumulate in the haemolymph, leading to a respiratory acidosis (Taylor & Butler, 1978). The effects of this accumulation on the acid-base balance of the crab, however, are smaller than those effects resulting from the accumulation of L-lactate in the fully immersed crabs. It has been reported that in partially emersed animals, much of their carbon dioxide is excreted into the hypoxic water, that is maintained in their branchial chambers (Wheatly &

Taylor, 1979; Burnett & McMahon, 1987). This reduces the Pco_2 of the haemolymph and helps minimise the respiratory acidosis.

There are situations, however, in which either the crab is not able to become emersed (e.g. deep rock pool with steep walls) or in which it would be dangerous to do so (e.g. high predation risks). Fully immersed crabs exhibit certain physiological responses which enable them to survive exposure to hypoxia. Initially, the crab maintains its respiratory independence by hyperventilating, which involves an increase in the frequency of beating of the scaphognathites, resulting in an increase of the ventilatory flow. Since hyperventilation causes CO_2 to be excreted from the gills at a greater rate than under normoxic conditions, a respiratory alkalosis is often observed. The alkalosis, causes a Bohr shift resulting in an increase in the oxygen affinity of the haemolymph. Obviously, an increase in the frequency of the scaphognathites or heart beat, requires a greater expenditure of energy. Once the environmental Po_2 decreases to below the critical oxygen tension (Pc point), it becomes energetically impractical to maintain this high ventilatory rate. Below the Pc, the scaphognathite rate declines and the crabs are no longer able to maintain a rate of oxygen uptake that is sufficient to sustain the normal rate of oxygen consumption.

As the degree of hypoxia becomes more severe, the biochemical responses associated with hypoxia and anoxia assume a greater importance. The concentration of L-lactate starts to increase, indicating the utilisation of anaerobic metabolism. During the early stages of severe hypoxia the transphosphorylation of ADP by phospho-l-arginine helps to supplement the glycolytically derived energy, but this is rapidly depleted since initial concentrations of this phosphagen are fairly small. When conditions became anoxic ($<$ 2 Torr), the heart rate was observed to continue to beat at a very low rate.

Anaerobic metabolism is energetically very wasteful, owing to the incomplete

oxidation of the carbohydrate precursor, resulting in a very much lower efficiency of ATP production than under aerobic conditions. If an animal's anoxic energy demand remains at the pre-anoxic rate, then the rate of glycolysis must be enhanced (i.e. 'Pasteur effect'), which would rapidly deplete the carbohydrate pool. The need for this Pasteur effect could be overcome by reducing the energy demand of the animal. In the present study, a metabolic depression was shown to occur during exposure to anoxia (Chapter 6, section 6.3.). Although this has been previously reported, studies have tended to concentrate on molluscs (Pamatmat, 1980; Gnaiger, 1981; Famme *et al.*, 1981). In the present study, after 2 hours of anoxia, the metabolic rate in *Carcinus maenas* had decreased to 16 % of the rate under aerobic conditions. This low metabolic rate is consistent with the low rates of L-lactate accumulation observed in *C. maenas* (Table 3.4) and helps to explain the high LT_{50} of 15.8 h .

It appears that the metabolic depression during anoxia is probably the most important response of *Carcinus maenas* to explain the crab's high anoxia-tolerance. The mechanism by which this depression occurs has only really been investigated over the last decade, with attention focusing mainly on the role of certain key glycolytic enzymes under anaerobic conditions. The following enzymes have been identified as being rate limiting in the glycolytic pathway: hexokinase (HK) (EC 2.7.1.1.), glycogen phosphorylase (GP) (EC 2.4.1.1.), phosphofructokinase (PFK) (EC 2.7.1.11.) and (possibly) pyruvate kinase (PK) (EC 2.7.1.40.).

Initially, studies concentrated on the allosteric effect of phosphagens and adenylate nucleotides on the activity of these rate-limiting enzymes (Atkinson, 1969; Jaffe *et al.*, 1971; Grieshaber & Gäde, 1976; Storey, 1976; de Zwaan & Ebberink, 1978; Storey & Storey, 1978, 1979; Lesicki, 1980). For example, HK and PFK are inhibited by high concentrations of phospho-l-arginine and ATP, while being activated by high concentrations of ADP, AMP, Pi. Some enzymes have also been

shown to respond most strongly to the relative proportions to each other of these metabolites (e.g. [ATP]/[AMP] and [ATP]/[ADP]) (Atkinson, 1969).

In the present study, the concentrations of phospho-l-arginine and ATP in the tissue of *Carcinus maenas* decreased during anoxia, whilst the concentration of AMP increased (Chapter 3, section 3.3.3.2.). This would be expected to cause an activation of HK, PFK and PK and thereby result in a Pasteur effect. As described earlier, however, results from calorimetric techniques have shown a pronounced depression of the metabolic rate of *Carcinus maenas* during anoxia. Although this reduction in the metabolic rate has also been observed by other authors, it has only really been investigated in the molluscs. Ebberink & de Zwaan (1980) observed that in the adductor muscles of *Mytilus edulis*, the concentration of fructose-6-phosphate increased whilst the concentration of fructose-1,6-bisphosphate decreased during initial exposure to anoxia. This is indicative of PFK inhibition, and occurs despite a reduction in the levels of the allosteric inhibitors, together with an increase in the activators.

Hue (1982) stated that fructose-2,6-bisphosphate regulates PFK by controlling the use of carbohydrate for biosynthetic purposes under aerobic conditions and that during exposure to anoxia these effects are withdrawn. This means that, during anoxia, anabolic reactions are largely stopped, leaving only a minimal level of metabolism required to maintain basic energy requirements. This would have the effect of greatly reducing the glycolytic rate. A decrease in the concentration of fructose-2,6-bisphosphate, in soft tissues, during anoxia has been observed in mammalian hepatocytes (Hue, 1982) and in the muscle tissue of *Ostrea edule* and *Mytilus edulis* (Storey, 1985b). Storey (1984) demonstrated that in *Busycotypus canaliculatum*, fructose-2,6-bisphosphate is a less effective activator of PFK in anoxic muscle as compared with aerobic muscle.

The action of fructose-2,6-bisphosphate does not explain the absence of the Pasteur

effect in the presence of allosteric activation. Storey (1985a) postulated two mechanisms of higher level control of PFK and other glycolytic enzymes, which overrides allosteric control. Firstly, covalent modification of enzymes by protein phosphorylation and secondly, enzyme association/dissociation with subcellular particles.

i) Covalent modification of enzymes.

Cohen (1980) described covalent incorporation of phosphate as being widespread and extremely important in metabolic control. It provides a means of bringing about transient changes to enzyme activities. Currently, most of the work on covalent modification has been carried out on mammalian tissues (Engstrom, 1978; Cohen, 1980; Sakakibara & Uyeda, 1983).

Work concerning covalent modification in invertebrates has again concentrated on molluscs. Storey (1984) found that there were two forms of PFK: (i) dephosphorylated (aerobic) and (ii) phosphorylated (anaerobic) forms. The phosphorylated enzyme was more sensitive to inhibition by ATP and citrate and less sensitive to the effects of the activators. This was directly comparable with the mammalian system (Foe & Kemp, 1982). During anoxia, therefore, the effects of PFK phosphorylation overrides the action of the allosteric activators.

Evidence for the existence of two forms of PK in anoxia-tolerant marine molluscs has been increasing for several years (Siebenaller, 1979; Holwerda *et al.*, 1981, 1983). Plaxton & Storey (1986) isolated both anoxic (phosphorylated) and aerobic (dephosphorylated) forms of PK from *Busycotypus canaliculatum*. They found that the anoxic variant exhibited a lower maximal activity, a reduced affinity for phosphoenolpyruvate (PEP), a reduced activation by fructose-1,6-bisphosphate and, finally, an extremely potent inhibition by alanine. All these kinetic differences are comparable to the mammalian L-type PK (Engstrom, 1978).

ii) Enzyme association/dissociation with subcellular particles.

There is increasing evidence to suggest that there are associations between many of the enzymes of glycolysis and membrane fractions, glycogen particles and structural proteins (Knull, 1978; Wilson *et al.*, 1982). These associations appear to be important in regulating rates of aerobic and anaerobic glycolysis (Walsh *et al.*, 1980; Clarke *et al.*, 1984; Storey, 1985a). Plaxton & Storey (1986) separated soluble and particulate fractions of the muscle of the gastropod *Busycotypus canaliculatum*. They found that exposure to anoxia led to a decrease in the association of glycolytic enzymes with subcellular particles, but that this decrease was reversible on the return of *B. canaliculatum* to normoxic conditions. During anoxia, the reduction in particle associations, led to a decrease in enzyme and pathway organisation, resulting in a reduction in the rate of anaerobic glycolysis.

A great deal of work needs to be carried out before the mechanism of metabolic depression in anoxia-tolerant crustaceans can even begin to be fully understood. In molluscs, metabolic depression appears to be brought about by (i) reduction of physical activity, (ii) cessation of anabolic reactions through a reduction in the concentration of fructose-2,6-bisphosphate, (iii) phosphorylation of rate-limiting glycolytic enzymes and, (iv) a transient decrease in the association of enzymes with subcellular particles. It remains to be seen if these are the regulatory mechanisms controlling metabolic depression in crustaceans.

It has been known for many years that decapod crustaceans accumulate L-lactate as an end product of anaerobic metabolism. Prior to this investigation, however, there had been no comprehensive study to investigate the possible existence in decapods of any of the other anaerobic pathways that have been found to be present in other invertebrate groups. A stoichiometric comparison revealed that some 92 % of the

catabolism of the carbohydrate pool (mainly glycogen and oligosaccharides), associated with exposure to anoxia, could be explained by the accumulation of L-lactate. It is possible that some of the remaining catabolised carbohydrate (8 %) was incorporated into the amino acid alanine, whose concentration was observed to increase in the tissues of *Carcinus maenas* during anoxic conditions. The use of High Performance Liquid Chromatography indicated that, despite a small increase in the concentration of fumarate in response to exposure to anoxia, organic acids were unimportant end products in the anaerobic metabolism of decapod crustaceans (Chapter 3, section 3.3.3.3.). The role of amino acids in the anaerobic metabolism of *C. maenas* was found to be of minor importance. There was some evidence to indicate the existence of the anaerobic pathway involving the transamination of aspartate to oxaloacetate, which might explain the observed increase in the concentration of fumarate and alanine (Fig. 3.14). The magnitude of these changes, however, were very small in comparison to the increase in the concentration of L-lactate.

The results from the present study show that the only anaerobic pathway of any importance in decapod crustaceans is that culminating in the production of L-lactate. This is in contrast to other invertebrate groups, in which anaerobic metabolism can be composed of several different pathways within the same species (Livingstone, 1983). Further evidence confirming the dominance of L-lactate as the anaerobic end product in decapod crustaceans, was obtained from radiolabelling experiments (Chapter 4). In these experiments, it was found that during exposure to anoxia, the incorporation of radioactivity from D-[U-^{14}C]-glucose into the weak acid fraction (mainly L-lactate), followed exactly the same time course as the accumulation of L-lactate (Fig. 4.8). In addition, there was little or no incorporation of radioactivity into the TCA fraction (containing organic acids).

One of the initial aims of this project was to determine the fate of L-lactate

following a period of exposure to anoxia. A stoichiometric comparison revealed that about 89 % of the carbohydrate (mainly glycogen and oligosaccharides) accumulating during recovery could be explained by the depletion of L-lactate. This indicated the existence of some form of gluconeogenic pathway (Chapter 3, section 3.4.5.2.). This precursor/product relationship was later confirmed using radiolabelled L-[U-^{14}C]-lactate, for it was demonstrated that radioactivity from the L-lactate was incorporated into glycogen and later into amino acids (Chapter 4, Table 4.2). It was also demonstrated that radioactivity from L-[U-^{14}C]-lactate, became incorporated into carbon dioxide and bicarbonate, indicating the complete oxidation of L-lactate as a possible means of the elimination of this anaerobic end product (Chapter 4, Fig. 4.7). It is conceivable, however, that this labelled carbon dioxide could have originated from glucosyl units, formed via a gluconeogenic pathway from radiolabelled L-lactate. No excretion of labelled L-lactate by *Carcinus maenas* during recovery was detected (Chapter 4, Table 4.3). It appears, therefore, that end product elimination in decapod crustaceans relies mainly on the formation of the initial precursors, and to a limited extent, the complete oxidation of L-lactate to carbon dioxide. These findings are consistent with those of Gäde *et al.* (1986), who investigated the fate of L-lactate during recovery in the stone crab, *Menippe mercenaria*.

During recovery from exposure to anoxia, 'overshoots' were measured in the heart rate, oxygen consumption and in heat dissipation rate, probably associated with the repayment of the 'oxygen debt' (Chapter 5, Fig. 5.4 and Chapter 6, Figs. 6.2 and 6.3). This is consistent with the observation, based on calorimetric studies, that most of the energy being produced from respiratory metabolism during this period was being utilised in anabolic, endothermic reactions (Chapter 6, section 6.4.2.). During anoxia, individual *Carcinus maenas* were observed to be very inactive, but when the water was re-aerated, locomotor activity increased rapidly. After the first hour of recovery, oxygen consumption and heat dissipation rates decreased

significantly, but required a further 4 h before rates approached pre-anoxic values.

All these physiological and behavioural responses are typical of an aquatic animal recovering from exposure to hypoxia and have been previously reported by many authors (Thompson & Pritchard, 1969; Taylor *et al.*, 1977; Bridges & Brand, 1980; Shick *et al.*, 1986, 1988). Of particular interest in the present study, however, was that, in addition to the physiological and behavioural responses, the concentration of L-lactate in *Carcinus maenas* was observed to double during the first hour of recovery (Chapter 3, section 3.3.3.1.). This observation was corroborated by the experiment using D-[U-^{14}C]-glucose, in which the incorporation of radioactivity into the weak acid (mainly L-lactate) fraction was shown to double during the first hour of recovery (Chapter 4, Fig. 4.6). Head & Baldwin (1986) reported a similar phenomenon during recovery in *Cherax destructor*, although the magnitude of the L-lactate accumulation was smaller. The accumulation was thought to be the result of functional anaerobiosis in muscle tissue. It is possible, therefore, that the observed increase in the concentration of L-lactate could be the result of functional anaerobiosis in muscular tissue, associated with the increased locomotor activity that was observed during the first hour of recovery. Since L-lactate was determined in pooled tissue samples only, it is not possible to confirm this speculation from the present data. Indirect calorimetric measurements indicated that, during the first hour of recovery, anaerobic metabolism accounted for approximately 22 % of the total energy produced. These measurements were based on oxygen consumption for the aerobic component and the rate of L-lactate accumulation and depletion of phospho-l-arginine for the anaerobic component. The caloric equivalents used were -450, -60 and -55.2 mJ.umol^{-1} of oxygen, L-lactate and phospho-l-arginine (Shick *et al.*, 1983 and Gnaiger (1983).

In comparison to other invertebrate groups, the anaerobic metabolism of decapod crustaceans is comparatively simple, being based on the pathway culminating in the

production of L-lactate. It is clear, however, that such a pathway is sufficient to provide the majority of the energy required by *Carcinus maenas* to survive exposure to environmental anoxia and has in part contributed to the success of this species in being able to withstand the extreme conditions of the intertidal zone.

Although this study has answered its initial aims, it has also raised many questions, which could usefully form the basis of future work. The carbohydrate and L-lactate metabolism of specific tissues are areas of research that currently require attention. Similarly, further work is needed on the glycolytic regulation of metabolic depression in crustaceans, to increase our understanding of the metabolism of decapods under anoxic conditions.

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APPENDIX 1 - PREPARATION OF HAEMOLYMPH/TISSUE SAMPLES USING PERCHLORIC ACID EXTRACTION.

Reagents.

Liquid nitrogen.

Potassium bicarbonate: (2 M).

Perchloric acid (PCA): 0.6 M and 0.3 M (for haemolymph and tissue samples respectively).

Procedure.

Haemolymph Samples:

i) Haemolymph samples were taken and immediately mixed with an equal volume of chilled PCA (0.6 M), to precipitate the protein and inactivate enzymes.

ii) After mixing thoroughly, the mixture was centrifuged for 20 minutes at 10,000 g and the resultant supernatant removed.

iii) Dropwise addition of 1/20 vol. potassium bicarbonate (2 M) was used to neutralise the supernatant. The pH was checked using full range indicator paper.

iv) The precipitated potassium perchlorate was removed by centrifuging again at 10,000 g after cooling on ice for 10 minutes.

v) The clear supernatant was separated and frozen at (-20 ^{o}C) until used.

Tissue samples:

i) Frozen tissue was placed in a mortar of liquid nitrogen and ground into a powder using a pestle.

ii) Once the liquid nitrogen had evaporated away, 50 mg of the powder was transferred to an Eppendorf (1.5 ml plastic centrifuge tube) and 500 ul

of chilled PCA (0.3 M) was added.

iii) After mixing thoroughly on a vortex mixer, the mixture was centrifuged for 10 minutes at 10,000 g and the resultant supernatant (1) separated and stored on ice.

iv) A further 500 ul of chilled PCA (0.3 M) was added to the pellet, mixed again and centrifuged for a further 20 minutes at 10,000 g.

v) The resultant supernatant (2) was then added to the supernatant (1) and after mixing, neutralised using potassium bicarbonate (2 M).

vi) Having centrifuged the mixture for a final time to remove the precipitate of potassium perchlorate, the resulting supernatant was separated and stored at -20 ^{o}C until needed.

Application.

This haemolymph /tissue preparation method was used to estimate the following metabolites: D-glucose, glucose-6-phosphate, total hexose/pentose sugars, L-lactate, succinate, phosphoenolpyruvate (PEP), phospho-l-arginine, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), various organic acids and amino acids.

Storage and stability of samples.

Since nucleotides and phospho-l-arginine are so prone to degradation, samples were stored at -70 ^{o}C and the number of times that a sample was defrosted and refrozen was kept to minimum.

Comments.

a) Care was taken to ensure that all liquid nitrogen had evaporated from the homogenate before the 50 mg sample was removed, to ensure accurate weighing.

b) The total volume of potassium bicarbonate required to neutralise each sample was recorded.

c) When potassium bicarbonate is added to the PCA sample mixture carbon dioxide is generated. The lid of the Eppendorf tube was therefore opened occasionally, to release any build up of pressure which might have led to a loss of material.

Reference.

Gäde G., E. Weeda & P.A. Gabbott (1978). Changes in the level of octopine during the escape responses of the scallop, *Pecten maximus* (L.). J. comp. Physiol. 124B, 121-127.

APPENDIX 2 - METHOD FOR TOTAL HEXOSE/PENTOSE SUGAR DETERMINATION.

This method is based on that of Carroll *et al.* (1956), and involves the acid hydrolysis of glycosidic bonds to give monosaccharide units, by the concentrated acid in the anthrone reagent. These in turn are dehydrated to furfural and its derivatives. The furfural reacts with anthrone (10-keto-9,10-dihydro-anthracene) to give a blue-green complex, which shows an absorbance maximum at 620 nm.

Reagents.

Anthrone reagent: 72 ml of concentrated sulphuric acid was added to 28 ml distilled water (Care was taken to slowly add the acid to the water and to prevent the solution from becoming too hot). 50 mg of anthrone was then dissolved into the 72 % sulphuric acid, allowed to cool and stored at 0-5 ^{o}C. Any reagent not used within 7 days was discarded.

D-glucose/glycogen: The following standards were run: 5 mM, 2.5 mM, 1 mM and 0.5 mM (90 mg.100 ml^{-1} = 5 mM and 81 mg.100 ml^{-1} = 5 mM, for D-glucose and glycogen respectively).

Haemolymph/Tissue Preparation.

See Appendix 1 for details.

Assay.

1 ml of Anthrone reagent and 50 ul of sample/standard, were added to a 5 ml test tube and mixed well. A reagent blank was produced by substituting 50 ul of distilled water, for the 50 ul of sample/standard.

Procedure.

i) The assay mixtures were placed in a boiling water bath for 10 minutes, and then cooled on ice for a further 10 minutes.

ii) After this time the contents of the test-tubes were transferred to 1.5 ml spectrophotometric semi-micro cuvettes.

iii) The absorbance of each sample was then measured at a wavelength of 620 nm.

Interpretation.

A calibration line was constructed and a linear relationship was observed between the concentration of standards and their absorbances. Therefore if a blank was used to zero the spectrophotometer, any change in absorbance was regarded as being proportional to the concentration of total hexose/pentose sugars present in either the sample or the standard.

Comment.

i) The efficiency of the hydrolysis reaction was checked by comparing a 1 mM standard of D-glucose with a similar glycogen standard. It was found that there were no significant ($P < 0.05$) differences between the results obtained for similar concentrations of the 2 sugars.

ii) Care was taken to ensure that the assay mixtures were well mixed before placing them in the water bath.

Reference.

Carroll N.V., R.W. Longley & J.D. Roe (1956). The determination of glycogen in liver and muscle by use of anthrone reagent. J. biol. Chem. 220, 583-593.

APPENDIX 3 - METHOD FOR GLYCOGEN AND FREE OLIGOSACCHARIDE /MONOSACCHARIDE DETERMINATION IN TISSUE SAMPLES.

This method is based on that of Keppler and Decker (1974) and involves the hydrolysis of the 1-4 & 1-6-glycosidic bonds of glycogen, by the enzyme 1-4, 1-6-amyloglucosidase (EC 3.2.1.33). The resultant glucosyl units are then assayed, using Hexokinase (EC 2.7.1.1) and Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), as described in Appendix 4.

Assay.

1) Glycogen or $(\alpha\text{-glucosyl})_n + H_2O \longrightarrow (\alpha\text{-glucosyl})_{n-1}$ + D-glucose

2) D-glucose + ATP $\longrightarrow$ Glucose-6-phosphate + ADP

3) Glucose-6-phosphate + $NADP^+ \longrightarrow$ 6-phosphogluconate + NADPH + H^+

Reagents

Liquid Nitrogen

Potassium hydroxide: 30 g.100 ml^{-1} (30 %).

Ethanol: Absolute.

Acetate buffer: 2.4 ml. of 96 % acetic acid and 4.87 g of sodium acetate (35 mM) were made up to 1000 ml. using distilled water. The pH was adjusted to 4.8 with NaOH (5 M).

Tris buffer: 2.42 g of Tris and 0.240 g of magnesium sulphate (100 mM and 10 mM respectively) were dissolved in 200 ml of distilled water. The pH was adjusted to 7.4, using HCl (5 M).

D-glucose and glycogen: The following standards were run: 1 mM, 0.5 mM, 0.25 mM and 0.1 mM (18 mg.100 ml^{-1} = 1 mM and 16.2 mg.100 ml^{-1} = 1 mM for D-glucose and

glycogen respectively).

ATP: 121 mg.10 mg^{-1} (22 mM)

$NADP^+$: 168 mg.10 ml^{-1} (22 mM)

Hexokinase: Dilution of 1 in 25 (i.e. 180 $U.mg^{-1}$ protein).

G6P-DH: Dilution of 1 in 25 (i.e. 300 $U.mg^{-1}$ protein).

1-4,1-6-Amyloglucosidase: Used without dilution (i.e. 77 $U.mg^{-1}$ protein).

Procedure (including preparation of tissue)

i) Frozen tissue was placed in a mortar of liquid nitrogen and ground into a powder using a pestle.

ii) Once the liquid nitrogen had evaporated away, 50 mg of the powdered tissue was transferred to an Eppendorf tube and 400 ul potassium hydroxide (30 %) added.

iii) After mixing, the Eppendorf tube was put into a boiling water bath for 20 minutes, and then removed and allowed to cool.

iv) After adding 1 ml of absolute alcohol to give a final concentration of 70 %, the Eppendorf tubes were left on ice for 2 h, to allow the glycogen to precipitate out.

v) After centrifuging the Eppendorf tubes for 10 minutes at 10,000 g, the supernatant was decanted off.

vi) Both the supernatant and the pellet were stored separately at -20°C.

- **Free oligosaccharide/monosaccharide estimation:**

a) 100 ul of the supernatant and 10 ul of the 1-4,1-6-amyloglucosidase was added to 500 ul of the acetate buffer, mixed well and then incubated in a water bath at 34 °C for 2 h . (The alkalinity of the supernatant was found not to alter the pH of the overall mixture).

b) At the end of this time 100 ul of the solution was mixed with 700 ul of Tris buffer, 100 ul of ATP and 100 ul $NADP^+$.

c) The fluorescence of NADPH was measured using a

spectrofluorophotometer (excitation and emission wavelengths were 340 nm and 457 nm respectively). ----- E1

d) Hexokinase and glucose-6-phosphate dehydrogenase (10 ul of each) were added and left for 5 minutes. ----- E2

- Glycogen estimation

a) 1000 ul of acetate buffer and 10 ul of 1-4,1-6-amyloglucosidase were added to the pellet obtained in (vi), mixed thoroughly and incubated at 37 ^{o}C for 2 h .

b) 100 ul of this mixture was added to 700 ul of Tris buffer, 100 ul of ATP and 100 ul of $NADP^{+}$.

c) The fluorescence of NADPH was measured using a spectrofluorophotometer (excitation and emission wavelengths were 340 nm and 457 nm respectively). ----- E3

d) Hexokinase and glucose-6-phosphate were added (10 ul of each) and left for 5 minutes. ----- E4

Interpretation

E2 - E1 - Change proportional to the concentration of oligosaccharides / monosaccharides present in the sample/standard.

E4 - E3 - Change proportional to the concentration of glycogen present in the sample/standard.

Comments

- When the tissue was being boiled in potassium hydroxide (iii), the lid of the Eppendorf tube was pierced with a needle in order to prevent any increase in pressure.
- Care was taken not to disturb the pellet fraction in (vi), when the supernatant was being decanted.
- The monosaccharide fraction was estimated separately by directly assaying the

supernatant obtained in (vi), prior to the hydrolysis by 1-4,1-6-amyloglucosidase.

Reference

Keppler D. and K. Decker (1974). Glycogen determination with amyloglucosidase. *In*: Methods of enzymatic analysis, 2nd ed. pp. 1127-1131. Ed. by H.U. Bergmeyer. New York : Academic Press.

APPENDIX 4 - METHOD FOR GLUCOSE-6-PHOSPHATE AND D-GLUCOSE DETERMINATION.

This method, based on that of Kunst *et al.* (1981), involves a 2 step reaction using the enzymes hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The hexokinase catalyses the phosphorylation of glucose, whilst glucose-6-phosphate dehydrogenase catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate.

Assay

1) Glucose-6-phosphate + $NADP^+$ $\longrightarrow$ 6-Phosphogluconate + NADPH + H^+

2) D-glucose + ATP $\longrightarrow$ Glucose-6-phosphate + ADP.

Reagents

Tris Buffer: 2.42 g of Tris and 0.240 g of magnesium sulphate (100 mM and 10 mM respectively) were added to 200 ml of distilled water. The pH was adjusted to 7.4, using HCl (5 M).

D-glucose: The following standards were run: 1 mM, 0.5 mM, 0.25 mM and 0.1 mM (18 mg.100 ml^{-1} = 1 mM).

Hexokinase: A dilution of 1 in 25 (i.e. 180 $U.mg^{-1}$ protein).

G6P-DH.: A dilution of 1 in 25 (i.e. 300 $U.mg^{-1}$ protein).

ATP: 121 mg.10 ml^{-1} (22 mM).

$NADP^+$ 168 mg.10 ml^{-1} (22 mM).

Haemolymph/Tissue Preparation

See Appendix 1 for details.

Procedure

The following reagents were added into a 1.5 ml semi-micro cuvette:

Tris Buffer	700 ul
ATP	100 ul
$NADP^+$	100 ul
Sample/standard	100 ul
G6P-DH	10 ul
Hexokinase	10ul
	1020 ul

The analysis could be carried out using either a Spectrophotometer (Philips PU 8700) (absorbance wavelength = 340 nm) or a Spectrofluorophotometer (Shimadzu RF 5000) (at a wavelength of 340 nm and 457 nm for excitation and emission respectively). The latter was more sensitive and was used when the concentration of either of the metabolites was very low. Absorbance/fluorescence values are represented by E1 to E3.

i) Tris buffer, ATP, $NADP^+$ and standard/sample were mixed ---- E1

ii) Glucose-6-phosphate dehydrogenase was added ---- E2

iii) Hexokinase was added ---- E3

Readings were taken 5 minutes after the addition of each enzyme.

Interpretation

E2 - E1 = Change proportional to the concentration of glucose-6-phosphate present in the sample/standard.

E3 - E2 = Change proportional to the concentration of D-glucose in the sample/standard.

Comment

The Tris buffer and both enzymes were refrigerated when not being used (the buffer was never kept for longer than a week). The ATP and the $NADP^+$ solutions were kept frozen, and discarded after 2 weeks.

Reference

Kunst A., B. Draeger & J. Ziegenhorn (1981). UV-methods with hexokinase and glucose-6-phosphate dehydrogenase. *In*: Methods of enzymatic analysis, 3rd ed. pp. 117-123. Ed. by H.U. Bergmeyer. New York : Academic Press.

APPENDIX 5 - METHOD FOR L-LACTATE DETERMINATION.

This method is based on that of Gutmann and Wahlefeld (1974) and can be used on both tissue and haemolymph samples. The L-lactate is oxidised to pyruvate in a reaction that is catalysed by lactic dehydrogenase (EC 1.1.1.27).

Assay

$$\text{L-lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH$$

Reagents

Glycine-hydrazine buffer:	3.75 g of glycine, 0.5 g of EDTA and 2.0 ml of hydrazine-hydrate were mixed with 98 ml of distilled water. The pH was adjusted to 9.0, using NaOH (1 M).
Lactic acid:	The following standards were run: 2 mM, 1 mM, 0.5 mM and 0.25 mM (38.4 mg.200 ml^{-1} = 2 mM).
NAD^+:	26.5 $mg.ml^{-1}$ (40 mM)
LDH:	Dilution of 1 in 2 (i.e. 600 $U.mg^{-1}$ protein).

Haemolymph/tissue preparation

See Appendix 1.

Procedure

The analysis can be carried out using either a Spectrophotometer (absorbance wavelength = 340 nm) and a Spectrofluorophotometer (at wavelengths of 340 nm and 457 nm for excitation and emission respectively). The latter was more sensitive and was used when the concentration of the L-lactate was low. The following reagents were mixed in a 1.5 ml semi-micro cuvette:

Glycine-Hydrazine buffer:	1000 ul
NAD^+:	50 ul
Sample/standard:	50 ul
LDH:	5 ul

1) The reaction mixture was mixed thoroughly and then incubated in a water bath at 37 ^{o}C for 2 hours.

2) Blanks were run by substituting 50 ul of distilled water for the sample/standard ----- E1

3) Sample/standard absorbance/fluorescence ----- E2

Interpretation

E2 - E1 = Change proportional to the concentration of L-lactate present in the sample/standard.

Comment

a) The EDTA was included in the buffer to remove free copper ions, the presence of which has been shown to interfere with the end point of the reaction (Engel and Jones, 1978).

b) Since L-lactic acid is present in human skin, gloves were worn to avoid contamination.

References

Engel P. & J.B. Jones (1978). Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD^{+} in alkaline hydrazine buffers: Improved conditions for the assay of L-glutamate, L-lactate and other metabolites. Anal. biochem. 88, 475-484.

Gutmann I. & A.W. Wahlefeld (1974). L-(+)-lactate. Determination with lactate dehydrogenase and NAD^{+}. *In* Methods of enzymatic analysis, 2nd ed. pp. 1464-1468. Ed. H.U. Bergmeyer. New York: Academic Press.

APPENDIX 6 - METHOD FOR SUCCINATE AND PHOSPHOENOLPYRUVATE (PEP) DETERMINATION IN TISSUE SAMPLES.

This method, is based on those of Beutler (1974) and Lamprecht & Heinz (1981) and involves the conversion of succinyl-CoA with the coenzyme A (CoA) and guanosine 5'- triphosphate (GTP) in the presence of succinate thiokinase (EC 6.2.1.4-5). The resultant guanosine 5'-diphosphate (GTP), reacts with phosphoenolpyruvate (PEP) to form pyruvate. The pyruvate is then estimated as described in Appendix 8.

Assay

1) Succinate + GTP + CoA $\longrightarrow$ Succinyl CoA + Pi + GDP

2) GDP + PEP $\longrightarrow$ GTP + Pyruvate

3) Pyruvate + NADH + $H^+ \rightleftharpoons$ L-lactate + NAD^+

Reagents

Tris buffer: 2.42 g of Tris, 0.24 g of magnesium sulphate and 0.25 g of ethyldiaminetetracetic acid (EDTA) were added to 200 ml of distilled water (100 mM, 10 mM and 4.2 mM respectively). The pH was adjusted to 7.4, using HCl (5 M).

Acetyl CoA mixture: 1.81 mg.10 ml^{-1} of Acetyl CoA, 12.3 mg.10 ml^{-1} of GTP and 25.7 mg.10 ml^{-1} of PEP (0.11 mM, 21 mM and 1.1 mM respectively).

Succinate The following standards were run 1 mM, 0.5 mM, 0.25 mM and 0.1 mM (16.2 mg.100 ml^{-1} = 1 mM).

PEP The following standards were run: 1 mM, 0.5 mM, 0.25 mM and 0.1 mM (23.4 mg.100 ml^{-1} = 1 mM).

NADH: 28.4 mg.10 ml^{-1} (4 mM).

LDH: 2.1 $U.ml^{-1}$

Pyruvate Kinase: 630 $mU.ml^{-1}$

Succinate thiokinase: 90 $mU.ml^{-1}$

Tissue sample preparation

See Appendix 1.

Procedure

The analysis can be carried out using either a Spectrophotometer (absorbance wavelength of 340 nm) or a Spectrofluorophotometer (at wavelengths of 340 nm and 457 nm for excitation and emission respectively). The following reagents were mixed in a 1.5 ml semi-micro cuvette:

i) 850 ul Tris, 50 ul NADH, 50 ul Acetyl CoA mixture and 50 ul sample/standard ------E1

ii) 10 ul of both LDH and PK ------E2

iii) 10 ul of succinate thiokinase (left for 30 minutes) ------E3

Interpretation

E1 - Reagent blank.

E2 - E1 - Change proportional to the concentration of PEP in the sample / standard.

E2 - E3 - Change proportional to the concentration of the succinate present in the sample.

Comment

i) Owing to the high cost of the succinate thiokinase, succinate was only estimated in a few samples.

ii) It is sometimes difficult to obtain satisfactory results using the

spectrofluorophotometer in the succinate assay, owing to the interference caused by high levels of GTP.

References

Beutler H.O. (1974). Succinate. *In:* Methods of enzymatic analysis, 2nd ed. pp. 25-33. Ed. by H.U. Bergmeyer. New York: Academic Press.

Lamprecht W. & F. Heinz (1981). D-glycerate 2-phosphate and phosphoenolpyruvate. *In:* Methods of enzymatic analysis, 3rd ed. pp. 555-561. Ed. by H.U. Bergmeyer. New York: Academic Press.

APPENDIX 7 - METHOD FOR ADENOSINE TRIPHOSPHATE AND PHOSPHO-L-ARGININE DETERMINATION.

These methods are based on those of Trautschold *et al.* (1981) and Heinz & Weiber (1981) with a number of modifications. Arginine kinase (EC 2.7.3.3) catalyses the de-phosphorylation of phospho-l-arginine. The phosphate group then produces ATP from ADP. The ATP is estimated according to the method described in Appendix 4, with the exception that ATP, rather than D-glucose, becomes the limiting factor.

Assay

i) Phospho-l-arginine + ADP⟶ Arginine + ATP

ii) ATP + D-glucose ⟶ Glucose-6-phosphate + ADP

iii) Glucose-6-phosphate + $NADP^+$ ⟶ 6-Phosphogluconate + NADPH + H^+

Reagents

Tris Buffer:	2.42 g of Tris and 0.24 g of magnesium sulphate (100 mM and 10 mM respectively) were added to 200 ml of distilled water. The pH was adjusted to 7.8, using HCl (5 M)
ATP/Phospho-l-arginine:	The following standards were run: 1 mM, 0.5 mM, 0.25 mM, and 0.1 mM (65 mg.100 ml^{-1} = 1 mM and 27.6 mg.10 ml^{-1} = 1mM, for ATP and phospho-l-arginine respectively).
D-Glucose:	0.99 g.10 ml^{-1} (0.55 M).
$NADP^+$:	15 mg.3 ml^{-1} (6.5 mM).
ADP:	12 mg.1 ml^{-1} (24.6 mM).
Hexokinase (HK):	Dilution of 1 in 25 (i.e. 180 $U.mg^{-1}$ protein).
G6P-DH:	Dilution of 1 in 25 (i.e. 300 $U.mg^{-1}$ protein).
Arginine kinase:	100 ul in 1 ml of 0.7 M mercapto-ethanol (i.e. 75 $U.mg^{-1}$ protein).

Haemolymph/Tissue preparation

See Appendix 1

Procedure:

The analysis could be carried using either a Spectrophotometer (absorbance wavelength = 340 nm) or a Spectrofluorophotometer (at wavelengths of 340 nm and 457 nm, for excitation and emission respectively). The latter is more sensitive and was used when the concentration of either of the metabolites was very low. The following reagents were added to a 1.5 ml semi-micro cuvette:

i) 1 ml Tris buffer, 50 ul $NADP^+$, 10 ul ADP, 50 ul D-glucose ---- E1

ii) Add 10 ul HK and 10 ul G6P-DH (leave 5 minutes) ---- E2

iii) 100 ul of sample/standard were added and left for 5 minutes ---- E3

iv) 20 ul arginine kinase were added and left for 15 minutes ---- E4

Interpretation

E1 - Regarded as the reaction blank.

E2 - E1 - Change proportional to the concentration of ATP contamination of the ADP.

E3 - E2 - Change proportional to the concentration of ATP present in the sample/standard.

E4 - E3 - Change proportional to the concentration of the phospho-l-arginine present in the sample/standard.

Comment

a) Phospho-l-arginine is highly labile and was therefore kept frozen at -70°C, when not being used.

b) Internal standards were necessary, because unknown tissue factors caused partial inhibition of the reaction. It was found that only 80 % of the phospho-l-arginine was estimated. (An internal standard represented the addition of a

known amount of standard, to a reaction mixture that already contained a tissue sample).

c) The arginine kinase was left in the 0.7 M mercapto-ethanol for at least 5 minutes to ensure full activation of the enzyme, before being used in the assay.

d) Mercapto-ethanol is both extremely poisonous and possesses an unpleasant smell. Therefore the use of protective gloves and glasses, together with an efficient fume cupboard, were necessary.

References

Trautschold I., W. Lamprecht & G. Schweitzer (1981). UV-method with hexokinase and glucose-6-phosphate dehydrogenase. *In*: Methods of enzymatic analysis, 3rd ed. pp. 346-357. Ed. by H.U. Bergmeyer. New York: Academic Press.

Heinz F. & H. Weiber (1981). Creatine phosphate. *In*: Methods of enzymatic analysis, 3rd ed. pp. 507-514. Ed. by H.U. Bergmeyer. New York: Academic Press.

APPENDIX 8 - METHOD FOR PYRUVATE, ADENOSINE DIPHOSPHATE AND ADENOSINE MONOPHOSPHATE DETERMINATION.

This method is based on that of Jaworek & Welsch (1981) and involves a 3- step reaction. Firstly, the pyruvate is reduced to L-lactate in the reaction catalysed by lactic dehydrogenase (EC 1.1.1.27). ADP is estimated from the reaction, catalysed by pyruvate kinase (EC 2.7.1.40), in which ATP is produced from the ADP via the de-phosphorylation of phosphoenolpyruvate (PEP) to pyruvate. Finally ADP is produced from the phosphorylation of AMP with ATP, which is catalysed by myokinase (EC 2.7.4.3).

Assay

1) 2 Pyruvate + 2 NADH + 2 $H^+ \rightleftharpoons$ 2 L-lactate + 2 NAD^+

2) 2 ADP + 2 PEP $\longrightarrow$ 2ATP + 2 Pyruvate.

3) AMP + ATP $\longrightarrow$ 2 ADP

Reagents

Tris buffer: 2.42 g of Tris, 0.24 g of magnesium sulphate and 0.75 g of potassium chloride (100 mM, 10 mM and 50 mM respectively) were added to 200 ml of distilled water. The pH was adjusted to 8.0, using HCl (5 M).

NADH: 28.4 mg.10 ml^{-1} (4 mM).

PEP: 41.2 mg.10 ml^{-1} (20 mM).

EDTA: 74.4 mg.10 ml^{-1} (20 mM).

ATP: 121 mg.10 ml^{-1} (20 mM).

Pyruvate: The following standards were run: 1.0 mM, 0.5 mM, 0.25 mM and 0.1 mM (11.9 mg.100 ml^{-1} = 1 mM).

ADP: The following standards were run: 1.0 mM, 0.5 mM, 0,25 mM and 0.1 mM (47.1 mg.100 ml^{-1} = 1 mM).

AMP: The following standards were run: 1 mM, 0.5 mM, 0.25 mM and

0.1 mM (34.7 mg.100 ml^{-1} = 1 mM).

LDH: Dilution of 1 in 25 (i.e. 600 $U.mg^{-1}$ protein).

PK: Dilution of 1 in 25 (i.e. 72 $U.mg^{-1}$ protein).

MK: Dilution of 1 in 25 (i.e. 1300 $U.mg^{-1}$ protein).

Haemolymph/tissue preparation

See Appendix 1.

Procedure

The analysis can be carried out using either a Spectrophotometer (absorbance wavelength = 340 nm) or a Spectrofluorophotometer (at wavelengths of 340 nm and 457 nm for excitation and emission respectively). A cuvette of distilled water was used as the reagent blank (E1). The following reagents were mixed in 1.5ml semi-micro cuvettes:

i) 750 ul Tris buffer, 50 ul EDTA, 50 ul PEP, 50 ul ATP, 50 ul NADH and 50ul sample /standard -----E2

ii) 5 ul of LDH (left for 1 minute) -----E3

iii) 5 ul of pyruvate kinase (left for 5 minutes) -----E4

v) 5 ul of myokinase (left for 10 minutes) -----E5

Interpretation

E1 - Reagent blank.

E2 - Absorbance/emission of 50 ul 4 mM NADH.

E3 - E2 - Change proportional to the concentration of pyruvate present in the sample/standard.

E4 - E3 - Change proportional to the concentration of ADP present in the sample/standard.

E5 - E4 - Change proportional to the concentration of AMP present in the

sample/standard.

Comment

a) High concentrations of the metabolites can lead to NADH becoming the limiting factor. Care was taken to avoid this by ensuring NADH was in excess.

b) Since the concentration of AMP was very low, it was necessary to use the spectrofluorophotometer.

Reference

Jaworek D. & J. Welsch (1981). Adenosine 5'-diphosphate and adenosine 5'-monophosphate: UV-method. *In*: Methods of enzymatic analysis, 3rd ed. pp. 365-370. Ed. by H.U. Bergmeyer. New York: Academic Press.

APPENDIX 9 - CONCENTRATIONS OF AMINO ACIDS DURING ANOXIA AND SUBSEQUENT RECOVERY.

Amino acid	A^+	B^+	C^+	D^+	E^+
Asp.	1.25 ± 0.34	0.52 ± 0.06	0.50 ± 0.13	0.62*	0.93 ± 0.14
Thr.	3.78 ± 3.04	1.83 ± 1.05	3.40 ± 0.52	3.25*	3.66 ± 0.53
Ser.	4.81 ± 2.36	1.86 ± 1.65	1.23 *	0.64*	0.85 ± 0.13
Glu.	4.59 ± 2.32	1.84 ± 0.49	2.58 ± 0.18	2.82*	2.22 ± 0.68
Pro.	9.55 ± 3.88	6.87 ± 1.29	11.7 ± 0.84	5.96*	7.06 ± 2.70
Gly.	13.9 ± 1.39	12.8 ± 3.16	13.0 ± 2.80	12.7*	13.1 ± 3.40
Ala.	5.77 ± 0.70	8.16 ± 0.61	11.9 ± 1.13	7.59*	7.72 ± 0.60
Val.	0.78 ± 0.23	0.53 ± 0.26	0.52 ± 0.22	0.45*	0.38 ± 0.12
Met.	0.45 ± 0.29	0.40 ± 0.16	0.52 ± 0.07 *		0.25 ± 0.05
Ile.	0.73 ± 0.28	0.60 ± 0.37	0.56 ± 0.12	0.29*	0.36 ± 0.12
Leu.	1.18 ± 0.37	0.81 ± 0.61	0.85 ± 0.18	0.35*	0.64 ± 0.24
Tyr.	1.34 ± 0.32	0.74 ± 0.23	0.96 ± 0.18	0.78*	1.31 *
Phe.	0.79 ± 0.06	0.48 ± 0.24	0.68 ± 0.12	0.40*	1.15 ± 0.62
His.	0.37 ± 0.07	0.36 ± 0.14	0.36 ± 0.06	0.33*	0.45 ± 0.2
Lys.	3.91 ± 0.85	2.63 ± 0.49	3.72 ± 0.85	2.19*	1.67 ± 0.13
Arg.	9.67 ± 0.41	7.49 ± 1.4	7.53 ± 1.30	6.22*	7.08 ± 0.64
Tau.	24.3 ± 0.56	21.7 ± 4.15	21.7 ± 2.80	19.6*	19.1 ± 3.10

+ - umol.g^{-1} fresh wt.
* - n=4 except where marked by an asterisk, which indicates that n=1.
A - Normoxia.
B - After 12 hours of anoxia.
C - After 1 hours recovery.
D - After 9 hours recovery.
E - After 12 hours recovery.

APPENDIX 10 - PREPARATION OF RADIOLABELLED TISSUE SAMPLES FOR USE IN ION EXCHANGE CHROMATOGRAPHY.

Reagents

Liquid Nitrogen

Potassium hydroxide: 30 g.100 ml^{-1} (30 %).

Ethanol: 75 %

Procedure

i) Grind up animals in liquid nitrogen, using pestle and mortar.

ii) Take duplicate 50 mg tissue samples and put in pre-cooled Eppendorf tubes.

iii) Add 0.3 ml of potassium hydroxide (30 %) and boil for 20 minutes.

iv) Add 0.7 ml of ethanol (75%), mix thoroughly and put on ice for 2 h.

v) Spin for 10 minutes (12,000 g), remove the supernatant and freeze until needed (contains fractions F2, F3, F4 and F5).

vi) Redissolve the pellet fraction in water; spin for 5 minutes (12,000 g), remove the supernatant and freeze until needed (fraction containing glycogen - F1).

Comment

i) Prior to boiling the sample in potassium hydroxide (iii), the lid of the Eppendorf tube was pierced with a needle in order to prevent any increase in pressure.

ii) Since the tissue contained radioactivity, all the preparation was carried out in a controlled radiation area. In addition, all pieces of apparatus and work surfaces were subsequently washed with decon (90 %).

APPENDIX 11 - METHOD FOR THE ESTIMATION OF PRIMARY AMINO ACIDS USING THE NINHYDRIN REACTION.

The concentration of pooled amino acids were estimated using this method, to assess the efficiency of separation and recovery of standards, eluted from the ion exchange columns described in Chapter 4.

Reagents

Citric buffer (1): Add 21 g of citric acid (1M) and 0.8 g of hydrated stannous chloride to 200 ml of sodium hydroxide. Dilute to 500 ml with a 1:1 mixture of water and ethanol. The pH was adjusted to pH 5.

Ninhydrin (2): Add 1 g of ninhydrin to 30 ml of ethylene glycol.

Ninhydrin reagent: Take 30 ml of (2) and add to 10 ml of (1). Store in brown bottle. Any reagent not used within 7 days was discarded.

Standards: 1 mM standards of any amino acid in water.

Sample preparation

See Figure 4.2.

Assay

1 ml of Ninhydrin and 50 ul of sample/standard, were added to a 5 ml test tube and mixed well. A reagent blank was produced by substituting 50 ul of distilled water, for the 50 ul of sample standard.

Procedure

i) The assay mixtures were placed in a boiling water bath for 20 minutes, and then cooled on ice for a further 10 minutes.

ii) After this time, the contents of the test-tubes were transferred to a 1.5 ml spectrophotometric semi-micro cuvette.

iii) The absorbance of each sample was then measured at a wavelength of 570 nm.

Interpretation

A calibration line was constructed and a linear relationship was observed between the concentration of standards and their absorbances. If a blank was used to zero the spectrophotometer, any change in absorbance was regarded as being proportional to the concentration of the amino acid(s) present in either the sample or standard.

APPENDIX 12 - COMPOSITION of *CARCINUS* RINGER.

i) Final ionic composition of the *Carcinus* Ringer.

Ion	Concentration (mM)
Na^+	523
Ca^{2+}	12.8
K^+	11.2
Mg^{2+}	19.8
SO_4^{2-}	14.6
Cl^-	549

After J.D. Robertson (1960) and A. C. Taylor (pers. comm.)

The above ionic composition of *Carcinus* Ringer was obtained by adding the following to 1 litre of deionised water:

Salt	$g.l^{-1}$
NaCl	28.9
$CaCl_2.\ 2H_2O$	1.87
KCl	0.84
$MgCl_2.\ 6H_2O$	4.03
Na_2SO_4	2.06

APPENDIX 13 - METHOD TO FRACTIONATE ACID-SOLUBLE TISSUE PHOSPHATES BY PRECIPITATION.

This method is based on that of Kaplan & Greenberg (1944) and Sacks (1949) and relies principally on differences in the solubilities of the barium salts of the phosphates. The present assay was used to separate glycolytic phosphates from TCA acids, in the radiolabelling experiments described in Chapter 4.

Reagents

Barium acetate: Add 25.5 g of barium acetate (1 M) to 100 ml of distilled water. The pH was adjusted to 8.4.

Ethanol: 75 %

Hydrochloric acid: 1 M.

Sample preparation

See Appendix 10 and Figure 4.2. The strong acid fraction (F5) was then used in the following procedure.

Procedure

i) Take 100 ul of F5 and 400 ul of saturated barium acetate and add 500 ul of ethanol. Mix thoroughly.

ii) Spin for 10 minutes (12,000 g) and remove supernatant (F5t).

iii) Redissolve the pellet sample in 200 ul hydrochloric acid (F5g).

Interpretation

F5t ----- Tricarboxylic acid cycle acids (e.g. fumarate, citrate, malate).

F5g ----- Glycolytic phosphates (e.g. PEP, glucose-6-phosphate).

Ecoscint (5 ml) was then added to the 2 fractions (F5t and F5g) and the radioactivity in each determined by scintillation counting (Chapter 4, section 4.2.2.3.).

References

Kaplan N.O. & D.M. Greenberg (1944). Studies with radioactive phosphorus of the changes in the acid-soluble phosphates in the liver coincident to alterations in carbohydrate metabolism. I. Separation and nature of the organic acid-soluble phosphates of liver. J. biol. Chem. 156, 511-524.

Sacks J. (1949). A fractionation procedure for the acid-soluble phosphorus compounds of liver. J. biol. Chem. 181. 655-666.

APPENDIX 14 - EFFICIENCY OF SEPARATION AND RECOVERY OF STANDARD METABOLITES FOLLOWING ION EXCHANGE CHROMATOGRAPHY.

A mixture of the following pure commercial standard metabolites (all 1 mM) was passed through cation and then anion exchange columns as described in Figure 4.2 - glycogen, glutamic acid, alanine, arginine, D-glucose, L-lactate, glucose 6-phosphate, phosphoenolpyruvate and fumarate. The strong acids were then separated into glycolytic and TCA acids using the method described in Appendix 13. The efficiency of separation and recovery of each of these standards was then determined, using a range of spectrophotometric and spectrofluorophotometric techniques. The 3 amino acids were collectively estimated using the ninhydrin method (Appendix 11). The recoveries of glycogen, D-glucose, L-lactate and glucose 6-phosphate were determined using methods described in Appendices 3, 4, 5 and 4 respectively. In the last 3 assays the samples were neutralised using potassium bicarbonate (2 M). Fumarate was estimated by spectrophotometrically measuring the absorbance of its double bond at 250 nm (quartz semi-micro cuvettes were used). The following table presents the percentage recoveries of these standards in each of the fractions (F1 - glycogen; F2 - amino acids; F3 - neutral compounds; F4 - weak acids (mainly L-lactate); F5 - strong acids (divided into glycolytic acids and TCA acids)).

	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 GP* (%)	F5 TCA* (%)
Glycogen	95	-	2.2	-	-	-
Amino acids	-	86	-	1.4	-	-
D-glucose	-	-	98	-	-	-
L-lactate	-	-	-	114	-	-
Glucose 6-phosphate	-	-	-	-	94	2.5
Phosphoenolpyruvate	-	-	-	-	92	-
Fumarate	-	-	-	-	-	94

* GP - Glycolytic phosphates; TCA - Tricarboxylic acid cycle acids.