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# THEOREMS OF ENVIRONMENTAL ADAPTATION

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**
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# THEOREMS OF ENVIRONMENTAL ADAPTATION

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## PREFACE

**The Central Marine Fisheries Research Institute,** through the Centre of Advanced Studies in Mariculture, strives to enhance the availability of competent and qualified personnel to undertake both basic and applied research in the field of mariculture. The science of mariculture is new to India and concerted R & D efforts were initiated by the Institute in the early seventies. To evolve suitable mariculture techniques, appropriate technologies from various disciplines need to be selectively applied wherein the importance of environmental physiology and biochemistry is vital. Dr. Peter William Hochachka, Professor, Department of Zoology and Sports Medicine Clinic, University of British Columbia, Vancouver, British Columbia, Canada, an internationally acclaimed scientist in this field, spent some time at the Institute on our consultancy programme to train the scientists. As a result of this consultancy, a manual on the theorems in environmental biochemstry was prepared. The manual lucidly deals with the problems related to levels of oxygen tension and temperature extremes. It is a simplified exposition of problems concerning the studies of environmental physiology. I am sure the manual would be useful to the Scientists and research workers concerned with the environmental interactions with animal life. This book is being published as the follow up of the workshop conducted during the consultancy of Dr. Hochachka as a companian volume of the manual dealing with the methods pertaining to studies of environmental physiology.

I express my sincere appreciation to Dr. Hochachka for his efforts to project and analyse the problems of environmental physiology and biochemistry in this book. I thank Shri D. C. V. Easterson, Scientist of this Institute who served as the counterpart, for ably managing the consultancy programme. I am thankful to Dr. K. Rengarajan, Scientist for editing the manual.

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## ABBREVIATIONS USED

| | |
|---|---|
| ADP | adenosine 5-diphosphate |
| AMP | adenosine 5-monophosphate |
| ATP | adenosine 5-triphosphate |
| CBF | cerebral blood flow |
| CHO | carbohydrate |
| $CMRO_2$ | cerebral metabolic rate of oxygen uptake |
| DPG | 1, 3-bisphosphoglycerate |
| ECF | extracellular fluid |
| ETS | electron transfer system |
| FFA | free fatty acids |
| F 1, $6P_2$ | fructose 4, 6-bisphosphate |
| F 2, $6P_2$ | fructose 2, 6-bisphosphate |
| GPDH | glyceraldehyde 3-phosphate dehydrogenase |
| ∝GPDH | alphaglycerol-1-phosphate dehydrogenase |
| G3P | glyceraldehyde 3-phosphate |
| G6P | glucose 6-phosphate |
| $H^+$ | Hydrogen ion ( = proton) |
| HK | hexokinase |
| ICF | intracellular fluid |
| $K_d$ | dissociation constant for enzyme-substrate complex (affinity of an enzyme for its substrate is equal to the inverse of $K_d$ for enzyme-substrate complex) |
| $K_m$ | Michaelis-Menten constant (substrate concentration (moles/litre) at which half maximal reaction velocity is reached) |
| LDH | lactate dehydrogenase |
| mTAL | medullary thick ascending limb |
| MDH | malate dehydrogenase |
| $NAD^+$ | nicotinamide-adenine dinucleotide |
| NADH | reduced form of $NAD^+$ |
| NMR | Nuclear magnetic resonance |
| $P_i$ | inorganic phosphate |

| | |
|---|---|
| PE | phosphatidyl ethanolamine |
| PEP | phosphoenol pyruvate |
| PFK | phosphofructokinase |
| 3PGA | 3-phosphoglycerate |
| PGK | phosphoglycerate kinase |
| PK | pyruvate kinase |
| $P_aO_2$ | partial pressure of oxygen |
| $Q_{10}$ | Van't Hoff coefficient-ratio of chemical reaction velocities at 10°C temperature (T°) intervals |
| SMR | standard metabolic rate |
| SR | sarcoplasmic reticulum |
| $T_b$ | body temperature |
| $\dot{V}O_2$ | volume of oxygen consumed (= metabolic rate) per unit time |

# 1

## INTRODUCTION

Organisms are perpetually buffetted by the external environment, yet of course somehow manage its effects. In ectothermic species in particular the external environment impinges directly upon cellular and subcellular functions; *i.e.*, directly upon cell chemistry and cell metabolic machinery. Adaptive adjustments in response to environmental change activated in one tissue therefore must be balanced with adjustments at other sites so as not too seriously (i) to disrupt functions at other sites or (ii) to disrupt cooperative metabolic interactions between tissues and organs in the integrated physiology of the whole organism. Making problems worse are simultaneous changes in more than one parameter of the environment. Not only must organisms then cope with differential effects of each parameter on specific metabolic processes or upon specific tissues and organs; they must also successfully adjust to interacting effects of the external environmental parameters which may drive the same processes or pathways in the same or in opposite directions. How do animals manage such problems? What trade-offs are required? How are adjustments made to more than one environmental factor at a time?

It has been commonly assumed that organisms harness three general mechanisms for solving their adaptational problems. These include:

i. adjustments in the kind or amount of macromolecular (especially enzymatic) components of cell metabolism,

ii. adjustments in the intracellular (or in "micromolecule") microenvironment within which macromolecules function, and

iii. adjustments in the outputs of metabolism on a moment-by-moment basis.

Whereas the meaning of such adjustments or adaptations in solving standard biological problems (such as fasting, growth, reproduction, differentiation, migration and locomotion) is essentially clear, it is by no means as evident how they contribute to solving environmental problems *per se*. Why would such adjustments even be made during adaptation to parameters such as hypoxia or hypothermia? What roles are played by enzyme kind or content adjustments in response to $O_2$ lack? What general or specific functions do such adjustments serve which enable organisms to survive changes in any one environmental parameter or in more than one factor changing simultaneously?

In addressing these problems, our strategy in this account is to take advantage of two kinds of organisms - those, in general, tolerant to environmental change and those sensitive to it, and specifically, those tolerant to hypoxia and hypothermia *vs* those sensitive to these two environmental parameters. Closely contrasting their respective responses to environmental changes leads to three demonstrable, but not previously evident, principles of environmental adaptation. Two of these surprisingly simple theorems are parameter-specific, while the third is general.

**The hypoxia adaptation theorem states that to be able to sustain prolonged periods of hypoxia, organisms (organs, tissues or cells) must balance the rates of nonspecific ion leaks across cell membranes with an enforced decline in rates of ion pumping (due to partial metabolic arrest and thus reduced ATP synthesis rates) and that this biological state is achievable only if controlled, partially, arrested metabolism is closely coupled with reduced membrane permeability (reduced channel density) of proportionate magnitude.**

**The hypothermia adaptation theorem states that to be able to sustain prolonged periods of hypothermia (defined as a species-specific state of body temperature being below levels which are distinctly suboptimal and at which indefinite survival is not probable) organisms must balance rates of nonspecific ion leaks across cell membranes with an enforced decline in rate of ion pumping (due to cold-induced reductions in ATP synthesis rates) and that this biological state is achievable only if downward adjustments in regulated metabolism are closely coupled with reduced membrane permeability (reduced channel density) of similar magnitude.**

**The third theorem is a general extension of the first two and states that to be able to sustain prolonged periods of environmental change, organisms must balance rates of nonspecific ion leaks across cell membranes with enforced changes in rates of ATP synthesis and thus in rates of ATP-dependent ion pumping, and that this biological state is achievable only if upwards or dowards adjustments in regulated metabolism are coupled with permeability changes of proportionate magnitude and direction.**

Although as we shall discuss in depth below these theorems imply quite specific underlying metabolic and membrane mechanisms, yet their main virtue is that they supply simple unifying cellular homeostatic goals for adaptational processes already well described in the literature. The theorems in effect identify a distinct homeostatic function (balanced metabolic and membrane processes) for environmental adaptation and rationalize more fully than ever before what standard adaptations (in enzyme content or kind, in intracellular mileu and in metabolic output) are used for, and why they are necessary in managing environmental change.

The data supporting these theorems of environmental adaptation have been in the open literature now for some time, but have been overlooked, it turns out for understandable reasons: They are by no means self-evident. Our own recognition of their significance, moreover, does not come as a flash of insight from on high. Rather it arises from a detailed analysis of defence strategies against hypoxia and hypothermia in widely diverse ectotherms and in endotherms. Such anlysis, to which we shall now proceed, is complex and at times difficult, but its unexpected reward is that it in effect charts a deductive pathway leading to the two specific theorems of hypoxia and hypothermia adaptation; these in turn set the stage for the general theorem and hopefully for a rather more precise understanding of how animals interact with their environment and of how we can intervene to protect them when environmental insults are too extreme for them to manage.

# 2

# DEFENCE STRATEGIES AGAINST HYPOXIA

## Lessons from Animal Extremists on How to Survive $O_2$ Lack

Although most animals, including man, are sensitive to even modest $O_2$ limitations, numerous species are known to be profoundly resistant to hypoxia. Detailed study of such animals indicates that they typically rely on only one or two broad categories of adaptive response. In the first case, typified in animals by high altitude adapted species, and in man by patients suffering from chronic hypoxia, metabolic strategies are directed towards *sustained oxidative function* despite potentially chronic $O_2$ limitations. In this adaptive response, there is little or no extension in tissue anaerobic capacities (Hochachka, 1985). In contrast, numerous invertebrates and lower vertebrates under hypoxic stress rely critically on metabolic strategies directed towards *sustained anaerobic function;* many of these species have developed such effective mechanisms of protection against hypoxia that they are often referred to as 'good' animal anaerobes or more precisely as facultative anaerobes (Hochachka, 1983 a; Hochachka and Somero, 1984). Although theoretic and clinical insights may derive from the improved understanding of both groups of organisms and both metabolic strategies, it is the latter that have developed the most effective protective mechanisms against tissue hypoxia and even anoxia. What are the rules of metabolic design that make these organisms so extremely effective at sustaining prolonged periods of $O_2$ lack? Can these rules be translated or transferred to protect hypoxia-sensitive systems against $O_2$ limitation? Although these kinds of questions are still being actively researched, it is already clear (Hochachka, 1983 a; Hochachka and Somero, 1984) that in this adaptive response, the two mos critical metabolic problems are (i) conservation of fermentable substrate and (ii) avoidance of self-pollution by accumulation of

undesirable end products; while the membrane problem is how to maintain ion regulation in the face of $O_2$ lack. The metabolic problems arise from the energetic inefficiency of anaerobic metabolism, for the yield of adenosine triphosphate/mole of substrate fermented is always modest compared to oxidative metabolism. For this reason in most animal tissues carbohydrate (CHO) utilization rates vary inversely with $O_2$ availability (a response termed the Pasteur Effect*); *if demands for ATP remain unchanged during anoxia,* CHO consumption rates necessarily have to drastically rise (Racker, 1980; Hochachka, 1983 a).

Potentially very large depletions of glycogen from central stores are minimized in faculative anaerobes by (i) storing more glycogen, (ii) utilizing more efficient fermentation pathways and/or (iii) depressing ATP turnover rates (*i.e.* energy demand) during $O_2$ limiting periods. All three mechanisms can be shown to be theoretically capable of extending anoxia survival time (Hochachka, 1983 a); however, the first two mechanisms even in principle could not extend hypoxia tolerance by more than a 3 to 4 fold factor. In contrast, metabolic arrest mechanisms in *Mytilus* can increase anoxia tolerance by some 20 fold; in diving turtles, by some 60 fold; and in brine shrimp embryos, by orders of magnitude. Brine shrimp embryos in estivation express this strategy at its limit for their maximum anoxia tolerance coincides with entrance into a fully arrested or ametabolic state (Hochacka and Somero, 1984). Even from the less extreme animal anaerobes, however, two instructive conclusions are derived: (i) *reversing the classical Pasteur Effect so as to allow ATP turnover rates to drastically decrease during anoxia apparently constitutes the most effective strategy for solving the problem of substrate conservation and (ii) this metabolic arrest strategy, in principle at least, may be universally applicable, while mechanisms such as energetically improved fermentation pathways are phylogenetically restricted and therefore even in theory could not represent realizable mechanisms of hypoxia adaptation in species lacking the appropriate enzyme pathways, such as in most ectothermic and endothermic vertebrates* (Hochachka and Somero 1984). This is why current estimates of substrate-sparing advantages of metabolic arrest strategies for surviving periods of $O_2$ lack may actually be on the conservative side, on the assumption that other factors (end products, for example) do not become limiting.

Analysis of the 'end products' problem is more complex, because it involves estimating (i) the relative effects of organic (usually

* Please see page 7

anionic) end products *vs* those of $H^+$, (ii) the metabolic sites of $H^+$ production, (iii) the pathways for subsequent proton disposition, (iv) the $H^+$ stoichiometry of different fermentation pathways (Hochachka, 1983 b; Hochachka and Somero, 1984) and (v) the metabolic effects, if any, of net change in strong ion concentrations, such as $K^+$, $Na^+$, $Ca^{++}$ and $Cl^-$. Nevertheless it is now evident that the potentially undesirable effects of $H^+$ and organic anion accumulations are minimized in 'good' animal anaerobes by only a handful of mechanisms. These include (i) utilizing fermentation pathways which allow more ATP to be turned over per mole of $H^+$ accumulated than in classical glycolysis, (ii) tolerating proton accumulation by improved tissue buffering capacity, (iii) minimizing end product accumulation by recycling it for further metabolism or excretion, (iv) utilizing $H^+$ consuming reaction pathways and (v) depressing metabolic rates during anoxia (Hochachka, 1983 a; 1983 b).

Whereas mechanisms (i-iv) of course are advantageous, potentially yielding upto about a several-fold improvement in tolerance of $O_2$ lack, it is evident that by depressing demands for ATP during anoxia, mechanism (v) above, an organism not only reduces the depletion rates of glycogen (glucose) in relatively useless fermentation, it also automatically reduces rates of formation of anaerobic end products, including $H^+$. In the case of the anoxic goldfish example, the rate of proton production is reduced by 5 fold due to metabolic depression, while in the turtle it is recuced by around 60 fold (Hochachka and Somero, 1984).

The picture that emerges from such analyses of phylogenetically rather diverse groups of animals is that several processes contribute to the hypoxia tolerance of 'good' anaerobes, but of these *metabolic arrest mechanisms yield by far the most effective protection against $O_2$ lack. Of the known protection strategies, it alone supplies resolution to both the problem of substrate conservation during anoxia and the problem of end product accumulation.* That is why we concluded some time ago (Hochachka, 1983 a, 1983 b; Hochachka and Somero, 1984) that for prolonged survival in hypoxia or anoxia, anaerobic life-support systems (whether considered at the organismal, organ or cellular level) *must be able to metabolically switch-down or even switch-off.* For purposes of simplicity, we term this the metabolic arrest concept of defence against hypoxia.

At the cell metabolism level, the only clue we have to aid in unravelling how this can be achieved is the occurrence of a reversed Pasteur Effect in facultative anaerobes. To appreciate the mystery of the missing Pasteur Effect and gain some clues on possible mechanisms of its reversal, it seems necessary to rather closely examine current attempts to understand mechanisms underlying a standard Pasteur Effect. We should begin with some clear definition of states. The Pasteur Effect, named for its discoverer who worked out the basic phenomenon with yeast in 1861, is defined as the inhibition of carbohydrate (glucose) consumption when $O_2$ concentrations are high and includes the opposite situation: increased anaerobic glycolysis when $O_2$ is limiting. A reversed Pasteur Effect is defined as *decreased or unchanging glycolytic flux when $O_2$ is limiting*, and must be carefully distinguished from the Crabtree Effect, defined as the inhibition of $O_2$ consumption by activated carbohydrate fermentation. In terms of modern metabolic biochemistry, then, a Pasteur Effect is activated glycolysis when $O_2$ is limting; a reversed Pasteur Effect is simply the absence of such activation, or in the extreme, actual inhibition of anaerobic glycolysis at low $O_2$ levels. Although underlying mechanisms are still not fully resolved, current approaches seem in fall naturally into three categories: (i) allosteric regulation of glycolysis, (ii) covalent modification of key regulatory enzymes, and (iii) enzyme and pathway functional compartmentalization. We shall look at each of these in turn (Srivastava and Berhard, 1985; Hochachka and Guppy, 1986).

## PASTEUR EFFECT AS DIRECT OUTCOME OF CONVENTIONAL METABOLITE MODULATION

Historically the most interesting hypothesis attempting to explain the Pasteur effect emphasizes $P_i$ in regulating glycolysis. The idea is that glycolysis and oxidative phosphorylation compete for inorganic phosphate so that in the presence of oxygen, the latter limits the availability of $P_i$ for glycolysis. This simple and brilliant idea suffers a serious deficiency. It only explains an inhibition of lactate formation which is dependent on $P_i$, but it does not explain the original Pasteur observation of $O_2$-dependent inhibition of glucose phosphorylation which is independent of $P_i$ and requires only ATP, a product of oxidative phosphorylation.

Racker, reviewing how our concepts here developed (Racker, 1980) points out that when this problem with the $P_i$-based model was realized, some time went by before evidence for allosteric models

began to accumulate and lead to a somewhat more sound concept of coordinated control of glucose metabolism as a function of $O_2$ availability. The fundamental framework for this approach was initially and still is, centered on an interplay between key regulatory enzymes in the pathway. Usually the emphasis is on hexokinase (HK) and phosphofructokinase (PFK), for HK is inhibited by glucose 6-phosphate (G6P), and PFK is inhibited by high concentrations of ATP. These two enzymes are inhibited at physiological concentrations of the allosteric effectors and the two intermediates thereby influence the rate of glucose utilization. The Pasteur Effect can thus be formulated as the result of a set of sequential and cascading events initiated as $O_2$ availability for oxidative phosphorylation increases: (i) depletion of $P_i$ and ADP required for glycolysis and increase in the ATP level, (ii) inhibition of phosphofructokinase by ATP resulting in G6P accumulation and (iii) inhibition of hexokinase by G6P.

In yeast, in contrast, because HK is not under G6P product inhibition, this mechanism cannot contribute to control. So it is not surprising that $^{31}P$ and $^{13}C$ NMR spectroscopic studies in yeast can *fully* account for an observed Pasteur Effect (of about a 2-fold increase in glycolytic flux on transition to anoxia) by changes in flux at the PFK locus; these are mediated by increased F2, $6P_2$, FI, $6P_1$, ADP, AMP, $P_i$ and $H^+$ concentrations. In a different kind of preparation, permeabilized yeast cells metabolizing G6P, increased glycolytic flux is sustained by the pathway being coupled to AMP deaminase; the latter under conditions of elevated ATP demand is thought to serve in stabilizing adenylate ratios (by adenylate depletion) with the undesirable effect that this takes away at least three potential PFK activators (relatively elevated levels of AMP, ADP, and $P_i$). In the absence of a secondary or back-up glycolytic activator, AMP deaminase stabilization of the adenylate ratios might be expected to slow down glycolytic flux, but this does not occur because $NH_4^+$ (which is a well known allosteric PFK activator) accumulates enough under these conditions to contribute to the sustained glycolytic flux and the observed Pasteur Effect.

One of the more interesting Pasteur Effects noted in the literature is that activated by sperm during reversible transitions between anaerobiosis and aerobiosis. Because sperm display a simplified metabolism, it is possible to measure both the *in situ* flux rate at specific enzyme catalyzed steps as well as the overall net flux and net ATP yield. Such data, analysed from the literature by Regen and Pilkis (Srivastava and Berhard, 1985) can be quantified using the Kacser-Burns metabolic control theory to specify the contri-

bution of different sites to the Pasteur Effect. The principle of such analysis, worked out independently by Kacser and Burns in the United Kingdom and by Heinrich and Rapaport in East Germany, is straight forward and involves several theorems showing how the sensitivity of pathway flux to the activity of any component enzyme depends upon coefficients of the pathway. The sensitivity expression commonly used is *the fractional change of pathway rate per fractional change of a specific enzyme activity*, an expression termed the "sensitivity coefficient" or more commonly the "control strength" for that enzyme step. Each enzyme in a given pathway has its own characteristic control strength or z value and the sum of all z values is unity (fractional change in pathway rate by definition can never be exceeded by the sum of fractional changes in enzyme activities). In the case of sperm under normoxia, HK is not very rate determining despite its position at the head of the pathway, because of a large "elasticity" (a large fractional change in the HK net rate per fractional change in metabolite concentrations). As a result, control is passed from the HK step to subsequent steps in the pathways, mainly to PFK, which is the enzyme to which glycolytic flux is most sensitive under $O_2$ saturating conditions. As glycolysis is accelerated by the use of specific ETS inhibitors (equivalent to a natural Pasteur Effect), PFK control strength is reduced while that of hexokinase is increased to values about three times those for PFK. These subtle changes in the rate limitingness of HK and PFK on transition from low to high glycolytic flux rates are generated by entirely conventional mechanisms with G6P deinhibition at the HK step and with adenylate (mainly AMP) activation at the PFK step.

To the degree that the above kind of analysis of the Pasteur Effect is empirical, it is valid irrespective of other controlling inputs. Nevertheless, the implicit assumption in all analyses like the above (whether they be made for yeast, sperm or whole organisms) is that changes in flux of the pathway are *caused* by specific modulator-mediated changes in catalytic rate at specific, identifiable enzyme steps in the pathway. However, this need not be the case because activity changes of enzymes in pathways may be caused by adjusting the ratio of active/inactive forms of enzymes, which brings us to an alternative way of looking at the problem.

## Pasteur Effect as an Outcome of Covalent Modification of Enzymes

One of the most common ways known for modifying the active/inactive ratios of enzymes (Srivastava and Berhard, 1985; Hocha-

chka and Guppy, 1986) involves controlled (*i.e.* enzyme catalyzed) phosphorylation and dephosphorylation cascades. The best worked-out example in mammalian tissue is glycogen phosphorylase which occurs in a dimer low activity *b* form which, in the presence of *b* kinase, $Ca^{++}$ and ATP, can be rapidly phosphorylated to a more active, tetramer *a* form. In tissues which store glycogen, it is obvious that the equivalent of a Pasteur Effect would, in the absence of $O_2$, lead to increased consumption of glycogen, rather than glucose. As it turns out, hypoxia-sensitive tissues and organs (which typically display reasonably marked Pasteur Effects) typically sustain significant $Ca^{++}$ accumulation, which undoubtedly could contribute to *b* kinase activation, followed by phosphorylase $b \longrightarrow a$ conversion and a net increase in glycolytic flux (see discussion below and Fig. 3).

Similarly, recent studies of hypoxia-tolerant molluscan species indicate that two glycolytic enzymes (PFK and pyruvate kinase) are under phosphorylation control. In this system, environmental anoxia leads to the phosphorylation of both enzymes; PFK as a result is somewhat altered in its regulatory behaviour while PK catalytic capacity is profoundly reduced. The net effect in this kind of organism is a reversed Pasteur Effect: As $O_2$ availability declines, PFK regulation favours normal (rather than activated) flux, while at the phosphoenolpyruvate branchpoint, phosphorylating and so blocking PK favours channeling PEP carbon through a low-activity PEP carboxykinase step towards the succinate fermentation pathway (improving the yield of ATP/mole glucosyl unit as a result). Two advantages of this control system arise: The first favours a general reduction in glycolytic flux, while the second favours flow into a more energetically efficient pathway (Hochachka and Guppy, 1986).

## Pasteur Effect as an Outcome of Enzyme 3-D Control of Metabolism

A third approach to unravel the mechanisms of the Pasteur Effect places particular emphasis on evidence showing that the enzymes of glycolysis are not really functional in a so-called cytosol or soluble phase, subject to simple, mass action dominated control; these data support the concept of cell metabolism as a highly organized matrix of pathways, *where physical position* (as in bound to cell ultrastructure *vs* free in solution; or as in enzymes interacting specifically with other enzymes) is a critical, perhaps the most critical, determinant of activity (Hochachka and Guppy, 1986). Hypoxia and ischemia in mammalian heart, for example, lead to an increased binding of glycolytic enzymes, a process leading to

increased enzyme activity and increased glycolytic flux, thus partially contributing to a positive Pasteur Effect. Whereas in this kind of conceptualization, a kind of 3-dimensional or 3-D theory of metabolic control, metabolite concentration changes are still certainly predicated and are still obvious sources of information about flux through the pathway, they are *effects, not causes, of change in enzyme activity*. This conceptualization in other words, turns things around completely. Support for these kinds of control concepts is of two forms: On the one hand, numerous workers have obtained evidence for significant degree of enzyme localization and enzyme order in cell metabolism, which we refer to as structual evidence. On the other hand, more recent studies have marshalled evidence showing that even in solution, enzyme - enzyme interactions are potent determinants of pathway function and this line of support we shall refer to as functional evidence for an important 3-D component to metabolic control.

## Structural Basis of 3-D Metabolic Control Concept

A rather unexpected source of information about how many enzymes of the total complement exist free in solution in eukaryotic cells comes from studies analysing the forms and behaviour of intracellular water. An impressive and rather overlooked early example is that involving stratifying *Euglena* (a unicellular eucaryote) by ultracentrifugation (100,000 X gravity for an hour). Cytochemistry of macromolecules and enzyme-specific cytochemistry show that no macromolecules of any kind, including a variety of classical 'soluble' enzymes, occur at detectable levels, in the aqueous band; the cells, which in this treatment serve as their own centrifuge tube, retain viability after such treatment, indicating that the outcome may be taken as an indication of events as they exist in the cell. Analogous work on stratified *Neurospora* and *Artemia* cells also indicates that intact cells contain very few truly soluble enzymes or macromolecules. That is, the equilibrium

$$\text{soluble enzyme} \rightleftharpoons \text{bound enzyme}$$

is shifted far to the right under these conditions.

In a totally different approach, studies of intracellular diffusion of endogenous and injected proteins (using a reference phase technique) indicate that most intracellular enzymes and proteins do not diffuse at all (presumably due to fairly tight binding to intracellular sites) over time course of upto several hours and this appears true in both amphibian eggs and mammalian fibroblasts. More

recently, electron spin resonance measurements show that the microviscosity of normal cells and cells 50% dehydrated behave as if the cytoplasm contained only minor amounts of dissolved macromolecules (or none at all). In this context, when L - cells in tissue culture are placed in 2M sorbitol, they may lose upto 80% of intracellular water with hardly a measurable effect on glucose metabolism, an utterly implausible result if the pathway of glycolysis were operative in a simple aqueous solution.

Most workers in this field (Hochachka and Guppy, 1986) consider that the explanation for the above kinds of data is that water in cells exists in various forms; of these, bulk water serves more as a communication channel than it does as a solvent for 'soluble' glycolytic enzymes and other so-called cytosolic pathways.

But if glycolytic enzymes are largely out of solution, where are they to be found? The answers here are varied and depend in part on the cell or tissue type under analysis. Trypansomes, for example, contain an actual membrane-bound microbody or organelle, termed the glycosome, which houses most of the enzymes of glycolysis. In skeletal and cardiac muscle, myofibrills have long been considered to possess specific binding regions for glycolytic enzymes. Red blood cells, which in mammals are powered entirely by glycolysis, bind several glycolytic enzymes specifically to band 3 protein. In addition, Clegg has argued in support of a binding role for the microtrabecular lattice, the MTL, an enormously branched series of proten-rich structures that remify throughout the cell. For all these workers the implications are the same: Pathways such as glycolysis, classically considered to operate simply in cytosolic solution, are probably structurally compartmentalized. One possible (and extreme) functional consequence is that different isozyme complexes are structurally strung together each forming its own semi-independent, functionally discrete pathway. This could be the basis for the apparent simultaneous coexistence in arterial smooth muscle of one pathway for glucose fermentation to lactate (in the presence of $O_2$), which is coupled to $Na^+$ $K^+$ ATPase function and another for the complete oxidation of glycogen (or glucose) which supports muscle work. As a means of fermentation of glycogen to lactate, the latter pathway is not used in the presence of $O_2$ (*i.e.* displays a standard Pasteur Effect). In essence this work implies that within a single smooth muscle cell two pathways of glycolysis are structurally and functionally discrete enough so that glucosyl units derived from endogenous breakdown of gly-

cogen do not enter the same pool of glycolytic intermediates that are used in the aerobic production of lactate from exogenous glucose and vice versa. The picture emerging -- *two lactate forming pathways, semi-independent, with discrete starting substrates, and nonmixing pools of chemically identical intermediates, one showing a Pasteur Effects, the other a reversed Pasteur Effect* - is a far cry from the model of glycolysis and similar enzyme pathways that we have in mind when we look at crossover plots, at control strengths, or at elasticity.

Do any of the current 3-D metabolic control theories adequately account for the Pasteur Effect when it is observed, or its reverse in other cases? In one sence, it is evident that in principle the *only* condition needed to cause a Pasteur Effect is one shifting the equilibrium

bound glycolytic enzymes $\rightleftharpoons$ soluble glycolytic enzymes
active forms  less active forms

to the left whenever $O_2$ limiting conditions prevail. A reversed Pasteur Effect could then be caused by shifting that equilibrium to the right under hypoxic conditions. In ischemic or hypoxic mammalian heart, as mentioned above, a Pasteur Effect is observed and the equilibrium

myofibrillar bound glycolytic enzymes $\rightleftharpoons$ solubilizable glycolytic enzymes
(more active)  (less active)

is shifted to the left. In contrast, in anoxic bivalve molluscs, the equilibrium is shifted to the right, consistent with the reversed Pasteur Effect that is the hallmark of these kinds of organisms. These are promising beginnings, yet we should emphasize that thus far no single study has been able to show that these effects could be large enough to fully account for the magnitude of the observed Pasteur Effect, nor actually do any of these kinds of studies show us why bound enzymes (for example myofibrillar complexed aldolases) should be better catalysts than the same enzymes would be in simple solution. This kind of information necessarily comes from more functional studies.

## Functional Basis for 3-D Metabolic Control Concept

Perhaps the most direct reason for rethinking the way metabolic pathways such as glycolysis are organized *in vivo* comes from

the relative concentrations of proteins and metabolites in cells. Despite a great deal of cell-to-cell variability in the concentrations of specific proteins, the total concentration (in mg/ml or in molarity of peptide bonds) is remarkably uniform. Estimated localized concentrations of 200 - 400 mg/ml are only modestly lower than the concentration in protein crystals used in X-ray crystallography, which indeed is consistent with the high viscosity of all cell cytoplasms.

In view of such high protein concentrations, it is fair to ask (i) how much room remains for the metabolites of the cell? and (ii) how are substrate metabolites transferred from enzyme site to site in such a highly viscous medium? The answer to the first question (not much, which is why concentrations must be low) and to the second (by direct enzyme-to-enzyme transfer at least in some cases implies that *structural interactions within the cell are the framework of metabolism.* This can be nicely illustrated by maintaining our focus on glycolysis. Rough estimates of cellular concentrations of individual glycolytic enzymes of muscle sarcoplasm (Table 1) indicate that two glycolytic components, aldolase and GPDH, by themselves, represent 40 - 50% of the total glycolytic enzymes or over 10% of the protein content of typical protein crystals. In contrast, the concentrations of intermediary metabolities of glycolysis are much lower on a molar basis (Table 2). Enzyme concentrations at the same level as or even higher than, substrate concentraions is just the reverse of conditions for most *in vitro* studies of enzyme catalysis and this reverse situation places the problem of transfer of metabolic from one enzyme site to another in a new light. The total concentrations of $NAD^+$ and NADH in particular are less than the total concentration of dehydrogenase binding sites in the muscle and liver cytosol, NADH occurring at only a small fraction of the total dehydrogenase binding sites. With most of the intermediates in glycolysis being enzyme bound, the clear cut exceptions being starting fuels (hexose phosphates and phosphocreatine) and end products (lactate and possibly ATP), the implication is that *excessive enzyme site concentrations rather than excessive metabolites concentrations control substrate distribution.* One can therefore well appreciate the strength of selective pressures favouring some order to enzyme function in this kind of metabolic field. For example, it is difficult to see how the transfer of bound NADH among four cytosolic dehydrogenases - glyceraldehyde-3-P dehydrogenase, alphaglycero-P-dehydrogenase, lactate dehydrogenase and malate dehydrogenase - could be achieved with out some sort of special mechanism. Studies by Srivastava and Berhard (1985) suggest that the special mechanism is an NADH

TABLE 1. *Concentrations of individual glycolytic enzymes in rabbit muscle sarcoplasm**

| Enzyme | Concentration (mg/ml) | Site Concentration ($\mu$M) |
|---|---|---|
| Phosphoglucomutase | 1.98 | 31.9 |
| Aldolase | 30.35 | 809.3 |
| $\alpha$ -glycerol-P dehydrogenase | 1.78 | 61.4 |
| Triose-P-isomerase | 5.82 | 223.8 |
| Glyceraldehyde-3-phosphate dehydrogenase | 50.7 | 1398.6 |
| Phosphoglycerate kinase | 5.81 | 133.6 |
| Phosphoglycerate mutase | 6.37 | 235.9 |
| Enolase | 22.98 | 540.7 |
| Pyruvate kinase | 9.94 | 172.9 |
| Lactate dehydrogenase | 11.10 | 296.0 |

*From Srivastava and Berhard (1985).

TABLE 2. *Concentrations of precursors, coenzymes and glycolyric intermediates**

| Metabolite | Concentration ($\mu$M)** |
|---|---|
| Glucose-1-P | 240 |
| Glucose-6-P | 3,900 |
| Fructose-6-P | 1,500 |
| Fructose-1, 6-$P_2$ | 80 |
| Dihydroxyacetone-P | 68-160 |
| Glyceraldehyde-3-P | 80 |
| 3-phosphoglycerol-GPDH | 800 |
| 1, 3-diphosphoglycerate | 50 |
| 3-phosphoglycerate | 152-200 |
| 2-phosphoglycerate | 20 |
| Phosphenol pyruvate | 65 |
| Pyruvate | 380 |
| Lactate | 3,700 |
| Creatine-P | 26,600 |
| ATP | 8,050 |
| ADP | 926 |
| AMP | 43 |
| $P_i$ | 8,000 |
| $NAD^+$ | 541 |
| NADH | 50 |

*In resting rat muscle except for the concentrations of coenzymes which are for liver. From Srivastava and Berhard (1985).

**Values based on assumption that these intermediates are restricted to the cytoplasmic fluid.

direct transfer enzyme-to-enzyme (rather than a dissociation of NADH into solution followed by an enzyme competition for

limiting coenzyme). Their concept is of such significance to understanding metabolic control in general, as well as the Pasteur Effect specifically, that we must examine it more closely.

### The Handoff vs The Fumble : Two Ways to Transfer Metabolites from Enzyme to Enzyme

In principle, metabolites can be transferred from their site of synthesis to their site of utilization by one or two mechanisms: Either by dissociation from their site of synthesis in to solution, then by diffusion (Eq. 1), or by direct transfer from site of synthesis to site of utilization without the intervention of the aqueous solvent (Eq. 2):

$$E_1 \cdot S_2 \rightleftharpoons E_1 + S_2 + E_2 \rightleftharpoons E_2 \cdot S_2 \rightarrow \text{Products} \quad (1)$$

(Random diffuison)

$$E_1 + S_2 \rightleftharpoons E_1 - S_2 - E_2 \rightarrow \text{Products}$$

(Direct transfer) (2)

Srivastava and Berhard (1985) refer to the latter as a handoff, the former as a fumble; for at least two kinds of enzyme couples in glycolysis (PGK transfer of 1,3-phosphoglycerate (DPG) to GPDH and GPDH tranrfer of NADH to LDH), kinetic evidence now strongly favours the handoff alternative. Experiments designed to test this all involve *the reduction of metabolite concentration in solution by the formation of enzyme-metabolite complex in the presence of an excess of enzyme over metabolite.* To illustrate the case with PGK, when the kinase is in excess of the substrate, DPG, virtually all of the metabolite is bound to the enzyme, since the $k_d$ for the DPG-enzyme complex is smaller than $10^{-8}$M; as a result, the aqueous DPG concentration is exceedingly low and the reaction can be written:

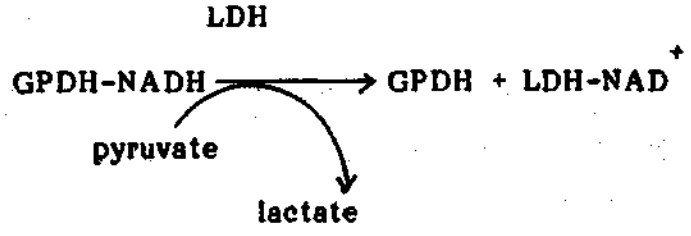
$$\text{PGK–DPG} + \text{NADH} \xrightarrow{\text{GPDH}} \text{PGK} + \text{G3P} + \text{NAD}^+ + P_i$$

In tests designed to compare PGK-DPG *vs* free DPG as substrates for GPDH, the concentration of GPDH utilized is low, comparable to the usual concentrations of enzyme catalyst employed in enzymological assays (of the order of nanomolar concentrations); thus, any aqueous DPG formed by dissociation of the PGK-DPG complex almost invariably would be reabsorbed by PGK rather than by GPDH. *The rate of reaction of the metabolite should therefore be substantially slowed down if aqueous DPG is required for the GPDH-catalyzed reaction (due to its low buffered concentration or*

*to its very slow rate of dissociation from PGK).* However this does not occur and the actual rate for the above reaction is far higher than can be accounted for either by rates of PGK-DPG dissociation or by rates of DPG diffusion. These and other results are therefore consistent with the handoff or direct transfer mechanism of metabolite transfer between two adjacent enzymes in the pathway.

An interesting feature of the coupled transfer reaction between PGK-DPG and GPDH is that, at PGK-DPG *saturation, the rate can be further enhanced almost* 20 *fold by the presence of saturating* 3-*phosphoglycerate.* At effective 3PGA concentrations, this metabolite is neither an inhibitor of the GPDH-catalyzed reaction, nor is it an effective competitive inhibitor of the interaction of DPG with phosphoglycerate kinase. This means that 3*PGA is a specific effector of the transfer process itself.* The activation is achieved because DPG can be transferred within the enzyme-enzyme complex either towards the GPDH catalyzed reaction or back to the kinase site. However, *if the vacant kinase site is already occupied by* 3*PGA, the ability of DPG to transfer back to the kinase will be inhibited whereas the reduction reaction at the GPDH site can proceed unaltered; the net effect is a powerful activation of the overall reaction rate.*

Analogous data on handoff *vs* fumble mechanisms for transfer of NADH between cytosolic dehydrogenases show that the following kind of reaction proceeds at rates substantially faster than if the coenzyme is supplied from solution:




In the context of this chapter, the NADH transfers that are of major interest are (i) GPDH→LDH, (ii) GPDH→MDH, (iii) LDH→ GPDH, (iv) LDH → $\alpha$ GPDH and (v) LDH→MDH. Whether or not NADH transfer between any two dehydrogenases is possible depends upon whether the two are of the "A" or "B" type. "A" type dehydrogenases catalyze the exchange of hydrogen

from the A face of nicotinamide, at the $C_4$ position, while "B" type catalyze the exchange from the B face. "A" type dehydrogenases directly transfer NADH to enzyme sites which allow for hydrogen transfer via the B face of nicotinamide. NADH transfer is not possible between two dehydrogenases of the same chiral specificity, but enzymes of opposite chiral specificity can handoff NADH in either direction (A → B or B → A).

Whereas the functional advantages of the kinds of transfer mechanisms are instantly obvious, the problem of how they are possible is not so clearly evident. However, information on the quaternary structure of dehydrogenases (LDH, for example) suggest that binding of NADH results in the closure of the enzyme binding site cleft; in this holoenzyme conformation there is no apparent way in which bound coenzyme can escape to the surrounding aqueous environment. On the other hand, there is sufficient space for coenzyme to enter and to leave the active site when the enzyme protein is in its unliganded (or apo) form. Presumably, the holoenzyme→apoenzyme transition allows for the free passage of NADH out of, and into, the binding sites of all dehydrogenases. When the surfaces around the active sites are juxtaposed for a pair of "A" type enzymes, the conformations of the bound NADHs are the same; in order for NADH to transfer from site to site, it would have to effect a molecular rotation of 180° which is impossible. That is presumably why the transfer of NADH between dehydrogenases of the same chirality necessarily involves dissociation from the first site into the aqueous medium, followed by diffusion to the second site. In contrast, when the dehydrogenases of opposite chirality are juxtaposed, *the nicotinamide ring of one dehydrogenase site has a mirror image relationship to the nicotinamide binding site of the other dehydrogenase.* The coenzyme molecule bound to one site can transfer its nicotinamide to a second structurally complementary site by translation alone without rotation and without consequent dissociation into the aqueous medium. Thus dehydrogenase direct transfer of NADH can be brought into line with current understanding of dehydrogenase structure and we are now in a position to see how these properties may come into play in the Pasteur Effect.

## Pasteur Effect as an Outcome of GPDH Handoffs to PGK and LDH

From the above considerations it is evident that during activated glycolysis GPDH must be involved minimally in a dual coupling *for the effective transfer of its two products (DPG and NADH) to two target enzyme sites, PGK and LDH* respectively. As sum-

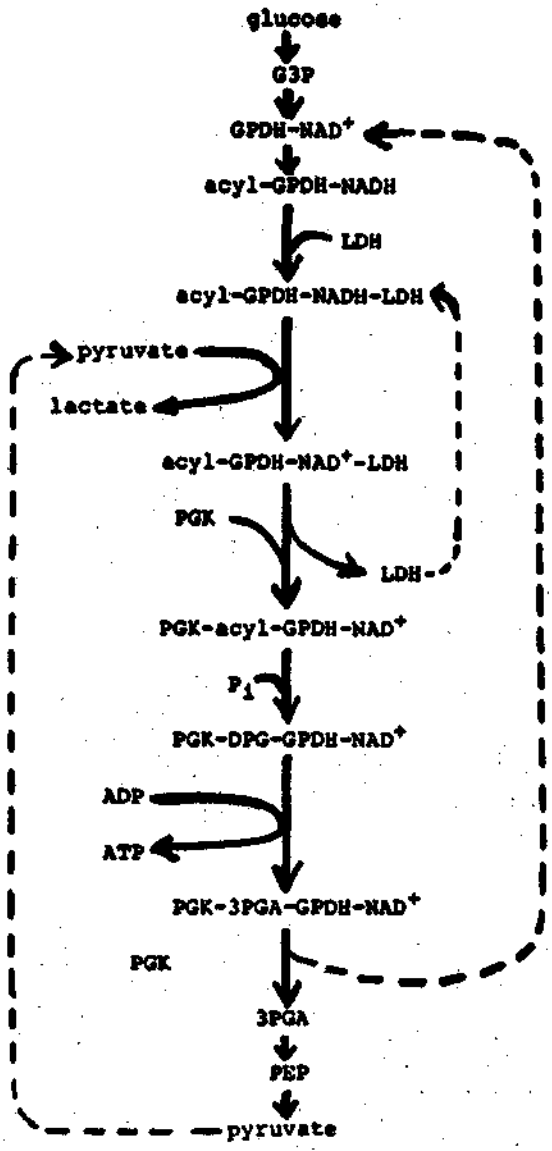



Fig. 1. Model of the Pasteur Effect based upon the transition from solution-dominated glycolytic function to direct-transfer dominated glycolysis. Modified from Srivastava and Bernhard (1985).

marised in Fig. 1, this dual coupling in essence initiates a series of glycolytic cycles with LDH and PGK both entering and leaving at prescribed steps in the process. Pyruvate is reduced to lactate while LDH is cycling through; phosphorolysis of the acyl GPDH and subsequent ATP synthesis occur while PGK is cycling through; NADH and $NAD^+$ never need to enter the aqueous medium, for GPDH-$NAD^+$, which initiates the entire sequence is regenerated as 3PGA is released for the next step in the glycolytic path. In this model, the Pasteur Effect (activation of glycolytic flux by low $O_2$ availability) is viewed as simply a transition from solution-dominated glycolysis during normoxia to direct transfer or handoff-dominated glycolysis when $O_2$ is limiting, a process leading to some 10-fold increase in flux assuming no other input.

Although an elegant model of how the Pasteur Effect might be mediated, we hasten to add that it has not been proven *in vivo*. As with the model based on ratios of bound/soluble glycolytic enzymes, there is no information available on how large a Pasteur Effect could be generated *in vivo* by this system. Nor can this interpretation at this stage at least account for why some cells show very large Pasteur Effects (*e.g.* sperm), some show intermediate ones (brain cells), while others show only modest ones. Furthermore, there is no easy way to see how this system might differ in facultative anaerobes which show a reversed Pasteur Effect. Thus, a lot remains to be done; in particular, what needs to be brought onto the same track is this functional line of research and binding studies. How, for example, might the bindng of GPDH, PGK and LDH to myofibrillar sites (in muscle) or band 3 protein (in red blood cells) influence handoff mechanisms? To this date, these two lines of research have been progressing in parallel and it is our hope they will come together at some near future.

## Pasteur Effect May Be Cell Line and Species Specific

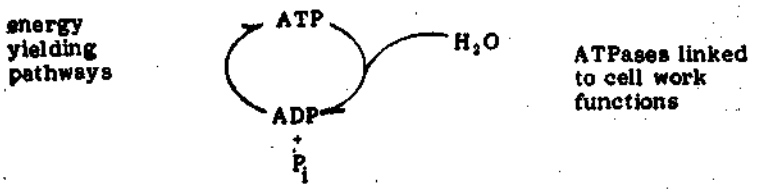
In summary, then, it appears that the most effective defence strategy against hypoxia used by good animal anaerobes involves metabolic arrest of variable degree. We are not sure what the main mechanisms utilized may be, but their net effect is expressed as a reversed Pasteur Effect. Standard Pasteur Effects appear to be mediated (i) by conventional allosteric regulation, (ii) by covalent modification of key enzymes in glycolysis or (iii) by more subtle control modifying the 3-D pathway structure and thus function of anaerobic glycolysis. Or some combination of all three mechanisms. The most judicious interpretation of the available data is that there is no single mechanism accounting for the Pasteur

Effect; both its magnitude and its mechanism may well show a great deal of cell line specificity arising from cell specialisations and adaptations for specific functions. In the case of smooth muscle of arterial walls, the Pasteur Effect may even be pathway-specific: one pathway (glucose→lactate) being fully operational in the presence of $O_2$ while a second (glycogen→lactate) appears to be normally inhibitable by high $O_2$ tensions.

With this degree of cell line and species specificity of the standard Pasteur Effect, it is reasonable to expect a similar degree of specificity for the reversed Pasteur Effect in animal anaerobes. Nevertheless, reversal of each of the above positive Pasteur mechanisms should yield a reversed Pasteur Effect of similar magnitude which is apparently consistent with those few data already available for hypoxia-tolerant bivalve molluscs.

## Metabolic Arrest as an Intervention Strategy : Current Limitations

In principle, metabolic arrest could be achieved not only by slowing down the rate of ATP synthesis, but by *slowing down the rate of ATP utilization:*




A reversed Pasteur Effect illustrates an example of the former (left arm of the ATP–ADP cycle above). Since the underlying mechanisms of this metabolic arrest process are still only poorly understood, it is not surprising that, when applied to hypoxia-sensitive systems, metabolic arrest strategies that have been used so far for protecting tissues against $O_2$ lack have depended upon showing down the rate of ATP utilization. Although earlier 'arrest'–type concepts of protection against hypoxia are evident in the scientific literature one stivation, torpor and hibernation (Faleschini and Whitten, 1975), attempts to directly test such concept appear only in clinical literature, mainly in studies of cardiac arrest, stroke and acute renal failure (Farber *et al.*, 1981). Important insights arise from comparing these studies to the ones analysed above, so we will examine them closely. A well developed literature deals with the heart, where it is widely accepted that ischemic myocardial damage is

influenced by work load or metabolic rate. Accordingly, several intervention procedures all aim at minimizing damage by reducing myocardial requirements during ischemia, and all, to a lesser or greater degree, can be shown to be helpful (Herse *et al.*, 1980), and thus consistent with the metabolic arrest concept. However, the most convincing test of the hypothesis probably is provided by recent studies using ischemic rat kidney preparations as models of acute renal failure (Brezis *et al.*, 1984 a - d). During ischemic acute renal failure large reductions in renal blood flow occur with consequent reduction in delivery of $O_2$ and substrates. Since the main ATP-requiring processes of the kideny involve membrane-coupled ion translocations, our hypothesis would predict that experimentally reducing the demands for ATP by ion pumps should yield a proportionate increase in tolerance to limiting $O_2$ availability. In mammalian kidney, the medullary thick ascending limb (mTAL) of Henle's loop is the most hypoxia-sensitive segment of the nephron and during perfusion of the isolated organ, complete disruption of mTAL cells occurs by 90 minutes; interestingly, this hypoxia sensitivity can be fully eliminated by perfusion with ouabain, a specific inhibitor of $Na^+$ $K^+$ ATPase or by reducing ion pumping work by preventing glomerular filtration. Conversely, polyene antibiotics increase membrane permeability, increase the energy requirements of ion transport and consequently increase the hypoxia sensitivity of mTAL tubules, but again the hypoxia-induced damage is prevented if reabsorptive transport is inhibited with ouabain. Even KCN-induced lesions can be eliminated by simultaneous ouabain inhibition of $Na^+$ $K^+$ ATPase (Brezis *et al.*, 1984 a - d).

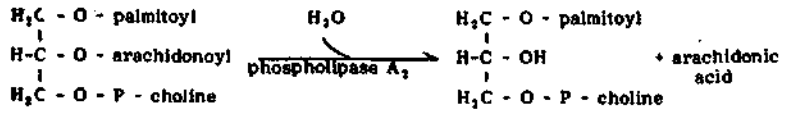
In effect, these provocative studies represent empirical attempts to establish a mammalian organ hypoxia tolerance that is more characteristic of hypoxia-adapted lower vertebrates and invertebrates. Even if these manouevres are seemingly effective and support the general predictions of our hypothesis, they also emphasize an important difference; namely, that metabolically arrested endothermic systems so far described are profoundly less hypoxia-tolerant than are hypoxia-adapted lower animals (Hochachka, 1983 b) Something, therefore, is still missing in our analysis of defence strategies against hypoxia in animal anaerobes. Further analysis is our next chapter indicates that the missing element is to be found at the coupling between metabolism and membrane functions and that a final provision for expanding hypoxia tolerance is the stabilisation of membrane function during hypoxic exposure.

# 3

# METABOLISM - MEMBRANE INTERFACE

## Balancing Metabolism with Membrane Functions

To illustrate the critical significance of close coupling between cell metabolism and cell membrane process, we can find no more convenient a system than the mammalian brain which perhaps is the most hypoxia-sensitive organ in the mammalian body and for which events during various kinds of energy perturbations are well charted (for reviews Siesjo, 1981; Hansen, 1985). As we shall argue, however, the broad outlines should be the same for *all* hypoxia-sensitive systems. In complete cerebral ischemia, then, as occurrs in cardiac arrest, the brain becomes isoelectric within 15–25 seconds. This electrically silent period precedes a massive outflux of $K^+$ from the neurons and a flux of $Na+$ into the neurons, which occur when the membrane ion pumps fail in the absence of sufficient energy for their functioning (at regional cerebral blood flow, CBF, of about 10% of the normal). At an ECF ($K^+$) of about 12–13 mM, the membrane potential change apparently becomes large enough to activate (or open) voltage-dependent $Ca++$ "gates" and develop a largely uncontrollable influx of $Ca^{++}$ (Siesjo, 1981), which at abnormally high cytosolic levels is essentially toxic (Siesjo, 1981; Trump *et al.*, 1981; Fleckenstein *et al.*, 1983; Nayler, 1983; Nayler *et al.*, 1983; Ruigrok *et al.*, 1983; Rasnussen and Barrett, 1984; Hearse *et al.*, 1984; Hansen, 1985). Although high cytosolic ($Ca^{++}$) may disrupt many intracellular functions (Rasmussen and Barrett, 1984), its activation of phospholipases $A_1$ and $A_2$ is considered to be the most damaging under hypoxic conditions (Siesjo, 1981). For $A_2$, this activation can be written as follows:




phosphatidylcholine (PC) lysoPC

When uncontrolled, the reaction leads to membrane phospholipid hydrolysis, to the consequent disruption of cell and mitochondrial membranes, to the release of telling free fatty acids (such as arachidonic acid) and to the further potentiation of ion redistributions (Siesjo, 1981). The overall metabolic impact can be summarized as in Fig 2.

In myocardial ischemia, energy defficiencies and membrane failures are also evident as indicated by intra-and extracellular changes in ($Na^+$) and ($K^+$) as well as by a large influx of $Ca^{++}$, a loss of sarcolemmal $Ca^{++}$ and a disruption of mitochondrial $Ca^{++}$ homeostasis. In addition, analogous membrane failure with associated translocation of ions between intra-and extracellular pools is found in liver under $O_2$ limiting conditions and in acute renal failure in mammals and is presumably general *in all hypoxia-sensitive animals and all hypoxia-sensitive organs and tissues during $O_2$ lack* (Farber *et al.*, 1981; Trump *et al.*, 1981). In sharp contrast, in the brain and other organs of 'good' ectothermic anaerobes during hypoxia (Sick *et al.*, 1982; Jackson and Heisler, 1983; Surlykke, 1983), *such breakdown of membrane function does not occur at all or the failure develops relatively slowly, despite metabolically depressed states in anoxia and proportionately lower ATP turnover rates* (Hochachka and Guppy, 1986). Obviously, something about cell membranes in 'good' ectothermic anerobes is different; under hypoxic conditions, either these membranes are more impermeables to ions or ionic pumping capacity can pace thermodynamic drift to electrochemical equilibrium. Stabilized membrane function almost certainly *cannot be due to acclerated ion pumping* because ATP turnover rates are lowered in the metabolically arrested states typical of animal anaerobes in anoxia. For this reason, we argue that the above membrane-based differences in the effects of hypoxia are mainly *due to different permeability barriers* and are an expression of a basic difference between cell membranes of hypoxia-tolerant, eurythermic ectotherms *vs* hypoxia-sensitive stenothermic ectotherms and endotherms. For convenience and because membrane permeability to ions depends upon densities of ion specific channels (see below), we term this the channel arrest concept of defence against hypoxia. This concept explains several previously poorly

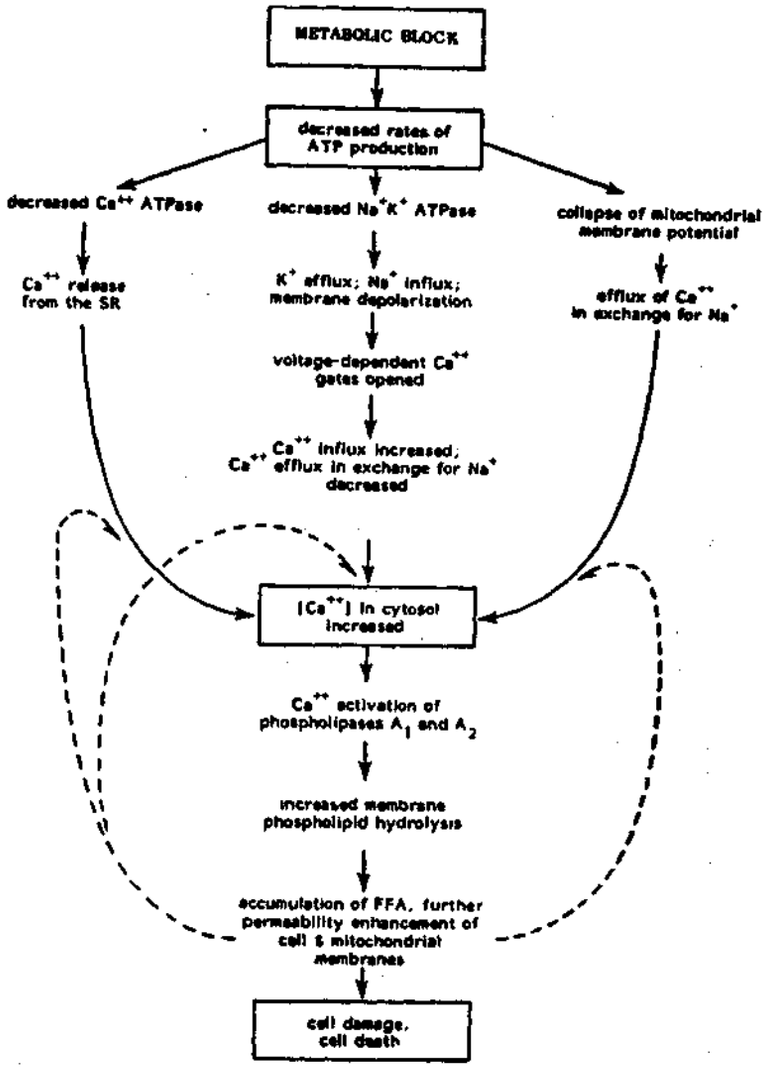



Fig. 2. A summary of probable metabolic events progressing in hypoxia-sensitive cells from the initial energetic consequences of $O_2$ limitation to cell damage and cell death. The summary is based on analysis in the text and is constructed from various studies of hypoxia-sensitive cells, tissues, organs and organisms by different authors.

understood observations rather well. In the first place, it explains (indeed predicts) the above-mentioned comparative studies showing that even if ion gradients do decline in hypoxia-tolerant ectotherms in hypoxia, the process is much slower than in endotherms (Sick *et al.*, 1982) and that in the extreme, ion gradients obviously remain stable after days of anoxia (Surlykke, 1983), even if ATP turnover rates are too low to explain the stability with ion pumping (Hochachka and Guppy, 1986).

Secondly, this interpretation helps to explain a rather long-standing paradox in environmental biochemistry and physiology on how organisms (both ectothermic and endothermic) respond to $O_2$ availability in the external medium. In order to clarify this, we need to describe the effect of $O_2$ availability on basal or standard metabolic rate (SMR).

## Where SMR Depends on Oxygen Availability

It is not surprising of course that the SMR of any given species depends to some extent on the general status of the organism. Analyses of determinants of SMR often focus on variables such as organ work rates, hormonal status, nutritional status, ionic concentration and composition and so forth. Any changes in SMR related to these parameters can be largely accounted for by changes in the rates of ATP turnover at the cell and tissue level; such SMR changes are readily understandable as arising from alterations in cellular work rates. Somewhat more perplexing and more relevant to our theme, however, are observations showing that some tissues and organs of animals at rest display $O_2$ consumption rates ($\dot{V}O_2$) which vary with $O_2$ availability; typically the higher the $O_2$ availability, the higher the $\dot{V}O_2$ reaching a plateau in some cases, but not in others (such as skeletal muscles). The key to this metabolic response may supply us with a key to the role of membrane function in controlled SMR and thus controlled metabolic arrest states.

To be sure, not all tissues and organs show this response and the brain is perhaps the best example of such an opposite pattern, with the cerebral metabolic rate ($CMRO_2$) being maintained nearly constant over quite large changes in $PaO_2$. When $O_2$ availability becomes low, $CMRO_2$ necessarily falls, but the energetic shortfall is at least partially made up by anaerobic glycolysis (by activation of a Pasteur Effect). Activation of a Pasteur Effect at the cell level can be viewed as a means for maintaining near-normoxic ATP turnover rates despite reduced $O_2$ availability. Glucose fermentation to lactate generates 2 mol ATP/mol glucose, compared to 36

mol ATP/mol glucose fully oxidised in aerobic metabolism, so to glycolytically make up the shortfall in oxidative ATP production, about 18 times more glucose must be consumed per unit time per unit mass of tissue. However, such large Pasteur Effects are rarely seen; for example, as mentioned in Chapter 2, a 14-fold activation of glucose consumption in anoxic sperm is one of the largest Pasteur Effects we have been able to find recorded in the literature. By contrast, over short (6-10 min) periods of $O_2$ limited function, the Pasteur Effect in the mammalian brain makes up only 50% of the $O_2$ deficit (which would represent about a 9-fold increase in glucose consumption rates under these conditions). During longer periods of brain anoxia, the magnitude of the Pasteur Effect declines and makes up for even a smaller fraction of the energetic shortfall.

Organs and organisms whose $O_2$ consumption rates are largely independent of $O_2$ availability over broad ranges are termed $O_2$ regulators, while those whose $\dot{V}O_2$ varies directly with $O_2$ availability are termed $O_2$ conformers. The metabolic response of the mammalian brain to changes in $PaO_2$ fits the pattern of $O_2$ regulators and represents an extreme end of a spectrum of responses observable in vertebrate tissues. Liver expresses an intermediate pattern while skeletal muscle is perhaps the most $O_2$ conforming of all mammalian tissues. In the case of liver, as $O_2$ delivery declines, liver $\dot{V}O_2$ initially remains constant, but even at fairly high $O_2$ delivery rates, $\dot{V}O_2$ begins to decline. This complex pattern of $\dot{V}O_2$ varying with $O_2$ availability up to some critical value is probably the most common pattern for mammalian cells and tissues. Even at the whole-organism level, at least in several small mammals, $\dot{V}O_2$ increases as $PaO_2$ increases towards a critical (usually species-specific) value, above which $\dot{V}O_2$ is essentially constant no matter how much more $O_2$ tensions or delivery are increased. Similar $O_2$ conforming patterns are common amongst numerous invertebrate groups and amongst ectothermic vertebrates. In some invertebrates, $\dot{V}O_2$ appears to increase with $PaO_2$ essentially indefinitely, but this does not seem to ever be evident in mammals. However, the $\dot{V}O_2$ of skeletal muscle appears to increase with no apparent limit; no plateau in $O_2$ consumption is reached even at very high $O_2$ tensions or very high $O_2$ delivery rates (Hochachka and Guppy, 1986).

$O_2$ conforming metabolic responses at the organ, tissues and cell levels present us with a thus far unresolved paradox which revolves around the way mitochondrial metabolism interacts with

molecular $O_2$. In all isolated mitochondria thus far studied, the apparent $K_m$ for $O_2$ is very low, perhaps in the 0.1–0.5 $\mu$M range. The paradox is that probably in all these $O_2$ conforming cases *the $O_2$ concentration in arterial plasma exceeds by large factors (10 fold or more) the value that would be required to fully saturate mitochondrial metabolism. Why then should $O_2$ conforming tissues and organisms behave as if they were truly $O_2$ limited?*

The paradox is not yet resolved and is really beyond the scope of this account. Suffice to emphasize that there seem to be three possible explanations for this discrepancy between *in vitro* and *in vivo* $O_2$ concentration dependence of aerobic metabolism: (i) *in vivo* mitochondrial metabolism may be truly $O_2$ limited, because of intracellular *diffusional* limitation even at fairly high arterial plasma $O_2$ levels, (ii) *in vivo* mitochondrial cytochrome oxidase is regulated to display a low $O_2$ affinity so that respiration varies with $O_2$ concentration even at fairly high $O_2$ tensions, or (iii) *in vivo* mitochondrial metabolism is regulated via an $O_2$ receptor, with $O_2$ concentration serving as a *set point* for mitochondrial metabolism, while regulating $O_2$ uptake by conventional metabolite signals (such as ADP availability) rather than by true substrate limitation. At this time, it is not possible to choose between these alternative. What is already evident, however, is that $O_2$ conformity implies some very considerable adjustments in membrane functions.

## Why $O_2$ Conformity Requires Membrane Adjustments

The reason we raise the issue of $O_2$ conformity in the first place is because in all $O_2$ conforming systems (be they cells, tissues, organs or organisms) *SMR obviously must decrease as $O_2$ availability decreases.* But then what happens to necessary, energy-consuming processes (defined as maintenance metabolism, to which ion pumping is one of the largest contributors)? One possibility is that the processes contributing to SMR also are turned down or off, a second possibility is that these operational costs are paid for by anaerobic ATP generating metabolism; in the latter event, a Pasteur Effect would be necessarily activated. The instructive insight is that *$O_2$ conforming systems typically do not show a Pasteur Effect.* Indeed, where $O_2$ conforming patterns are most numerous, namely amongst invertebrate groups and ectothermic vertebrates, a *reversed* Pasteur Effect is the rule, as discussed in Chapter 2. *In the absence of a Pasteur Effect, it is evident that the processes normally contributing to SMR in $O_2$ conforming organisms or cells are themselves necessarily downward adjusted as a function of $O_2$ availability.* As mentioned above the most important single

contribution to SMR probably is the maintenance of disequilibrium between intracellular and extracellular ion pools; *i.e.* ion pumping driven by ATP hydrolysis (Hochachka and Guppy, 1986). This energetic cost arises because, to communicate with the ECF or the external environment, cells must maintain ion-specific transport channels or pores in their membranes and these raise the spectre of osmotic or compositional equilibrium with the outside medium; as such an equilibrium is clearly incompatible with life, cells harness ATP-coupled, ion-specifie pumping mechanisms to maintain a normal physiological state far removed from equilibrium. *To maintain the same disequilibrium state at reduced SMR and thus at reduced pumping rates necessarily requires permeability adjustments* (closing of ion-specific channels). When these adjustments are realized, then-and only then-can $O_2$ conforming behaviour be expressed at the cell and tissue level. A point of emphasis is that such behaviour *and* such adjustments are most common and most striking in hypoxia-tolerant invertebrates and in hypoxia-tolerant endothermic tissues, as would be predicted by the channel arrest concept.

## Role of Membrane Permeability in Endothermy

A third problem area which the channel arrest interpretation puts into a somewhat clearer focus concerns the origins of endothermy. In particular, the concept is closely consistent with recent comparisons of mammals and reptiles (Else, 1981) which indicate (i) that ATP turnover rates and ouabain sensitivities of homologous tissues in mammals are about 5 times higher than in ectothermic reptiles, but (ii) that the 'leakiness' of mammalian cell membranes (measured as $Na^+$permeability) is also some several fold greater than in reptiles. Hulbert and Else (1981) suggest that the latter explains the former; *i.e.* the cost of endothermy is a higher rate of thermogenesis arising in part at least from 'leaky' membranes and from the consequent necessity for higher ion pumping rates and higher ATP turnover rates. Leaky membranes thus may be adaptive in endothermic tissues since they are a part of an $O_2$ fueled biological furnace, while nonleaky membranes are adaptive in anaerobes *because they allow metabolic arrest without the risk of a breakdown in ion regulation and membrane functions generally.* The implication *that leaky membranes may be inherent to endothermy, but result in an increased sensitivity to hypoxia in tissues of endotherms* is exactly what is predicted by the channel arrest concept and appears to supply the element - stabilized membrane function - missing in earlier attempts to extend the hypoxia tolerance of hypoxia sensitive tissue and cells (see Chapter 2).

## HYPOXIA-TOLERANCE : METABOLIC ARREST COUPLED WITH CHANNEL ARREST

Now the penny has dropped: this is the kind of insight or generalisation we have been waiting for and working towards. The insight specifies that a minimal requirement for establishing in hypoxia sensitive tissues the hypoxia tolerance of ectothermic anaerobes is to couple the reversed Pasteur Effect with nonleaky membranes : *couple metabolic arrest with channel arrest.* It seems simple enough a strategy, it seems eminently successfuli n ectothermic anaerobes; but is it experimentally realizable? Although the answer is not in yet, it is interesting that procedures aimed at precisely such a goal *have* been attempted. In these manoeuveres, the channel being targetted is the $Ca^{++}$ channel in order to block uncontrolled $Ca^{++}$ fluxes. The intervention is designed, in effect, to block one or more of the steps leading to a rise in cytosolic $Ca^{++}$ or to phospholipase activation as shown in Fig. 2.

$Ca^{++}$channel arresı, thus, is a first line of defence designed to protect hypoxia sensitive tissues against $O_2$ lack and it is often coupled with hypothermia-induced metabolic arrest. To our knowledge, this procedure applied to any mammalian tissue or organ so far investigated supplies a 'protective' effect against hypoxia for short time periods, but, unfortunately, prolonged hypothermia by itself strongly perturbs membrane-based and metabolic functions in mammal tissue and presumably in all low temperature sensitive cells or organisms. That is, hypothermia *per se* is damaging and its disrupting effects in combination with hypoxia may well be exaggerated. That is why hypothermia is not the metabolic arrest mechanism of choice for extending hypoxia tolerance of tissues sensitive to $O_2$ lack. To fully understand this, it is necessary to examine the environmental stress of hypothermia more closely, using the same analytical approach as in our analysis of hypoxia.

# 4

# DEFENCE STRATEGIES AGAINST HYPOTHERMIA

## Metabolism - Membrane Decoupling in Cold Sensitive Systems

It has long been realised that most endotherms and cold sensitive ectotherms are unable to survive well, or at all, at low body temperature ($T_b$) for extended time periods, but why should this be so when functions at $T_b$ near 0°C is so commonly observed in cold-tolerant ectotherms and mammals capable of torpor or hibernation? While there may well be many biochemical 'problems' at low $T_b$ which cold sensitive organisms cannot solve (Hochachka and Somero, 1984), the two most important ones thus far indentified are (i) maintaining regulated and adequate metabolic rates and (ii) maintaining ion gradients. Because the first of these has recently been analyzed (Musacchia, 1984), we here need only emphasize that two facets of metabolism are disrupted in hypothermia: metabolic rate and metabolic regulation, partly as a result of simple $Q_{10}$ effects and partly as a result of substrate limitation. Tissue-specific metabolic pathways may be expected to display unique $Q_{10}$ values (Hochachka and Somero, 1984), so if hypothermic metabolic depression is largely unregulated, the functional decline of one organ (*e.g.* heart) need not parallel that of others (*e.g.* respiratory system). Not surprisingly, in hypothermic animals, respiratory failure may occur more quickly and at higher temperatures than does cardiac failure while these functions are depressed in synchrony during the *regulated* metabolic depression sustained in the hypothermia of cold-tolerant species (Willis, 1979; Musacchia, 1984).

A disruption in metabolic regulation is also indicated by disrupted glucose homeostasis: cold-sensitive animals during imposed hypothermia may experience a hypoglycemia so severe that plasma

glucose concentrations fall far below normal, sometimes an order of magnitude below normal! Yet the immediate precursor of plasma glucose, liver glycogen, is retained at near-normal levels, a situation, clearly indicating severe perturbation of normal regulatory mechanisms (Musacchia, 1984). While much has yet to be learned about the details of such metabolic disarrangements, it is clear that imposed hypothermia is characterised by a serious mismatch between substrate availability and substrate delivery and ultimately between ATP requirements and ATP generating capacities. In terms of cell survival, one of the critical functions influenced by this mismatch is ion regulation.

The regulation of $K^+$, $Na^+$, $Ca^{++}$, of $Mg^{++}$ concentrations in ICF and ECF is in fact often used as a criterion of cell survival in the cold, because such ion regulation is essential for maintenance of cell volume and specific metabolic processes (*e.g.* protein synthesis) and can be used as an indicator of cell integrity and sustained function. In principle, most cold-sensitive cells and tissues lose $K^+$ at low temperature because the *temperature coefficient for leakage of $K^+$ is smaller than the temperature dependence of ATP-dependent, active accumulation* (or of energy metabolism *per se*). As a result, $K^+$ efflux at low cell temperatures is blocked less than inward $K^+$ pumping; *efflux therefore exceeds $K^+$ influx* so that ICF and ECF $K^+$ concentrations drift towards their equilibrium positions (Willis, 1979; Kamm *et al.*, 1979). $Na^+$ moves in the opposite way, and if this process is not brought under control, it presumably leads to partial depolarisation, the opening of voltage-dependent $Ca^{++}$ channels (Siesjo, 1981; Rasmussen and Barrett, 1984), and the influx of $Ca^{++}$ (Goto *et al.*, 1978; Baller *et al.*, 1983). $Ca^{++}$ influx may also be facilitated by $Na^+/Ca^{++}$ exchange or by damaged (*i.e.* low temperature modified) $Ca^{++}$ channels behaving as $Na^{++}$ channels, a process which would increase $(Na^+)$ in the ICF and set the stage for activated $Na^+/Ca^{++}$ exchange. In addition, a low temperature characteristically leads to a loss of $Ca^{++}$ from the sarcoplasmicreticulum (SR), which is partly due to an imbalance between rates of SR $Ca^{++}$ uptake (by $Ca^{++}$ ATPase) and rates of $Ca^{++}$ efflux (Newbold and Tume, 1977); *i.e.*, an imbalance between 'leak' and pump, as in the plasma membrane $K^+$ regulation. In addition, because a drop in cell temperature alters the fractional dissociation of imidazole groups on proteins, the intracellular pH rises (White and Somero, 1982) and directly activates $Ca^{++}$ efflux from the SR (Newbold and Tume, 1977). The net effect of all these processes would seem to necessarily lead to gradually increasing $(Ca^{++})$ in the cytosol and to some (perhaps all) of the metabolic perturbations shown in Fig. 3.

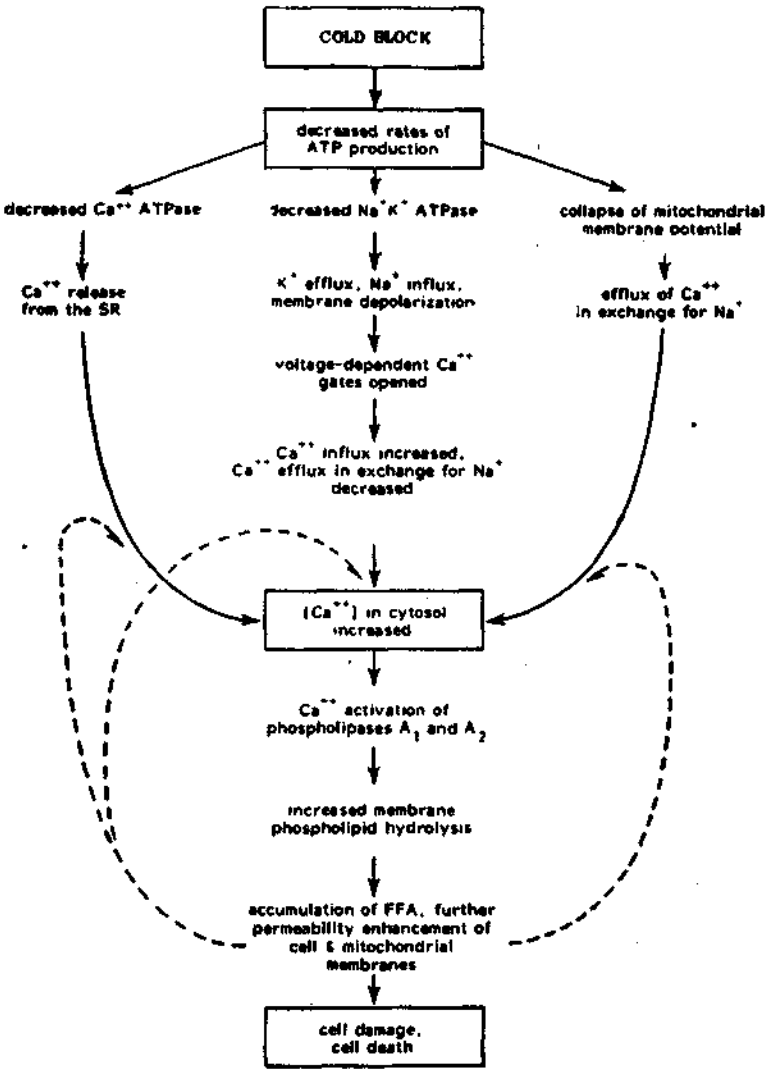


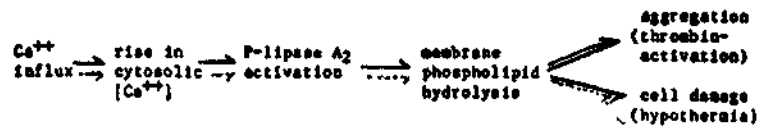

Fig. 3. A summary of probable metabolic events progressing in hypothermia sensitive cells from the initial cold block to energy metabolism, to consequent perturbation of $Ca^{++}$ homeostasis, and finally to cell damage and cell death. The summary is constructed from various studies of cold-sensitive cells, tissues, organs and organisms from different authors.

In platelets (Imai *et al.*, 1982, 1984), for example, hypothermia indirectly appears to differentially facilitate the following reactions:

$$\text{phosphatidylcholine (PC)} \xrightarrow{H_2O} \text{lysoPC} + \text{arachidonic acid}$$

$$\text{phosphatidylethanolamine (PE)} \xrightarrow{H_2O} \text{lysoPE} + \text{arachidonic acid}$$

The end products of these reactions normally are formed after thrombin activation of platelets, but during hypothermia the above reactions proceed without thrombin, indicating a low temperature-mediated 'activation' of phospholipase $A_2$ (Imai *et al.*, 1982, 1984). From parallel studies it is known that the influx of $Ca^{++}$ occurs prior to the onset of lysoPC and lysoPE formation and the liberation of arachidonic acid (Imai *et al.*, 1982, 1984). Thus either one of two conditions (hypothermia or thrombin activation) is able to initiate the same chain of metabolic events:




As in hypoxia, the pathogenic pathway favoured by prolonged hypothermia may be autocatalytic in the sense that cell membrane damage may in turn accelerate further dissipation of ion gradients. Any cellular defence mechanisms requiring activated ion pumping would also require increased ATP turnover rates, so *an energy-depressed state (as may be expected in hypoxia), if prolonged, would probably aggravate the low temperature problem.* Thus hypothermia coupled with hypoxia is probably an unsatisfactory combination.

## Lessons from Animal Extremists on How to Survive Hypothermia

From the above analysis, it is evident that the cells and tissues of hypothermia-tolerant ectotherms and endotherms must main tain (i) regulated metabolism and (ii) regulated membrane functions despite low cell temperatures. Although mechanisms employed are still not fully understood and are being actively researched (Goldman and Willis, 1973; Ellory and Willis, 1978; Willis *et al.*, 1980), it has

long been appreciated that metabolism during the hypothermia of cold torpor, estivation or hibernation is not a simple resultant of thermodynamics; metabolism is slowed down, but nonetheless it is clearly and closely regulation in both ectothermic and endothermic systems (Willis, 1979;Musacchia, 1984; Hochachka and Somero 1984). More recent studies (Goldman and Willis, 1973;Ellory and Willis, 1978; Willis, 1979; Willis *et al.*, 1980) furthermore indicate that, as was earlier assumed, membrane functions also are maintained during hypothermal stress despite a greatly depressed metabolism. Regulated metabolism and regulated membrane functions maintain myocardial function in deep hibernation of ectotherms and endotherms, which thus sustains adequate blood pressure and contributes to rapidly expandable heart rates and cardiac outputs. Similarly, sustained contractions of skeletal muscles are allowed for maintained respiratory movements; in rodents, skeletal muscle metabolism also provides much of the heat for periodic arousal (Willis, 1979). The functions of other cells and tissues, particularly the brain and peripheral nerves, also are potently protected on a long-term basis during the hypothermia of deep torpor and hibernation (Willis, 1979). Although some ionic redistribution occurs [for example, $Mg^{++}$ availability in the ECF rises (Al-Badry and Taha, 1983)] the uncontrolled dissipation of ion gradients between different intracellular and extracellular compartments typical of cold-sensitive cells does not occur in these animals. If it did, none of the above membrane-based functions could be sustained. Obviously, *something* about cell membranes of cold-tolerant species differs dramatically from cold-sensitive cells. Either, as in the ectotherm anaerobe case mentioned above, cold-tolerant cells are fundamentally *less permeable* during hypothermia than cold-sensitive cells or they maintain higher ion pumping capacities. We do not consider the latter mechanism very likely because metabolic rates during hibernation and cold torpor may be reduced by nearly two orders of magnitude (Musacchia, 1984; Hochachka and Somero, 1985). Moreover, recent of $Na^+$ $K^+$ ATPase studies (Goldman and Willis, 1973; Ellory and Willis, 1978; Willis *et al.*, 1980) are illuminating: although the enzyme is 'cold-adapted' in hibernators (displays higher catalytic capacities at low temperature, higher affinities for $Na^+$ and $K^+$, and probably higher affinity for ATP), as indeed would be expected for many enzymes in ectotherm and endotherm hibernators (White and Somero, 1982; Hochachka and Somero, 1984), no single feature nor combination of kinetic features is adequate to account for the low-temperature viability differences between cold-sensitive and cold-tolerant species Thus we are led to the conclusion that cold-tolerant cells in hypothermia are able to maintain near-normal membrane functions (*i.e.* maintain near-normal

TABLE 3. *Summary of several fundamental features of cells tolerant to hypoxia and hypothermia* vs *cells which are sensitive to these two environmental parameters*

| HYPOXIA TOLERANT | | HYPOXIA SENSITIVE |
|---|---|---|
| 1. Metabolic arrest capacity (reversed Pasteur Effect) | intervention mechanisms ← | 1. No metabolic arrest capacity (standard Pasteur Effect) |
| 2. Low-permeability membranes Low $Na^+$ $K^+$ ATPase | transition from eurythermy to stenothermy → | 2. High permeability membranes High $Na^+$ $K^+$ ATPase |
| 3. ATP synthesis rates = ATP utilisation rates | | 3. ATP synthesis rates cannot pace energy requirements for ion pumping |
| 4. Stable membrane functions; stable ion gradients | | 4. Disrupted membrane function; dissipated iongradients; $Ca^{++}$ influx |

| HYPOTHERMIA TOLERANT | | HYPOTHERMIA SENSITIVE |
|---|---|---|
| 1. Regulated metabolic depression | intervention mechanisms ← | 1. Metabolic depression simply by $Q_{10}$ effects |
| 2. Low-permeability membranes | transition from hibernation to eurythermy → | 2. High-permeability membranes |
| 3. ATP synthesis rates = ATP utilization rates | | 3. ATP synthesis rates cannot pace energy requirements for ion pumping |
| 4. Stable membrane functions; stable ion gradients | | 4. Disrupted membrane function; dissipated ion gradients; $Ca^{++}$ influx |

ion concentration differences between various internal and external compartments in the face of greatly reduced cell metabolic rates) in large part *because they maintain cell membranes fundamentally less leaky than those in cold-sensitive cells.* For convenience, we term this "the channel arrest concept of hypothermia tolerance." It describes the same strategy that is utilized in anoxia by "good" animal anaerobes and in both cases channel arrest can be viewed as a solution to a common problem: *ATP synthesis capacities being potentially more depressed than are membrane leaks.* In hypothermia-tolerant ectotherms and endotherms, the drop in ATP synthesis rates is due to a regulated metabolic depression at reduced cell temperatures, while in hypoxia in animal anaerobes it is due to reduced $O_2$ availability and the reversed Pasteur Effect. To keep ion pump demands for ATP in balance with reduced ATP synthesis rates, natural selection seems to favour low-permeability (channel arrested) membranes in response to both environmental parameters (Table 3). The final problem we need to consider therefore is how the two common adaptations are achieved. This in turn may supply us with insights (i) into the application of analogous intervention strategies for protection against hypoxia (metabolic arrest mecha nisms *other than hypothermia*) and (ii) into ways to assess stress on cultured aquatic species due to combined environmental parameters.

## How Membrane Permeability is Regulated

Because moment-to-moment regulated changes in membrane permeabilities are requisite for normal function in most tissues and organs, any large permeability differences between homologous cold-tolerant and cold-sensitive tissues must be due to fundamental specialisations in the way membranes are structured or in their regulated function. From what is currently known about membrane properties (Hille, 1984) for an excellent overview) such basic differences could arise from (i) change in the phospholipid composition and consequently in functional properties of membranes, (ii) change in the ratio of functional/non-functional ion-specific channels by change in the recruitment of channels from nonfunctional pools or "storage" sites or (iii) change in the abundance of "pores", *i.e.* in the density of ion-specific protein channels.

The first of these, requiring adjustments at the level of bilayer composition, is probably of minimal importance since a major function of bilayer adjustments is to lower the temperature for phase transition thus, allowing *normal membrane fluidity and normal membrane functions despite lower cell temperatures.* In all animals such "homeoviscous adaptations" in effect contribute to functionally

similar membrane functions at different cell temperatures and thus, probably do not contribute heavily to any tissue permeability differences between cold-tolerant and cold-sensitive animals, a conclusion consistent with recent tests finding no correlation between membrane fluidity *per se* and permeability to ions (Costanzo *et al.*, 1983). Thus we are left with the ion channels option.

## Ion Channel Density Regulation

In assessing this matter, we should emphasize at the outset, as Hille (1984) has done, that even if bilayer adjustments influence permeability properties, they presumably do so mainly by changing the properties of proteins called ion channels. To this point, we have referred to channels rather loosely. Now we should clarify that ions move across cell membranes through voltage-regulated or receptor-regulated aqueous pores or ion-specific channels each of which has characteristic permeability, selectivity and kinetics. In skeletal muscle and nerves, electrical excitation involves voltage- and time-dependent changes in $Na^+$, $K^+$ and $Ca^{++}$ permeabilities through $Na^+$, $K^+$ and $Ca^{++}$ specific ion channels. At rest, $Cl^-$ channels and other $K^+$ channels carry the dominant conductances (Stefani and Chiarandini, 1982) for example. Perhaps best understood of identified ion channels, the $Na^+$ channel from at least two tissues is now described down to subunit composition and is being analyzed at the subunit level. When solubilized, the $Na^+$ channel protein from both muscle and nerve (Catterall, 1984) is about 316,000 daltons in size and consists of three nonidentical subunits. Transmembrane $Na^+$ flux is mediated by a hyrophilic pore containing a selective ion co-ordination site. By the selective use of neurotoxins which bind with high affinity to specific sites on the $Na^+$ channel protein, it has become evident that $Na^+$ conductance through the channel is regulated or "gated" by controlling the rate and voltage-dependence of opening and closing of the channel (Hille, 1984; Catterall, 1984).

It is generally believed that such fundamental features as protein size, oligomeric structure, subunit composition, ion coordination site, pore size and neurotoxin binding sites, are common to the $Na^+$ channel protein in all cell membranes, from different tissues and from different species; that is, *like channel proteins in general $Na^+$ channels in particular appear to be highly conservative* (Rogart, 1981; Hille, 1984; Catterall, 1984). *Channel density per $\mu m^2$ of membrane surface, however, varies between and within tissues and in different functional states within the same tissue.* The number of channels per $\mu m^2$ in rat brain, for example, ranges from about 100 in unmyelinated axons to possibly over 10,000 at the nodes of Ranvier in

myelinated nerves, where the highest $Na^+$ fluxes are required (Rogart, 1981; Catterall, 1984). In addition to the number of channels per unit area of membrane being regulated by long term mechanisms, channel density may be modifiable on a moment-to-moment basis during transitions between different metabolic and physiological states. For example, recent studies of toad bladder indicate that antidiuretic hormone and aldosterone may influence the density of ion channels by controlled recruitment of performed channels possibly stored in the cytosol (Li *et al.*, 1982; Palmer *et al.*, 1982). Although additional studies on shorter-term regulation of numbers of functional channels in membranes clearly are needed, the evidence already available indicates that (at least in the long term) *regulating channel density may be a universal way of meeting tissue-, cell- and ion-specific permeability requirements* in different microenvironments or different metabolic states In such event, the common utilisation of this strategy may explain the observed permeability differences in cell membranes between cold-tolerant and cold-sensitive cells (Goldman and Willis, 1973; Ellory and Willis, 1978; Willis, 1979; Willis *et al.*, 1980; Baller *et al.*, 1983) and between homologous tissues in "good" anaerobes *vs* hypoxia-sensitive endotherms (Sick *et al.*, 1982; Surlykke, 1983). Furthermore, exactly the same considerations hold in principle for membranes of mitochondria and of other intracellular organelles (Miyahara *et al.*, 1984). Although systematic, channel-density studies of homologous cold-sensitive and cold-tolerant tissues designed to quantitatively test this idea are not available, it may be mentioned that, in the rabbit, $Na^+$ channel densities in unmyelinated nerves (vagus) average about 110 pores per $\mu m^2$, while in analogous axons in ectotherms (lobster and garfish), channel densities are 90 and 35 per $\mu m^2$ respectively (Catterall, 1984). Similarly, in rat fast, slow and diaphragm muscle, $Na^+$ channel densities of 557, 371 and 421 $\mu m^2$ respectively contrast with densities in frog skeletal muscle membranes as low as 195 per $\mu m^2$ (Rogart, 1981). The occurrence of lower $Na^+$ channel densities, not coincidentally, correlates with lower $Na^+$ $K^+$ ATPase activities (lower $Na^+$ pump densities) in cell membranes from ectotherms compared to endotherms (Hulbert and Else, 1981; Else, 1984). Similar differences in $Ca^{++}$ channel densities may be the reason, why, as observed by Ruigrok *et al.* (1983), the '$Ca^{++}$ paradox' is expressed in the mammalian heart, but not in the amphibian one. If these considerations are correct, they supply a simple explanation for the apparent permeability differences in hypoxia and hypothermia sensitive *vs* tolerant cells. 'Channel arrest' in the latter simply means fewer functional, ion-specific channels, reduced ion leakage and thus reduced requirements for ATP-dependent ion pumping.

# 5

# ENVIRONMENTAL ADAPTATION THEOREMS

## Coupled Metabolic and Membrane Adaptations

An important outcome of our analysis of hypoxia and hypothermia is that several common processes in cellular adaptations for tolerance of these two environmental stresses emerge. In both conditions, an unavoidable (if sometimes desirable) depression in ATP synthesis rates is potentially greater than any associated change in the passive leak of ions across membranes. In the absence of any additional adjustments, ATP synthesis rates cannot match ATP requirements for sustained stable membrane functions. In cold-sensitive and anoxia-sensitive cells, this problem does not appear to be resolvable and during prolonged anoxic or hypothermic exposure ion gradients dissipate, intracellular $Ca^{++}$ concentrations rise and activate membrane phospholipid hydrolysis in a process which ultimately leads to cell damage or cell death. Anoxia-tolerant and hypothermia-tolerant cells avoid this $Ca^{++}$ mediated pathogenic process by maintaining low permeability membranes (possibly by means of lower ion-specific channel densities) so that the energy cost of ion pumping can be matched by the rates of ATP synthesis realizable under sustained hypoxic or hypothermic conditions (Table 3).

An instructive insight arising from this interpretation is that critical provisions for tolerance of hypoxia overlap with provisions for tolerance to hypothermia. Cells and tissues protected against hypoxia should automatically be at least partially protected against hypothermia. Interestingly, it has been known for many years that the foetus and neonate in mammals display a greater hypoxia tolerance than the adult (Mott, 1961; Haddad and Mellins, 1984); our analysis would predict, and it is indeed observed, that they are

also more tolerant of hypothermia (Sinclair, 1976). The reverse situation (mechanisms for protecting tissues against hypothermia being automatically protective against hypoxia) need not hold since other requirements (such as appropriate regulation of anaerobic metabolism, appropriate means for solving end products problems and so forth) may not be realisable. Still, it is perhaps worth noting that fishes, amphibians and reptiles even in modest cold torpor or hibernation have been long known to be unusually hypoxia-tolerant compared to the same species when normothermic. A part of this tolerance may arise from the metabolically depressed state caused by reduced temperatures; another part may well arise from the maintenance of less leaky cell membranes at least in those tissues and organs whose sustained function is most crucial to successfully surviving the stress period.

While the above is simply a short summary of the indepth analysis of Chapter 2, it can be more quantitatively defined as a specific theorem of hypoxia-hypothermia adaptation in the following way: To be able to sustain prolonged periods of hypoxia or hypothermia, *organisms must balance the rates of non-specific ion leaks with an enforced decline in rates of ion pumping, a biological end which is achievable if, and only if, downward adjustments in regulated metabolism are step-to-step coupled with downward adjustments in membrane functions.* That is, to sustain prolonged periods of depressed metabolism (reversed Pasteur Effect and metabolic arrest in hypoxia; cold-induced metabolic arrest in hypothermia), the following relationships must be realised or at least approached.

$$t_H = k_1 (1/M_{ATP}) \text{ or } t_C = k_2 (1/M_{ATP})$$

$$P = k_3 (M_{ATP})$$

$$J_{(leak)} = J_{(pump)}$$

where $M_{ATP}$ is the metabolic rate in $\mu$mol ATP cycled $g^{-1} min^{-1}$, $t_H$ is the hypoxic survival period (a measure of the severity of metabolic arrest required), $t_c$ is the hypothermic survival period, $k_1$, $k_2$ and $k_3$ are constants, P is the effective, energy dependent membrane potential, and $J_{(leak)}$ and $J_{(pump)}$ are the ion leakage rates and ion pumping rates respectively (no specific pumping mechanisms being assumed except that they be coupled to ATP hydrolysis).

## Testable Predictions of Specific Theorem

Theorems are useful if they help to explain previously perplexing and poorly understood data and if they help in charting the way to further studies; as specified above and in Table 3, ours is helpful by the first criterion, but is it useful by the second? Only future experiments can fully answer this question, yet at the least our analysis is heuristic and supplies some new guidelines. One experimental approach for example would interfere with processes utilised for sustained anoxia survival by "good" anaerobes. It would aim to block or reverse metabolic arrest mechanisms typical of the anoxic state and preferentially open up ion-specific membrane channels so as to facilitate rapid dissipation of ion gradients. Our theorem would then predict that these manipulations must necessarily convert anoxia-tolerant organisms, tissues or cells *simultaneously* into anoxia-sensitive and hypothermia-sensitive ones. In metabolic terms, it would be tantamount to moving left along the arrows in Table 3.

A second testable prediction, the flip side of the first, assumes that if ATP turnover rates in stenothermic or endothermic systems can be significantly curtailed and if 'leaks' (*i.e.* dominant ion-specific membrane channels) can be effectively blocked (so that ATP production rates can continue to pace rates of ATP utilization by ion pumping), then both anoxia tolerance and hypothermia tolerance should be greatly expanded. In metabolic terms, these manipulations would be analogous to moving right along the arrows in Table 3. If successful, studies along both experimental lines would have extensive theoretical and practical implications.

## Environmental Adaptation : The General Theorem

Only a moment's contemplation is required to realise that the above hypoxia - hypothermia specific adaptation theorem can be generalised, presumably to *all* environmental parameters. In this view environmentally sensitive cells, tissues, organs or organisms display a cascading series of mutually reinforcing responses when their environment changes: Firstly, metabolic rate processes change strictly according to thermodynamic effects *i.e.* metabolic regulation is disrupted and rates of ATP synthesis drop. Secondly, membrane structural and functional adjustments are not realisable, so changes in leak rates are also determined strictly by thermodynamics. Since ion leakage is a physical process (diffusion) its coefficient of change is never the same as the coefficient of change for ATP generating metabolic pathways; that is, change in rates of ion

leakage with environmental change cannot be appropriately balanced by changes in ATP-dependent ion pumping. For convenience, we say the leak = pump flux equivalence cannot be maintained, or the leak/pump flux ratio cannot be maintained at unity. Uncontrollable ion fluxes develop leading to further reductions in ATP synthesis rates, which further unbalance leak and pump rates and so forth. A self-reinforcing, positive feedback cascade develops which if left uninterrupted for long enough terminates in cell damage and cell death.

In cells or organisms tolerant to environmental change selective pressure is clearly to reverse the downhill cascading cycle: breakdown in metabolism and metabolic regulation→altered leak/pump ratio uncorrectable by adjustments in membrane structure or by change in ATP-dependent pumping rates→membrane failure→uncontrollable influxes→cell damage→cell death. The key adaptations of organisms tolerant to environmental change must, in this view, include (i) metabolic changes closely tuned to the environmental change, but under equally close modulator regulation and (ii) associated adjustments in membrane permeability properties (possibly by channel density adjustments) so as to keep changes in leak (a physical process) equal to changes in pump (a chemical process driven by metabolism). As a result, the ATP $\rightleftharpoons$ ADP + $P_i$ cycle can be maintained in equilibrium in the new environmental condition as can electrochemical potentials across cell membranes, the two together in effect allowing sustained tolerance of the environmental change.

In more quantitative terms, a general theorem of envirnomental adaptation can be stated as follows: To be able to sustain prolonged periods of environmental change, *organisms must balance rates of non-specific ion leaks with enforced changes in rates of ATP synthesis and thus in rates of ion pumping, a biological end which is achievable if, and only if, upward or downward adjustments in regulated metabolism are step-to-step coupled with adjustments in membrane functions.* That is, in organisms able to sustain prolonged environmental change, the following relationships again appear to be selected for:

$$t_s = k_4 \, (1/M_{ATP})$$

$$P = k_5 \, (M_{ATP})$$

$$J_{(leak)} = J_{(pump)}$$

where $t_S$ is survival time during exposure to the environmental stress parameter (usually the greater the reduction in $M_{ATP}$, the longer the survival time, all else being equal), $k_4$ and $k_5$ are constants and the other symbols are as specified above. Obviously, the general theorem is a direct extension of the parameter-specific ones.

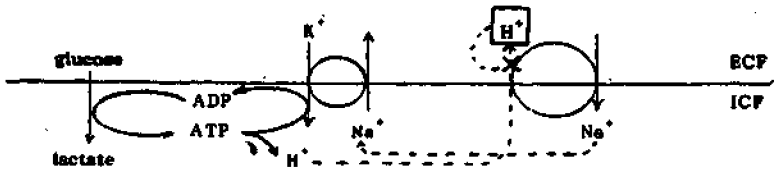
Although we acknowledge that there are many other facets to biochemical adaptation to the environment (Hochachka and Somero, 1985), it is surprising how many of these can be readily explained as subserving the above defined homeostasis: *balanced metabolic and membrane functions*. Thus adaptations directed towards facilitating this homeostasis would include a host of environmentally induced adjustments in enzyme catalytic and regulatory properties, in enzyme content and in enzyme pathway participation (in effect, facilitating adjustment in metabolism for preventing membrane failure, for keeping changing rates of membrane leaks balanced with ion pumping rates). Other adaptations in this category would also include a host of membrane-based adjustments in ion pump densities, in ion channel densities, or in membrane bilayer composition (facilitating maintenance of a leak/pump ratio of unity and thus balancing the energetic demands of membrane-based ion regulation with cellular metabolic capacities for generating ATP during the environmental change). Giving functional meaning to previously well known adaptations is thus one satisfying outcome of our dedutcive analysis, but it is not the only one. Another is the fresh possibilities for new defence strategies against environmental change that become evident.

## General Defence Strategies Against Combined Environmental Stresses

In broader terms or more specifically, in aquaculture terms, this new theorem of adaptation suggests that *any* combination of environmental factors causing *differential* changes in leak *vs* pump (*i.e.* in a physical process *vs* a metabolic one) may lead to potentially perturbing or even lethal states. One such combination, for example may be the *simultaneous* occurrence of salinity increases with temperature decrease, as may be expected in brackishwater during high tide, during storms, or in other situations. Under these conditions, steeper concentration gradients for numerous ions favour higher passive leaks into cells, thus *demanding higher ion pumping rates, higher of ATP synthesis and higher substrate plus $O_2$ fluxes when metabolism is being simultaneously depressed by low temperatures*. Thus low temperature aggravates problems generated by high salinity and *vice versa*.

Another obviously undesirable combination of environmental stress is the occurrence simultaneously of hypoxia plus increased salinity. In this situation, steeper ion concentration gradients again increase leak and demand for ATP-dependent ion pumping to keep the leak/pump flux ratio at unity. However, simultaneously ATP synthesis rates (for the required ion pumping) are depressed by hypoxia, again raising the spectre of entrance into the self-reinforcing self-destruct patterns typified by uncontrollable fluxes of unwanted ions into cells.

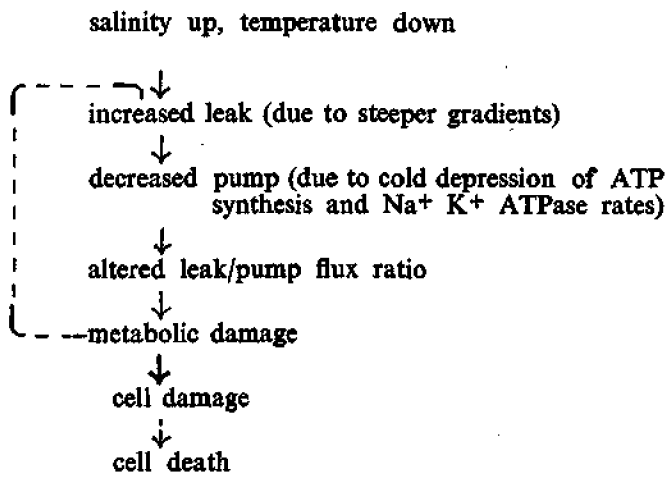
A third debilitating, if rather interesting, combination of environmental stresses is the simultaneous occurrence of low external pH (high $H^+$ concentrations) and low temperature. Under normal circumstances, $H^+$ efflux from cells is considered to depend upon a cotransport of $Na^+$ inwards. In some cells, the $Na^+$ $K^+$ ATPase may be preferentially driven by glycolytically-derived ATP, because this linkage generates $H^+$ which on efflux supply $Na^+$ for the $Na^+$ $K^+$ ATPase:




Elevated concentrations of external $H^+$ by mass action effects necessarily slow down efflux of internal $H^+$, thus slowing down $Na^+$ delivery to $Na^+$ $K^+$ ATPase and ultimately slowing the rate of ion pumping. With a simultaneous low temperature block, this situation is further aggravated and the rate of ion pumping is further reduced, because of fairly high $Q_{10}$ values normally found for ATP-generating pathways and for $Na^+$ $K^+$ ATPases *per se*. The $Q_{10}$ for diffusion in contrast is close to unity, so ion leaks are only modestly reduced when temperature declines. The net effect of low external pH plus low temperature therefore is a large depression in ATP synthesis rates and in $Na^+$ $K^+$ATPase rates with only a small effect on ion leakage. Leak/pump flux ratios cannot be maintained at unity and thus the stage is set for the familiar perturbing consequences of a membrane failure made assuredly irreversible because of further ion-induced metabolic blocks.

In all such potentially risky combinations of environmental stresses, the problems generated for the organism are mutually reinforcing and it is evident that natural selection would strongly favour processess that would tend to reverse or block such mutua-

lism. To take a specific example, for organisms at frequent risk of simultaneous insults from large increases in salinity coincident with decreases in temperature, natural selection would potentially favour processes which would block or reverse the ion-based uncontrollable spiral:




For more applied settings such as aquaculture, this analysis supplies us with a clue for design of a general intervention strategy. As with selection in natural systems, the goal clearly is to reverse the above kinds of pathogenic pathways. However, the only way such self-destruct cycles can be blocked is by *decoupling the mutually reinforcing environmental stresses*, thus breaking the linkage necessary for rapid alteration of leak/pump flux ratios. This can be illustrated by pursuing the example of high salinity combined with low temperature where an effective intervention strategy is to *compensate for decreased temperature by controlled reduction in salinity*. In effect, the aim is to couple reduced pump fluxes (due to low temperature) with reduced need for pumping; *i.e.* with reduced leak (by lowering the ion concentration gradients between the ECF and ICF). The reverse coupling of course is where the problem lay. In the case of hypoxia combined with increasing salinity, our intervention strategy would compensate for increased salinity with controlled increase in $O_2$ availability or *vice versa*. In the case of low temperature combined with low pH, the intervention strategy should aim to compensate for decreased temperatures by controlled increase in pH or *vice versa*, whichever alternative is more readily achieved.

While these interventions may sound theoretically straightforward, in practice they may represent rather difficult tasks (which are beyond the scope of this essay). Nevertheless, despite obvious difficulties and because the effects of environmental interactions are now on sound theoretical footing, interventions should be considered on practical planes. In classical agriculture and in medicine, intervention is the name of the game, the means by which the system under examination is brought under control. Perhaps, the time is now ripe for aquaculture to move in the same direction.

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