CMFRI SPECIAL PUBLICATION
Number 25

A PRACTICAL MANUAL FOR STUDIES OF ENVIRONMENTAL PHYSIOLOGY AND BIOCHEMISTRY OF CULTURABLE MARINE ORGANISMS

ISSUED ON THE OCCASION OF THE WORKSHOP ON
METHODS AND DESIGNS OF EXPERIMENT RELATING TO ENVIRONMENTAL BIOCHEMISTRY AND PHYSIOLOGY
ORGANISED BY
THE CENTRE OF ADVANCED STUDIES IN MARICULTURE
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
Indian Council of Agricultural Research
HELD AT COCHIN ON 7TH AND 8TH JUNE, 1985

The CENTRE OF ADVANCED STUDIES IN MARICULTURE was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate Agricultural Education and Research'. The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to:

— provide adequate facilities to carry out research of excellence in mariculture/coastal aquaculture;

— improve the quality of post-graduate education in mariculture;

— make available the modern facilities, equipments and the literature;

— enhance the competence of professsional staff;

— develop linkages between the Centre and other institutions in the country and overseas;

— undertake collaboration programmes; and

— organise seminars and workshops.

Under the programmes of the Centre, Post-graduate courses leading to M.Sc. (Mariculture) and Ph.D. are offered in collaboration with the University of Cochin since 1980.

A PRACTICAL MANUAL FOR STUDIES OF ENVIRONMENTAL PHYSIOLOGY AND BIOCHEMISTRY OF CULTURABLE MARINE ORGANISMS

Prepared by

P. W. HOCHACHKA

Department of Zoology,
University of British Columbia, Vancouver, B. C.,
Canada V6T 2A9

CMFRI SPECIAL PUBLICATION
Number 25

ISSUED ON THE OCCASION OF THE WORKSHOP ON
METHODS AND DESIGNS OF EXPERIMENT RELATING TO ENVIRONMENTAL BIOCHEMISTRY AND PHYSIOLOGY

ORGANISED BY

THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
Indian Council of Agricultural Research
HELD AT COCHIN ON 7TH AND 8TH JUNE 1985

(LIMITED DISTRIBUTION)

Published by : **Dr. P. S. B. R. James**
Director,
Central Marine Fisheries Research Institute,
Cochin 682 031.

Edited by : **Dr. K. Rengarajan**
Scientist,
Central Marine Fisheries Research Institute,
Cochin 682 031.

PRINTED IN INDIA
AT PAICO PRINTING DIVISION, ERNAKULAM, COCHIN 682 031

CONTENTS

PREFACE	... ...	iii
CHOICE OF STUDY ORGANISMS	...	1
ANIMAL MANIPULATIONS	...	2
PHYSIOLOGICAL MEASUREMENTS	...	6
BIOCHEMICAL PREPARATIONS AND MEASUREMENTS		20

ABBREVIATIONS USED IN THIS MANUAL

acetyl Co A	acetyl coenzyme A (= active acetate)
ADP	adenosine 5-diphosphate
ALD	aldolase
AMP	adenosine 5-monophosphate
APK	arginine phosphokinase
ATP	adenosine 5-triphosphate
BSA	bovine serum albumen
BW	body mass
Ca_{O_2}	oxygen content of arterial blood
Cv_{O_2}	oxygen content of venous blood
CPK	creatine phosphokinase
cpm	count per minute
Crp	creatine phosphate
CS	citrate synthase
DA	dorsal aorta
DMO	5, 5-dimethyl-2, 4-oxazolidinedione
dpm	distintegrations per minute
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
ECF	extra cellular fluid
ECFV	extra cellular fluid volume
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylenebis (oxyethylenenitrilo) -tetraacetic acid
ETS	electron transfer system
F-1-P	fructose 1-phosphate
F-1, 6-P_2	fructose-1, 6-bisphosphate (=FBP)
F-6-P	fructose 6-phosphate
GAP	glyceraldehyde - 3-phosphate
GDH	glutamate dehydrogenase
GOT	glutamate-oxaloacetate transaminase
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
α GPDH	alpha glycerol-1-phosphate dehydrogenase
HEPES	4- (2-hydroxyethyl) -1 piperazineethanesulfonic acid
HK	hexokinase
HOAD	hydroacyl acetyl coenzyme dehydrogenase
HTO	tritiated water
ICF	intracellular fluid
ICFV	intracellular fluid volume
I.U.	international unit
Kd	dissociation constant (for enzyme substrate complex)

LDH	lactate dehydrogenase
MCR	metabolic clearance rate
MDH	malate dehydrogenase
MS 222	tricaine methane sulfonate
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of NADP
OD	Absorbance (= optical density)
P_i	inorganic phosphate
PCA	perchloric acid
PE	polyethylene
PFK	phosphofructokinase
6PG	6-phosphoglyconate
PGM	phosphoglycomutase
PK	pyruvate kinase
pH_i	intracellular pH
pH_o	extracellular pH
pK_i	dissociation constant (kH) of intracellular fluid
pK_o	dissociation constant (kH) of extracellular fluid
Q_h	cardiac output
QX-314	quaternary ammonium drug
S	substrate
SA	specific activity
SMR	standard metabolic rate
STX	Saxitoxin
TBW	total body water (in ml)
TCA	trichloroacetic acid
TTX	Tetrodotoxin
U_{crit}	critical activity *i.e.* maximum sustainable speed
VA	ventral aorta
V_{O_2}	volume of oxygen consumed

PREFACE

Though man has excelled as cultivator and animal husbandman of terrestrial biota, over aquatic life, he remains more a hunter. The pressing demand for additional food resources has compelled us to take seriously cultivation of aquatic life and the management of the vast marine biome. The **Central Marine Fisheries Research Institute** took up mariculture oriented programmes in the early seventies. In mariculture the understanding of environmental physiology and biochemistry is vital for the manipulation of cultivable marine organisms.

Dr. Peter William Hochachka, Professor, Department of Zoology and Sports Medicine Clinic, University of British Columbia, Vancouver, British Columbia, Canada has considerable research expertise and has authored three books, namely, "Biochemical Adaptation", "Living Without Oxygen" and "Metabolic Arrest in the field of Environmental Physiology and Biochemistry". During the consultancy, Dr. Hochachka had a number of group discussions, gave four seminars and conducted a short workshop. This manual is being issued as the follow up of the workshop conducted. It is hoped that this manual will be useful to the scientists working in the field.

I express my sincere thanks to Dr. P. W. Hochachka for writing this useful manual in which he has lucidly explained the procedures. I thank Shri D. C. V. Easterson, Scientist, who has competently served as the counterpart from the Institute. I also thank Dr. K. Rengarajan, Scientist for editing the manual.

Dr. P. S. B. R. JAMES
Director,
Central Marine Fisheries
Research Institute.

Cochin - 682 031,
5-6-1986.

1

CHOICE OF STUDY ORGANISMS

For the applied biologist interested in mariculture, choosing the 'right' organism may mean the difference between success and failure. Generally decisions require a compromise between ease of handling (a function of how much has already been done by others) and desirability of the cultured product. In the Cochin area of India, the use of mullet, milkfish and *Tilapia* for mariculture, for example, represents such a compromise, which is evidently effective, presumably in large part because of the years of experience and data which have gradually accumulated on these species. However successful such choices may be, they should not rule out a continuous search for other, perhaps more advantageous, species: ones which are more hardy, grow faster, sell faster and taste better!

For unravelling problems in environmental physiology and biochemistry, strategies for choosing the 'right' organism are also pragmatic, but in this case the criteria should be related to the parameters under investigation. For example, hypoxia-sensitive species can help us identify the most debilitating problems of O_2 lack while hypoxia-tolerant species can yield powerful insights into how these problems can be solved. The same is true for other environmental parameters. Thus, a judicious choice of study organisms would usually settle on at least two, and preferably more, species with varying tolerance to the parameters of interest.

2

ANIMAL MANIPULATIONS

Holding Facilities

Our own experience is based mainly upon studies with trout, salmoon, goldfish, carp, lungfish and tuna. For hypoxia-sensitive species such as tuna and salmonids holding facilities with flow-through fresh water usually are found to yield the best results; for hypoxia-tolerate species, this is not as important. Temperature and salinity controls, while not a necessity, obviously are advantageous, if they can be arranged.

Anaesthesia

Probably the most widely used fish anesthetic is MS 222 (tricaine methane sulfonate) which is rapidly effective at 1:2000 w/v concentrations. With invertebrates, near 0°C hypothermia is usually an acceptable alternative, although survivability may sometimes be compromised. In some cases, it may be necessary to perform the entire experiment under totally controlled conditions, in which event the initial deep anaesthesia may be gradually lightened to some operationally determined lower level; in tuna, for example, at 1:15,000 w/v, MS 222 maintains a light level of anaesthesia for prolonged experimental periods.

Catheterization

If animals are large enough, their metabolic and physiological responses to environmental parameters can be much more precisely monitored, if sequential blood sampling is made possible. This is most easily achievable by catheterization of either the ventral or dorsal aorta (VA or DA respectively). Procedures for this are now

rather routine, thanks to the contributions of Smith and Bell (1967). Fish are anaesthetised as above, then placed in a V-shaped operating table usually positioned on their backs with a hose inserted into the mouth for irrigating the gills. After the aorta is punctured with a Medicut No. 16, the needle is removed, leaving a plastic sleeve in place. A polyethylene (PE) 50 cannula filled with heparinized Cortland saline (10 I.U. sodium herapin/ml) is inserted through the sleeve and advanced some 1–2 cm into the DA. The cannula may at this time be secured with a stitch in the roof of the mouth, then exteriorized through a short nose cone or collar of PE 190 tubing, which is previously inserted through a hole puncutured between the nares. Patency may be verified by pressure recordings and by measurements on blood samples obtained. Between samples, blood in the cannula is replaced with heparinized saline and the free end of the cannula is plugged with a sterilized metal pin. VA cannulations are performed through the floor of the mouth by placing the cannula (PE 50 tubing tipped with a No. 23 Yale needle and filled with heparinized saline) into the ventral aorta midway between the second and third gill arches. The cannula in this case may be secured with a stitch through the side of the mouth. Generally, if the VA cannulation is performed before the DA one, blood loss is reduced. These kinds of preparations in our hands can be secured for periods of days and even weeks if necessary, with appropriate precautions for avoiding clotting and avoiding infection. With these kinds of preparations, changes in environmental parameters may be monitored by measuring blood gases, pH, metabolities, ions, or hormones. Furthermore, in the case of environmental toxicology, the effects of specific toxicants can be assessed by direct introduction via DA or VA catheters of the compound or compounds under investigation in precisely definably aliquots, allowing much more precise assessment of the organism's metabolic defence strategies, of target tissues and of ultimate metabolic fate of the introduced material.

Control of Activity States

Since one of the largest determinants of metabolic rate is physical activity, in all studies of environmental physiology and biochemistry control of the activity state of the animal is desirable. For example, determining the standard metabolic rate (SMR) for aquatic ectotherms is by no means an easy task, while determining their maximum metabolic rate, $\dot{V}O_{2\,(max)}$, is equally troublesome. We have found that SMR can best be found (in salmonids at least) by maintaining the animal in small, darkened metabolic chambers with no access to the surface (to avoid air gulping or surface skim-

ming in hypoxia experiments, for example) and with at least 24 hours of habituation time. Under these conditions we find that the animal gradually arrives at a stable, reproducible metabolic state, by criteria which include plasma metabolite measurements, plasma gas tensions, plasma pH, and whole organism VO_2 (or O_2 uptake). For other activity states, such as variable swim speeds, it is necessary to develop some sort of swim mill. These have been described and perfected by various workers notably by Fry and Brett in Canada (Brett, 1965).

A typical protocol for assessing exercise costs and performance in trout may run as follows: Firstly, the fish used for these experiments must be pre-conditioned to swim in continuous exposure, in our case, to a mean water velocity of 20 cm sec^{-1}. (zero cm sec^{-1} in centre; 35-40 cm sec^{-1} at edge) in a large, circular holding tank equipped with a pump. The training or conditioning time is never less than two weeks. Selected fish are then introduced into a water tunnel through an access port and allowed to habituate for at least 18 hr at a water velocity of approximately 8.7 cm sec^{-1}, the lowest speed on the pump, before being tested.

The respiratometer/water tunnel incorporates a cylindrical Plexiglass chamber (126.5 cm^2 cross-sectional area) through which a variable-speed water pump can maintain the speed at any desired value, with the water being renewed at a rate of 1 L min^{-1}. The temperature is controlled ($\pm$0.5°C) by a heat exchanger system. The partial pressure of oxygen of the water flowing into the swim mill must always be held close to saturating levels.

The critical velocity swimming test is a measure of exercise performance. In the typical experiments, U_{crit} (the maximum sustainable speed) is directly determined as follows:

$$U_{crit} = u_1 + (t_1/t_2 \times u_2)$$

where u_1 is the highest velocity maintained for the prescribed 30 min period (cm sec^{-1}),

u_2 is the velocity increment [which was approximately 0.5' fork length' sec^{-1} (fl sec^{-1})],

t_1 is the time (min) that each fish swims at the 'fatigue' velocity,

t_2 is the prescribed period of swimming (30 min).

In these kinds of experiments, fatigue is defined as the point at which a fish is unable to remove itself from a electrified downstream grid in the water tunnel after repeated efforts and electrical stimulation. Because this may be a difficult state to identify, one procedure, once a fish has fallen against the grid, is to reduce the water velocity by 0.25 fl sec^{-1}. If the fish then continues to swim, even after the 'fatigue' velocity is regained, the first failure is ignored. However, the subsequent failure terminates the run and defines the time to 'fatigue.' This is the time used in the calculation of U_{crit}. All swimming speed experiments are performed over the same time priod of the day, to overcome possible diel differences in exercise performance.

For some types of research, it is important to monitor voluntary activity cycles in the natural environment. For useful measurements, this need can best be satisfied by the use of mobile field laboratories (for example, a small laboratory outfitted into the back of a jeep or onto a small boat). For some species, even more elaborate field laboratories are obviously necessary, such as oceanographic vessels.

3

PHYSIOLOGICAL MEASUREMENTS

Metabolic Rate

Estimates of metabolic rates of organisms in theory can be obtained by measurements of O_2 uptake, CO_2 production, or heat production, the first usually being the easiest. In addition, the rates and percent participation of specific metabolic pathways can be assessed by the judicious use of ^{14}C–, ^{3}H–, ^{32}P–, ^{15}N–, or ^{35}S– labelled substrates. The latter may or may not be directly related to overall metabolic rate. Again, these kinds of measurements are meaningful if – and only if – the activity state of the animal is definable, which means the nature of the metabolic chamber used for making the measurements in the first place is of utmost importance. In the case of experiments with isotopes, the labelled substrates can be introduced as a single bolus (for example, via a DA or VA catheter, or even intraperitoneally or intramuscularly) or can be delivered continuously with the use of a peristaltic pump attached to the incoming catheter. For fishes, the bolus technique at least to date has been the more successful of the two, perhaps because of the fragility of such preparations.

Cardiac Output and Blood Distribution

The volume of blood pumped by the heart per unit time, the **cardiac output,** can be quantified in a number of ways. One common method depends on the simple fact that all the oxygen consumed ($\dot{V}O_2$) by the animal is carried by the blood ejected from the heart. From the difference in the oxygen content of arterial and venous blood the cardiac output ($\dot{Q}_h$) can be calculated from the following equation:

$$\dot{V}O_2 = \dot{Q}_h \ (C_aO_2 - CvO_2)$$

To determine the cardiac output, the oxygen consumption, the oxygen content in arterial blood and oxygen content in venous blood are measured.

Assessing cardiac output according to the Fick principle can be carried out only in animals that have a complete separation between arterial and venous blood in the heart, as do mammals and birds. This approach cannot be used in amphibians and reptiles, where there is a possibility for mixing arterial and venous blood in the heart. In fish the approach is perfectly valid since the venous blood sample can be taken from the VA near the heart (but before the gills) and arterial blood from the DA after the gills.

Other methods for determining cardiac output are based on a variety of principles. A frequently used earlier method consists of injecting an easily measureable dye at a known moment in a vein leading. to the heart. The dye will shortly afterward appear on the arterial side, and by integrating the curve representing the concentration of the dye as it rapidly increases and again decreases at the arterial sampling post, it is possible to calculate the cardiac output. Thermal dilution is based on the same principle.

Nowadays, the use of radiolabelled materials to measure cardiac output and blood volume distribution is widespread and probably the method of choice. Carbonized microspheres (obtainable from 3M Co., Nuclear Products, St. Paul, Minnesota, U.S.A.) varying in diameter from 15 to 50 μm can be labelled with ^{85}Sr, ^{141}Ce, ^{125}I or ^{46}Se and are injected as a single bolus, over an approximately 1 min period, preferably into the DA. The principle of this technique is that the microspheres lodge in the first capillary bed they find and thus the number of spheres/gm of tissue (*i.e.* cpms/gm tissue) are directly proportional to flow (and to fractional cardiac output). In such an experiment, blood is withdrawn at a constant rate beginning just *before* injection of microspheres. Cardiac output can then be computed as the product of DA withdrawl rate *times* total injected radioactivity *divided by* the DA blood sample radioactivity.

In fish, $^{86}Rb^+$ may be particularly useful, because it is easier to handle than microspheres. Elemental rubidium and its isotope $^{86}Rb^+$ have biogical properties which are similar to those of K^+. When $^{86}RbCl$ of known activity is administered intravenously as a single injection, the amount of the indicator in an organ remains

essentially unchanged for an appreciable time interval after the first circulation. Since $^{86}Rb^{+}$ equilibrates and mixes with the blood and is distributed throughout the body in the same proportions as the blood flow itself, the subsequent fraction of the total injected counts found in any excised tissue sample will be the same as the fraction of the total blood flow (or cardiac output) directed to that tissue under different conditions (different environments or different activity states). In trout, $^{86}Rb^{+}$ and microsphere experiments yield similar estimates of both cardiac output and blood distribution (Daxboeck, 1981).

A typical experimental protocol may run as follows:

Some 24 hours after recovery from surgery, a total of 5 or 6 fish (each tested individually) are forced to swim against an incrementally increasing water current upto an estimated 80% $U_{crit.}$ After 25 min swimming at the highest velocity, 0.05 mCi $^{86}RbCl$ (0.30 ml Cortland saline vehicle + 0.5 ml 'cold' saline wash) are introduced into the circulation via the DA cannula. Five minutes are allowed for tracer equilibration within the body and then the fish is sacrificed by a 0.20 ml injection of KCl.

Another group of fish matched for size, are prepared as above, but are allowed to recover in darkened perspex holding boxes, supplied with running aerated water at the same temperature as the swimming fish. After recovery (for 18 has a minimum) these 'resting' fish are injected with ^{86}Rb and are sacrificed in the same manner as before.

Several more fish are sacrificed by overanaesthesia and fresh excision of selected tissues is made, to determine the relative percentage of the total body weight accounted for by each sample. Care is taken to blot excess blood and other fluids from all tissues. Gut contents are removed before weighing, as is the blood contained within the heart.

After sacrifice, 200 mg samples of various tissues are carefully excised and weighed into low potassium, borosilicate glass scintillation vials (22 ml capacity). Care is taken not to cross-contaminate any sample with blood or fluid from another. To each vial is added 1.5–2.0 ml of Protosol tissue solubilizer (0.5 M quaternary ammonium hydroxide solution) and the capped contents are allowed to digest at 55°C for 18–24 hr in a temperature controlled shaking water bath.

Once digested, coloured solutions are partially decolourised by the dropwise addition of 0.1–0.5 ml 30% H_2O_2 at room temperature, then rewarmed and shaken for an additional 30 min at 55°C. 50 μl glacial acetic acid are added to these vials per 0.5 ml Protosol. Once cooled again, 10 ml Econoflour (NEN–a premixed scintillation solution with characteristics similar to those of toluene base cocktails) is added, mixed well and counted in a scintillation counter using standard techniques. Each sample is counted for at least 10 min in a ^{32}P window setting of the counter. Data are converted to counts per minute per gram tissue (cpm g^{-1}). Since the percentage body weight of all tissues and the weights of each tissue sample are known (usually 200 mg), the total cpm's in the fish can be calculated. This value is compared to the injected cpm's for each fish to obtain the fractional distribution of cardiac output.

TABLE 1. *Blood volume* distribution to various tissues from normal, resting rainbow trout* (N= 6; 371.0 ± 36.7 *g*)

Tissue	Blood Volume ml.g $^{-1}$	Percent Body Weight	Percent Total Blood Volume ml. 100g^{-1}
Mosaic muscle	0.008	66	0.51
Lateral red muscle	0.021	2.5	0.05
Gills (2nd arch)	0.159	3.9	0.62
Liver	0.225	1.15	0.26
Spleen	3.088	0.22	0.70
Ventricle (emptied)	0.064	0.13	0.01
Pseudobranch	0.256	0.12	0.03
Stomach and oesophagus	0.020	1.27	0.02
Intestine and caeca	0.032	1.64	0.05
Gonads	0.023	4.22	0.10
Skin	0.016	4.0	0.07
Eyes	0.029	1.12	0.03

Pre-injection Hct	16.25%	(at time = 0)
Sample Hct	17.72%	(at time = +2hours)
Blood volume	6.08%	body weight

*All volumes determined from dorsal aortic (arterial) blood sample reference CPM's. Modified from Daxboeck (1981).

Representative blood volume distributions at rest and during heavy exercise are shown in Tables 1 and 2, while the fractional cardiac outputs delivered to various tissues are shown in Table 3.

TABLE 2. *Blood volume* distribution to various tissues from rainbow trout swimming at 80% U_{crit} ($N = 7$; 445.0 ± 27.7 g)*

Tissue	Resting Blood Volume ml.g^{-1}	Exercise Blood Volume ml.g^{-1}
White muscle	0.008	0.008
Lateral red muscle	0.021	0.125
Liver	0.225	0.065
Spleen	3.088	1.546
Kidney	0.326	0.117
Stomach and oesophagus	0.020	0.012
Intestine and caeca	0.033	0.034
Skin	0.016	0.018
Haematocrit	20.5%	26.4% (at t = 25 min at 80% U_{crit})
Total blood volume	6.08%	3.72% body weight (at t=25 min at 80% U_{crit})

*All volumes determined from dorsal aortic (arterial) blood sample reference CPM's. Modified from Daxboeck (1981).

TABLE 3. *Percentage of cardiac output to various tissues from rainbow trout at rest, expressed as percentage of total injected CPM's*

Weight (g)	Mosaic Muscle	Red Muscle	Spleen	Liver	Stomach	Intestine	Gonad	Kidney	Cheek	Brain	% Injected CPM's Recovered
Using ^{86}Rubidium											
384.4	36.4	6.51	0.74	8.73	1.86	5.82	1.99	9.26	0.16	0.02	82.6
Using Radiolabelled Microspheres											
429.4	49.0	11.2	1.06	4.5	7.40	10.0	4.5	8.3 skin			89.3

Ion Regulation, Ion Channels and Ion Pumps

Physiologists and biochemists traditionally divide transport mechanisms into two classes – carriers and pores. Carriers include energy-linked pumps (such as the Na^+ and Ca^{++} pumps, which work against concentration gradients) and simpler facilitators (such as the glucose transporter which do not work against diffusion gradients and are not energy requiring). Although much is known about the functional properties of carriers, the specific mechanisms of transport by such important carrier devices as the $Na^+ - K^+$ pump, the Ca^{++} pump, $Na^+ - Ca^{++}$ exchange, $Cl^- - HCO_3^-$ exchange, glucose transport, the Na^+–coupled co– and counter-transporters, and so on, remain unknown.

On the other hand, the water-filled pore as the other class of transport mechanisms has now been firmly established for ionic channels of essentially all cell membranes. In the period since 1965 a valuable interplay between studies of excitable membranes and studies on model pores, such as the gramicidin channel in lipid bilayers, accelerated the pace of research and greatly improved out understanding of this transport function. The major technical advance of this time was the development of methods to resolve the activity of single channel molecules. This led to the discovery that the rate of passage of ions through any given open channel - often more than 10^6 ions per second – is far too high for any mechanism other than a pore.

Ionic channels are macromolecular pores in cell membranes. When they evolved and what role they may have played in the earliest forms of life we do not know, but today it is accepted that these channels bear the same relation to membrane function (to electrical potentials and signalling) as enzymes bear to metabolism. Although their diversity is less broad than the diversity of enzymes, there are still many types of channels working in concert, opening and closing to shape the signals and responses of cells. Although opening or closing of ion channels does not appear to require ATP, generally open channels lead to dissipation of ion and electrical gradients. Restoring these requires ion-specific pumps and these are ATP-dependent. Thus the functions of pores and pumps must always be balanced with metabolism, otherwise the system would rapidly run downhill.

From the point of view of environmental physiology or biochemistry, major problems arise when change in an external para-

meter (*e.g.* salinity, temperature, O_2 availability) causes a **differential** effect on channels and pumps. The problem is trivial if pump activity can outpace the demands arising from channel activity. However, whenever pumps cannot keep up with the demands arising from too many channels being open for too long, viability is at risk. This is commonly seen, for example, in hypoxia-sensitive and cold-sensitive cells at low O_2 and at low temperature, respectively. As we argue elsewhere, the commonest solution to this conundrum involves permeability adjustments; for example, hypoxia-tolerant animal anaerobes are able to survive at low ATP turnover rates in anoxia because of low permeability membranes. From the best available evidence, the adjustment of membrane permeability is achievable mainly, if not solely, by adjustments in densities of ion-specific channels. Hence analysis of channel functions and in particular of channel densities is of central importance to modern environmental biochemistry and physiology.

TABLE 4. *Reversible blocking agents for different channels*

Channel	Acting from Outside	Acting from Inside	Membrane-Permeant
Na^+	TTX, STX H^+	QX-314 Pancuronium Thionin dyes	Local anaesthetics Strychnine Diphenylhydantoin
Ca^{++}	Mn^{2+}, Ni^{2+}, Co^{2+}	Quaternary D-600	D-600, verapamil nifedipine
K^+ (Ca^{++})	TEA Cs^+ Apamin	TEA Na^+	Quinidine
Cl^-	Zn^{2+}	?	Anthracene-9-carboxylic acid
Endplate	QX-314 and many other quaternary or charged drugs	?	Local anesthetics

Tetrodotoxin (TTX) and saxitoxin (STX) are paralytic natural toxins which are exceptionally specific blockers of Na^+channels. The local anaesthetic, procaine, is a synthetic agent used clinically to block Na^+ channels. Tetraethylammonium ion (TEA) is a simple quaternary ammonium compound used experimentally to block K^+ channels. All these agents act reversibly. Modified from Hille (1984).

One of the best ways of getting information on the role of channels and pumps in environmental adaptation involves experimental

studies with channel-specific pharmacological inhibitors, or so-called channel blockers. Some common, commercially available compounds that can be used this way are summarised in Table 4. The situation can be illustrated with Ca^{++} channels. Voltage-sensitive calcium channels occur in many cells and open in response to membrane depolarization, permitting an influx of calcium ions. a process that may become uncontrolled in hypoxia-sensitive cells during times of O_2 lack. In hypoxia-tolerant cells, in contrast, such uncontrolled Ca^{++} fluxes are avoided, probably because of fewer functional Ca^{++} channels per unit surface area of membrane, a theory that may be testable with Ca^{++} channel binding agents. To date, three binding sites have been described in association with calcium channels, each able to find a particular group of organic molecules. Of the radiolabelled 1, 4-dihydropyridines which bind to the class I site, (^3H) azidopine is the most exciting. In skeletal muscle, for example, the affinity of azidopine for this site is particularly high, with a K_d of about 0.4 mM. Thus, (^3H) azidopine can be particularly useful in studies designed to irreversibly liable subunits of the channel thus permitting investigations on the structure of the ionic pore, as well as upon its density per gram of tissue or per unit of surface area.

As channel blocking agents are not yet commonly used in studies in environmental physioloy and biochemistry, specific protocols are not available. It is now urgent to obtain information concerning (i) the relative permeabilities of hypoxia-sensitive *vs* hypoxia-tolerant cells and organs tested *in vitro* and *in vivo* if possible, (ii) the relative channel densities and the quantitative relationship between channel densities and permeabilities, and (iii) the relationship between channel densities and tolerance to specific environmental parameters such as O_2, temperature and perhaps especially salinity.

To test the relationship between Ca^{++} channels and hypoxia tolerance, for example, a possible experimental protocol might run as follows:

1. Prepare slices of 200–500 mg of the organ or tissue under study (cubes of about 1 mm diameter or less).

2. Incubate several control flasks in Ringer's solution (5 or 10 ml total volume) containing 5 mM glucose at controlled temperatures.

3. After 0, 30 and 60 min, remove 1.0 ml samples of Ringer's, filter through cheese cloth or filter paper to remove debris and red blood cells, then use the filtrate directly for assays of glucose, lactate, K^+ and Ca^{++}. If possible, O_2 uptake should be measured simultaneously.

4. Repeat in the presence of inhibitors of the electron transfer system (ETS) such as 0.1 μM antimycin A or 5 μm KCN to assess the magnitude of the Pasteur Effect.

5. Repeat in the presence of antimycin A or KCN with 0.5 μM nifedipine or 5 μM verapimil, specific Ca^{++} entry blockers. If uncontrolled Ca^{++} entry contributes to the hypoxia sensitivity, then some protection against hypoxia should be achieved by the Ca^{++} channel blockers, and the Pasteur Effect should be **reduced.**

6. Repeat in the presence of ouabain (0.5 μM), a specific Na^+ pump inhibitor. Generally, the cost of the Na^+ pump constitutes a major fraction (30–60%) of maintenance metabolism in animal tissues; this may be substantially reduced in hypoxia-tolerant ectothermic tissues.

7. Repeat in the presence of ouabain (0.5 μM) + ETS inhibitors. If hypoxia sensitivity is due to decoupling of metabolism and membrane functioning, ouabain should aggravate this while simultaeously **reducing** the Pasteur Effect (the increment in lactate production caused by ETS inhibitors). Thus under these conditions, ECF (K^+) should rise while ECF (Ca^{++}) should decline simultaneously with a decline in glycolytic flux (measured as a lactate increment).

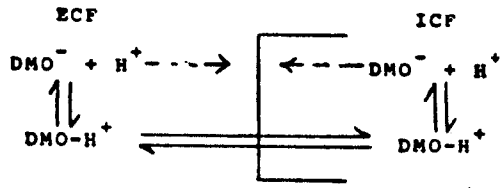
To assess the densities of functional channels per unit area of membrane, electrical methods must be utilised (Hille, 1984). However, to measure **total** content of functional channels per unit amount of membrane or per gram of tissue, advantage can be taken of the 1:1 stoichiometry between channel blockers and channels. Thus, the content of Na^+ channels can be quantified using labelled TTX or STX, while the content of Ca^{++} channels can be measured by determining the cpm's ^{14}C-nifedipine or ^3H-azidopine (as described above) per gram tissue. Utilising methods such as these should allow one to test the hypothesis for example that any given organ in an environmentally tolerant species contains **fewer** Ca^{++} and K^+ channels than does the homologous organ in environmentally sensitive species.

Although pH is now recognized as a dependent, rather than an independent variable, it is closely regulated in both the ECF and ICF. Plasma pH or ECF pH is normally measured with pH specific electrodes. Although intracellular pH (pH_i) can also be measured with small intracellular electrodes, for many tissues and in many metabolic states, this is impossible. A popular and reliable alternate technique for measuring pH_i employes the distribution of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) across the cell membrane (Roos and Boron, 1981). The major advantages of the DMO method lie in its simplicity when using ^{14}C-isotopes and in its application to a variety of *in vivo* and *in vitro* preparations. Its major limitations are: (i) only one measurement per animal tissue can be made, therefore many animals are required for a time course study and (ii) an apparently slow distribution of the weak acid across the membrane hinders accurate measurement of actual transients of intracellular pH. The latter point is particularly important during the initial equilibration period following injection of the weak acid, ^{14}C-DMO solution into the animal or following immersion of *in vitro* preparations into ^{14}C-DMO-containing solutions. The initial whole body equilibration times in living mammals is about 30 to 60 minutes. Unfortunately, there are few *in vivo* data concerning the duration of equilibration with a disturbance in acid-base balance following the initial DMO equilibration. Recent studies utilizing rainbow trout (Milligan and Wood, 1984) and the sea mussel *Mytilus edulis* (Lindinger *et al.*, 1984) have indicated that the DMO equilibration time during acid-base disturbance may be less than 10 min *in vivo*. Neely *et al.* (1975), using the isolated perfused rat heart preparation, found that the distribution of DMO within the tissue was complete within 3 min and this was without prior equilibration of DMO in the animal before the start of perfusion.

Simultaneous measurement of the ECF space in the tissue often relies on using ^{3}H-mannitol, a substance which is not metabolized and is impermeable to cell membranes. This also permits quantifications of fluid volume changes in the ECF and ICF, necessary for the accurate determination of electrolyte and metabolite fluxes.

The simple principle of the DMO method, as with other weak acid or base methods, is that the cell membrane is relatively impermeable to DMO^- anion, while the uncharged DMO molecule is much more permeable. Thus, pH can be derived from the steady

state distribution of DMO if ECF pH (pH_o) and the apparent pK_a values in the ECF and ICF (pK_o and pK_i) are known:



Therefore:

$$[DMO\text{-}H^+]_o = [DMO\text{-}H^+]_i = [DMO\text{-}H^+]$$

in the steady state. Then:

$$pH_o = pK_o + \log\left\{\frac{([DMO^-]+[DMO\text{-}H^+])_o - [DMO\text{-}H^+]}{[DMO\text{-}H^+]}\right\} \quad (1)$$

and

$$pH_i = pK_i + \log\left\{\frac{([DMO^-]+[DMO\text{-}H^+])_i - [DMO\text{-}H^+]}{[DMO\text{-}H^+]}\right\} \quad (2)$$

Combining these two equations to solve for pH_i:

$$pH_i = pK_i + \log\left\{\frac{([DMO^-]+[DMO\text{-}H^+])_i}{([DMO^-]+[DMO\text{-}H^+])_o\ (10^{pH_o - pK_o+1})-1}\right\} \quad (3)$$

Additionally, if we assume that $pK_o = pK_i$ then the relationship experssing the degree of dissociation of DMO in the ECF and ICF to the pH_o and pH_i becomes:

$$\frac{([DMO^-] + [DMO\text{—}H^+])_i}{([DMO^-] + [DMO\text{—}H^+])_o} = \frac{{}_{10}pH_i - pK_{+1}}{{}_{10}pH_o - pK_{+1}} = \frac{[DMO^-]_i}{[DMO^-]_o}$$

This expression indicates that when $pH_i < pH_o$, the weak acid DMO is more concentrated in the ECF than ICF.

In a typical experimental protocol, the experimental animal (fish, mussel, crustacean) is injected with 23 μCi/kg ^{14}C-DMO and 35 μCi/kg ^{3}H-mannitol to give final concentrations of about 0.05 μCi/ml and 0.20 μCi/ml, respectively, in the blood. The injection value for DMO (23 μCi/kg) is based on the relationship relating the degree of dissociation of a weak acid extra - and intracellularly to the pH_o and pH_i as derived by equation 4. Using the values 7.04, 7.40, and 6.13 for pH_i, pH_o, and pK' at 37°C respectively, the ratio $DMO_i/DMO_o = 0.4648$; in other words, DMO_o is 2.15 fold greater than DMO_i. Thus to obtain an ECF concentration of ^{14}C-DMO=0.05 μCi/ml, and hence an ICF (^{14}C-DMO) = 0.4648 x 0.05=0.023 μCi/ml: where ECFV=175 ml/kg; ICFV=600 ml/kg

$$(^{14}\text{C-DMO})_{\text{ECF}} = 0.05\ \mu\text{Ci/ml} \times 175\ \text{ml/kg} = 8.75\ \mu\text{Ci/kg}$$

$$(^{14}\text{C-DMO})_{\text{ICF}} = 0.023\ \mu\text{Ci/ml} \times 600\ \text{ml/kg} = 13.92\ \mu\text{Ci/kg}$$

Thus total ^{14}C-DMO = 22.65 μCi/kg. The injection value for ^{3}H-mannitol is simply the product of the desired final ECF concentration and the ECFV, since mannitol does not penetrate the intracellular compartment and is not metabolized by the animal. Muscle ECFV is calculated from the distribution of ^{3}H-mannitol by: ECFV (ml/kg) = tissue ^{3}H (dpm/g)/perfusate ^{3}H (dpm/g), where dpm is disintegrations per minute of the isotope. Thus muscle ICFV can be calculated as the difference between total tissue water (as determined by drying) and ECFV.

Using the principles outlined above, pH_i can be calculated from the distribution of DMO according to equation 2 of Hinke and Menrad (1978):

$$pH_i = pK' + \log\left\{\left(\frac{[B_H]V_t}{D_H} - 1\right)^{-1}\left(\frac{[B_H]D_C}{[B_C]D_H} - 1\right)\left[{}_{10^p}H_o - pK' + 1\right] - 1\right\}$$

where:

B_H = ^{3}H—mannitol in dpm/unit ECF volume,
B_C = ^{14}C—DMO in dpm/unit ECF volume,
D_H = total ^{3}H (dpm) in muscle tissue,
D_C = total ^{14}C (dpm) in muscle tissue, and
V_t = total tissue fluid volume.

Standard techniques of liquid scintillation counting are used for measurement of ^{3}H and ^{14}C in plasma and tissue extracts.

Field Measurements of Metabolic Rate

Two techniques, to my knowledge, are now most popularly used in the measurement of average metabolic rates of free-ranging organisms. In one approach, $^{3}H_2O$ or HTO is used to measure the turnover rate of water (stoichiometrically coupled to O_2 uptake), while in a second approach, the turnover rate of Na^+ is measured (it too is directly proportional, but not stoichiometrically coupled, to O_2 uptake, the proportionality presumably arising from the large contribution to SMR that is due to Na^+ pump activity). The theoretical basis and derivation of equations required for calculating the turnover rate of water (or the rate at which tritated water molecules are replaced by unlabelled water molecules) are given in greater detail by Nagy and Costa (1980). Suffice here to include the following equations which are typically used (Davis *et al.*, 1983):

1. TWB (ml) $= N_0$

   $N_0 = CPM_{(inj)}/SA_{(t=0)}$

   a) $CPM_{(inj)}$ = activity (mCi) of HTO injected

   b) $SA_{(t=0)}$ = specific activity ($mCi.ml^{-1}$) of HTO in blood wate after a 90 min equilibration period

2. Fractional clearance rate (k, day^{-1}) and half-time ($t_{1/2}$, day) for TBW turnover

   $k = \ln (SA_0/SA_1)/\Delta t^{-1}$

   $t_{1/2} = (\ln 2)/k$

   a) SA_0 and SA_1 = specific activity of HTO in blood water at times t_1 and t_2

   b) $\Delta t = t_2 - t_1$

3. Water flux rate, r_w, for constant body mass (BW) and TBW. Body mass changes of less than $\pm 10\%$ are considered constant.

$$r_w \text{ (ml/day/kg)} = k \times TBW \div BW$$

4. Water influx, $r_{w,in}$, and efflux, $r_{w,out}$, for changes in body mass greater than $\pm$ 10%

$$r_{w,out} = \frac{(N_t - N_0) \cdot \ln (SA_0 \cdot N_0 / SA_t - N_t)}{\ln (N_t / N_0) \cdot \Delta t}$$

$$r_{w,in} = r_{w,out} + \frac{N_t - N_0}{\Delta t}$$

a) N_0 and N_t = TBW (HTO space) measured initially and after recapture with a second injection of HTO.

b) SA_0 and SA_t = specific activity of HTO in blood water initially (t_i) and after recapture (t_2).

c) Δt = time between blood samples.

These techniques have been validated for organisms as different as desert beetles and bees, or as different as desert ungulates and aquatic mammals, and should be readily applicable to fishes and invertebrates in mariculture settings.

4

BIOCHEMICAL PREPARATIONS AND MEASUREMENTS

Isolated Cell Preparations : Hepatocytes

As already impiled above, many fundamental problems in environmental physiology and biochemistry can best be explored using cell suspensions rather than tissues, organs, or whole organisms. Hepatocytes are one of the most useful of such preparations and probably the easiest to prepare. Interestingly enough, although this technique is now very widely used in mammalian and fish studies, it was first described only 15 years ago for rat liver, while the isolation of fish liver cells became routine only about 5 years ago. The basic principle of the technique involves isolating the liver, perfusing it until all blood is replaced with perfusing medium, then digesting the intercellular matrix with collagenase to release free liver cells.

In a typical protocol, the experimental fish is anesthetized as described above, a ventral incision is made and the intestinal or hepatic portal vein is catheterized. Using a peristaltic pump, the liver is cleared of blood by perfusion with well oxygenated Medium A (Table 5), the flow rate being adjusted to assure good oxygenation for the liver cells during the course of the isolation (2 ml min^{-1} g of liver). At this point, some minor massage of the liver may enhance blood clearance, decrease red blood cell contamination and increase the yield of liver cells.

When the perfusate exiting the liver drainage is clear (free of RBC) the perfusion medium is switched to include collagenase (Medium C, Table 5). The concentration of collagenase is chosen (usually less than 0.6 mg ml^{-1}) to deliver a well digested liver within

30 to 45 min of perfusion. Perfusion with Medium C is maintained, sometimes with recirculation, until the liver begins to disintegrate. The liver is transferred to a petri-dish containing ice-cold Medium A, sliced into a mince with a razor blade, then poured through two successive screens of plankton netting (253 and 73 μm respectively). Cells are collected by refrigerated centrifugation (40 to 100 x g) for 2–3 min. The resulting supernatent is decanted and the cells are resuspended in Medium B (Table 5). The hepatocytes are washed 3 times with Medium B, each time being harvested by centrifugation. The cells are then suspended in a small volume of Medium B, weighed, counted in a hemocytometer and tested for viability (Table 2). For metabolic measurements, 25–50 mg fresh weight of liver cells are used per ml of suspension medium.

TABLE 5. *Perfusion media for the isolation of viable salmonid hepatocytes. Modified from French* et al. (1981)

MEDIUM A (for perfusion and for first isolation)
176 mM NaCl
5.4 mM KCl
0.81 mM $MgSO_4$
0.44 mM KH_2PO_4
0.35 mM Na_2HPO_4
4 to 8 mM $NaHCO_3$, depending upon fish species*
10 mM HEPES as a buffer
*$NaHCO_3$ is added after gassing the medium with 0.5% to 1% CO_2 (balance O_2) for 30 min. pH is adjusted to pH 7.6 at 20°C.
MEDIUM B (for washing and for incubation in metabolic experiments)
Medium A to which is added
2% bovine serum albumin, fatty acid free
1 mM $CaCl_2$
MEDIUM C (for digestion)
Medium A to which is added
0.6 mg/ml collagenase *(Clostridium* sp.)

A number of tests for viability of hepatocytes are commonly utilized (Table 6); of these, the maintenance of intracellular ATP levels is usually considered the most sensitive criterion for viability. In liver cell preparations from the American eel, ATP content increases slightly during incubation as does energy charge due to slight decreases in both ADP and AMP (Moon *et al.*, 1985).

Those fish species which have been analysed tend to rely mainly of protein and lipid as energy sources, but poorly utilize carbohydrates. White muscle, the major tissue mass of fish, is poorly perfused and anaerobic, and relies on glycogen breakdown to main-

tain contractile activities. The ultimate source of muscle glycogen is glucose produced by gluconeogenesis from precursors including amino acids (exogenous and endogenous) and lactate (from anaerobic glycolysis). Liver is one of the main sites of gluconeogenesis in vertebrates, including fish.

TABLE 6. *Some useful criteria for assessing quality of hepatocyte preparations*

1. dye exclusion (trypan blue)
2. exclusion of succinate (assayed by lack of succinate sparking of O_2 uptake)
3. low rates of leakage of cytosolic and mitochondrial enzymes into the incubation medium
4. ATP concentrations not significantly different from the freeze-clamped liver
5. maintenance of interacellular hydrogen ion concentration (PH_i)
6. maintenance of intracellular potassium ion concentration
7. polysomal profiles in sucrose gradient centrifugation

In assessing gluconeogenic flux in fish hepatocytes it is important to correct for the continuous conversion of relatively high amounts of glycogen to glucose during incubation. Generally this requires use of isotopically labelled glucose precursors. Glycogenolysis can be modified by hormones, calcium, substrate availability and pH, but fasting at least in cold-water fish species does not deplete reserves as in mammalian liver cells. Gluconeogenic fluxes are shown for a number of precursors in a number of fish species in Table 7. By and large, flux from lactate exceeds that from any amino acid in any given species (Table 7). As in mammals, alanine and serine are generally the preferred amino acid substrate, probably because they can easily be transformed into pyruvate. From the point of view of environmental physiology and mariculture in tropical waters, the urgent need is to assess (i) environmental conditions most suitable for gluconeogenic function in tropical species and (ii) how specific environmental parameters influence the process.

OTHER CELL PREPARATIONS

The same advantages arising from working with liver cells instead of the intact organ also acrue in studies of other tissues. Generally, the latter are not as easy to prepare. Myocytes, however, can be prepared by collagenase digestion of cardiac muscle or skeletal muscle from juvenile animals. Similarly, isolated renal tubules can be prepared from the kidney. Advantage can sometimes also be taken of tissues whose cell components are naturally dispersed (*e.g.* sperm, RBC or other blood cells).

TABLE 7. *The flux of labelled carbons into glucose, CO_2 and other products by fish hepatocytes. Units are μmols product per g wet weight per hour of incubation (generally at 10°C). L, lipids**

Species	Glucose	CO_2	Lipid
Rainbow trout			
lactate	1.2 to 20.3	11.1, 25.1	—
pyruvate	10.3	—	—
alanine	0.2 to 8.8	2.2 to 5.8	—
serine	1.0 to 5.7	8.5	—
others	1.0 to 5.7		
Sockeye salmon			
lactate	0.9 to 16.5	4.4 to 12.0	—
alanine	0.4 to 4.7	0.2 to 5.7	—
serine	0.5 to 1.5	1.4 to 2.8	—
palmitate	—	0.3 to 1.4	—
Coho salmon			
lactate	7.7	27.6	—
alanine	1.2	7.6	—
Atlantic salmon			
lactate	1.1	3.8	—
alanine	0.04	0.8	—
Sea raven			
glucose (C–1)	—	0.04	—
lactate	0.12	0.8	—
alanine	0.11	0.7	—
serine	0.69	3.7	—
oleate	—	0.005	—
Japanese eel			
lactate	23.8	—	—
pyruvate	15.9	—	—
alanine	11.4	—	—
American eel			
lactate	0.6 to 13.0	0.3 to 1.9	4.9
glycerol	19.4	1.1	4.7
alanine	0.1 to 12.4	0.5 to 6.9	7.4
aspartate	0.07 to 1.1	nil to 0.9	4.5
Rat (25 °C)			
lactate	107	—	—
glycerol	78	—	—
alanine	64	—	—
serine	39	—	—

*Modified from Moon *et al.* (1985)

Mitochondria also can be used to conveniently monitor environmental stresses on aquatic organisms. For example, the salinity adaptations of mitochondria from marine organisms differ from those in homologous tissues of freshwater organisms; trimethylamine and urea effects on mitochondria from elasmobranchs probably differ substantially from effects found with preparations from teleosts. Mitochondrial function also may show a great deal of tissue and species specificity with respect to environmental changes.

TABLE 8. *Buffer media for heart or muscle mitochondria from fish*

1.	Isolation Buffer, pH 7.3 at 25 °C
	210 mM mannitol
	70 mM sucrose
	10 mM EDTA
	100 mM Tris-HCl or 125 mM HEPES
	0.1% BSA (optional)
2.	Assay Buffer, pH 7.3 at 25 °C
	210 mM mannitol
	70 mM sucrose
	10 mM EGTA
	10 mM KH_2PO_4
	100 mM Tris-HCl

Note: 0.1% BSA helps to stabilize mitochondria, particularly when yields are low. If BSA is not included in initial isolation procedure, final mitochondrial suspension should include 0.1% BSA. 5 mM$MgCl_2$ sometimes stabilizes muscle mitchondria.

A typical protocol for the isolation of mitochondria from cardiac muscle or red muscle in fish might run as follows, with the entire procedure preferably done on ice or at less than 4°C.

1. About 2–5 g tissue are dissected out and placed in ice-cold isolation buffer (Table 8), then chopped and decanted (as much blood as possible is cleared away).

2. The preparation is then taken up in about 30 ml isolation buffer containing 22.5 mg NAGARSE (or Sigma Protease P 5255), then incubated on ice for 10 min, with occasional stirring.

3. The tissue is then homogenized **very gently** using a hand homogenizer (teflon pestle, loosefitting); 2 passes are usually adequate.

4. The homogenate is centrifuged 600 x g for 5 minutes (at this stage, the mitochondria remain in supernatant, which is decanted). The mitochondrial phase is then centrifuged at 9000 g for 7–10 min in a refrigerated centrifuge.

5. The supernatant now is discarded and the pellet is carefully resuspended in isolation buffer; this is again centrifuged at 9000 g for 7–10 min.

6. Step 5 is repeated 4 more times.

7 (a). If no BSA has been included to this point, the pellet is suspended in BSA-free isolation buffer, usually 1 ml (aliquots are taken for protein or enzyme determinations). An identical volume of isolation buffer (1 ml) containing 0.2% BSA is added so that the final concentration of BSA in suspension is 0.1%.

(b). If isolation buffer initially contained 0.1% BSA, then the pellet is suspended in isolation buffer containing BSA (usually 1 ml aliquots are taken for protein or enzyme determinations as is an aliquot of the isolation buffer to determine its protein content). An identical volume of isolation buffer (1 ml) containing 1.0% BSA is added so that the final concentration of BSA in suspension is 0.1%.

For white muscle or smooth muscle in fish, essentially the same procedure is used except much more tissue is required for the same mitochondrial yield. Modifications in our laboratory (Donaldson, pers. comm.) are as follows:

1. About 50 g of white muscle (instead of 2–5 g) are chopped up initially.

2. The batch is divided in half (25 g each) and each half is put in 60 ml isolation buffer with 45 mg NAGARSE (or Sigma Protease P 5255) and incubated for 14 min.

3. Usually these will have to be further divided for homogenization. For example, we routinely divide each lot (25 g) into 3 fractions divide (approx. 8 g) and homogenize in 25-30 ml of isolation buffer.

Mitochondrial Protein Determination

Usually the Biuret method is used with Na-deoxycholate to solubilize mitochondrial membranes:

50 µl mitochondrial suspension
100 µl 10% Na-deoxycholate, pH 13.2 (KOH)
50 µl water
800 µl Biuret reagent (and store in brown bottle)

1.5 g $CuSO_4$. $5H_2O$	
4.9 g Na.K.Tartrate	1 litre
7.5 g NaOH	

The cocktail is heated in a water bath for about 1 min, then the OD at 540 nm is determined, using BSA of varying concentrations as standards.

It should be noted that this assay is insensitive below 0.25 mg, therefore with 50 µl samples, the lower limit is 0.5% protein. Also, Tris buffers interfere with the assay and thus should not be used. If mitochondrial yields are low enough to make the Biuret assay untenable, then modified Lowry method can be used as described by Bensadown and Weinstein (1976). The assay strategy of this method is to precipitate protein, then redissolve it and react it with Lowry and Folin-Ciocalteu reagents, a process which removes interference from any number of chemicals, buffers, etc. We have further modified this strategy to meet needs of mitochondrial assays. In the first place, we include high (Na-deoxycholate) at the start to solubilize the membranes; (Na-deoxycholate) must then be reduced to an appropriate degree for protein precipitation. The medium is then adjusted to bring final concentration within range of the assay (5–50 µg).

A typical protocol might include the following steps:

1. Mitochondrial samples (30–50 µl) are diluted to 100 µl; *i.e.* about 2–3 fold.

2. To the 100 µl sample, Na-deoxycholate is added to a 10% concentration and incubated 1–2 min until membranes are solubilized.

3. Aliquots (10–15 µl) are then removed to 12 x 100 mm culture tubes and diluted upto 3 ml with distilled water, mixed vigourously then let to stand for 15 min.

4. At this time, 1 ml of 24% TCA is added and mixed, then it is centrifuged at 3300 g for 30 min in Swinging Bucket Rotor.

5. The supernatant is then aspirated with a Pasteur pipette to remove as much liquid as possible.

6. The pellet is dissolved in 1.5 ml Lowry reagent C and mixed vigourously for 10–30 sec. The composition of Lowry C reagent is:

61.7% $Na_2Co_3.H_2O$
35.7% distilled H_2O
1.3% $CuSO_4.5H_2O$
1.3% $NaKC_4H_4O_6.4H_2O$

Then 0.15 ml Folin-Ciocalteu reagent is added (diluted 1:1 with water just before use).

7. The colour is developed in the dark for 45 min, then the OD is read at 660 nm, again using BSA as a standard.

Tissue and Blood Sampling and Preparation

For measurements of metabolically or chemically labile metabolites, as well as for ions whose distribution between compartments is linked to intact membrane and metabolic functions, it is desirable to instantaneously 'stop' metabolism; the goal is in effect to obtain a 'snap shot' of metabolism at a given instant in time. Of course this goal cannot actually be achieved, but it is approximated by various quick freezing techniques followed by standard perchloric acid (PCA) extraction.

To fix tissue rapidly, one uses flat tongs with aluminium jaws which can be pre-cooled with liquid air or liquid nitrogen; these are called 'quick-freeze' tongs or clamps and can be built in any workshop. The tissue gripped between the cold jaws is compressed to form a thin layer and protruding tissue is broken off. Tests of this method indicate that 1.6 g of guinea pig kidney can be cooled to -160°C in 0.5 sec. Most tissue can be stored in the frozen state (at -80°C) for at least week-long periods. At -20°C, storage time is reduced for highly labile metabolites, but even several weeks of storage would be fine for stable metabolites.

For subsequent analysis, a weighed amount of frozen tissue is powdered (with periodic additions of liquid N_2) with a mortar and pestle, then added to a known amount of 6% PCA (about 0.2 g tissue per ml PCA). The PCA extract can then be further homogenized (using an Ultraturax homogenizer, for example), although for most metabolites this does not increase the yield.

The homogenate is then spun in any available centrifuge and the supernatant is decanted. For stable metabolites, such as lactate and glucose, this PCA extract can be directly used for assay in a well buffered assay medium. For unstable compounds, such as CrP and ATP, the PCA extract is neutralized with known volumes of 1 M KOH; the K^+ salt of perchlorate precipitates out leaving a clear supernatant which can be used for metabolite assays.

In the case of blood metabolites, the freezing steps above may be omitted and the blood sample is added directly to a known volume of PCA (*e.g.* 1 ml blood to 2 ml 6% PCA). The brown precipitate is spun down and the supernatant can be used directly for metabolite assays, or it is first neutralized, then used for analysing the concentrations of intermediates of interest. After corrections for dilution, these procedures yield information on metabolite content in **whole** blood; for plasma concentrations, the component cells must first be centrifuged down and only the plasma used for metabolite assays. For many assays, plasma may be used directly, but in some cases, it is useful to PCA extract the plasma as well.

Principles of Enzyme Coupled Metabolite Assays

If a metabolite is involved in an enzymatic reaction, the reaction can be used for the quantitative assay of the metabolite by enzymatic analysis of reactants or products. If the conversion of the metabolite is practically complete, enzymatic analysis is particularly simple and the result can be easily calculated with the aid oft he known physical constants of the substrate (*e.g.* the extinction coefficient in the case of light-absorbing substances).

The most widely used assays are those coupled to NAD^+-dependent and $NADP^+$-dependent dehydrogenases. In general, these reactions can be described as follows:

$$S_1 + \text{coenzyme} \longrightarrow P_1 + \text{coenzyme H} + H^+$$

$$\text{or } S_2 + \text{coezyme H} + H^+ \longrightarrow P_2 + \text{coenzyme}$$

Two specific examples might include:

$$\text{ethanol} + NAD^+ \longrightarrow \text{acetaldehyde} + NADH + H^+$$

$$\text{or pyruvate} + NADH + H^+ \longrightarrow \text{lactate} + NAD^+$$

By sequential addition of several enzymes, accurate determinations can be made of several metabolites in one cuvette. In general, several auxilliary reactions are carried out successively as a 'chain', with one substrate reacting in each of these reactions at a time (Fig. 1).

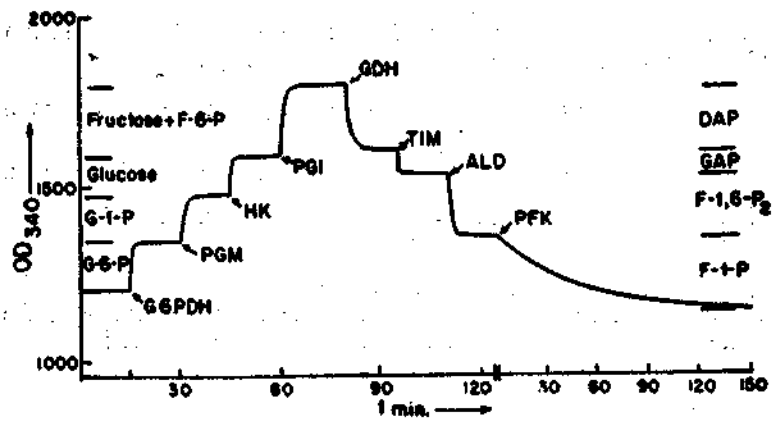


Fig. 1. Determination of a mixture of sugars and sugar phosphates in a single cuvette. Modified from Bergemeyer (1974).

The following symbols are commonly used:

ε extinction coefficient (6.22 cm² / µmole for NADH or NADPH)
E extinction

ΔE extinction change
V assay volume (ml)
v volume of sample used in assay (ml)
t time (min)

Δt interval between measurements (min)
d light path (cm)
c concentration (µmole/ml), (µg/ml)
MW weight of one micromole (µg)

From Lambert-Beer law,

$$c = \frac{E}{\varepsilon \times d} \text{ (µmole/ml)}$$

For enzyme reactions,

$$c_1 - c_2 = \frac{E_1 - E_2}{\varepsilon \times d}; \quad \Delta c = \frac{\Delta E}{\varepsilon \times d} \ (\mu\text{mole/ml})$$

If conversion is complete ($c_2 = 0$),

$$c = \frac{\Delta E}{\varepsilon \times d} \ [\mu\text{mole/ml}] \qquad \text{(in the cuvette)}$$

To calculate the concentration of the tissue or blood PCA the ratio of assay volume: sample (V:v) is to be considered:

$$c = \frac{\Delta E \times V}{\varepsilon \times d \times v} \ [\mu\text{mole/ml}] \quad \text{(in the PCA extract)}$$

$$c = \frac{\Delta E \times V \times MW}{\varepsilon \times d \times v} \ [\mu\text{g/ml}] \quad \text{(in the PCA extract)}$$

Finally, (tissue or plasma) $= \left[c_{\text{(Extract)}}\right]$ (dilution factor).

Enzymatic analysis for lactate, for *e.g.* depends upon LDH, available commercially from several sources (or preparable in the home laboratory). For example, a typical cuvette contains.

0.10 ml NAD^+
0.10 ml PCA extract (with unknown amount of lactate)

0.75 ml glycine-hydrazine buffer (pH 9.2)
0.05 ml LDH solution (empirically determined minima amounts)

The reaction is allowed to go to completion, usually 5–10 min will do; change in optical density (OD) at 340 nm may be monitored continuously or periodically. A typical protocol for the determination of, say, lactate, in fish liver may run as follows. To prepare the initial extract, 1 g of fresh liver is homogenized in 7.25 ml of 6% PCA. With a value of 75% for the fluid content of the liver, the volume of the extract is 7.25+0.75 = 8.00 ml. To neutralize and remove perchlorate, 0.2 ml of K_2CO_3 or KOH is added to 6 ml of the extract. The volume of the perchlorate-free extract is thus 6.2 ml. The dilution factor for the extract is 6.2/6.0–1.033 and that for the tissue is 8 x 6.2/6.0 = 8.264. The experimental data must be multiplied by these values to express the results per ml of acid extract or per g of tissue water. Assuming that the measured change in extinction at 340 nm ($E = 6.22\ cm^2/\mu mole$) is $\Delta E = 0.065$, the volume of the assay solution is 1 ml, the volume of sample is 0.1 ml, and the light path is 1 cm, then the concentration in the perchlorate-free extract used for the assay is

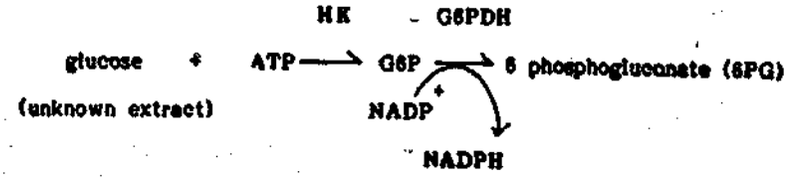
$$c = \frac{0.065 \times 1}{6.22 \times 0.1} = 0.105 \ \mu\text{mole lactate/ml}$$

Multiplication by the dilution factor gives the concentration in the PCA extract:

$$c = 0.105 \times 1.033 = 0.108 \ \mu\text{mole lactate/ml}$$

The content of lactate in the tissue is
$0.105 \times 8.26 = 0.158$ μmole 0.867/g of tissue water
If no correction is made for the tissue water content, this value would be only 75% of the estimate concentration of 1.147 μmole lactate per g tissue.

For glucose, exactly the same procedure is utilized except that 2 enzymes are required:



The reaction cuvette (total volume 1 ml) now contains:

0.1 ml $NADP^+$ (buffered to the assay pH, final concentration, 1 mM)
0.1 ml ATP (buffered to the assay pH, final concentration 5 mM)
0.65 ml buffer (Tris HCl, 50 mM, Mg^{++} 10 mM, pH 7.5)
0.05 ml G6PDH (in excess, again empirically determined, usually about 1 unit/ml)
0.1 ml unknown extract

The reaction is run to the first new steady absorbance (usually only a few minutes are required for the endogenous G6P to be converted to 6PG). Then 0.05 ml or about 1 unit of HK is added to kick off the phosphorylation of glucose. When the final OD reading is obtained, the glucose concentration is calculated as above for lactate.

For glycogen, the assay principle is the same as for glucose, but the extracted glycogen must first be hydrolyzed to glucose. Two

procedures are commonly used: PCA extraction of glycogen or extraction in boiling 30% KOH followed by precipitation with 65% ethanol. In the case of PCA extraction, the extract must be neutralized (so also removing the perchlorate). Quantitative hydrolysis of the extracted glycogen is obtained with amyloglycosidase (available commercially) at pH 4.8, 40°C, incubated with shaking for a period of 2 hours. Acetate buffer (0.2 M, pH 4.8) can be used for this purpose by mixing 4.8 ml 96% acetic acid and 9.75 g sodium acetate in distilled water and making up to 1000 ml total volume. The incubation is stopped by addition of a known volume of PCA. As before, dilutions must be carefully monitored and appropriate corrections must be made for the final calculations of glycogen as glucosyl units in μmole/g tissue.

If amyloglucosidase is unavailable, the glycogen extract can be hydrolyzed by boiling in 1 N H_2 SO_4 for at least 2 hours. The hydrolyzate is then neutralized and the released glucose can be assayed as above.

Extraction of Soluble Enzyme

For many studies in environmental physiology and biochemistry a knowledge of the amount of enzyme or isozyme kind present in a given tissue under a given environmental condition is extremely useful. As in metabolite studies, it is useful in enzyme studies to work with tissue as soon as possible after sacrificing the organism. For example, for assessing total glycolytic enzyme content, soluble enzyme content and bound enzyme content in fish hearts the following protocol may be useful:

Either whole hearts or samples of heart tissue are homogenized in 3 vol of ice-cold 0.25 M sucrose containing 1 mM dithiothreitol for 15 sec using an Ultraturrax homogenizer at full speed. Homogenates of 1 ml volume are immediately centrifuged for 4 min in an Eppendorf microcentrifuge at 23000 x g. After centrifugation the supernatant is diluted with 4 vol of a stabilization buffer containing 0.1 M potassium phosphate, 1 mM EDTA, 2 mM dithiothreitol, 0.1 mM fructose, 1,6-bisphosphate (fructose bisphosphate) and 0.1 mM ATP, pH 7.5. Enzyme activities in this supernatant are taken as a measure of the amount of soluble enzymes. The pellet is twice extracted with 1 ml of stabilization buffer by resuspension and centrifugation. This procedure usually is sufficient to extract all previously bound enzymes. The enzyme activities in the combined extracts are taken as a measure of the amount of enzyme bound. As a control, a further 1 ml of the homogenate is diluted to 5 ml

with stabilization buffer and kept for measurement of total enzyme activity to check on recovery in the supernatant and pellet fractions. Recoveries range from 95–105%. Although this protocol is useful for all glycolytic enzymes, as described, it is designed to stabilize PFK activity (without perturbing other glycolytic enzymes).

Extraction of Enzymes Loosely Bound to Membranes

Enzymes such as citrate synthase (CS), hydroacyl CoA dehydrogenase (HOAD), and glutamate dehydrogenase (GDH), while membrane bound, can usually be solubilized relatively easily either by means of a freeze-thaw cycle or by repeated extractions of homogenates prepared in dilute buffers (which break up organelles). We routinely use 50 mM imidiazole buffer, pH 7, containing 50 mM KCl, 7–10 mM $MgCl_2$ and 5 mM EDTA. The homogenate is centrifuged at about 3000 g for 10 or 20 min and the supernatant decanted and saved for enzyme assay; the pellet is resuspended, rehomogenized after freezing and thawing, to extract any remaining enzyme. Detergents at low concentration (*e.g.* 1% Triton X-100) can be used to solubilize membrane bound acetylcholinesterases.

More tightly bound membrane-based enzymes are difficult to work with; the interested student should search the primary literature for means of solubilization, problems and possible resolutions (for example Hazel, 1984).

Basis for Interpreting Enzyme Activity Levels

It was first clearly documented some 2 decades ago that glycolytic enzyme activities are elevated in tissues adapted for anaerobic function, while enzymes of the Krebs cycle and β-oxidation, in particular CS and HOAD, correlate with oxidative capacities. These studies form the basis for the concept of 'constant proportion groups of enzymes' and have received refinement in later studies. Thus, although CS activities/g supplies a good index of a tissue's maximum oxidative capacity both in inter-tissue and in interspecies comparisons, HOAD does not always co-adapt with CS. This is strikingly evident in systems like bee flight muscle, where capacities for fat oxidation are extremely low, but vigorous glucose oxidative capacities are expressed and where HOAD/CS ratios are consequently vanishingly small. On the other hand, in tissues with a strong fat dependent, HOAD may be more noticeably elevated than CS activity. Because of such obvious needs for adjustment in mitochondrial enzymatic composition, CS activity is now more commonly related to oxidative capacity while HOAD

activity is used more specifically as an index of relative capacity for oxidation of fatty acids. To facilitate comparisons of tissues displaying widely differing absolute enzyme activities, lactate dehydrogenase (LDH)/CS activity ratios are utilized to assess relative capacities for anaerobic *vs* aerobic metabolism, while HOAD/CS ratios supply an index of the relative potentials for fat oxidation *vs* overall aerobic catabolism. An indication of dependence upon aerobic glycolysis *vs* anaerobic glycolysis is sometimes difficult to assess, because all the enzymes in the pathway, except LDH, are utilised in both processes. However, LDH activity/g has been found to correlate well with anaerobic capacities of homologous tissues in interspecies comparisons. On the other hand, in comparing systems where the LDH/CS ratios are the same, the activity ratios of pyruvate kinase (PK)/LDH, or indeed the activity of any preceding glycolytic enzyme/LDH, may supply a relative index of aerobic glycolytic *vs* anaerobic glycolytic capacity. Thus to obtain a qualitative impression of metabolic organization and metabolic capacity in tissues of organisms adapted to different environmental circumstances, it may be acceptable as a first approximation to assume:

1. that CS activity/g yields an approximate measure of relative oxidative capacities of homologous tissues,

2. that LDH/CS activity ratios yield a measure of relative anaerobic *vs* aerobic metabolic capacities,

3. that, where LDH/CS ratios are similar, PK/LDH activity ratios yield a measure of the relative capacities for aerobic glycolysis,

4. that HOAD activity/g yields a measure of relative capacities for fatty acid oxidation, and

5. that HOAD/CS activity ratios yield a measure of how closely β-oxidation and Krebs cycle maximum capacities are co-adapting.

While for qualitative interspecies comparisons of homologous tissues these assumptions are not controversial, the reader may find it useful to examine the other experimental and theoretical bases for them elsewhere (Hochachka *et al.*, 1982, for literature in this area).

Typical Assay Protocols for Some Metabolic Enzymes

As a large number of enzymes and tissues may come under study in any large environmental physiology study of mariculture species, it is not possible to anticipate all protocols which may be

desirable. However, a number of potentially useful enzyme assay conditions are presented below. Usually, activity is monitored at 340 nm using the appearance or disappeance of NADH or NADPH (with the exception of citrate synthetase which is monitored at 412 nm with 5,5'–dithiobis (2–nitrobenzoic acid) (DTNB) as the optically active substance). All assays are done in a Unicam SP 1800 spectrophotometer plus recorder at 25°C, pH 7.0, in 50 mM imidazole buffer, with the exception of citrate synthase which was assayed in 50 mM Tris-HCl buffer, pH 8.0.

Phosphorylase: 50 mM sodium phosphate buffer, pH 7.0, 0.4 mM NADP, 4 μM glucose-1,6–diphosphate, 1.6 mM AMP, 10 mM MgCl, 2 mg/ml glycogen (omitted for control), excess phosphoglucomutase and excess glucose-6-phosphate dehydrogenase.

Phosphofructokinase (PFK): 10 mM $MgCl_2$, 100 mM KCl, 0.15 mM NADH, 2 mM ATP, 5 mM fructose-6-phosphate (omitted for control), excess alphaglycerophosphate dehydrogenase, aldolase, and triosephosphate dehydrogenase.

Aldolase: 0.15 mM NADH, 0.02 mM fructose-1,6-diphosphate (omitted for control), and an excess of alpha-glycerophosphate dehydrogenase and triosephosphate isomerase.

Pyruvate kinase (PK): 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM NADH, 5 mM ADP, 5 mM phosphoenolpyruvate (omitted for control) and excess lactate dehydrogenase.

Lactate dehydrogenase (LDH): (high pyruvate) 10 mM pyruvate (omitted for control), and 0.2 mM NADH; LDH (low pyruvate), 1 mM pyruvate, and 0.2 mM NADH.

Adenylate kinase: 2 mM AMP (omitted for control), 2 mM ATP, 0.5 mM phosphoenolypyruvate, 4 mM $MgSO_4$, 150 mM KCl, 0.2 mM NADH, and excess pyruvate kinase and lactate dehydrogenase.

Glycerol-1-phosphate dehydrogenase (aGPDH): pH 7.8, 0.2 mM NADH, 0.4 mM dihydroxyacetone phosphate (omitted for control).

Citrate synthase: 0.1 mM DTNB, 0.3 mM acetylCoA (omitted for control), 0.5 mM oxaloacetate. The assay is monitored at 412 nm, the DTNB and acetylCoA being added first to obtain a measure of acetylCoA deacylase activity and the oxaloacetate

added last to start the reaction. The extinction coefficient of DTNB under this condition is 13.6 $cm^2/\mu mole$.

β-hydroxy butyrylCoA dehydrogenase (HOAD): 0.2 mM NADH 0.1 mM acetoacetylCoA (omitted for control).

Glucose-6-phosphate dehydrogenase: 7 mM $MgCl_2$, 0.4 mM NADP, 1 mM glucose-6-phosphate (omitted for control).

Malate dehydrogenase (MDH): 0.2 mM NADH, 0.5 mM oxaloacetate (omitted for control).

Glutamate-oxaloacetate transaminase (GOT): 40 mM aspartate, 7 mM α-ketoglutarate (omitted for control), 0.2 mM NADH and excess malic dehydrogenase.

Glutamate-pyruvate transaminase: 0.2 mM alanine, 10 mM α-ketoglutarate (omitted for control), 0.2 mM NADH and excess lactate dehydrogenase.

Glutamate dehydrogenase (GDH): 250 mM ammonium acetate, 0.10 mM NADH, 0.1 mM EDTA, 1 mM ADP, 14 mM α-ketoglutarate (omitted for control).

Fructose-1,6-diphosphatase (FDPase): 6 mM $MgCl_2$, 0.02 mM fructose-1, 6-diphosphate (omitted for control), 0.2 mM NADP, and excess phosphoglucoisomerase and glucose-6-phosphate dehydrogenase.

Creatine phosphokinase (CPK): 50 mM CrP, 0.5 mM ADP, 10mM glucose, 1.0 mM $NADP^+$, HK, and G6PDH in excess.

Arginine phosphokinase (APK): 50 mM arginine phosphate; other conditions as in CPK assay. Otherwise, arginine release can be monitored with octopine dehydrogenase. The latter unfortunately is not available commercially and therefore has to be prepared in the homel aboratory.

A third APK assay includes 20 mM L-arginine (omitted for control), 5.0 mM ATP, 1.0 mM phosphoenolpyruvate, 0.05 mM NADH, 8 units lactic dehydrogenase and 24 units pyurvate kinase, in imidazole buffer (50 mM) pH 7.40. The rate of reaction in the absence of arginine (ATP-hydrolysis) was less than 10% of the values given in Table 3.

Proline dehydrogenase: 20 mM L-proline (final concentration), (omitted for control), 10 μM rotenone, 4 μM cytochrome *c* in 75 mM KCl, 12.5 mM potassium phosphate, 0.25 mM EDTA, 5 mg/ml bovine serum albumin, pH 7.40. Final volume after the addition of homogenate 1.0 ml. After terminating the reaction through addition of 50 μl perchloric acid (70%), the liberated pyrroline carboxylate was determined spectrophotmetrically with *o*-aminobenzaldehyde.

Ornithine δ-aminotransferase: 60 mM L-ornithine (omitted for control), 21 mM 2-oxoglutaric acid, 25 μM pyridoxal phosphate in 50 mM imidazole pH 7.40, final volume 1.0 ml. The reaction is stopped with 50 μl perchloric acid and pyrroline carboxylate is measured as above. Glutamate was measured with glutamate dehydrogenase and acetylpyridine dinucleotide.

Pyrroline-carboxylate reductase: 1.05 mM pyrroline carboxylate (omitted for control), 0.15 mM NADH, in imidazole buffer (100 mM) at pH 7.00. Final volume was 1.0 ml and the decrease in absorbance at 340 nm was recorded continuously. DL-pyrroline carboxylate was liberated from its 2,4-dinitrophenylhydrazone (14) and measured with *o*-aminobenzaldehyde before each assay.

Octopine dehydrogenase: 15 mM arginine (omitted for control), 1.0 mM pyruvate, 0.15 mM NADH, imidazole buffer (50 mM) pH 7.0

Isocitrate dehydrogenase: 0.6 mM *threo*-isocitrate (omitted for control), 10 mM $MgCl_2$, 0.4 mM $NADP^+$, imidazole buffer (50 mM) pH 7.0.

Arginase: 250 mM L-arginine (omitted for control), 1 mM $MnCl_2$, at pH 9.50. Final volume 1.0 ml. The reaction is stopped with 50 μl perchloric acid, and urea is measured with 1-phenyl-1,2-propanedione-2-oxime.

For purposes of calculating enzymes activities in μmols per min, the rate of substrate conversion or product accumulation (equivalent to coenzyme oxidation or reduction) is calculated, usually as a change in concentration per time unit (min). In the assay mixture the enzyme activity per ml is determined as follows:

$$\text{activity} = \Delta c/\Delta t = \frac{\Delta E}{\varepsilon \times d}/\Delta t \quad [\mu\text{mole/ml per min}]$$

$$\text{activity} = \frac{1000}{\varepsilon \times d} \times \Delta E/\Delta t \quad [\text{U/L}] \text{ (in assay mixture)}$$

Correcting for dilution factors in preparing the initial homogenate, one obtains enzyme activity in μmole/g per min or in international units (U)/g per min at specified temperatures (at least one temperature used should always be 25°C to facilitate comparisons with other studies in the literature).

Kinetic Tests for Direct Enzyme-to-Enzyme Substrate Transfer

Finally in this section, we should bring attention to recent techniques for distinguishing between direct transfer of substrates from enzyme to enzyme *vs* dissociation of product of enzyme-1 into solution, followed by competition by enzyme-2 for that metabolite as its substrate. All experiments along these lines, at least thus far, involve the diminution of the aqueous metabolite concentration by the formation of an enzyme-substrate complex in the presence of excess enzyme over metabolite. For dehydrogenases, for example, the kinetic approach can be generalized by the equation

$$E_1 - NADH + S_2 \xrightarrow{E_2} E_2 - NAD^+ + P_2$$

In each experiment, one dehydrogenase is utilized in high substratelike concentration as a potential carrier of substrate quantities of NADH (E_1-NADH) to participate in the specific reduction reaction catalyzed by nanomolar quantities of a different dehydrogenase (E_2), for example, the LDH-catalyzed reduction of pyruvate by NADH in the presence of a molar excess of GPDH over NADH:

$$\underset{\substack{\text{(substrate-like}\\ \text{concentration)}}}{GPDH - NADH} + \text{pyruvate} \xrightarrow{LDH} \underset{\substack{\text{(nanomolar}\\ \text{catalytic}\\ \text{concentrations)}}}{LDH - NAD^+} + \text{lactate}$$

Obviously in such reactions NADH free in solution is a competent substrate (coenzyme) for carrying out the specific catalyzed reduction. Thus, the kinetic alternatives are either of the following: (i) Addition of a stoichiometric excess of E_1 over NADH may reduce the free aqueous NADH concentration to a low buffered value. In this event, the rate of reduction of S_2 catalyzed by E_2 is greatly reduced according to known Michaelis parameters for the E_2-catalyzed reaction. (ii) The carrier dehydrogenase-NADH complex (E_1 - NADH) may itself be a substrate in the E_2 - catalyzed

reduction of S_2. If E_1-NADH is utilized with comparable efficiency to the aqueous NADH, the direct transfer mechanism may be apparent due to the very large excess of E_1-NADH over unliganded NADH. To make such a distinction, it is important to have precise estimates of the dissociation equilibrium constants for E_1-NADH plus the K_m (for NADH) and V_{max} for each E_2-catalyzed reaction. The equilibrium dissociation constants (K_d) and K_m for NADH are both solvent dependent, so that these must be empirically determined in each new study. The main experimental problem with this approach is that, since the carrier enzyme (E_1) is present in nearly 10^6-fold molar excess over the catalytic enzyme (E_2), it is necessary to check that any particular carrier enzyme preparation be free from E_2 contamination at a level $<<10^6$. Such impurities in the carrier enzyme can be quantitatively observed, even at exceedingly low contamination levels. Thus these experiments require the highest degree of enzyme purity.

In general, the expectation is that if NADH is the only competent substrate, the presence of substratelike concentrations of E_1-NADH will lead to a reduction in the reaction velocity (because solvent NADH concentration is reduced because most of it is liganded to E_1). In this event, reaction velocities are determined by the rate of E_1-NADH dissociation and are always observed to be very slow. On the other hand, with appropriate pairs of dehydrogenases, it is observed that reaction velocities are so much faster than would otherwise be found that the direct transfer of NADH from E_1 to E_2 is considered to be necessarily operative (Srivastava and Bernhard, 1985).

Replacement or Turnover Rates of Plasma Metabolites

Perhaps the most useful kinds of data one can obtain in environmental physiology and biochemistry are those describing the flux rates or turnover rates of specific metabolites. O_2 consumption rates are of course equivalent to O_2 flux rates through the plasma compartment and no one argues about how useful such measurements are; that is why they are so abundant in the literature. Surprisingly, the flux rates of other plasma metabolites, particularly substrates for metabolism, are rarely measured. For example, there are only a few measurements of the turnover rates of lactate and glucose in the literature, and only one that we are aware of describing the turnover rate of an amino acid (Abe and Hochachka, 1986). In theory there are two techniques readily available for measuringt he turnover rates of metabolites (single bolus injection *vs* continuous infusion of the labelled compound being stu-

died); in both, what is being measured is the rate at which injected 'hot' molecules are being replaced by endogenous 'cold' molecules. For culturable marine organisms only the bolus injection technique is applicable, since it requires the sampling of a smaller total volume of blood.

For fishes, a typical protocol used successfully in our laboratory in studies of tuna and trout run as follows:

When the fish is fully anaesthetized (using MS 222 at 1:22000 w/v), it is placed ventral side on an operating table. The gills are irrigated with aerated 24°C recirculating seawater containing MS 222 (1:15000) to maintain anaesthesia. A catheter (SURFLO I. V. catheter of required dimensions) is inserted at a 45° angle about 2 cm in front of the pelvic fins, directed towards the head for the percutaneous cannulation of the ventral aorta just anterior to the heart. The catheter is connected to a pressure transducer for pressure verification of catheter position. A 40 cm piece of PE 160 is attached to the catheter and sutured to the underside of the animal. Double cannulation (ventral and dorsal aorta) may also be used, with the dorsal aorta catheter establishment through the roof of the mouth and exterioized through a nose cone.

Catheters, PE tubing, and syringes are always rinsed with heparinized saline (10 units/ml) before use. Total catheterization time usually does not exceed 20–35 min. Once the catheter(s) are in place, heart and blood pressure are monitored via the transducer. In the case of tuna, the fish now may be righted, strapped to a plexiglass holder and submerged. The anasthetic concentration is then reduced to approximately 1: 30000 and is adjusted individually for each fish throughout the turnover experiments. In the case of trout, the fish at this point may be allowed to recover for 18–24 hours directly within a swim mill or a metabolic chamber. Steady glucose and lactate levels, steady heart rates, and steady O_2 uptake rates indicate that the assumptions of steady state kinetics required for the bolus injection technique are in fact met.

At this point, a bolus of 25 to 35 μCi U-^{14}C-lactate (specific activity > 100 mCi/m mol), 10 μCi U-^{14}C-glucose (4 mCi/m mol), 25 to 35 μCi 6-^{3}H-glucose (300 mCi/m mol), or any other metabolite of interest is injected via the catheter at time 0. The catheter line is flushed with 2–3 ml heparinized saline immediately after injection. Blood samples (0.5 ml) are drawn starting 1 min after injection to allow the bolus to equilibrate in the rapidly mixing pool. Samples are drawn about every 40 sec during the first 5 min, and

then at appropriate intervals (usually 20–30 min intervals are fine). The catheter is flushed with 0.5 ml saline between samples. The total amount of blood withdrawn throughout any given experiment must never exceed 10% of the animal's blood volume. Blood samples are deproteinized immediately in 1 to 2 parts cold perchloric acid (8%), and spun down. The supernatant is stored at -4°C. Lactate and glucose concentrations are determined enzymatically at 340 nm following the procedure described above. Tissue glycogen of the control fish is determined by the amyloglucosidase hydrolysis technique. All metabolite assays are performed in duplicate, usually within the first few days after sampling.

Blood glucose must be separated to determine its radioactivity. A known volume (200 µl) of blood perchloric acid extract is incubated with 4 ml glucose (1 M) and 0.6 g Amberlite MB-3 resin (Sigma). The mixture is shaken for 2 hr at 25°C, allowing all the charged compounds to bind to the resin. The resin is then spun down and 1 ml of the supernatant is counted. Over 90% of the total glucose is recovered after separation as determined with 6—^{3}H-glucose standards.

Lactate activity is measured by counting 40 µl perchloric acid extract and correcting for the activity found in glucose. Scintillation counting is performed using any modern scintillation counter, preferably allowing for 2-channel (^{3}H and ^{14}C) counting. The specific activities of metabolites such as glucose and lactate decline as labelled molecules are replaced by unlabelled ones, and such decay curves are fitted by multi-exponential functions either by computer assistance or simply graphically. The surface areas under these curves are determined by counting squares or by integrating the functions between 0 and the time when 5% of the maximum specific activity is reached. The maximum possible activity of each metabolite is calculated by dividing the dose injected by the volume of the rapidly mixing pool estimated as 8% of the body volume. Under steady state conditions, the rate of appearance (R_a) equals the rate of utilization (R_d) and is called turnover rate. In such studies, turnover rate is determined by dividing the dose injected (DPM) by the surface area under the specific activity decay curve (in DPM µmol^{-1} min); the units obtained for R_a or R_d are µmol kg^{-1} min^{-1} and are a measure of flux through the plasma compartment. Metabolic clearance rate (MCR) is calculated as turnover rate divided by the steady state metabolite concentration; the units obtained are ml of blood cleared of a given metabolite per minute per kg body weight.

The turnover rates of plasma metabolites are known to be a function minimally of two parameters: metabolite concentration (or availability) and perfusion rate. In addition, the endocrine status of the organism may influence the replacement rates of metabolites and, of course, numerous environmental parameters are likely to do the same. The information becomes far more useful than are simple concentration determinations for the same metabolites.

Turnover Rates of Tissue Metabolites

For certain classes of metabolites, it is more useful to determine the turnover rate, not in the plasma, but in specific tissue compartments. We have had experience along these lines, for example, in examining the turnover and metabolism of histidine-based dipeptides in skeletal muscle of trout and tuna. The main function of thsee compounds is probably in intracellular buffering of H^+ production orconsumption and they are understandably very stable compounds which turn over extremely slowly. Exactly the same principles of metabolite turnover are utilized in these measurements as in the plasma metabolite studies, except that in this case the tissue metabolite pool is sampled, rather than the blood Abe and Hochachka, 1986).

Turnover Rates of Tissue Proteins

Sometimes in environmental physiology and biochemistry, it is important to assess the turnover rates of tissue proteins. Again the principle of this technique is as above: the monitoring of the rate at which labelled molecules are replaced by unlabelled ones. Although more difficult, it is readily achievable with culturable marine organisms.

Turnover Rates of Specific Proteins

Finally, along these lines, it should be mentioned that the turnover rates of individual proteins are sometimes also most useful in environmental adaptation studies. A relatively easy way to get estimates for the turnover rate of any given enzyme is to follow the time course of concentration change during the transition from one steady-state to another (Schimke and Doyle, 1970). The turnover rates of oxidative enzymes (such as citrate synthase or cytochrome oxidase) can be studied in skeletal muscle and heart of fish for example by following the return to normal levels from elevated concentrations established by a prior training regime. For this

kind of experiment, about 50 animals should be used, previously trained for sustained exercise by holding the animals in constant-current conditions. Groups of animals are sacrificed at timed intervals; the enzymes under study are extracted and their concen-, trations are quantified (either by activity measurements, if these can be converted back to molar units, or by immunochemical ones), The data are analysed by a first-order kinetic model using a nonlinear least-squares procedure. The half-life is calculated from the relationship:

$$t_{1/2} = \ln 2/k$$

where k = the derived slope (of enzyme concentration plotted vs time in days).

Metabolic Biochemistry of Free Ranging Animals

The last topic that needs at least a brief mention is one of broad interest to environmental physiology and biochemistry as a whole: the metabolic state of the organism while operating voluntarily in its natural ecosystem. Until recently, the only approach available in attempting to reach this goal wast hat of measuring water turnnover or Na^+ turnover. While useful (see above), this approach at best yields crude, average metabolic rates; everything the animal does is lumped into one kind of average metabolic cost. With the advent of smaller and smaller microcomputers, microprocessors small enough for free-ranging animals become available. The first such microprocessor, designed specifically for environmental physiological and biochemical studies, is that of R. D. Hill (1986). This package, which was designed specifically for work with diving marine animals, for the first time ever allowed arterial blood samples to be obtained from animals free ranging as far as 600 m below the surface of the sea. Such achievements are made possible by designing and building a dedicated microcomputer and pressure-resistant peristaltic pump for remote measurements of electrocardiograms and for sampling arterial blood. In principle, the microcomputer backpack can command withdrawal of one blood sample per excursion or sequential samples per excursion at programmable combinations of excursion time, excursion depth, environmental temperature, O_2 content, or heart rate. The backpack can communicate with a laboratory computer (for program adjustments with time and for 'dumping' data), via briefly connected fibre optic lines; or via periodic transmissions by conventional means (*e g.* radio transmission) or by less conventional ones (*e.g.* via satellite monitoring of backpack signals).

Although our vision is not acute enough to see fully the potential applications and impact of this kind of novel technology, we are fully convinced that in the future, major advances in environmental physiology and biochemistry will necessarily depend upon sophisticated data collected on the free-ranging organism, so it is our hope that progress in the application of these microcomputer techniques will continue unabated, or even at an ever increasing developmental pace. Although to date this technology has only been applied to aquatic mammals, it is clearly readily transferrable to studies of fishes and reptiles; aquatic turtles, for example, would be ideal experimental subjects for studies of metabolism and physiology of free-ranging individuals using such backpacks. In the future, we have no doubt that a similar system will be transferrable even to studies of the environmental physiology and biochemistry of invertebrates. In environmental biology this technology holds up the same kinds of promises for new insights as does nuclear magnetic resonance spectrometry in mainstream biochemistry for the noninvasive monitoring of metabolic processes in living tissues.

References

Abe, H. and P. W. Hochachka 1986. Turnover of ^{14}C-labelled L-histidine and its incorporation into carnosine and anserine in rainbow trout. *Mol. Physiol.* (in press).

Bensadown, A. and D. Weinstein 1976. *Anal. Biochem.*, **70** : 241-250.

Bergemeyer, H. U. 1974. Methods of Enzymatic Analysis. Academic Press, N.Y.

Brett, J. R. 1965. The relation of size to rate of oxygen consumption and sustained swimming speed of sockeye salmon *(Oncorhynchus nerka)*. *J. Fish. Res. Bd. Canada*, **22**: 1491-1501.

Davis, R. W., G. L. Kooyman, and J. P. Croxall 1983. Water flux and estimated metabolism of free-ranging gentoo and macaroni penguins at South Georgia. *Polar Biol.*, **2**: 41-46.

Daxboeck, C. 1981. A study of the cardiovascular system of the rainbow trout *(Salmo gairdneri)* at rest and during swimming exercise. *Ph. D. Thesis, Univ. of British Columbia, Vancouver, Canada.*

Hazel, J. R. 1984. Effects of temperature on the structure and metabolism of cell membranes. *Amer. J. Physiol.*, **246**: R 460-R 470.

Hill, R. D. 1986. Microcomputer monitor and blood sampler for freely diving seals. *J. Appl. Physiol.* (in press).

Hinke, J. A. M. and M. R. Menard 1978. Evaluation of the DMO method for measuring intracellular pH. *Resp. Physiol.*, **33**: 31-40.

HOCHACHKA, P. W., C. STANLEY, J. MERKT AND J. SUMAR-KALINOWSKI 1982. Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: An interpretive hypothesis. *Ibid.*, **52**: 303–313.

LINDINGER, M. I., D. J. LAUREN AND G. D. MCDONALD 1984. Acid-base balance in the sea mussel *Mytilus edulis* III. Effects of environmental hypercapnia on intra- and extracellular acid-base balance. *Marine Biology Letters*. (in press).

MILLIGAN, C. L. AND C. M. WOOD 1984. Intracellular pH transients in rainbow trout tissues measured by DMO distribution. *Am. J. Physiol.* (in press).

MOON, T. W., R. J. WALSH AND T. P. MOMMSEN 1985. The fish hepatocyte: A model metabolic system. *J. Fish. Aquatic Sciences*, (in press).

NEELY, J. R., J. T. WHITMER AND M. J. ROVETTO 1975. Effect of coronary blood flow on glycolytic flux and intracellular pH in isolated rat hearts. *Circ. Res.*, **37**: 733-741.

ROOS, A. AND W. F. BORON 1981. Intracellular pH. *Physiol. Rev.*, **61**: 296-434.

SCHIMKE, R. T. AND D. DOYLE 1970. *Annual Rev. Biochem.*, **39**: 929-976.

SMITH, L. S. AND G. R. BELL 1964. Anaesthetic and surgical techniques for Pacific salmon. *J. Fish. Res. Bd. Canada*, **24**: 1579-1588.

SRIVASTAVA, D. K. AND S. A. BERNHARD 1985. Enzyme-enzyme interactions and the regulation of metabolic reaction pathways. *Current Topics Cellular Regulation*, **28** (in press).

Manuals of Research Methods issued under the Centre of Advanced Studies in Mariculture, Central Marine Fisheries Research Institute, Cochin.

1. Manual of research methods for crustacean biochemistry and physiology. CMFRI Special Publication No. 7, 1981, 172 pp.

* 2. Manual of research methods for fish and shellfish nutrition. CMFRI Special Publication No. 8, 1981, 125 pp.

3. Manual of research methods for marine invertebrate reproduction. CMFRI Special Publication No. 9, 1982, 214 pp.

* 4. Approaches to finfish and shellfish pathology investigations. CMFRI Special Publication No. 11, 1983, 43 pp.

5. Application of genetics in aquaculture. CMFRI Special Publication No. 13, 1983, 90 pp.

6. Manual of research methods for invertebrate endocrinology. CMFRI Special Publication No. 14, 1983, 114 pp.

7. Production and use of *Artemia* in aquaculture. CMFRI Special Publication No. 15, 1984, 74 pp.

8. Manual on marine toxins in bivalve molluscs and general consideration of shellfish sanitation. CMFRI Special Publication No. 16, 1984, 100 pp.

9. Handbook on diagnosis and control of bacterial diseases in finfish and shellfish culture. CMFRI Special Publication No. 17, 1984, 50 pp.

10. Mariculture research under the Centre of Advanced Studies in Mariculture. CMFRI Special Publication No. 19, 1984, 109 pp.

11. Water quality management in aquaculture. CMFRI Special Publication No. 22, 1985, 96 pp.

12. A practical manual for studies of environmental physiology and biochemistry of culturable marine organisms. CMFRI Special Publication No. 25, 1986, 45 pp.

13. Theorems of environmental adaptation. CMFRI Special Publication No. 26, 1986 (In press).

---

*Out of print