PRINCIPLES OF MEDICAL BIOLOGY

Edited by

E. EDWARD BITTAR

NEVILLE BITTAR

CELL CHEMISTRY AND PHYSIOLOGY: PART I

Cell Chemistry and Physiology: Part I

PRINCIPLES OF MEDICAL BIOLOGY
A Multi-Volume Work, Volume 4

Editors: **E. EDWARD BITTAR,** *Department of Physiology, University of Wisconsin, Madison*
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Principles of Medical Biology
A Multi-Volume Work

Edited by **E. Edward Bittar,** *Department of Physiology, University of Wisconsin, Madison* and
Neville Bittar, *Department of Medicine, University of Wisconsin, Madison*

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Cell Chemistry and Physiology: Part I

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PREFACE

This is the first of a 4-volume module that is an introduction to the study of cell chemistry and physiology. It is not intended to be encyclopedic in nature but rather a general survey of the subject with an emphasis on those topics that are central to an understanding of cell biology and those that are certain to become of increasing importance in the teaching of modern medicine.

We have followed what appeared to us to be the logical divisions of the subject beginning with proteins. Allewell and her colleagues stress the point that proteins fold spontaneously to form complex three-dimensional structures and that some of them unfold with the help of proteins called chaperones. Michaelis-Menten kinetics are shown by Nelsestuen to describe the behavior of enzymes in the test tube. The formalism is particularly useful in the search for agents of therapeutic value, as exemplified by methotrexate. Uptake by mammalian cells of substrates and their metabolic conversions are discussed by van der Vusse and Reneman. However, both Welch and Savageau expound the view that the cell is not simply a bagful of enzymes. The biologist is urged by Savageau to abandon Michaelis-Menten formalism and apply the Power Law. The biologist is also told that the approach to arriving at a theory of metabolic control would have to be one of successive approximations requiring the use of the computer. Information gained from comparative biochemistry is shown by Storey and Brooks to have shed new light on mechanisms of metabolic rate depression and freeze tolerance, and to be applicable to organ transplantation technology. We are reminded that enzyme adaptation is

partly the result of the presence of a hydrating shell of vicinal water that stabilizes conformation of the enzyme. Vicinal water, according to Drost-Hansen and Singleton, lies adjacent to most solids and protein interfaces. The kinks or breaks observed in the slope of the Arrhenius plot are attributed to structural changes in vicinal water. Regulation of cell volume is shown by Hempling to involve regulation of cell water. It could be that the osmo-receptor or volume detection system is a protein that links the cytoskeleton to specific K and Cl channels. Additionally, it is interesting that aquaporins, which are water channel-forming membrane proteins, are now known to exist in both renal and extra-renal tissues. One of the renal porins is affected by vasopressin.

We then pass on to protein synthesis (Rattan) and other important topics including protein glycosylation (Hounsell), methylation (Clarke), ADP-ribosylation (Pearson) and prenylation (Gelb). Among the four types of lipids attached to membrane proteins are the prenyl groups. Ford and Gross in their chapter on lipobiology drive home the point that there is an accumulation of acyl carnitine and lysophospholipids during myocardial infarction.

It goes without saying that we owe these scientists a special debt of gratitude for their scholarly contributions. We also take this opportunity of thanking the editorial and production staff members of JAI Press for their courtesy and skill.

E. EDWARD BITTAR
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Chapter 1

Proteins: An Introduction

NORMA M. ALLEWELL, VINCE J. LICATA, and XIAOLING YUAN

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INTRODUCTION

Functions of Proteins

Proteins are the molecular machines of the cell. Without enzymes and their cofactors, none of the reactions of cellular metabolism would occur on a time scale that could sustain life as we know it. Enzymes catalyze the reactions that generate the DNA and proteins of chromatin, the primary genetic material of the cell. They transcribe RNA from DNA and catalyze the synthesis of the lipids and proteins of cell membranes and the carbohydrates of the cell surface. Membrane proteins signal the presence of hormones and pump ions through membranes; they also recognize other cells, viruses, bacteria, and macromolecules, and trigger cellular responses to those recognition events. The proteins of the cytoskeleton serve both active and passive roles, moving cellular organelles and the cell itself, and forming the scaffold against which such movement is leveraged. DNA- and RNA-binding proteins regulate gene expression by binding to specific nucleotide sequences.

Often a single protein is responsible for the primary function of highly specialized cells; for example, actomyosin in muscle mediates muscle contraction, rhodopsin in the eye absorbs light, and hemoglobin in red blood cells binds and transports oxygen. Specific proteins on the surface of nerve cells recognize neurotransmitters and generate nerve impulses. Specific proteins on the surface of cells of the immune system bind foreign antigens and initiate the immune response.

Proteins need not be contained within a cell in order to play a physiological role. Extracellular proteins break down macromolecules in the intestine, and bind and transport materials in the blood. The proteins of the extracellular matrix provide the framework upon which tissues are laid down. Antibodies in blood recognize and bind foreign antigens. Viral coat proteins package the nucleic acids of viruses. Proteins secreted by foreign organisms sometimes trigger dramatic physiological reactions such as those produced by toxic shock protein and cholera toxin from bacteria.

The seemingly bewildering array of protein functions can be simplified by noting that proteins fall into a few major functional classifications:

1. *Enzymes* such as DNA polymerase, papain and other digestive enzymes, and all of the enzymes of intermediary metabolism.
2. *Binding proteins* such as hemoglobin and myoglobin, antibodies, and cell surface receptors.
3. *Signaling proteins* such as insulin and other polypeptide hormones.
4. *Structural proteins* such as collagen in connective tissue and keratin in hair and nails.

Some proteins, such as the antifreeze proteins in the bloodstream of Antarctic fish or the heat shock proteins found in eukaryotes, seem to defy classification. Many proteins perform more than one of these general functions, but typically, such proteins have only one primary function which is modulated, assisted, or regulated by one or more secondary functions.

How do proteins perform all of these varied functions? As we shall see, the ability of proteins to function as molecular machines is closely linked to their ability to fold spontaneously to form complex three-dimensional structures in which structure is intimately related to function.

How Many Proteins are There?

The total number of proteins found in nature is extremely large. The 1992 edition of the *Enzyme List* (Webb, 1992) lists 3196 enzymes. The total number of proteins in a eukaryotic cell has been estimated to be between 10,000 and 20,000 (Alberts et al., 1989), based upon the number of different messenger RNAs in a typical eukaryotic cell. This number does not reflect the thousands of natural mutants of any one protein that may occur. (Approximately 500 naturally occurring mutants of human hemoglobin have been identified to date.) Nor does it take into account the fact that there are often very substantial differences in homologous proteins that perform the same function in different species. A minimal estimate of the number of different proteins in living systems on earth would be on the order of $10{,}000 \times 500 \times$ the number of species; i.e., literally, billions.

Specific proteins that mediate and regulate complex functions in higher organisms are being discovered every day. Just in the last few years, new proteins have been discovered that are involved in vision (Chen et al., 1993), in olfaction (Raming et al., 1993), and in the formation of the extracellular matrix of the brain (Iwata and Carlson, 1993). There are generally several distinct stages in the scientific study of a protein. Initially, all that may be known is that its presence or absence is correlated with a disease state. The first structural information is generally molecular weight as determined by polyacrylamide-gel electrophoresis. Further structural and functional characterization proceeds along experimentally distinct, but conceptually interdependent lines. In terms of structure, one begins by determining the sequence of amino acids comprising the protein and proceeds to determine the complete three-dimensional structure of the protein. In terms of function, one determines the chemical, thermodynamic, and kinetic features of the particular function the protein performs. After laying this groundwork, much more complex questions can be asked such as: How does the structure of a protein generate its functional characteristics? What is the relationship between the molecular process carried out by the protein and its physiological or clinical correlates?

Because we have been able to study a number of complex proteins in detail, it is now possible to design proteins not found in nature, to improve the function of

naturally occurring proteins, and to design proteins that will carry out functions that do not occur in nature. Many proteins yet to be discovered or designed will be important in clinical practice. Hence, an understanding of protein structure and function will be crucial to the practice of medicine in the future.

PRIMARY STRUCTURE

Proteins are polymers formed by linking together twenty different amino acids (Figure 1). The amino acids in Figure 1 are drawn showing their α-carbons as the center of a tetrahedron. Each amino acid has a carboxyl group, an amino group, a side-chain, and a hydrogen linked to the α-carbon atom. In proline, the side-chain and amino group of the amino acid are linked in a five-membered ring. The unique chemical properties of each amino acid depend on its side-chain. Several side-chains contain groups that are electrically charged under the conditions found in the cell. Glutamic acid and aspartic acid are negatively charged, while arginine, histidine, and lysine are positively charged. Asparagine, cysteine, glutamine, serine, threonine, and tyrosine contain groups that can form hydrogen bonds. The twenty side-chains differ in the extent to which they are nonpolar and in their tendency to sequester themselves in the interior of the protein, away from their aqueous environment. Several amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine) are entirely nonpolar. Others, like tyrosine, have both polar and nonpolar components. It is the chemical properties of the constituent side-chains that in large part determine the three-dimensional structures of proteins and make it possible for them to function as molecular machines.

The amino acids in proteins are connected by peptide bonds formed in a reaction in which the carboxyl group of one amino acid reacts with the amino group of a second amino acid to eliminate water (Figure 2). The sequence of amino acids in a protein is determined by the sequence of nucleotides in the corresponding gene, according to the rules of the genetic code. When the sequence of a protein is specified, three-letter or one-letter abbreviations for the amino acids are generally used. Each protein has a free α-amino group at one end (the amino terminus) and a free carboxyl group at the other end (the carboxyl terminus). Alternating α-carbon atoms and peptide bonds form the backbone of the protein, from which the side-chains extend. A single polypeptide chain generally contains between 100 and 500 amino acids; larger proteins are usually assembled from two or more separate polypeptide chains that form a multisubunit complex maintained by noncovalent bonds. The bond between the C of the CO group and the N of the NH group has partial double bond character; i.e., the double bond resonates between the peptide bond and the carbonyl group. As a result, the atoms of the CO and NH groups, as well as the two α-carbons to which they are attached, all lie in the same plane.

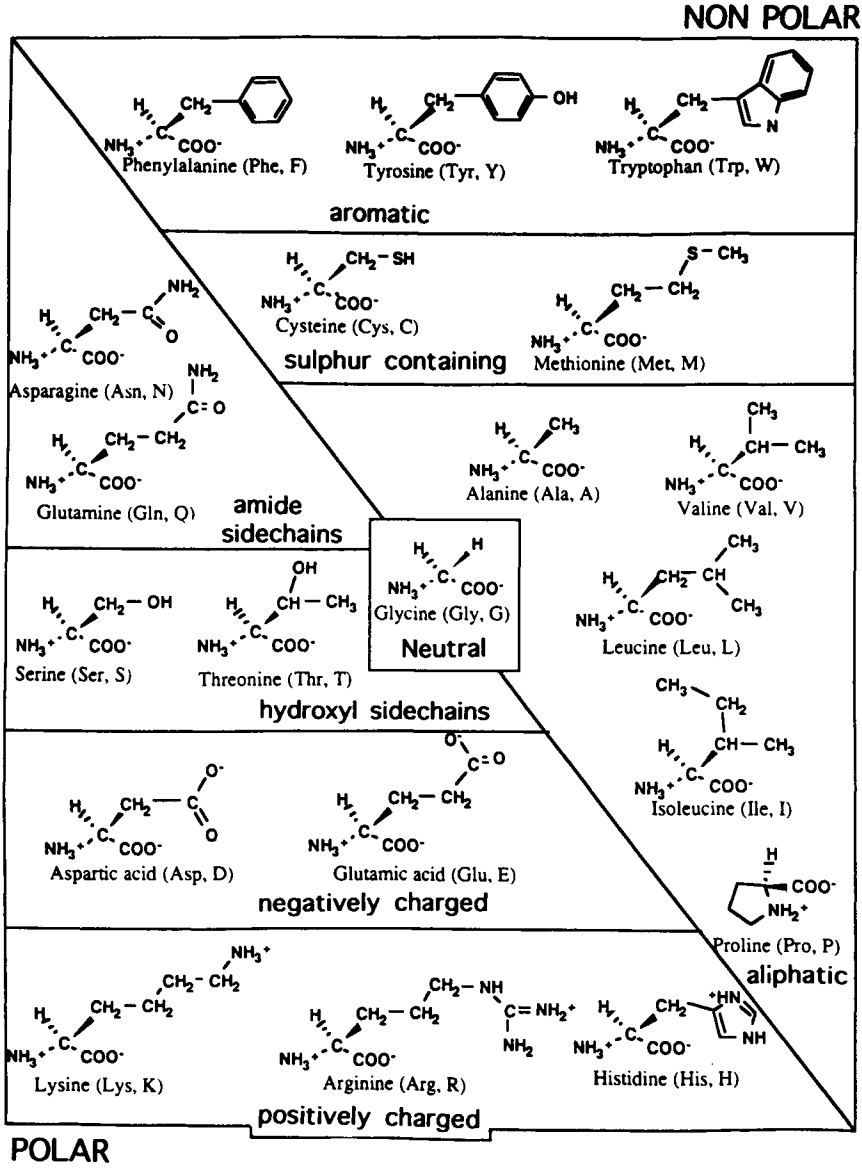

Figure 1. The twenty common amino acids found in proteins. The zwitterionic forms of the amino acids are shown as they exist in solution at pH 7. Amino acids, except for proline, differ only in their side-chains. Amino acids with similar side-chains are grouped together. They are also *roughly* organized according to polarity, from the most polar (lower left) to the most nonpolar (upper right). The polarity of the side-chains is a major determinant of the folded structure of a protein.

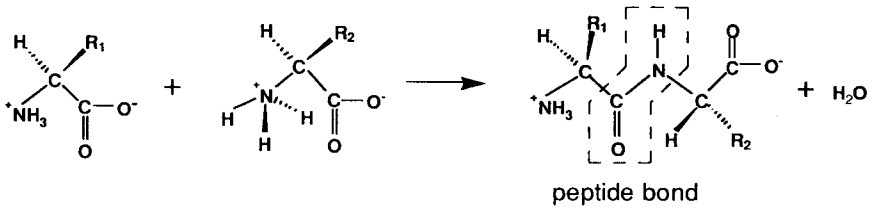

Figure 2. The formation of the peptide bond. The formation of a dipeptide is shown to illustrate how the carboxyl group of one amino acid reacts with the amino group of another.

THREE-DIMENSIONAL STRUCTURE

Elements of Secondary Structure

Because the atoms of the peptide group are coplanar, the only degrees of freedom in the peptide backbone are the angles of rotation, ϕ and ψ, about the bonds connecting each α-carbon to the adjacent N and carbonyl C atoms respectively (Figure 3) (Ramachandran and Sasisekharan, 1968). Thus, each α-carbon actually defines the corners of two adjacent planar quadrangles. Only a limited set of ϕ and ψ angles are allowed because of steric factors. When ϕ and ψ have the same values for successive amino acid residues, the backbone atoms form regular, repeating structures, which collectively form the secondary structure of the protein. The two most common types of secondary structure are the right-handed α-helix and pleated- or β-sheet (Figure 4). Whether a given stretch of backbone forms an α-helix or pleated-sheet is correlated with its amino acid sequence (reviewed by Fasman, 1989). Both structures are compact relative to a random-coil structure (see review by Dill, 1993), and are maintained by hydrogen bonds that link CO and NH

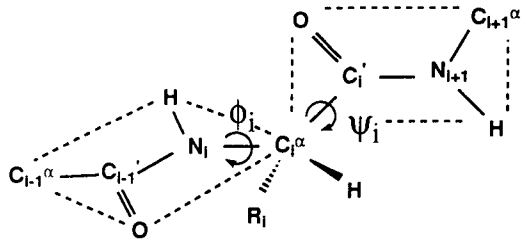

Figure 3. Phi (ϕ) and Psi (ψ) torsional angles. Free rotation can occur around the bonds between the α-carbon of an amino acid and the carbonyl carbon (ψ), and between the α-carbon and the nitrogen (ϕ). Resonance with the carbonyl group makes the peptide bond planar.

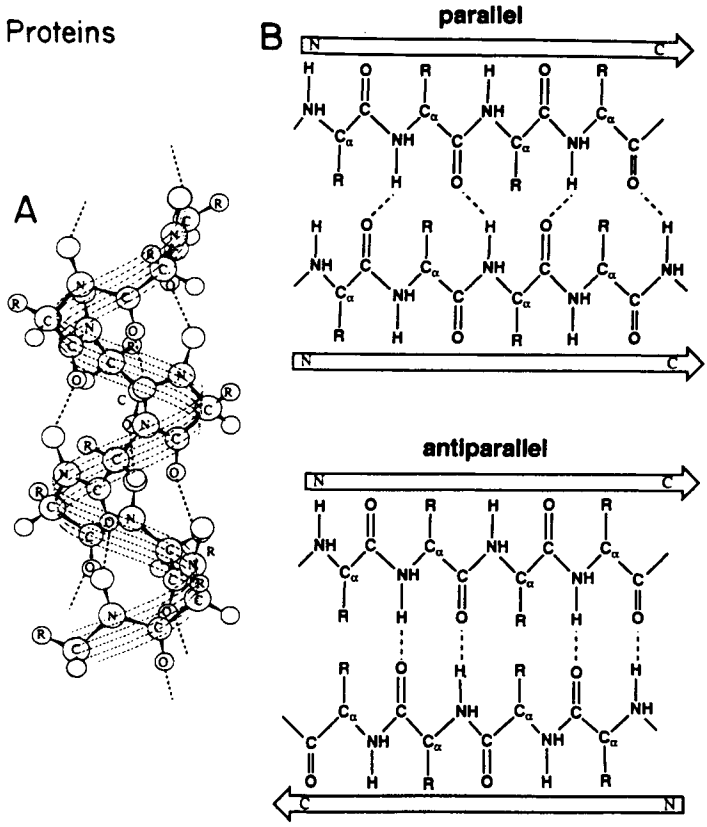

Figure 4. Elements of secondary structure. (A) The hydrogen-bonding pattern of an α-helix. Hydrogen bonds (denoted by dashed lines) form between residues four positions away from each other in the helix. Unmarked atoms are hydrogens. (**B**) Two-dimensional projections of the hydrogen-bonding patterns of parallel and antiparallel pleated sheets. Parallel and antiparallel pleated-sheets have different three-dimensional structures.

groups of the backbone. In the α-helix, the hydrogen bonds are parallel to the helix axis and link residues n and n + 4 (Pauling and Corey, 1951). In pleated-sheets, the hydrogen bonds are perpendicular to the strands of the sheet. Adjacent sheets may be either parallel, with the polypeptide chains running in the same direction, or antiparallel. When the structures of β-sheets are projected in two dimensions, one observes that the hydrogen bonds between strands are evenly spaced in parallel pleated-sheets and with alternate narrow and wide spacing in antiparallel pleated-sheets (Figure 4B).

The overall shape of the protein depends upon the location of loops in the protein backbone between elements of secondary structure. Because these loops cause the

backbone to change direction, they are generally located on the surface of the protein. Although their lengths are variable, their structures are known to have certain characteristic features. The best defined structure is the hairpin loop, in which two adjacent antiparallel β-strands are linked by only two residues.

Structural Motifs

The three-dimensional arrangements of secondary structural elements of proteins in turn form distinctive patterns or motifs. These are most readily recognized

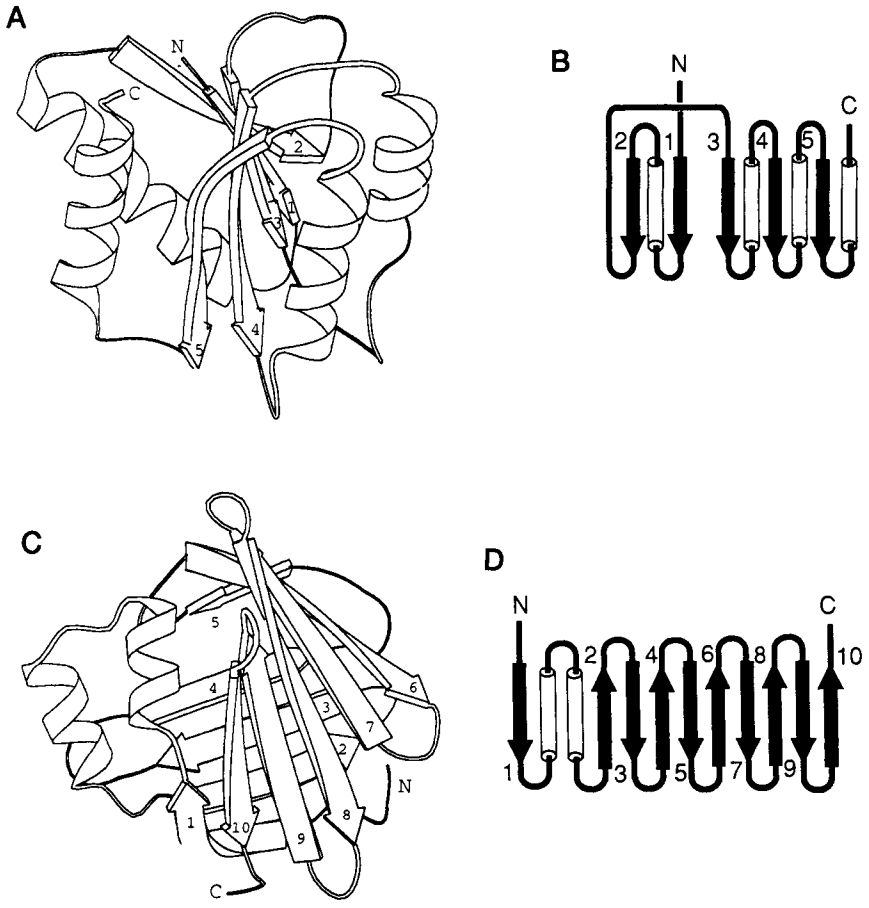

Figure 5. Three-dimensional and two-dimensional topological representations of flavodoxin (**A** and **B**) and adipocyte lipid-binding protein (**C** and **D**). In both projections, individual β-strands are numbered in order from the amino- to the carboxyl-terminus.

in structural diagrams in which α-helices are represented as helical ribbons, β-strands as flat arrows, and loops as tubes (Richardson, 1981) (Figure 5, A and C), or in two-dimensional topological diagrams, in which parallel/antiparallel relationships and connectivities are emphasized (Rossman and Argos, 1981; Chothia and Finkelstein, 1990) (Figure 5, B and D). Some biological functions seem to require particular structural motifs; for example, the helix-turn-helix and zinc-finger motifs are found in several DNA-binding proteins. Other structural motifs like the β-barrel are found in a variety of proteins with diverse functions.

Long polypeptide chains are likely to be organized into several domains, each with its own characteristic structural motif. For example, the first 50 amino acids of a single polypeptide chain may fold into a set of α-helices, while the second 100 residues form a β-barrel which flanks the α-helices. An example of this domain structure is exhibited by adipocyte lipid-binding protein in Figure 5 (note that there is a single "extra" β strand at the amino terminus). When proteins fold into multiple domains, the domains form sequentially from the amino terminus, i.e., in a two domain polypeptide, the first and second domains would be formed by the amino and carboxyl portions of the protein, respectively. This traditional definition of the term "domain" allows the sequence of the gene and the domain structure of the corresponding protein to be directly correlated. It is also consistent with the observation that amino acids that are close in the protein sequence *tend* to be close in the folded structure. In many multifunctional proteins, each domain is responsible for one of the multiple functions, although sometimes two domains of the protein cooperate to perform a single function.

In a number of proteins containing parallel pleated-sheets, α-helices alternate with strands of parallel β-sheets (see flavodoxin in Figure 5). The 2-D topological diagram in Figure 5B indicates that this protein consists of a single large domain called a parallel α/β structure (alternately, because of the long loop connecting β strands 2 and 3, one may also describe flavodoxin as consisting of two connected parallel α/β domains).

Determinants of Tertiary Structure

Many proteins, when unfolded by heat or chemical denaturants, have the ability to refold spontaneously to form native, biologically active structures when suitable conditions are restored. This phenomenon indicates that all of the information required to fold the protein correctly is encoded in the amino acid sequence and that, of all the structures that the polypeptide chain could fold into, the native structure has the lowest free energy; i.e., it is the most thermodynamically stable structure. It should, therefore, be possible to deduce the rules that determine the three-dimensional structure of a protein and to predict how a protein with a given sequence will fold. At this point, our understanding of the folding process is still incomplete, although rapid progress is being made.

Some complex proteins fold only in the presence of other proteins called *chaperones*. Although the ways in which chaperones facilitate protein folding is still under investigation, it appears likely that they prevent the formation of aggregates that interfere with normal folding.

Three major forces drive protein folding.

1. *Hydrophobic interactions.* Nonpolar amino acid side-chains pack tightly in the interior of the protein so that they are not in contact with water, and so there are no cavities between them. Because the shapes of nonpolar side-chains are complex, the number of ways in which they can be packed together without creating cavities is very limited.
2. *Hydrogen bonds.* Groups capable of forming hydrogen bonds must form them either within the protein or with the solvent. Interior hydrogen bonds serve to stabilize regions of the protein structure. Hydrogen bonds formed between protein and solvent may ensure that a specific type of interface is presented by the protein so that it may correctly interact with its ligands or with other macromolecules.
3. *Electrostatic interactions.* Within the protein interior, charged groups are almost always arranged so that groups with opposite charges are paired. Surface charges, again, may be involved in defining the surface of the protein, as well as in performing specific functional roles. Electrostatic dipoles may also drive folding; for instance, because the distributions of electrons in the CO and NH groups of the backbone are polarized, α-helices have electric dipoles which interact with charged groups in the protein and with each other.

Quaternary Structure

Folded polypeptide chains often assemble to form larger structures known as oligomers. Oligomerization is driven by the same forces that drive the folding of individual chains. The individual polypeptide chains comprising an oligomeric macromolecule are called *subunits*. While the topology of most monomeric proteins is highly asymmetric, the subunits of most oligomeric proteins are symmetrically arranged (Figure 6) to create shapes that have nearly classical geometry (spherical, cubical, toroidal, ellipsoidal, etc.). The arrangement of subunits within the oligomer and the interactions between subunits define the *quaternary structures* of these complexes.

Many complexes are able to switch between two or more quaternary structures, with slightly different arrangements of subunits and with different functional properties. Such changes in quaternary structure are usually driven by interactions with other molecules. The ability of multisubunit proteins to act as *molecular switches* underlies many mechanisms of cellular regulation. The archetypal mo-

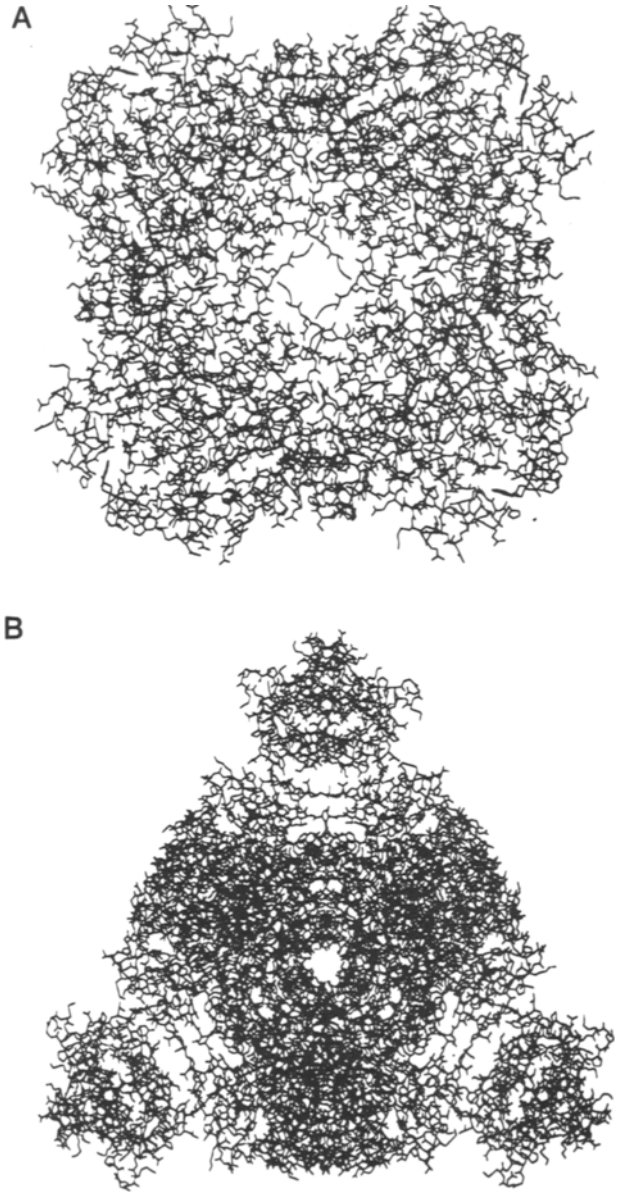

Figure 6. Illustrations of symmetry in oligomeric proteins. These views of glycolate oxidase (**A**, 8 subunits; Lindqvist, 1989), aspartate transcarbamylase (**B**, 12 subunits; Monaco et al., 1978), and glutamine synthetase (**C**, 12 subunits; Yamashita et al., 1989) illustrate the symmetry that can be achieved in oligomeric proteins. (*continued*)

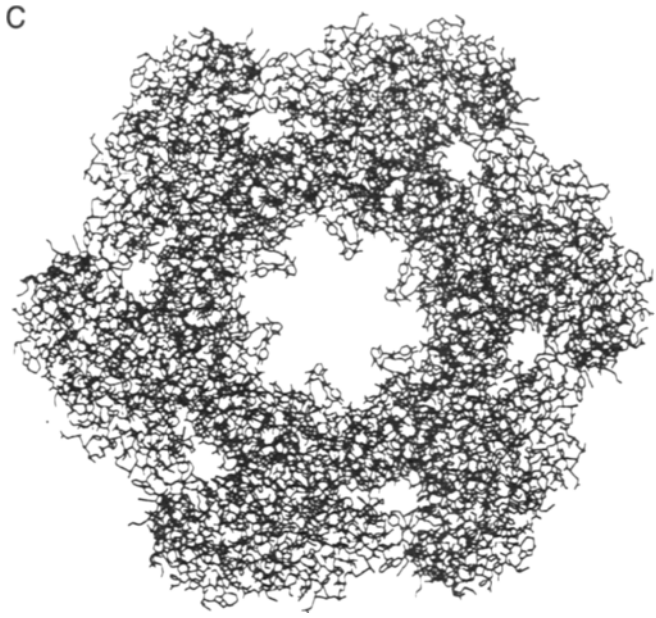

Figure 6. (Continued)

lecular switch is hemoglobin, whose subunit interactions change dramatically as a result of ligand binding (Ackers et al., 1992). Functions such as cooperative ligand binding or heterotropic allostery (in which binding of a regulatory *effector* molecule alters the ability of the protein to bind or act on its primary ligand) generally depend upon changes in quaternary structure. Although there are occasional exceptions, such as the cooperative binding of Ca(II) to the single polypeptide protein, calmodulin, cooperativity and heterotropic allostery most frequently involve oligomeric structures.

Protein-folding Pathways

There is great interest in determining the pathways by which proteins fold, because knowledge of these pathways would help a great deal in elucidating the rules of protein folding (reviewed by Kim and Baldwin, 1990; Goldenberg, 1992). A wide range of possibilities exists. If one surveyed the folding pathways of many individual molecules of the same protein, one might find that each separate molecule followed exactly the same pathway and passed through the same intermediate structures. On the other hand, there might be a great deal of variation in folding pathways among individual molecules, even though the entire population

of molecules eventually arrives at the same folded structure. Many proteins appear to pass through what is called a *molten globule* state, in which elements of secondary structure are loosely packed together, rather than being tightly packed, as they are in the native protein (reviewed by Kuwajima, 1989). This has led to the suggestion that the secondary structure forms first, either in a defined order or randomly, and directs the folding of the rest of the molecule. However, this is a very active area of research and a great deal remains to be learned.

MECHANISMS OF PROTEIN FUNCTION

The biological action of proteins depends upon their ability to bind tightly and specifically to other molecules on their surfaces. This generalization holds across the spectrum of protein functions, whether the protein binds specific ligands and catalyzes reactions between them, or plays a purely structural role by interacting with other protein molecules to form a structural array. When proteins fold, surfaces with complex patterns of nonpolar, hydrogen bonded, and charged groups are created. At a site where another molecule binds, the distribution of these groups is complementary to the interacting molecule. An example of the interactions that occur at the active site of an enzyme is shown in Figure 7.

Often proteins act on the molecules they bind. Enzymes catalyze the transformation of the bound molecules (their substrates) into another chemical species. Ion channels are able to pump bound ions from one side of the cell membrane to the other. Chains of myosin molecules pass actin along from one myosin molecule to another in muscle.

The ability of proteins to do work on the molecules they bind depends upon several factors. The active sites of enzymes bring reactive groups on both the substrate and protein together. Active sites often contain acidic or basic groups that act as chemical catalysts. They may also distort the structure of the substrate to make it resemble the transition state of the reaction being catalyzed. The flexibility of protein structures is also important because it allows the bound molecule to go through a sequence of interactions with the protein.

Doing work requires energy. Proteins obtain the energy they need to do work by coupling an energy-requiring process with an energy-releasing process. Frequently, the energy is supplied by the hydrolysis of a nucleotide triphosphate, usually ATP. The actual binding of ligands or effector molecules may also provide the energy to perform a function. When a ligand binds tightly to a protein, it is, in a thermodynamic sense, bringing useable energy into the system. In human hemoglobin, for example, 2,3-diphosphoglycerate binds tightly to deoxygenated hemoglobin and the energy of that binding is utilized to shift the molecule into a state of higher oxygen affinity. The reciprocal "price" for this type of internal energy transduction is that in its state of higher oxygen affinity, the affinity of hemoglobin for 2,3-diphosphoglycerate is decreased.

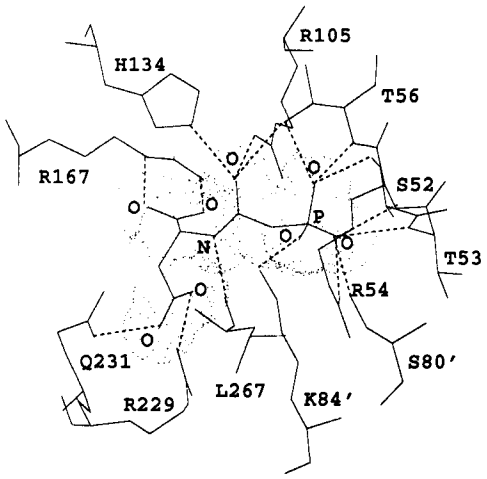

Figure 7. Interactions at the active site of aspartate transcarbamylase (ATCase). N-phosphonoacetyl-L-asparate (PALA) is a bisubstrate analog of the two natural substrates of ATCase, carbamyl phosphate and L-aspartate. PALA is shown bound in the active site of ATCase. Noncovalent interactions between PALA and side-chains of the protein are shown as dashed lines. Specific residues are indicated by their one letter abbreviation and by their position in the protein sequence (e.g., H134 = histidine at position 134). The active site is composed of residues from two separate polypeptide chains (denoted by primed and unprimed residue numbers). Note the complimentarity of the site and the ligand. The same interactions are used to align and catalyze the condensation of ATCase's natural substrates (Monaco et al., 1978).

REGULATION OF PROTEIN FUNCTION

The cell has many mechanisms of self-regulation that allow it to maintain homeostasis or adapt to changing conditions. Mechanisms for regulating protein function differ depending on how quickly the cell must respond and how long it must maintain the altered state. Long-term changes often depend upon changes in patterns of transcription or translation, or on the rates of RNA and protein degradation, which alter the concentration of particular proteins within the cell. Changes in the relative concentrations of isozymes within the cell may also be controlled at the level of transcription or translation. Isozymes are closely related enzymes which perform the same function, but with slightly different efficiencies. A number of isozymic families exist in higher organisms. Examples include the enzymes lactate dehydrogenase and cyclic AMP-dependent protein kinase, each of which has two different, but very similar forms in the human body. The number of different isozymes of a particular protein may number more than two, but is generally a small

number. Often the relative amounts of the various isozymes of a particular enzyme vary greatly from one organ to the next.

The long-term activity of a protein may also be influenced by changes in its chemical structure. Well-known examples of this type of regulation are the digestive enzymes produced in the pancreas as inactive precursors (e.g., trypsinogen, chymotrypsinogen, and pepsinogen) and activated in the intestine by the removal of an N-terminal peptide. This is a common mechanism for keeping a protein in an inactive state until it reaches the site where it is intended to function. Other posttranslational modifications are also used as regulatory strategies. The activities of many proteins are altered by the addition of complex sugar chains or lipids to the amino acid side-chains or amino terminus of the protein. Chemical modification of certain side-chains or the amino terminus through the addition of phosphoryl-, acetyl-, methyl-, amino-, carbamyl-, or prenyl- (Marshall, 1993) groups also occurs. Regulation by phosphorylation was first studied in the enzymes involved in glycogen metabolism (Fischer and Krebs, 1955), and is now recognized to be a widely used mechanism for regulating cell growth and division. While phosphorylation of metabolic enzymes generates rapid responses to changing concentrations of metabolic intermediates, changes in the phosphorylation state of oncogenic proteins may play a role in the induction of cancer over a much longer time frame. Thus, the response time for modulating protein function by phosphorylation spans a very wide range of possibilities.

Rapid cellular responses are generally achieved by modulating the efficiency with which a protein performs its biological function via a noncovalent mechanism. Because acidic and basic groups are often involved in protein function, changes in pH induced by metabolic changes will alter the charge state of the protein and often change the rate at which it performs its function. Most proteins have an optimum pH at which they work best. The activity of a protein also depends upon temperature, increasing with temperature up to the point at which the protein begins to unfold. While the internal temperature of mammals is fairly constant under normal conditions, temperature serves as a major regulatory mechanism in many organisms.

The activities of proteins are also regulated by the concentrations of a variety of other molecules which they bind noncovalently. The velocity of processes catalyzed by enzymes increases with substrate concentration until concentrations at which binding sites are saturated are reached. Conversely, competitive inhibitors that bind at the active site and block substrate binding reduce rates of protein catalyzed reactions, with the magnitude of the inhibition increasing with the concentration of competitive inhibitor. The ability of the products of many enzyme catalyzed reactions to bind at the active site provides a simple means of feedback regulation. When substrate concentrations are low and product concentrations are high, enzymatic activity will be diminished. Conversely, when substrate concen-

trations are high and product concentrations are low, the enzyme will rapidly convert substrate to product.

Many proteins are regulated by molecules which bind somewhere other than at the active site and either increase or decrease protein activity. These *allosteric effectors* are often quite specific and may have either a positive or negative effect upon protein activity. Classical *feedback inhibition* cycles in metabolism generally involve *heterotropic allostery*, in which a molecule produced near the end of a metabolic pathway acts as an allosteric effector to regulate a protein active earlier in the same pathway. Because of the need for very precise control of the energy charge of the cell, ATP and ADP serve as allosteric effectors for several of the proteins of glucose metabolism. Protons and ions act as allosteric effectors in many

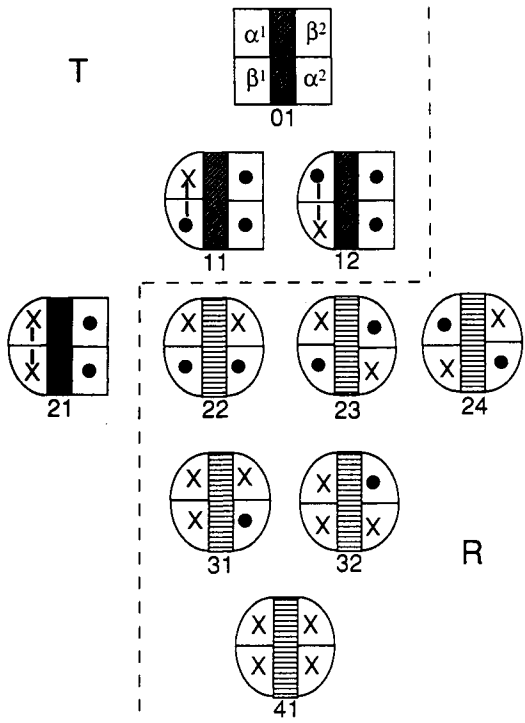

Figure 8. Quaternary structure changes are a major mode of functional regulation in proteins. Shown here are the intermediate ligation states of human hemoglobin. (The fact that hemoglobin consists of two identical dimers results in there being ten possible ligation forms rather than sixteen). Hemoglobin undergoes a switch of quaternary structure from a low-affinity structure (the T state) to a high-affinity structure (the R state). This switch occurs whenever there are ligands on both sides of the interface between the dimers (Ackers, 1992).

protein systems. Other allosteric effectors may be highly specific, acting on only one protein in a cell; for example, 2,3-diphosphoglycerate which increases the oxygen affinity of hemoglobin or fructose 2,6-bisphosphate which increases the enzymatic activity of phosphofructokinase. This type of regulation requires long-range communication between binding sites and generally involves a change in the three-dimensional structure of the protein and a switch in quaternary state (as discussed above in "Quaternary Structure").

Allosteric regulation serves as a paradigm for many different types of molecular recognition and communication. It is best understood in hemoglobin, although it has been studied in a number of different proteins. Figure 8 illustrates the recently discovered *symmetry rule* in human hemoglobin (Ackers, 1992). Hemoglobin consists of two identical $\alpha\beta$ dimers that associate roughly head to tail to form a tetramer. When ligands bind on opposite sides of the interface between dimers (i.e, are symmetrically bound) the entire molecule switches its quaternary structure to the high-affinity R-state. This is a large or *global* change in structure. When ligands are bound to only one dimer, there are some structural changes within that dimer, but the overall quaternary structure remains in the low-affinity T-state.

PROTEIN EVOLUTION

Because genes undergo mutations with time, the amino acid sequences of a given protein differ from species to species and sometimes even from individual to individual. Generally the more distantly related two species are, the greater will be the degree of divergence of their sequences. However, different regions of the genome evolve at rates that may differ by several orders of magnitude, with the result that some proteins appear highly conserved over a wide range of species, while other proteins exhibit less than 50% primary-sequence homology even between very closely related species. Residues that are central to the function of the protein tend to be conserved; in fact, one way of identifying residues that are potentially crucial to function is to compare sequences from many species and flag conserved residues. The correlation between conservation and function is not perfect, however, and the functional significance of residues flagged by sequence comparisons must be tested by site-directed mutagenesis.

Often specific mutations do not dramatically alter the shape or chemistry of the amino acid involved. An example of such a *conservative substitution* would be the replacement of glutamine by asparagine. When a mutation causes a large perturbation, a second mutation will sometimes be found to have compensated for the damage of the first mutation. For example, if a mutation eliminates the charge on the side-chain of one amino acid, a second mutation might eliminate the charge on the side-chain with which it is paired. This happens because an unpaired mutation would interfere with the ability of the protein to fold properly and perform its function and hence, the ability of the organism to survive and reproduce would be compromised. The unpaired mutation would, therefore, tend to be eliminated by

natural selection, either by reversion to the original amino acid or by a compensatory mutation such as described above.

As DNA sequences and three-dimensional structures of proteins have accumulated, new questions about protein evolution have arisen. One issue that has received a great deal of attention is the role of introns—DNA sequences that separate exons, the coding regions within genes. One possibility is that multidomain proteins arose from combining a relatively small set of exons in various ways (Gilbert, 1978). A second issue concerns the relationship between primary sequence, folding, and function. Many proteins with very different sequences and functions fold in essentially the same way. For example, flavodoxin (Figure 5) and domain 2 of phosphofructokinase both fold into nearly identical parallel α/β structures, yet they are very different proteins. How does this happen, and can this be considered in any way analogous to convergent evolution of whole organisms?

PROTEIN DESIGN

The development of methods to introduce mutations at specific sites in DNA plasmids, and machines that can synthesize polypeptides (reviewed in Kent, 1988) has made it possible to envision the design of proteins that perform functions that do not occur in nature, or that are more efficient than naturally occurring proteins (Kuntz, 1992; Fersht and Winter, 1992). The tremendous molecular specificity of antibodies has been used to engineer catalytic antibodies (Powell and Hanes, 1989; Lerner et al., 1991). Site-directed mutagenesis has been used to alter the specificity of proteases (Hedstrom et al., 1992). Peptide synthesis has been used to generate peptides that bind metal ions (Higaki et al., 1992) and synthetic ion channels (deGrado et al., 1989). Chimeric molecules that combine the molecular specificity of antibodies with the lethal properties of *Pseudomonas* exotoxin (a protein that kills animal cells by blocking protein synthesis) are beginning to be developed to eradicate cancerous cells (Brinkmann et al., 1991). These are all recent advances in a field which is undergoing very rapid development.

METHODS

Protein Purification

Most procedures for purifying proteins depend upon selective precipitation and chromatography. Proteins tend to precipitate at their isoelectric points (the pH at which they are electrically neutral) and in the presence of high concentrations of salts such as $(NH_4)_2SO_4$. Precipitation techniques are based on finding solution conditions which cause a protein to aggregate so that it falls out of solution. The precipitation of the protein must be reversible so that the protein does not suffer permanent damage. Because the conditions under which different proteins precipitate vary, a protocol can generally be developed to semi-selectively precipitate the

protein of interest. Precipitation increases the purity of a protein, since only a fraction of the other proteins present in a cell lysate or tissue homogenate will precipitate under the same conditions. Proteins can also be coprecipitated with antibodies. This type of "precipitation" is mechanistically different from the solvent-induced precipitation just discussed, because it depends on the specificity of the antibodies. An absolutely pure protein sample can be obtained in one purification step with a highly selective antibody, once the antibody has been removed.

Proteins can be separated chromatographically by *ion-exchange chromatography*, in which they bind selectively to charged groups on chromatographic resins; by *gel-permeation chromatography*, in which they sieve differentially by size through porous resins; or by *affinity chromatography*, in which a group for which a given protein has high affinity is chemically bonded to the resin. Proteins can also be separated on the basis of charge by electrophoresis, but this is generally not used as a purification method as the yields are extremely small relative to other preparative procedures.

Regardless of the purification scheme used, the protein must be "tracked" through the preparation. Enzymes are usually monitored by activity assays. Other proteins are sometimes monitored by assaying for their physiological function, although this may be tedious as for example, if the physiological function were to induce hair loss in mice! When activity cannot be assayed, protein purification can become extremely difficult. If a specific antibody is available, it may be used to track the protein of interest. Sometimes the protein can be tracked simply by noting its presence or absence in a polyacrylamide gel at each stage of the purification. Sometimes it is possible to selectively attach a radioactive label or chromophore to a small quantity of the protein which has been isolated by gel electrophoresis. When this labeled protein is then added to a large scale preparation of the protein, it serves as a marker for its kindred molecules.

During the purification, the *specific activity* of the protein is determined at each stage. Specific activity is defined as the amount of active protein present per unit weight of total protein. As purification proceeds, specific activity increases, although a substantial fraction of the protein of interest is always lost along the way.

The recent purification of HIV-1 protease to homogeneity serves as an example of how some of these elements are combined in a purification scheme (Margolin et al., 1991). The gene for HIV-1 protease was cloned into an expression vector and in *E. coli* bacteria. To purify the protease from the bacterial proteins, a series of four chromatographic steps were used as shown in Table 1. Since each chromatographic step involves a different type of interaction, every protein will be retained differently on each chromatographic column. The cell lysate, and hence all of the proteins present in the bacteria is first passed through the QAE-sepharose anion-exchange column under conditions where many proteins are negatively charged and thus stick to the positively charged QAE-sepharose. When the concentration

Table 1. Purification of HIV-1 Protease Cloned in *E. coli*[a]

Purification step (type of chromatography)	*Specific activity (umol/min/mg)*	*Recovery of original amount of HIV-1 protease (%)*
cell lysate	0.001	100
QAE-sepharose (anion exchange)	0.001	44
Hexyl-agarose (hydrophobic interaction)	0.003	27
Mono S (cation exchange)	0.07	17
Superose 6 (size exclusion)	1.12	13

Note: [a]Data are from Margolin et al., 1991.

of salt in the buffer flowing through the column is gradually increased, electrostatic interactions between the proteins and the column are weakened and the proteins elute from the column. The concentration of salt at which a particular protein elutes depends on its charge. As proteins elute, small aliquots called "fractions" are collected and assayed for HIV-1 protease activity by testing their ability to cleave a small peptide. Fractions containing the activity are then pooled and applied to the next column (hexyl-agarose). Since the hexyl-agarose column interacts with proteins via hydrophobic interactions, it will further separate the HIV-1 protease from other proteins. In Table 1, we can see that the most effective steps in this purification scheme are the Mono S cation exchange column and the Superose 6 size exclusion column. Specific activity is defined here as micromoles of small peptide cleaved per min per mg of total protein in the fractions exhibiting activity for that step. The elimination of proteins that are not the HIV-1 protease decreases the total amount of protein present and thus increases specific activity. We can see that the Mono S column increases the specific activity by 23-fold relative to the previous step. The Superose 6 column increases the specific activity another 16-fold relative to the Mono S step. The overall purification of the protease is 1,120-fold relative to its original concentration in the bacteria. Also note that there are the expected losses of protease at each step in the procedure. The yield of 13% for the HIV-1 protease obtained here is actually at the high end of the yields that are typically obtained.

Determining Primary Structure

Amino acid compositions are generally determined by hydrolyzing the protein with hydrochloric acid and separating the amino acids that are generated by chromatography, generally high performance liquid chromatography (HPLC). The specific sequence of amino acids is determined by cleaving amino acids one at a time from the amino terminus of the protein by a procedure known as Edman degradation in which the N-terminal amino acid is chemically modified so that it can be removed from the protein and analyzed. This cycle of modification, cleavage and analysis is successively repeated to determine the amino-terminal protein

sequence. Because Edman degradation gradually becomes unsynchronized (due to incomplete cleavage of some of the peptide chains during each cycle), only the first 30–40 amino acids can usually be determined. Complete proteins are sequenced by cleaving the chain into several fragments, separating the fragments, and determining the sequence of each fragment. Sequencing from the carboxyl-terminus of the protein is more difficult, although it can be performed with enzymes that cleave carboxyl-terminal peptide bonds. Because these enzymes continue to cleave amino acids from the end of the protein until it is fully degraded, the amino acid sequence must be deduced by monitoring the appearance of free amino acids with time. This very quickly becomes a complex and error-prone guessing game.

Complete amino acid sequences are often determined by sequencing the corresponding gene for the protein. This approach is generally more rapid and accurate than sequencing the protein. Usually when one is studying a new protein, the N-terminus is sequenced by Edman degradation to generate an oligonucleotide probe, a molecule of DNA whose base sequence corresponds to the amino-terminal amino acid sequence. This probe is then used to find and isolate the gene or messenger RNA coding for the protein. This is possible because a probe of sufficient length will only base pair with the gene corresponding to the protein among all of the DNA of the organism. Most of the protein sequence can then be determined from the nucleotide sequence, although frequently direct analysis of some segments of the amino acid sequence is required in regions where DNA or RNA splicing has occurred or when the protein has been posttranslationally modified.

Three-dimensional Structure Determination

Only two methods for determining the complete three-dimensional structures of proteins exist: nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. The former can be used to determine structure in solution, but only for proteins with molecular weights below approximately 15 kDa. The latter can be used to determine the structure of any protein that can be crystallized. The structure of a protein that is homologous to one that is already known can be determined by *molecular replacement methods*; structures that must be determined *de novo* require that heavy-atom derivatives be prepared. Although the possibility that crystallization would substantially distort the structure was initially a concern, in fact, the crystal structure generally closely approximates the time-averaged structure in solution. Both NMR and crystallographic methods are complex and require extensive computer analysis.

SUMMARY

Protein are polymers made up of amino acids linked by peptide bonds. Twenty different amino acids are commonly found in proteins. Most proteins contain

between 100 and 500 amino acids whose sequences are specified by the corresponding genes. A typical eukaryotic cell contains on the order of 10,000 proteins; the total number of proteins found in nature is much larger because of the existence of many mutant proteins and differences generated by evolution between homologous proteins in different species. Proteins fold spontaneously to form compact structures with complex surfaces built upon a framework of α-helices and pleated-sheets. Their ability to strongly and specifically interact with other molecules depends upon complementarity between binding sites on the surface of the folded protein, and the shapes and chemistries of the molecules to be bound. Proteins act as molecular machines, transforming or translocating the molecules with which they interact. Procedures for designing and producing novel proteins in the test tube are now being developed.

REFERENCES

Ackers, G. K., Doyle, M. L., Myers, D., & Daugherty, M. A. (1992). Molecular code for cooperativity in hemoglobin. Science 255, 54–63.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J. D. (1989). In: Molecular Biology of the Cell, Second Edition, p. 528, Garland Publishing, Inc., New York and London.

Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., & Pastan, I. (1991). B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. Proc. Natl. Acad. Sci. USA 88, 8616–8620.

Chen, T. Y., Peng, Y. W., Dhallan, R. S., Ahamed, B., Reed, R. R., & Yau, K. W. (1993). A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. Nature 362, 764–767.

Chothia, C., & Finkelstein, A. V. (1990). The classification and origins of protein folding patterns. Ann. Rev. Biochem. 59, 1007–1040.

Dill, K. A. (1993). Folding proteins: finding a needle in a haystack. Curr. Opin. Struct. Biol. 3, 99–103.

DeGrado, W. F., Wasserman, Z. R., & Lear, J. D. (1989). Protein design, a minimalist approach. Science 242, 622–628.

Fasman, G. D. (1989). Protein conformational prediction. Trends Biochem. Sci. 14, 295–299.

Fersht, A., & Winter, G. (1992). Protein engineering. Trends Biochem. Sci. 17, 292–294.

Fischer, E. H., & Krebs, E. G. (1955). Conversion of phosphorylase b to phosphorylase a in muscle extracts. J. Biol. Chem. 216, 121–132.

Gilbert, W. (1978). Why genes in pieces? Nature 271, 501.

Goldenberg, D. P. (1992). Native and non-native intermediates in the BPTI folding pathway. Trends Biochem. Sci. 17, 257–261.

Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992). Converting trypsin to chymotrypsin: the role of surface loops. Science 255, 1249–1253.

Higaki, J. N., Fletterick, R. J., & Craik, C. S. (1992). Engineered metalloregulation in enzymes. Trends Biochem. Sci. 17, 100–104.

Iwata, M., & Carlson, S. S. (1993). A large chondroitin sulfate proteoglycan has the characteristics of a general extracellular matrix component of adult brain. J. Neurosci. 13, 195–207.

Kent, S. B. H. (1988). Chemical synthesis of peptides and proteins. Ann. Rev. Biochem. 57, 957–990.

Kim, P. S., & Baldwin, R. L. (1990). Intermediates in the folding reactions of small proteins. Ann. Rev. Biochem. 59, 631–660.

Kuntz, I. D. (1992). Structure-based strategies for drug design and discovery. Science 257, 1078–1082.

Kuwajima, K. (1989). The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. Prot. Struct. Funct. Genet. 6, 87–103.

Lerner, R. A., Benkovic, S. J., & Schultz, P. G. (1991). At the crossroads of chemistry and immunology: catalytic antibodies. Science 252, 659–667.

Lindqvist, Y. (1989). Refined structure of spinach glycolate oxidase at 2 Å resolution. J. Mol. Biol. 209, 151–166.

Margolin, N., Dee, A., Lai, M., & Vlahos, C. J. (1991). Purification of HIV-1 protease which has been cloned and expressed in *E. coli*. Preparative Biochem. 21, 163–173.

Marshall, C. J. (1993). Protein prenylation: a mediator of protein–protein interactions. Science 259, 1865–6.

Monaco, H. L., Crawford, J. L., & Lipscomb, W. N. (1978). Three-dimensional structures of aspartate transcarbamylase from *Escherichia coli* and of its complex with cytidine triphosphate. Proc. Natl. Acad. Sci. USA 75, 5276–5280.

Pauling, L., & Corey, R. B. (1951). Configurations of polypeptide chains with favored orientations around single bonds: two new pleated sheets. Proc. Natl. Acad. Sci. USA 37, 729–740.

Powell, M. J., & Hansen, D. E. (1989). Catalytic antibodies—a new direction in enzyme design. Protein Eng. 3, 69–75.

Ramachandran, G. N., & Sasisekharan, V. (1968). Conformation of polypeptides and proteins. Adv. Prot. Chem. 23, 284–438.

Raming, K., Krieger, J., Strotmann, J., Boekhoff, I., Kubrik, S., Baumstark, C., & Breer, H. (1993). Cloning and expression of odorant receptors. Nature 361, 353–356.

Richardson, J. S. (1981). The anatomy and taxonomy of protein structures. Adv. Prot. Chem. 34, 167–339.

Rossmann, M. G., & Argos, P. (1981). Protein folding. Ann. Rev. Biochem. 50, 497–532.

Webb, E. C. (1992). Enzyme Nomenclature, Academic Press, Inc., San Diego.

Yamashita, M. M., Almassy, R. J., Janson, C. A., Cascio, D., & Eisenberg, D. (1989). Refined atomic model of glutamine synthetase at 3.5 Å resolution. J. Biol. Chem. 264, 17681–17690.

RECOMMENDED READINGS

Alber, T. A. (1989). Mutational effects on protein stability. Ann. Rev. Biochem. 58, 765–798.

Branden, C., & Tooze, J. (1991). Introduction to Protein Structure, Garland Publishing, New York and London.

Creighton, T. E. (1993). Proteins: Structure and Molecular Properties, Second Edition, W. H. Freeman, New York.

Richards, F. M. (1991). The protein folding problem. Sci. Am. 264, 54–63.

Rhodes, G. (1993). Crystallography Made Crystal Clear, Academic Press, Inc., San Diego.

Wuthrich, K. (1989). Protein structure determination in solution by nuclear magnetic resonance spectroscopy. Science 243, 45–50.

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Chapter 2

How Enzymes Work

GARY L. NELSESTUEN

INTRODUCTION

Enzymes are responsible for bringing about chemical reactions under the mild conditions that are essential to our existence. In the chemistry lab we often catalyze reactions with heat (refluxing or boiling water temperatures) and acid or base (often 0.1–6.0 molar H^+ or OH^-. However, these conditions are not compatible with life as we know it. While some unusual organisms do exist at high temperatures and in slightly acidic or basic medium, they are the exceptions. Furthermore, even these organisms have adapted by creating ways of keeping the pH inside the cell close to neutral (1×10^{-7}M H^+). Most organisms, including humans, live at low tempera-

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ture ($\leq 37°$ C) and close to neutral pH. Fluctuations of body temperature by only a few degrees or of pH by a few tenths of a pH unit can create serious conditions for humans.

Thus, living organisms require catalysts that can bring about chemical reactions under these mild conditions. In turn, the ways that these catalysts function are responsible for many of the most fundamental properties of living organisms. These properties also help explain the action of many medicines and drugs (as well as herbicides, pesticides, poisons, and all types of bioreactive molecules), and the phenomenon of 'biological specificity'. It should be stressed that this chapter takes the minimalist attitude about enzymes themselves. That is, it presents the smallest amount of enzymology needed to understand some basic features of biology and medicine. This presentation will briefly outline *some* of the mechanisms of enzyme catalysis and will spend most of the time describing the ramifications of these mechanisms for more overt biological properties. Enzyme behavior provides a foundation for understanding the mode of action of many of the chemicals used in medicine.

FREE ENERGY OF A CHEMICAL REACTION

There are two types of energy which are needed to describe chemical reactions; *free energy of the reaction* (ΔG) and *free energy of activation* ($\Delta G^{\ddagger}$). These are depicted in Figure 1. The first determines the direction in which the reaction will progress and the second determines the rate of the reaction. A third aspect of the energy diagram in Figure 1 is the *transition state*, or the structure at the top of the energy barrier. This is the most difficult structure to attain in the process of converting starting material to product.

If we take combustion of methane as an example of a chemical reaction, we all remember that fire has the requirements of heat, fuel, and oxygen. Methane is an example of a fuel and is a major component of natural gas. It obviously gives off heat when it is burned. This heat is a portion of the ΔG illustrated for the reaction in Figure 1. The energy of the products is lower than the energy of the reactants and this produces a ΔG value that is negative. In other words, *reactions will proceed in the direction that gives a negative value for* ΔG.

However, we know that methane can be isolated and, even in the presence of oxygen, it is unreactive unless combustion is stimulated by something like a spark or flame. The spark or flame serves as the heat/catalyst for the reaction. The reason for lack of reaction between methane and oxygen at room temperature is the high energy barrier that must be crossed during the conversion of reactants to products. The energy barrier is referred to as the free energy of activation or $\Delta G^{\ddagger}$ (Figure 1). The activation energy results from the fact that, in order for methane to combine with oxygen, chemical bonds must first be broken. Bond breaking always requires the input of energy. Release of energy is only realized when the new bonds are

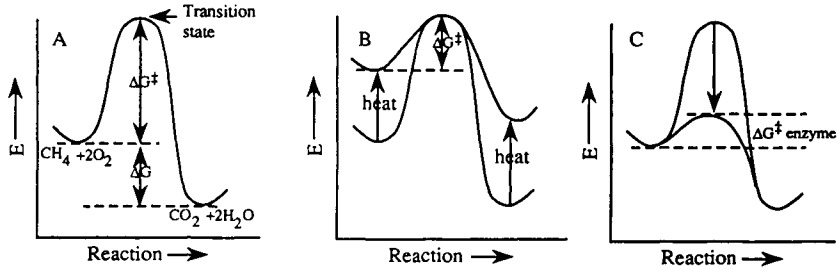


Figure 1. Energy diagram for a reaction. (**A**) The starting materials or substrates are shown on the left and the products on the right. In the case of methanol combustion, the energy of the products is lower than the energy of the reactants and so the reaction proceeds from left to right with a negative ΔG value, and the release of heat. The free energy of activation ($\Delta G^{\ddagger}$) consists of the difference between the energy contained in the average substrate molecule and that of the component that constitutes the transition state. The latter is the structure that exists at the top of the peak. (**B**) shows how heating the reaction components will increase their energy so the energy needed to reach the transition state is reduced. (**C**) shows that the free energy of activation is reduced in enzyme catalysis by reducing the energy of the transition state.

formed and the products are produced. The *rate at which the reaction will occur* is, therefore, determined by the height of the energy barrier to be crossed.

The energies of a population of molecules are a statistical distribution that has an average equal to the value given for the substrates in Figure 1. In order to form product, the molecules must acquire an energy that exceeds $\Delta G^{\ddagger}$. The percentage of molecules with this amount of energy will be determined by the height of $\Delta G^{\ddagger}$. Thus, at room temperature, methane appears to be stable even though there are always a few molecules that have enough energy to cross the barrier. The rate of such noncatalyzed reactions can be very slow. For example, another chemical reaction is the isomerization of L-aspartate to D-aspartate. This occurs at a rate that has been measured and is about 0.1% per year in the collagen of human tooth enamel. The proteins in tooth enamel do not turn over and are as old as the individual. Therefore, a person's age can be determined by measuring the amount of D-aspartate in tooth enamel. Obviously, biological systems cannot wait this period for a desired reaction product. If a reaction such as the conversion of L-aspartate to D-aspartate is desirable, it must be catalyzed by an enzyme and will then occur in a time span of milliseconds.

The energy barrier explains the need for a heat catalyst to combust methane. Heat is a catalyst for most reactions since it elevates the energy of the substrate molecules (Figure 1B) thereby reducing the height of the energy barrier ($\Delta G^{\ddagger}$) and increasing the number of molecules that contain adequate energy to cross the barrier

and form product. Again, large input of heat is not a viable mechanism for catalysis of reactions in biological systems.

Enzymes are catalysts that function by lowering the value of $\Delta G^{\ddagger}$ (Figure 1C) rather than by raising the free energy of the starting materials. However, any mechanism that decreases $\Delta G^{\ddagger}$ will increase the rate of the reaction. The mechanisms which enzymes use to lower $\Delta G^{\ddagger}$ are numerous and are not all thoroughly understood. However, some of the known mechanisms are responsible for some of the features most characteristic of biological systems.

STEP 1 OF ENZYME CATALYSIS

Enzymes Bind Substrates and Align Them for Chemical Reaction

A chemical reaction between two molecules can be illustrated by that catalyzed by the enzyme, alcohol dehydrogenase (Figure 2). This is the first step in the metabolism of ethanol in the human liver. This reaction consists of oxidation of carbon-1 of ethanol to an aldehyde with reduction of NAD^+ to NADH. In essence, the hydrogen from carbon-1 is transferred with its electrons to carbon-4 of NAD^+ as shown in Figure 2. In order for this reaction to occur, the ethanol and NAD^+ must first collide. At the low concentrations of reactants found in biological systems, these collisions are very rare and the uncatalyzed reaction is extremely slow because there are very few molecules close enough to allow the reaction to take place. The enzyme changes this by binding both NAD^+ and ethanol in close proximity to each other and with perfect orientation for the reaction to occur. The effective substrate concentration at the binding site on the enzyme approaches infinity.

The binding process requires that chemical groups on the enzyme interact with groups on each of the two substrates. For ethanol, there are very few sites for these contacts. For example, the hydroxyl group of ethanol can participate in a hydrogen bond to the protein and the methyl group can interact with a hydrophobic region on the protein. Each of these interactions is weak so that, in order to bind ethanol tightly, they must occur simultaneously. In other words, the hydrogen bonding site plus the hydrophobic region of the protein must be aligned perfectly on the enzyme so that both contacts with ethanol can occur simultaneously. Tight binding interactions require even more contact points and have the benefit that less substrate in

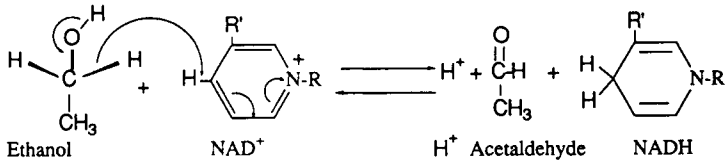


Figure 2. Reaction of ethanol and NAD^+, catalyzed by alcohol dehydrogenase.

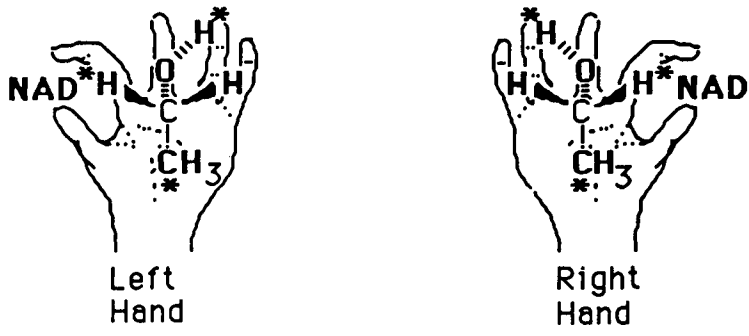


Figure 3. Illustration of an enzyme binding event with your hand. The drawing is of your hands as you would view them. A wedge-shaped bond represents one that projects out of the plane of the paper while a dashed bond represents one that is projecting into the plane of the paper. The asterisk represents a contact point for the ethanol molecule. Imagine that your hand represents a protein folded in a manner that creates binding sites for ethanol and NAD^+ and aligns them for the reaction shown in Figure 2. Imagine that the palm of your hand contains a binding site for a hydrophobic group such as the methyl group, while your ring finger contains a group that binds to hydroxyl groups. In fact, the hydroxyl group is known to interact with a zinc ion that is bound to the enzyme. This orients the ethanol molecule so the two hydrogens project out like the outstretched and slightly raised right and left arms of someone facing you. If NAD^+ binds to a site created by your thumb and index finger, an enzyme created from your left hand will react with the hydrogen that is on the left, while an enzyme created by your right hand will remove the other hydrogen from the 1-carbon of ethanol. Thus, binding of substrates to enzyme sites creates a high degree of 'specificity' that even includes selective removal of one hydrogen atom of a pair that are indistinguishable to conventional chemical reagents.

solution will be required to fill the binding site on the enzyme (see Saturation Kinetics, below). Thus, substrate binding to the enzyme is dependent on exact arrangement of groups on the enzyme with groups on the substrate. In other words, the protein must fold in such a manner that its amino acids cluster in a way that produces the interaction points for the substrate.

Only two contact points are needed to orient the ethanol molecule in space. You can imagine an ethanol binding site by thinking of your hand as an enzyme as shown in Figure 3. Imagine that the palm of your hand corresponds to a hydrophobic region on the enzyme that interacts with the methyl group of ethanol, your ring finger is a hydrogen-bonding group on the protein, and your thumb and index finger constitute the binding site for the NAD^+ molecule. The NAD^+ (thumb and index finger) will now remove a hydrogen from carbon-1 of ethanol. Because of distance restraints, you can see that there are two different hydrogens at carbon-1; one will be removed if you use an enzyme created by your left hand and the other will be removed if you use an enzyme created by your right hand. In effect, the enzyme is

Figure 4. L-alanine and D-alanine. Try to imagine how you might create a binding site specific for L-alanine from your left hand and one for D-alanine from your right hand. This could be accomplished by substituting L-alanine for ethanol in Figure 3. In this case, your thumb and index finger could interact with the α-amino group of alanine, the palm of your hand with the methyl group and your ring finger with the carboxyl group. With these contacts, your left hand should bind L-alanine and your right hand D-alanine.

specific for the substrate due to both the binding process for one substrate (the right types of groups must be in the correct locations) and its orientation to the other substrate. While chemical agents will treat the two hydrogens at carbon-1 of ethanol as identical, enzymes will react exclusively with one or the other.

Thus, the most basic characteristic of enzyme catalysis consists of binding substrates and aligning them with other reactive groups, thereby enhancing a chemical reaction. The binding process is dependent on the location of complementary groups on the substrate and enzyme. Substrates can thereby be discriminated by location of functional groups and by their 3-dimensional orientation when bound to the enzyme. Figure 4 shows the structures of D- and L-alanine. Using the analogy of your hand as an enzyme binding site (together with some imagination for contact points), describe how your left hand might favor the D-amino acid while your right hand would recognize only an L-amino acid. One idea is given in the figure legend.

Nature attempts to simplify its chemical environment and has selected L-amino acids as the 'natural' form. This has the saving feature that only one set of enzymes with binding sites for L-amino acids must be produced. However, D-amino acids do arise in nature from spontaneous isomerization of L-amino acids (see above) or from a very few sites in nature (the action of penicillin involves a structure that contains D-alanine, see below). While the enzymes that act on L-amino acids will almost never interact with D-amino acids and *vice versa*, there are rare examples of enzymes that interact with both isomers. In the latter case, the enzyme binding site must be 'ambidextrous' and have extra features that accommodate both isomers. In other words, enzymes naturally show stereoselectivity and avoidance of stereochemical selection is an exception.

Due to all of these features, stereochemical specificity has been described as a very 'deep evolutionary trait'. It is very difficult to transform an enzyme binding site for a D-isomer to that for an L-isomer. Such changes might be compared to changing the action of the tail flukes of a whale (horizontally mounted) to those of

fish (vertically mounted). Consider how many changes you would have to make in your left hand to accommodate a D-amino acid by the interactions outlined above. Thus, once biological specificity has been set, it is very difficult to change.

These very cursory examples illustrate the phenomenon of binding of molecules to others in a biological system. Although these types of binding events are described for enzymes—the topic of this chapter, there are many other binding events that do not result in a chemical reaction. However, all binding events are based on several points of contact between one molecule (often a protein) and another. In every case, the binding process produces specificity for the molecule that is bound. This makes specificity one of the foundation properties of biology.

Specificity generated by binding is used for many purposes, including recognition of one's own molecules and rejection of foreign molecules. Such recognition can be illustrated by the immune system which binds foreign materials and targets them for destruction. A high profile example is the rejection of tissue transplants and the need for tissue donors with compatible structures. However, biological organisms at all levels, including bacteria, display specificity. For example, many organisms produce DNA molecules with special properties or derivatives so they can recognize foreign DNA and degrade it. Thus, specificity and rejection of foreign material is common throughout biology.

Overall, while specificity has many purposes, it probably arose first because it is an unavoidable outcome of biological catalysis. You cannot have enzyme catalysis without binding of substrates and you cannot create a binding site without creating specificity.

Catalysis by an Enzyme Active Site Produces the Phenomenon of 'Saturation Kinetics'

Since the reaction only takes place when the substrates are bound to an enzyme, and since the number of enzyme molecules are finite, it logically follows that the reaction has some upper limit where all of the enzyme binding sites are filled with substrate. This produces a reaction rate versus substrate concentration plot with an upper limit. The approach to the upper limit can show several types of behavior such as a hyperbolic curve (Figure 5A) or a sigmoidal curve (Figure 5B). Simple enzymes give the hyperbolic curve while complex, or cooperative enzymes, give the sigmoidal curve. Sigmoidal-curve shape is very important for enzymes that are intensely regulated and is a very interesting phenomenon. It is a bit too far off the central topic of this chapter to be covered in detail here. Consequently, this discussion will emphasize the general concept of an *upper limit* to the reaction and some features of the *approach to saturation* which is true of both simple and complex enzymes.

The two plots in Figure 5A are both hyperbolic. Again, the upper limit is determined by the number of enzyme molecules in the reaction as well as by the

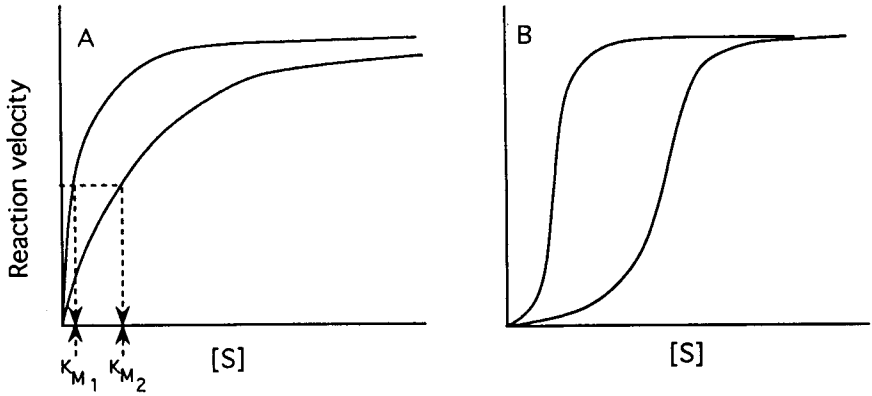


Figure 5. Saturation kinetics: the dependence of enzyme catalysis on the concentration of substrate. Reaction velocity represents the rate at which product is formed. (**A**) shows a 'hyperbolic' saturation curve for two hypothetical enzymes. One binds its substrate more tightly than the other and reaches saturation at lower substrate concentration. This enzyme has a lower K_m value, the substrate concentration where the reaction is half of maximum. The other binds the substrate more loosely and reaches the same velocity but requires higher substrate concentrations. (**B**) shows hypothetical velocities for cooperative enzymes. Although more complex, these enzymes also show the phenomenon of saturation.

rate at which each enzyme can convert a substrate molecule to a product. However, the two plots in Figure 5A show very different dependence on substrate concentration. That is, one enzyme reaches its maximum rate at a lower substrate concentration than does the other enzyme. The midpoint for these plots, the substrate concentration where the reaction rate is one-half the maximum is called the K_m and is a useful number to describe an important characteristic of an enzyme. The K_m is also a relationship between the free enzyme concentration [E], the free substrate concentration [S] and the complex concentration [ES]. The equation is: K_m = [E][S]/[ES]. From this relationship, one can easily see that K_m = [S], when [E]/[ES] = 1; that is, where half of the enzyme sites are filled.

The rate at which a compound will be converted to product is, therefore, a function of the enzyme concentration, the rate at which one enzyme can convert substrate to product plus the degree to which the enzyme is saturated with substrate. For illustration purposes, we might use the first enzyme involved in alcohol metabolism. We often hear that an average human adult can metabolize about one ounce of alcohol per hour. The legal definition of intoxication is often given as a blood alcohol level of 0.1%. This corresponds to about 0.017 moles/liter. If we assume that this same concentration exists in the liver (a relatively good assumption since ethanol can readily diffuse through both the aqueous medium and the membrane barriers of the cell), we can estimate the degree to which the substrate

saturates the liver enzyme. The enzyme that metabolizes alcohol in the human liver, alcohol dehydrogenase, has a K_m for ethanol of about 1×10^{-3}M. Thus, at the intoxication level, the alcohol concentration in the liver is probably many times greater than the K_m of the enzyme. The enzyme should be acting at its maximum rate, far out on the right of the velocity versus substrate concentration plot in Figure 5A. However, from the curve shown in Figure 5A, it should be obvious that as [S] declines, the velocity at which it is converted to product will also decline. A 6×10^{-3}% alcohol solution, corresponding to the K_m of the enzyme, should give a rate of alcohol metabolism that is about 0.5 ounce per hour. This is far below the intoxication level for alcohol and may be a level that has negligible impact on one's faculties. We can speculate that the liver enzyme has evolved so it can remove alcohol at the maximum rate until the alcohol level has reached safe proportions. This is clearly a speculation that is only appropriate for off-the-cuff discussions. However, this type of discussion and possible ramifications for the whole organism can be considered once one knows an enzyme's properties and the biological concentration of substrate.

This example of alcohol metabolism has been selected for emphasis in this discussion because the reaction is simple and also because so many people have experience with aspects of alcohol. However, the properties of this enzyme are typical of many others in biology and many of the properties outlined for alcohol dehydrogenase will apply to other enzymes as well.

One should be aware that the phenomenon of enzyme kinetic behavior can reach extreme levels of complexity and there are very few simple statements that will hold true in most cases. Two statements that most often are true about enzymes are the saturation behavior and existence of a K_m. For more in depth understanding of enzyme kinetic behavior, one needs to consult a much more comprehensive treatment of the subject. However, a few oversimplifications can give a general picture that is *often* correct. First of all, K_m is partially a function of substrate affinity for the enzyme binding site. A further simplification is that enzymes with higher numbers of contact points will have higher-affinity binding and lower K_m values. These will be characterized by the plot on the left in Figure 5A. Few interaction points between enzyme and substrate will produce low-affinity interaction and a curve such as that on the right in Figure 5A. Thus, enzymes having a strong interaction with their substrates will generally have curves that are saturated at low substrate concentration. High-affinity interaction is also consistent with many contact points between substrate and enzyme and with high specificity for the substrate.

Overall, this lengthy section as well as the following section illustrate that the enzyme–substrate binding event, an essential part of enzyme catalysis, has many ramifications for biological systems. In the next section, we discuss pharmaceuticals and other bioreactive molecules that function by binding to an enzyme and preventing the enzyme from binding its substrate. Obviously, the most active of

these materials will be those which bind tightest to the enzyme since these prevent its action at the lowest concentration. These materials might be compared to the two curves in Figure 5A. A highly active molecule will bind at low concentration and inhibit the enzyme (curve to the left in Figure 5A, but describing inhibition rather than activity), while a material with lower affinity will require a high concentration to bind to the enzyme (curve on the right in Figure 5A). The latter material will be less effective since a higher concentration must be maintained in order to inhibit the enzyme. Again, many bioreactive molecules bind to sites other than enzyme active sites and exert their influence in other ways. However, the binding process will be similar and will consist of several contact points between the two molecules (one is often a protein).

Once again, the alcohol dehydrogenase enzyme (Figure 2) can provide an introduction to the next section. If a person accidently ingests wood alcohol (CH_3OH) or antifreeze (1,2-ethanediol, $HOCH_2$-CH_2OH), the liver enzyme is capable of binding these materials and will oxidize them to formaldehyde (H_2CO) and glyoxal ($HOCH_2$-CHO), two very toxic materials. This is what makes wood alcohol and antifreeze poisonous. These two materials do not fit the binding site of the alcohol dehydrogenase enzyme as well as does ethanol, and the K_m for both methanol and ethanediol is about 0.03 M (Blair and Vallee, 1966). The medical treatment for wood alcohol or antifreeze poisoning is to introduce an inhibitor which prevents these compounds from interacting with alcohol dehydrogenase. The inhibitor chosen is ethanol. If there is enough ethanol in the body, it will fill all the binding sites on the alcohol dehydrogenase enzyme and prevent oxidation of the other substrates. The methanol and antifreeze can then slowly be excreted in their harmless forms through urine or sweat. This general principle of inhibition of a reaction by materials that bind to an enzyme is an extremely common approach for medical agents. This is illustrated further in the next section.

Use of Biological Specificity to Create Pharmaceuticals and Other Bioactive Molecules

Binding occurs between several sites on the substrate and on an enzyme. Obviously, one might be able to create materials that bind to an enzyme but lack the groups that undergo subsequent chemical reaction. Filling the active site with such a material will prevent an enzyme from functioning and this will have various impacts on the entire organism. Mimicry of normal compounds is common to many bioreactive molecules. One example, methotrexate, is shown in Figure 6. It binds to enzymes that normally utilize dihydrofolate. With methotrexate bound, the enzyme cannot accept dihydrofolate and catalyze its normal reaction. Enzyme catalyzed reactions involving dihydrofolate are important for DNA synthesis and the latter is critical to cells that are rapidly dividing. Such cells, including cancer cells, will be harmed more by methotrexate than cells that are growing slowly. Thus,

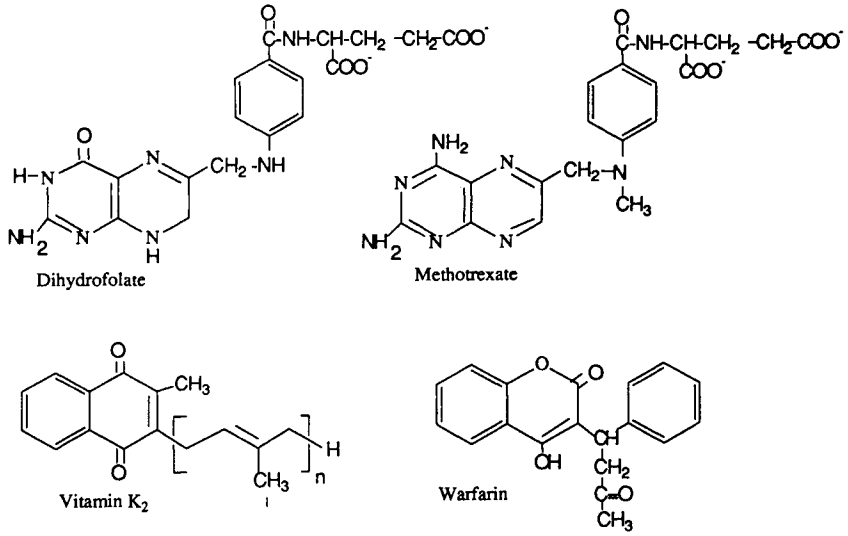


Figure 6. Some common pharmaceuticals that inhibit enzymes. Methotrexate binds to the enzyme that normally uses dihyrofolate. This binding prevents the enzyme from binding dihydrofolate reductase and carrying out its function. Inhibition of these enzymes is toxic for virtually all cells but has some selectivity for rapidly dividing cells. Warfarin binds to an enzyme site that is normally occupied by vitamin K. Again, with warfarin bound, liver enzymes that aid in synthesis of certain blood clotting proteins cannot function properly. As a result the blood clotting proteins produced are defective and there is reduced ability to clot blood.

methotrexate has been an important drug for treatment of some types of cancer as well as other medical problems. However, the potential for toxicity of methotrexate to all types of cells is obvious. Use of agents that target enzymes or other molecules that are critical to fast growing cells is common to many types of chemotherapy, especially for cancer.

Another example is the dicumarol family of molecules which are active in blocking enzymes that use vitamin K as a substrate. Vitamin K (Figure 6) is needed for enzyme-catalyzed modification of certain blood clotting proteins. Without the vitamin K-dependent modification, the blood clotting enzymes will not function. The reaction (the details are not important for this discussion) consists of carboxylation of glutamic acid residues of the blood clotting proteins to γ-carboxyglutamic acid. This process occurs in the liver. However, if the enzymes that use vitamin K contain a dicumarol derivative at the active site, the enzyme cannot catalyze the desired reaction as the relevant blood clotting proteins are not produced in adequate amount. Thus, administration of these vitamin K antagonists can be useful in many circumstances because they reduce the levels of several blood clotting proteins,

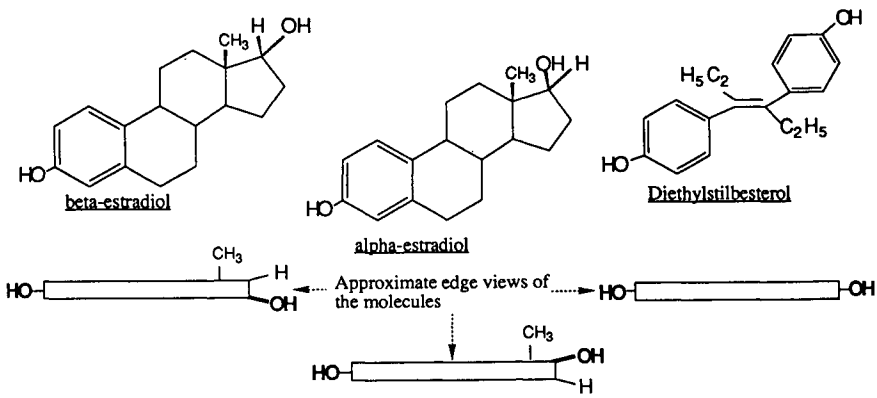


Figure 7. Active and inactive estrogens. β-estradiol is the normal active form of the hormone which binds to proteins and brings about many changes in cell behavior. The compound in the center, α-estradiol, is inactive. This difference in function is caused by a single change in the structure where a hydroxyl group projects out on the wrong side of α-estradiol. The latter is illustrated by the box drawings at the bottom where the molecules have been rotated 90° by picking up the bottom of the structures drawn in detail at the top of the figure. The 'box' view is also slightly from the right side. The third molecule shown, diethylstilbesterol, is very active as an estrogen because it contains hydroxyl groups that are located at the correct distance from one another and are available from the same side of the molecule (see edge view). The other parts of the estradiol molecule apparently have little to do with the binding event.

thereby reducing blood clotting ability. Reduced blood clotting can be useful when unwanted blood clots are a potential hazard. Again, if this is pushed too far, it can be lethal because individuals cannot stop any bleeding event. Both uses are made of these molecules as oral anticoagulants in medicine and as the most common rat poison, warfarin (Figure 6).

Overall, there are *many* examples of drugs that act by mimicry of another molecule, thereby blocking an enzyme binding site and preventing a reaction catalyzed by the enzyme. This same approach applies to other bioreactive molecules such as herbicides and pesticides. Again, while the target of these materials is not always an enzyme, the process of binding to a specific site is similar.

An example of a bioreactive molecule that targets binding sites other than enzymes is diethylstilbesterol (DES, Figure 7), an artificial estrogen. It has numerous uses in its history, some have been seriously questioned and discontinued. It has been a cattle feed additive, a chemical neutering agent for young roosters, a morning-after birth control pill, as well as many other uses. DES binds to the same site as the biological molecule, β-estradiol. An apparently strange phenomenon is that a slight modification of β-estradiol, known as α-estradiol, produces an inactive molecule. Yet, the very different structure of DES is highly active (Figure 7). This seems strange, but the answer must be found in the nature of the binding site. That is, the binding site for estradiol recognizes a few points on the molecule, consisting

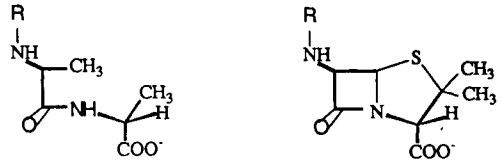
of two hydroxyl groups which are about 10 Å apart and which are available on the same side of a nearly planar, hydrophobic molecule. A simplified edge view of these molecules is shown in Figure 7. Both β-estradiol and DES satisfy the binding-site requirements. However, the fact that one of the hydroxyl groups of α-estrogen projects out on the wrong side of the molecule makes it impossible for the binding site to interact with both hydroxyls simultaneously. α-estradiol is, therefore, inert while DES is very active.

Artificial sweeteners are other examples of molecules that must bind to a nonenzymatic site (one that senses sweetness). The search for artificial sweeteners that do not have undesirable side-effects has been a long and intense one. The most effective artificial sweetener should have a high affinity for the sweetness receptors but not for other binding sites. The most effective commercial one is probably the material, L-phenylalanyl-L-aspartyl-methyl ester. Why this molecule should be so effective is not completely apparent, especially when one considers that certain sugars are also sweet. However, the binding sites recognize certain features of a molecule and the arrangement of the contact points cannot easily be predicted. Because of this, the search for bioreactive molecules can often be a nearly random process.

A common approach used when hunting for bioreactive molecules is to apply a mixture of compounds to an enzyme or biological system and to see if it influences the system. In this way, a few mixtures can test many molecules. If a certain mixture is found to influence the biological system in the desired manner, the individual compounds in that mixture are each tried to find the active ingredient. Some pharmaceutical companies have libraries of compounds that number in the hundreds of thousands. A variation of this approach has been used for ages when plants have been tried as medicines. Plants contain a wide range of molecules known as secondary metabolites. By chance, some of these molecules may interact with something in a human repertoire of binding sites and may function as a medicine, poison, or have other effects. Thus, the approach used by many modern chemical companies is just a more systematic version of a process that has been used by humans since the earliest times.

The existence of secondary metabolites that may fortuitously bind to proteins in the human body may also be illustrated in the nutrition field. Some recent research has investigated plant estrogens, molecules that may have hormonal activity in humans, despite the fact that they have no similar role in the plant. Thus, chance interactions of external molecules with human proteins is a major topic of consideration in all of biological sciences.

A major problem in the search for bioactive molecules is the need to have a limited effect. The compounds outlined above are used in a limited way by each organism. That is, dihydrofolate is needed for a limited number of enzyme reactions in the cell, vitamin K functions in a very limited number of cases, and estradiol has a very narrow set of functions. All of these molecules are used to influence normal processes in the human body.



Peptidoglycan to be crosslinked

Penicillin active structure

Figure 8. Penicillin (right) and the normal substrate for the enzyme that is inhibited by penicillin. Peptidoglycan is normally cross-linked in the bacterial cell wall to create a tough lattice of covalent bonds. However, if penicillin binds to and inhibits the enzymes needed for the cross-linking process, the bacteria will not survive.

Chemicals are also used to select between different biological systems and these include agents that selectively kill one of the organisms. Common examples are the antibiotics. The antibiotics that are most useful in medicine are those that target enzymes not found in the human. An example is penicillin (Figure 8). This antibiotic resembles a substrate used for the synthesis of the cell wall of many bacteria. Penicillin will bind to the enzyme and subsequent events will inactivate the enzyme permanently. In other words, penicillin not only binds to the active sites of the bacterial enzyme, but modifies that enzyme in a manner that makes it permanently inactive. Since humans do not make cell wall, there are no enzymes of this type to be influenced and penicillin should have little effect on human enzymes. This very selective manner of killing is extremely valuable in medicine. Obviously, since the antibiotics we use are isolated from other organisms, this approach was actually developed by other life forms.

Other antibiotics may not be quite as selective and may react with both human and bacterial enzymes, thereby making them somewhat toxic. An example of the latter is chloramphenicol, an antibiotic that targets the protein synthesis apparatus. Since both humans and bacteria synthesize proteins, the potential for some cross-reactivity is obvious. This antibiotic can have some deleterious side effects in humans. The level of the deleterious effect is a matter of debate and it is a duty of governmental regulatory agencies to monitor these properties.

In the case of viruses, the window for selective enzyme inhibition is particularly narrow. Viruses are not complete organisms in that they use the host's own enzymes for much of their required metabolism. Thus, selective targeting of viruses usually involves a very small number of enzymes that are unique to the virus itself. This is one of the reasons why viruses are very difficult to treat. In short, the opportunities to inhibit specific virus enzymes are very few. Some of the agents that are used for some news-worthy viruses include acylovir and dideoxyinosine (Figure 9). These are used to treat Herpes and HIV viruses, respectively. Apparently, certain viral enzymes will bind to these structures while the host's own enzymes will not. Attempts to target other viral enzymes is a current intense area of research.

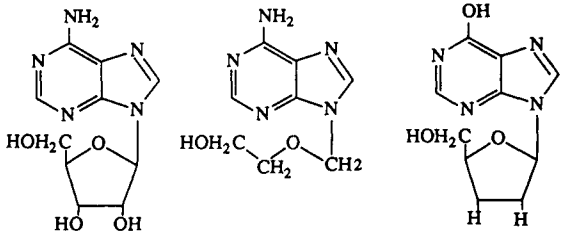


Figure 9. Adenosine and look-alike molecules that are used to inhibit viruses. The structural homology between these molecules is apparent. Since all cells contain enzymes that react with adenosine, a further requirement for function is that the agents bind better to the sites on enzymes produced in virus-infected cells than in normal cells. Acyclovir is used for treatment of Herpes while dideoxyinosine is used for the virus that causes AIDS. Several other related molecules such as AZT are also used for the virus that causes AIDS.

To carry this a step further, we know that organisms fight back. Through evolutionary processes, the binding sites on the various enzymes can be changed by substitution of the amino acids that make up the binding site. An altered binding site might not interact with the inhibitor as well and the organism will be less susceptible to that compound. Two examples will suffice to illustrate this property. Rat populations that are resistant to warfarin (Figure 6) are known. These rats arise when a population is exposed to the poison over long periods of time. A small portion of the rats contain a mutated binding site that does not bind the poison as well as it does vitamin K. Since the binding site is less effective and these rats also have a higher requirement for vitamin K in their diets, they do not thrive under normal circumstances. However, if normal rats are eliminated by the poison, the mutated rats have no competition in their ecological niche and can thrive. The same description can be made for various bacteria that become resistant to certain antibiotics. While there are a number of ways that antibiotic resistance can be produced, alteration of an enzyme binding site is one that has been observed. Again, selection for these antibiotic resistant strains will occur when the normal strains are systematically eliminated by high exposure to the antibiotic. Thus, limiting the use of antibiotics is an important way in which we can slow the rate at which resistant organisms appear in the environment. For this reason, antibiotics are distributed by a carefully regulated system.

Overall, the wide-ranging discussion in this section has been designed to show the connection between binding of substrate molecules, a process that is an unavoidable necessity of enzyme catalysis, and many approaches that are used in medicine and other areas. Substrate binding and the resulting specificity are responsible for many fundamental properties of natural systems and are the target of a large array of bioreactive molecules.

STEP 2 OF ENZYME CATALYSIS: BINDING IS FOLLOWED BY CHEMICAL REACTION

The simple process of binding substrates and creating close proximity is not sufficient to allow chemical reactions to occur under the mild conditions found in the cell. Enzymes must also facilitate the reactions in other ways. Again, the number of mechanisms is large and some produce secondary effects that help explain other aspects of medicine and biology.

There are several different types or 'families' of protease enzymes and one family is used to illustrate several other common factors of enzyme catalysis. The enzyme shown in Figure 10 is described as a 'serine protease' since it contains serine at its active site. Other families of proteases contain a different amino acid at the active site.

The peptide bond is an amide containing an sp^2 carbon atom. The amide is stabilized by electron resonance between the nitrogen and oxygen atoms. The uncatalyzed, or chemical hydrolysis of a peptide bond is illustrated at the top of Figure 10. The most difficult structure to form, or the *transition state*, consists of the *tetrahedral* carbon. This would be the structure at the top of the energy diagram in Figure 1.

The enzyme catalyzed reaction at the bottom of Figure 10 involves initial attack by the hydroxyl of a serine side-chain, a part of the enzyme, rather than by direct attack by hydroxyl ion as in the nonenzymatic reaction. The serine is assisted by a histidine which is located so it can accept the proton released from the serine. The serine and histidine are involved in the actual chemical reaction and are referred to as 'active site' amino acid residues. Thus, the protein must fold in a way that creates a substrate binding site along with exact alignment of these active site amino acids so they can catalyze the reaction. This first step in enzyme catalysis is then followed by attack by water, which is not shown in Figure 10. However, the first step in Figure 10 is adequate to illustrate several features of enzyme catalysis. First of all, enzymes sometimes use a series of reactions that differ from those of the nonenzymatic reaction. Several steps, each with low $\Delta G^{\ddagger}$, will be more rapid than a single step with high $\Delta G^{\ddagger}$.

Another mechanism for facilitating a reaction consists of stabilizing the 'transition state', the structure at the top of the energy curve in Figure 1. Stabilization of this most unfavored structure will lower the height of the barrier and the value of $\Delta G^{\ddagger}$ (Figure 1). One mechanism for stabilizing the intermediate is for the enzyme to simply bind the high-energy transition state substrate more tightly than the original low-energy substrate. The additional binding energy released when the intermediate is formed will decrease the difference between the energy of the original enzyme–substrate complex and that of the enzyme–transition state intermediate. In some protease enzymes, the binding site is known to prefer a tetrahedral carbon atom (sp^3 carbon) rather than the sp^2 carbon of the amide (Figure 10). Thus,

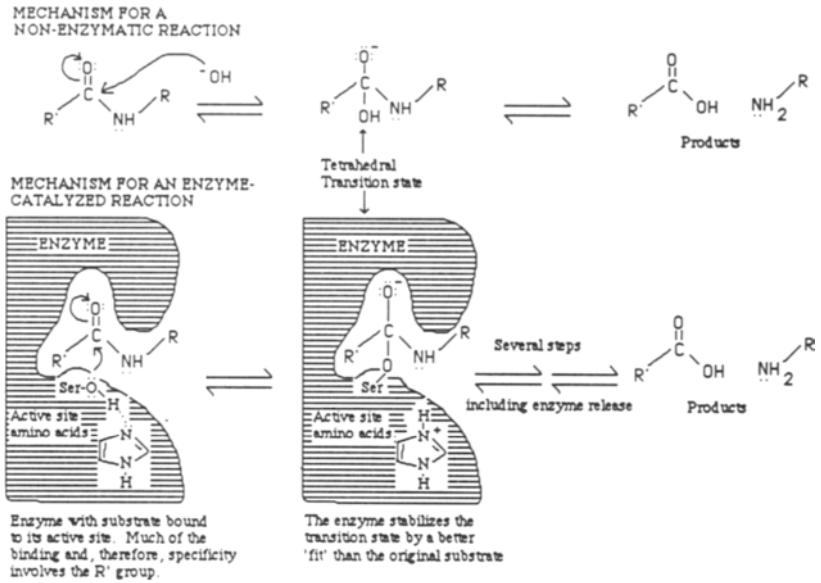


Figure 10. Enzyme catalyzed hydrolysis of a peptide bond. The top shows a general mechanism for chemical hydrolysis of a peptide bond. The conditions that are usually employed for this chemical reaction include 2M NaOH and 110° C. This is obviously incompatible with life. The bottom shows some aspects of enzyme catalysis of the same reaction. The first step, which is not shown, consists of binding the substrate to the enzyme. The shaded area represents the enzyme which is made up of polypeptide folded into a three-dimensional structure. The binding site is greatly abbreviated in the drawing here and there is no attempt to show the contact points between substrate and enzyme. The enzyme contains an active site serine which is aligned to interact with the carbonyl group of the substrate. The enzyme accommodates this attack in many ways and stabilizes the transition state by a better fit to the binding site. This achieves the most difficult part of a reaction by attaining the transition state (top of the energy diagram in Figure 1). Several steps are then needed to finally release the products from the enzyme. Note that the substrates and products of the enzyme catalyzed reaction are the same as those from the chemical reaction. The only difference is the pathway of the reaction and the nature of the intermediates generated.

by preferential binding of the transition state, the enzyme lowers $\Delta G^{\ddagger}$ and enhances the rate of the reaction.

As in the case of substrate binding to the enzyme, the fact that enzymes often stabilize the transition state is used often to create bioreactive molecules that are highly effective as enzyme inhibitors. An example of this is leupeptin, an antibiological molecule synthesized by a fungus. This molecule is a short peptide (the R′ group in Figure 11) that is terminated by an aldehyde. This peptide will bind to enzyme in the same manner as the normal substrate as shown in Figure 10. The

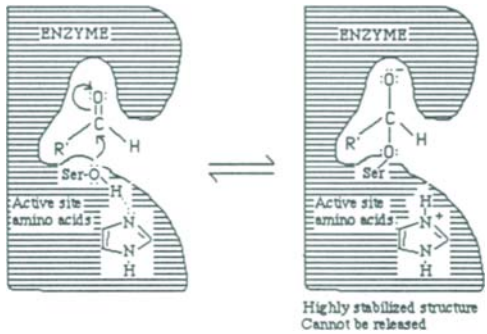


Figure 11. Mechanism by which leupeptin inhibits a protease enzyme. Leupeptin contains all the groups of the R′ structure that interact with the enzyme shown in Figure 10. However, it contains an aldehyde rather than an amide. The enzyme will attack the aldehyde and greatly stabilize the intermediate. However, the final stability of this structure is such that it cannot be released from the enzyme. The active site of the enzyme is permanently occupied and the enzyme is inhibited.

enzyme recognizes the R′ group and aligns the aldehyde with the active-site serine. However, unlike a peptide bond, an aldehyde is not stabilized by electron resonance between oxygen and nitrogen. The aldehyde readily assumes a tetrahedral shape. In this case, all of the energy that the enzyme expends to stabilize the tetrahedral becomes detrimental since the intermediate becomes so stable that it will not dissociate from the enzyme. The enzyme is, therefore, inhibited by very low amounts of leupeptin. Although leupeptin inhibits a broad range of enzymes and is, therefore, too toxic for medical use, it is possible that other molecules will be found that use this strategy and which selectively inhibit enzymes in a manner that will be a benefit for human medicine.

Other mechanisms for enhancing enzyme reactions can be proposed for the protease shown in Figure 10. Resonance stabilization of the peptide bond and the sp^2 carbon atom is dependent on a planar arrangement of the carbon, oxygen, and nitrogen atoms. If an enzyme can bend the C–N bond out of this plane, it will disrupt the stabilizing resonance and facilitate other reactions such as conversion to a tetrahedral. Often, substrate binding to an enzyme results in a conformational change in the protein. In several cases, the substrate-binding site is found in a crevice on the protein. Upon binding the substrate, the rest of the protein closes down and traps the substrate in the crevice. Such events can put strain on the various bonds of the substrate, as described above, and also help isolate the substrate from the aqueous environment.

Overall, a single enzyme will utilize many of these methods for catalyzing a reaction. The important point I have tried to stress in this chapter is that the basic mechanisms of enzyme action are responsible for many of the properties of biology, and for many approaches used in medicine and other situations that require bioreactive molecules. Virtually all forms of biology depend on binding and

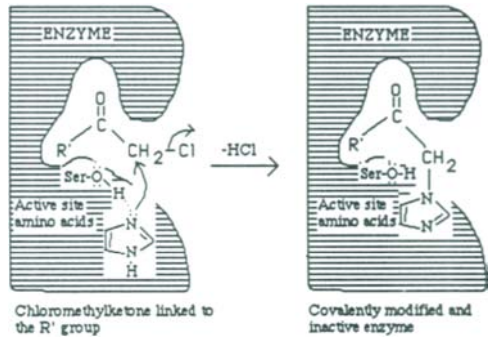


Figure 12. Chloromethylketone: a man-made enzyme inhibitor. Again, the compound is bound to the active site through contacts between the enzyme and the R′ structure. However, the structure at the active site is not an amide, the normal substrate. No reaction with active-site serine is possible. This molecule is, therefore, an inhibitor as it blocks the active site. However, a second, irreversible reaction can occur between the chloromethylketone and groups on the enzyme. This reaction will permanently fix the molecule to the active site and render the enzyme permanently inactive. The product is shown on the right. Chloromethylketones are, therefore, common enzyme inhibitors in man-made products.

specificity for many purposes other than just enzyme catalysis. Enzyme inhibitors used as toxins, medicines, or for other purposes, can bind to an enzyme (or other type of binding site) and resemble the substrate or the transition state. The latter, referred to as 'transition-state analogs' are often the most potent enzyme inhibitors.

Before leaving, we can point out that design of enzyme inhibitors sometimes is a totally human project. Figure 12 shows a common type of enzyme inhibitor that contains a time-bomb in the form of a highly reactive group that will chemically combine with amino acids near the active site of the enzyme. This chloromethylketone is attached to an appropriate R′ group that binds to the substrate site on the enzyme. Since the appropriate structure for subsequent enzyme catalysis (the amide) is not present, this compound will not undergo conversion to a different product. However, the chloromethylketone is chemically active and can alkylate various groups near the binding site, thereby rendering the enzyme permanently inactive due to a covalent derivatization that blocks the binding site. In the case of the protease shown, it is known that the chloromethylketone reacts with the histidine of the active site.

Thus, enzymes are critical to most reactions that occur in the biological environment. They function by a series of steps that lower the activation energy for a reaction. Virtually all aspects of enzyme catalysis have many ramifications for the overall properties of biological systems and for the practice of modern medicine and biotechnology in general.

SUMMARY

Enzymes are responsible for bringing about chemical reactions under the very mild conditions that are essential to the existence of biological organisms. A central feature of enzyme catalysis is the *free energy of the reaction* (ΔG), which describes the amount of energy released or taken up when the reaction occurs. This term will determine the direction in which the reaction will proceed and is not influenced by the enzyme. A second central feature of enzyme catalysis is the *free energy of activation* or $\Delta G^{\ddagger}$ which describes the energy barrier over which the reactants must pass in their transformation to product. The higher the energy of activation, the slower the reaction. Enzymes exert their influence by reducing the free energy of activation to levels that allow the reaction to proceed at low temperature. Finally, the third central feature of enzyme catalysis is the '*transition state*'. This is the structure that exists at the highest point of activation energy and it is the most difficult structure to attain throughout the course of the reaction. Thus, enzymes often function by lowering the energy of the transition state, thereby lowering $\Delta G^{\ddagger}$.

The first step of enzyme catalysis is responsible for many of the most outstanding properties of biological systems and explains the mechanism of action of many pharmaceuticals and other bioreactive molecules such as poisons, herbicides, pesticides, etc. The enzymes bind the reactants and align them for subsequent chemical reactions. The act of binding a molecule to a protein requires correct and complementary location of various functional groups on the protein and reactant. This requirement produces one of the most distinctive properties of biology which is *specificity*. Many pharmaceuticals and other bioreactive molecules are designed to take advantage of this specificity and to influence individual enzyme-catalyzed reactions. The binding process also explains why biological reaction kinetics display *saturation behavior*. That is, there is an upper limit to the rate at which a reaction can occur. The saturation point is determined in part by the nature of the binding site on the enzyme.

Overall, the biological need to exist under mild conditions of low temperature and neutral pH produces a requirement for enzyme catalysis. The enzymes in turn are responsible for many of the most distinctive properties of biological systems and for many approaches that are used in medicine.

REFERENCES

Blair, A. H. & Vallee, B. L. (1966). Biochem. 5, 2026–2034.

RECOMMENDED READINGS

Fersht, A. (1985). Enzyme Structure and Mechanism, Second Edition, W. H. Freeman and Co., New York.

Moran, L. A., Scrimgeour, K. G., Horton, H. R., Ochs, R. S., & Rawn, J. D. (1994). Biochemistry (2nd edition), pp. 1–44, Neil Patterson Publishers, Burlington.

Voet, D. & Voet, J.G. (1995). Biochemistry (2nd edition), pp. 331–410. John Wiley & Sons, Inc., New York.

Zubay, G. (1993). Biochemistry, Third Edition, pp. 197–278, Wm. C. Brown Publishers, Dubuque.

Chapter 3

Substrate Utilization in Mammalian Cells

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INTRODUCTION

Mammalian cells utilize substrates for anabolic and catabolic processes. These substrates are originally derived from food digested in the gastrointestinal tract and absorbed by the body. Part of the substrates undergo processing in one organ before being utilized in another. Substrates can be roughly divided into three categories: (i) carbohydrates, (ii) lipids, and (iii) amino acids.

Marked differences in substrate preference have been found among organs. Brain and nerve tissue utilize predominantly glucose and ketone-bodies. Cardiac cells consume preferentially lactate and fatty acids, while enterocytes in the intestinal wall use high amounts of glutamine for energy production. The various substrate categories will be separately discussed in this chapter. Because metabolic and regulatory inter-relationships exist between substrates in the different categories, their mutual relations will be discussed as well.

After extraction from the extracellular compartment, the bulk of the substrates is used for the production of energy. During rest in the fed state, the substrates taken up will be stored in intracellular depots from which they are released when the supply of exogenous substrates is limited. Part of the substrates absorbed will be utilized for the synthesis of macromolecules such as proteins and deoxyribonucleic acids (DNA), or the production of compounds crucial for cellular membranes, like phospholipids and cholesterol. The latter are typical examples of anabolic, energy consuming processes.

It is well known that the body consists of a host of specialized cells. Even within one organ, a variety of differently specialized cells can be found. In most cases these phenotype differences are associated with striking differences in metabolic properties reflected by differences in uptake, intracellular storage, and handling of substrates. If relevant, variations in metabolic properties between organs will be discussed in the sections below.

CARBOHYDRATES

Glucose is the most prominent carbohydrate supplied to mammalian cells from the vascular compartment. The carbon atoms of circulating glucose molecules are supplied to the body from digested food. After digestion of carbohydrate macromolecules, such as glycogen and starch, and the disaccharide sucrose in the lumen of the gastrointestinal tract, glucose is transported to the liver via the hepatic portal vein which drains the small intestines. A major proportion of glucose is extracted by the liver and used for replenishment of hepatic glycogen stores, synthesis of lipids, and energy production. Glucose molecules that escape extraction by the liver are taken up by other organs in need of carbohydrates.

Glucose levels in blood have to be maintained within safe limits. Under normal physiological conditions blood glucose concentration varies between 3 to 7 mmol/l blood plasma. Above and below these concentrations, the normal organ function

is at risk. Low blood glucose levels create a potential shortage of glucose for brain and nervous tissue, structures largely dependent on glucose for energy production. High circulatory levels of glucose may cause, among other effects, dehydration of tissue, glycosylation of proteins, and a favorable condition for bacterial infections. Hormones like insulin and glucagon are instrumental in keeping blood glucose levels within physiologically acceptable limits. The liver plays a crucial role in glucose homeostatic processes (see below).

Transmembrane Transport of Glucose

Due to its typical chemical structure, glucose is highly soluble in water. Therefore, no carrier system is required to transport glucose within the vascular compartment. However, cellular membranes consisting of a phospholipid bilayer are virtually impermeable for glucose. Nature has created special carriers in the cellular membrane to guarantee adequate transport of glucose into the mammalian cell. These glucose transporters or GLUT are proteins consisting of a chain of approximately 500 amino acids organized in 25 segments (Lienhard et al., 1992). Twelve segments are largely hydrophobic, while the remaining 13 have hydrophilic properties. The protein creates a channel in the cell membrane by arranging the predominantly hydrophobic segments in the phospholipid bilayer in such a way that a pore is formed. The hydrophilic segments are used for constructing an inlet and an outlet at the extracellular and intracellular sides, respectively, of the membrane. At present, at least five different types of GLUT are identified. GLUT1 is expressed in endothelial cells of the microvasculature of brain tissue and is thought to be involved in delivering sufficient amounts of glucose to brain cells. GLUT2 has been found in organs capable of releasing glucose into the blood, such as intestine, kidney, and liver, and also in the β-cells of the pancreas. The latter cells are known to respond to increasing blood levels of glucose by secreting the hormone insulin. Because the affinity of GLUT2 for glucose is relatively low, transport through the carrier protein is almost linearly related to the circulating level of glucose. GLUT3 is present in the cell membrane of brain tissue. GLUT4 has been identified in skeletal and heart muscle cells, and in fat cells of adipose tissue. This transporter protein has the ability to commute between intracellular stores and the plasma membrane. GLUT5 is mainly present in the epithelial cells of renal and small intestine tissue, absorbing glucose from the renal filtrate and the fluid in the lumen of the gut, respectively.

The GLUT-mediated transmembrane transport of glucose in liver, pancreas, intestines, kidney, brain, and nerve cells is insensitive to insulin. In sharp contrast, insulin strongly influences the transmembrane transport of glucose in cells possessing GLUT4 proteins. Within ten minutes after the binding of insulin to the specific insulin receptor in the cell membrane, the amount of GLUT4 in the same membrane is substantially increased. Concomitantly, the number of GLUT4 molecules in the

intracellular stores is decreased, indicating intracellular translocation of glucose transporting proteins (Lienhard et al., 1992). In addition to recruiting glucose transporters from intracellular stores, insulin is also thought to alter the capacity of a glucose transporter to channel glucose from the extracellular space into the intracellular compartment (Widdas, 1988). This theory is based on the presence of putative modifier sites, located on the hydrophilic segments of the transporter facing the extracellular space and the notion that clefts are present at the outer and inner openings of the pore bridging the cell membrane. As soon as extracellular glucose is bound to the outer cleft, the cleft closes behind the sugar molecule and glucose is actively released into the cytoplasmic compartment. The inner cleft closes and the outer cleft opens again to bind the next glucose molecule. The protein segments of GLUT forming the clefts are thought to possess high-affinity sites for glucose to ensure the binding of glucose to the transporter. Interaction with the modifier sites of the GLUT molecule might alter the affinity of the GLUT for glucose or the rate of opening and closing of the cleft, both of which result in enhanced transmembrane movement of glucose (Widdas, 1988). It should be realized that insulin not only acts on the transport of glucose across the cell membrane, but that this hormone also influences the intracellular utilization of the glucose moieties.

Intracellular Metabolism of Glucose

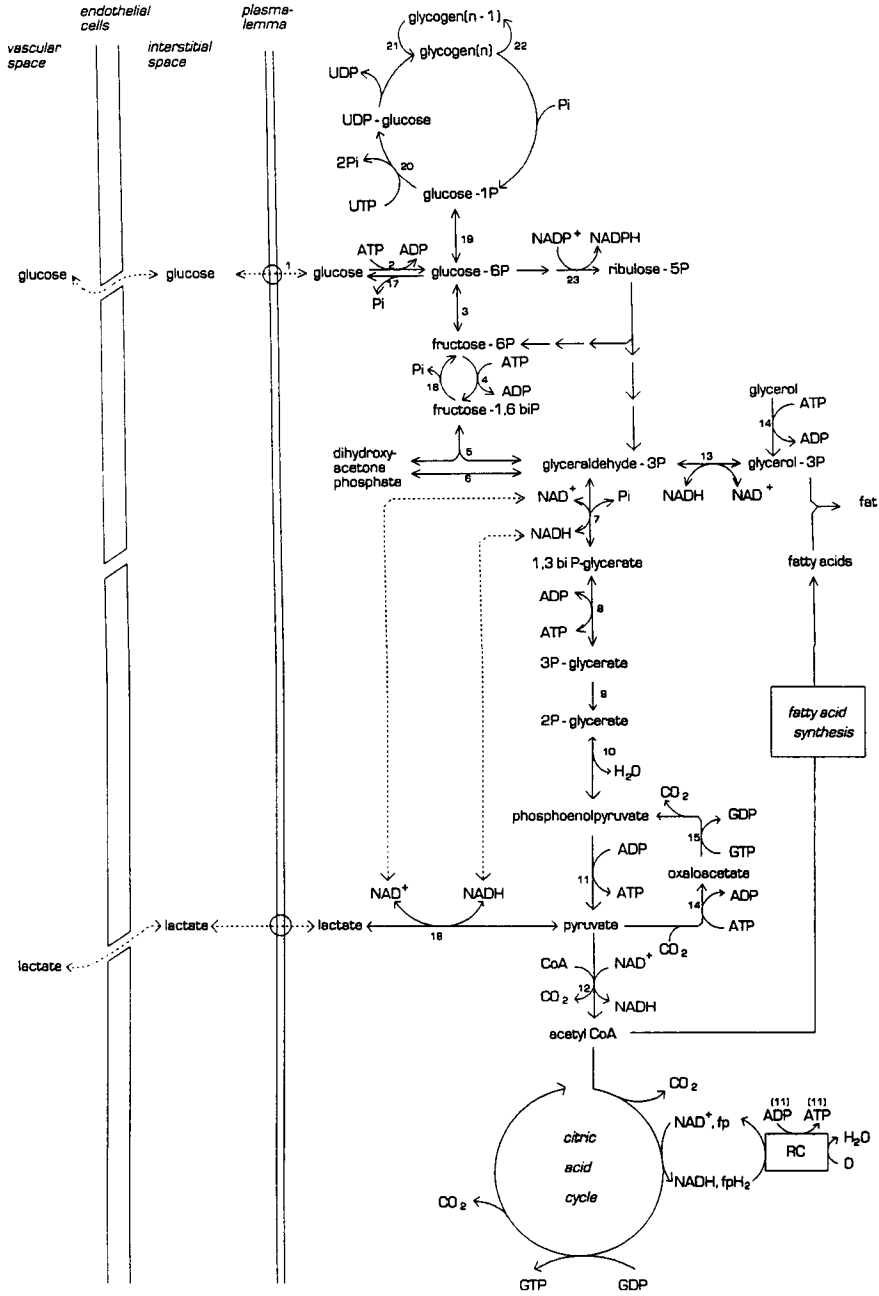
The metabolic fate of glucose in the cytoplasmic space depends on a variety of factors. Beside the type of cells by which glucose is extracted from the extracellular compartment, factors like state of feeding, level of exercise, and the concentration of blood hormones known to exert an action on glucose metabolism, are involved in cellular glucose homeostasis (Devlin, 1992).

Inside the cell, glucose can be stored as glycogen, or degraded in the glycolytic pathway to intermediates with 3-carbon atoms, like pyruvate (Figure 1). The latter substance can subsequently be used for the oxidative generation of ATP in the mitochondria or utilized for the *de novo* synthesis of fatty acids and amino acids. Moreover, glucose can be converted to 5-carbon carbohydrates in the pentose–phosphate shunt with a concomitant production of NADPH from $NADP^+$. Liver and skeletal muscle are the most prominent sites of glycogen formation. Glucose is mobilized from liver glycogen when blood glucose levels are declining and from muscle glycogen for its own use when intracellular consumption exceeds the supply from the blood. Almost all mammalian cells have the ability to glycolytically and oxidatively degradate glucose. Red blood cells are an exception as they lack mitochondria and rely completely on anaerobic glycolysis, that is, the conversion of glucose to lactate, as their sole source of energy. Liver cells have the unique capability of synthesizing glucose from smaller carbohydrates in the so-called gluconeogenic pathway.

Glycolytic Degradation of Glucose

Almost all mammalian cells display glycolytic activity. In the glycolytic pathway glucose is degraded stepwise to the 3-carbon carbohydrate, pyruvate (Figure 1). The first step in the glycolytic pathway is the conversion of glucose to glucose-6-phosphate (glucose-6P), a conversion catalyzed by glucokinase in liver and by hexokinase in extrahepatic tissue. Glucose-6P is used for the synthesis of glycogen (glycogenesis), the production of pyruvate (glycolysis), or is channeled into the pentose–phosphate pathway to provide the cell with NADPH and carbohydrates with 5-carbon atoms. In the glycolytic pathway, glucose-6P is converted to fructose-6P by the enzyme phosphoglucose isomerase. Fructose-6P is then converted to fructose-1,6biP by the enzyme, 6-phosphofructo-1-kinase. This first stage in the glycolytic pathway is followed by a step in which the 6-carbon carbohydrate is split into two 3-carbon intermediates, that is, dihydroxyacetone phosphate and glyceraldehyde-3P. The splitting is catalyzed by fructose biphosphate aldolase. Dihydroxyacetone phosphate can be converted to glyceraldehyde-3P and *vice versa* by triose phosphate isomerase. The third and final stage of the glycolytic pathway is characterized by a set of oxido-reduction and phosphorylation steps. Glyceraldehyde-3P is metabolized to 1,3-bisphosphoglycerate by the enzyme glyceraldehyde-3P dehydrogenase. The latter intermediate is converted to 3P-glycerate, a step catalyzed by phosphoglycerate kinase. The next step, the conversion of 3P-glycerate to 2P-glycerate, is catalyzed by phosphoglycerate mutase. 2P-glycerate, in turn, is converted to phosphoenolpyruvate (PEP). This reaction step is controlled by enolase. Pyruvate kinase stimulates the conversion of PEP to pyruvate.

Pyruvate can partake in a variety of metabolic pathways (Figure 1). From an energetic point of view, conversion to acetyl-CoA by pyruvate dehydrogenase and subsequent degradation of the acetyl residue in the citric acid cycle is of utmost importance. The overall reaction of the oxidative degradation of glucose to CO_2 and H_2O yields 38 mol ATP per mol glucose. In liver and adipose tissue, pyruvate can also be used as a precursor for fatty acid synthesis (see below). Moreover, pyruvate is converted to lactate by lactate dehydrogenase when the supply of molecular O_2 is limited or production of pyruvate exceeds the capacity of pyruvate dehydrogenase to convert pyruvate to acetyl-CoA. Reduction of pyruvate to lactate generates NAD^+ from NADH. NADH is formed during the glycolytic conversion of glucose to pyruvate (Figure 1). Since the amount of NAD^+ is limited in the cytoplasm, this cofactor has to be recovered continuously to ensure an unimpeded glycolytic flux. Under normal conditions, the majority of NAD^+ molecules is recovered from NADH by channeling the hydrogen atoms of NADH via a shuttle mechanism into the mitochrondrial matrix. When mitochondrial activity is impeded (lack of oxygen, low pyruvate dehydrogenase activity), NAD^+ is recovered from NADH by the conversion of pyruvate to lactate and, to a lesser extent, glyceralde-


endothelial cells
plasma-lemma
vascular space
interstitial space
glycogen(n - 1)
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glucose - 6P
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NADPH
ribulose - 5P
fructose - 6P
Pi
ATP
ADP
fructose - 1,6 biP
dihydroxy-acetone phosphate
glyceraldehyde - 3P
NAD+
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3P - glycerate
2P - glycerate
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NADH
NAD+
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fatty acid synthesis
CO2
citric acid cycle
NAD+, fp
NADH, fpH2
(11) ADP
(11) ATP
RC
H2O
O
CO2
GTP
GDP

hyde-3P to glycerol-3P (Figure 1). Glycerol-3P is used for the synthesis of triacylglycerols and phospholipids (see below). Anaerobic glycolysis, that is, the conversion of 1 mol glucose to 2 mol lactate, gives rise to the formation of 2 mol ATP instead of the 38 mol ATP generated in aerobic glucose degradation.

Regulation of Glycolytic Activity

In general, flux through the glycolytic pathway is regulated by the activity of hexokinase, 6-phosphofructo-1-kinase, and pyruvate kinase. These enzymes have in common that the metabolic steps they catalyze are non-equilibrium reactions. All other enzymes in the glycolytic pathway appear to catalyze a near-equilibrium reaction and are, therefore, not subject to regulation of the conversion of glucose to pyruvate or lactate.

Short-term regulation of the activity of the three above-mentioned enzymes is achieved by either allosteric effectors (such as intermediates in the carbohydrate converting pathways, energy-rich phosphates and related compounds, and ions) or covalent alterations of the enzyme under consideration. Allosteric effects are achieved when binding of an effector molecule to the enzyme results in structural changes in the catalytic center and, hence, alteration of the biological activity of the enzyme. The effectors themselves do not bind to the catalytic center, but to distant sites of the protein. Covalent alteration of enzymatic activity is commonly achieved by covalent attachment of a phosphate group to one or more amino acid

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Figure 1. Schematic overview of cellular carbohydrate metabolism. Note that basically, the metabolic properties of liver cells are shown; other cell types may lack one or more metabolic pathways (see text). Arrows with solid and broken lines refer to metabolic conversions and routes of transport, respectively. RC refers to respiratory chains, fp to flavoprotein, P_i to inorganic phosphate, UDP to uridine diphosphate, UTP to uridine triphosphate, and O to molecular oxygen. Numbers 1 through 23 refer to enzymes catalyzing the intracellular conversion of carbohydrates; 1, glucose transporter (GLUT); 2, glucokinase (in extrahepatic tissue, hexokinase); 3, phosphoglucose isomerase; 4, 6-phosphofructose-1-kinase; 5, fructose-biphosphate aldolase; 6; triosephosphate isomerase; 7, glyceraldehyde-3P dehydrogenase; 8, phosphoglycerate kinase; 9, phosphoglycerate mutase; 10, enolase; 11, pyruvate kinase; 12, pyruvate dehydrogenase; 13, glycerol-3P dehydrogenase; 14, pyruvate carboxylase; 15, phosphoenolpyruvate carboxykinase; 16, fructose-1,6-biphosphatase; 17, glucose-6-phosphatase; 18, lactate dehydrogenase; 19, phosphoglucomutase; 20, glucose-1P uridylyltransferase; 21, glycogen synthase (and 1,4-α-glucan branching enzyme); 22, glycogen phosphorylase (and debranching enzyme); 23, glucose-6P dehydrogenase, 6-phosphoglucolactonase and 6-phosphogluconate dehydrogenase, the first three enzymes of the pentose–phosphate shunt; [11] refers to the number of moles of ATP produced.

residues of the enzyme. For instance, phosphorylation and dephosphorylation represents an important way of regulating the activity of pyruvate kinase in liver. In addition, the activity of 6-phosphofructo-1-kinase is indirectly regulated by protein phosphorylation/dephosphorylation (see below). Long-term regulation of the glycolytic flux is achieved by induction of the synthesis of regulating enzymes, resulting in an increased number of enzymes per cell.

Hexokinase is strongly inhibited by its reaction product, glucose-6P. This feedback inhibition prevents the accumulation of phosphorylated hexoses and depletion of the cellular phosphate pool when degradation of phosphorylated hexoses cannot keep pace with their rate of synthesis. The regulation of the conversion of glucose to glucose-6P in liver is different. In hepatocytes, glucokinase, an enzyme with a low affinity for glucose but with a high-metabolic capacity, catalyzes this reaction step, and its activity is inhibited by fructose-6P and activated by fructose-1P. The amount of glucokinase in the hepatic cells is determined by, among other things, insulin. When the liver is exposed to high circulating levels of glucose, its glucose buffering capacity is increased as insulin induces the synthesis of the glucokinase protein.

Regulation of the activity of 6-phosphofructo-1-kinase is an intriguing example of a complex system designed for fine-tuning an important metabolic pathway in mammalian cells. Allosteric effectors are ATP, citrate, H^+, AMP, inorganic phosphate (P_i), fructose-2,6biP, and glucose-1,6biP. The first three compounds exert a negative action on 6-phosphofructo-1-kinase, while the last four stimulate the activity of the enzyme. The rationale of these adverse effects can be explained as follows. The energy state of the cell is involved in the regulation of metabolic processes leading to the production of high-energy phosphates. Therefore, high levels of cellular ATP will slow down glycolytic activity, whereas AMP and P_i, the concentration of which is increased in cells in need of energy in the form of ATP, will stimulate the flux through the glycolytic pathway. Citrate readily increases in the cytoplasmic space of the liver and probably also in muscle cells when fatty acid oxidation contributes largely to overall energy production. By blocking the glycolytic consumption of glucose in liver and muscle, the latter substrate is preserved for such organs as the brain for which glucose is the substrate of choice for the production of ATP. Hydrogen ions (H^+) are produced in cells with a high rate of anaerobic glycolysis. To prevent self-destruction of the cellular structures by severe acidosis, feedback inhibition of glycolytic activity by H^+ is an important measure to keep the cellular levels of hydrogen ions within acceptable limits.

Fructose-2,6biP is produced when the glycolytic intermediate fructose-6P is phosphorylated by the enzyme 6-phosphofructo-2-kinase. Fructose-2,6biP can be converted back into fructose-6P by the catalytic action of fructose-2,6 biphosphatase. Interestingly, fructose-2,6 biphosphatase/6-phosphofructo-2-kinase is a single enzyme that carries out both catalytic functions. The two activities of this enzyme are covalently regulated; that is, cyclic AMP-dependent protein kinase

promotes phosphorylation of the enzyme, resulting in enhanced phosphatase and decreased kinase activity. Dephosphorylation (by phosphoprotein phosphatase) of the enzyme evokes the opposite effect. The extent of phosphorylation/dephosphorylation is regulated by the blood plasma concentration ratio of insulin and glucagon. A high insulin/glucagon ratio results in dephosphorylation of the enzyme. Kinase activity will then prevail and the cellular content of 6-fructose-2,6biP increases, resulting in stimulation of 6-phosphofructo-1-kinase and enhanced glycolytic flux. A low insulin/glucagon blood plasma ratio decreases the flux through the glycolytic pathway by reducing the intracellular content of fructose-2,6 biP.

The activity of pyruvate kinase is substantially reduced by high intracellular levels of ATP. In liver, fructose-1,6biP exerts a feed-forward positive effect on pyruvate kinase activity. By doing so, a high production rate of glycolytic intermediates in the first part of the glycolytic pathway sets the stage for an enhanced conversion of PEP to pyruvate in the last part of the metabolic pathway. Covalent modifications of liver pyruvate kinase have a strong influence on its actual activity. Phosphorylation of the enzyme by a cyclic AMP-dependent protein kinase renders the enzyme less active. This occurs when glucagon levels in blood plasma are elevated. A high insulin/glucagon ratio in blood plasma results in a decreased phosphorylation state of the enzyme and enhanced flux through this step of the glycolytic pathway. A long-term effect of high-circulating glucose (and insulin) concentrations in blood is the induction of the synthesis of pyruvate kinase in hepatocytes, which increases the capacity to utilize sugar in the glycolytic pathway.

In addition to allosteric regulation and covalent modification of enzymes controlling the rate limiting steps in the glycolytic pathway, intracellular translocation of the enzymes most likely affects their catalytic activity (Beitner, 1993). In this regard, it is worthy to note that part of the hexokinase proteins is bound to contact sites between the mitochondrial inner and outer membranes, linking hexokinase activity to oxidative-phosphorylation. Unlike hexokinase, 6-phosphofructo-1 kinase and other glycolytic enzymes bind reversibly to the cytoskeleton. Binding to this intracellular structure enhances the activity of the enzymes and renders them less sensitive to allosteric effectors. Insulin promotes binding of glycolytic enzymes to the cytoskeleton and of hexokinase to the mitochrondrial membranes.

The Pentose–Phosphate Shunt

Glucose can serve as substrate in the pentose–phosphate shunt (Figure 1). After transformation to glucose-6P, the latter compound is not converted to fructose-6P but to 6-phosphoglucono-δ-lactone, the committed step in the pentose–phosphate shunt. This metabolic pathway provides NADPH, which is required for the biosynthesis of fatty acids (e.g., in liver cells and adipocytes) and to keep glutathione in the reduced state (e.g., in red blood cells). Moreover, the pentose–phosphate shunt provides ribose-5P which is necessary for the production of nucleotides.

Gluconeogenesis in the Liver

As mentioned above, the liver is unique in the sense that it is able to produce and release glucose in considerable amounts. Production is achieved from smaller size intermediates, such as lactate, pyruvate, glucogenic amino acids, and glycerol (Figure 1). Sugars like galactose and fructose can serve as substrate for glucose production as well. The liver can also secrete glucose by mobilizing the glucose residues stored in glycogen, that is, glycogenolysis (see below). In general, conditions that may lead to unphysiologically low blood plasma glucose levels promote the production and release of glucose by the liver. In this homeostatic process, hormones like insulin and glucagon play a critical role. In general, glucagon, released from the pancreas when circulatory glucose levels are low, stimulates the production and release of glucose from hepatocytes, while insulin stimulates its uptake by the liver by promoting intracellular consumption of glucose.

Gluconeogenesis from pyruvate is not equal to the reverse process of glycolytic degradation of glucose to this 3-carbon intermediate. The glycolytic pathway and the gluconeogenetic pathway deviate at three steps. The conversion of pyruvate to PEP is not mediated by pyruvate kinase due to the irreversible nature of this metabolic step. Pyruvate, derived from either lactate or alanine, is converted to oxaloacetate in the mitochondrial matrix. This step is catalyzed by pyruvate carboxylase. Oxaloacetate *per se* cannot pass the mitochondrial inner membrane. However, with the use of the malate–aspartate shuttle, the 4-carbon skeleton of oxaloacetate can be transferred into the cytoplasmic compartment. Then oxaloacetate is converted to PEP by the action of PEP carboxykinase (Figure 1).

The conversion of PEP to fructose-1,6biP is the reversal of the glycolytic pathway. Because 6-phosphofructo-1-kinase catalyzes a metabolically irreversible step, the enzyme fructose-1,6biP phosphatase is required to promote the generation of fructose-6P. The next step, the conversion of fructose-6P into glucose-6P, is fully reversible. Removal of the phosphate residue from glucose-6P is accomplished by glucose-6-phosphatase (Figure 1). The activity of this enzyme is relatively high in liver and kidney. Inside the cell, the enzyme is located in the endoplasmic reticulum. Hence, glucose-6P has to be transported into the lumen of the endoplasmic reticulum with the use of a specific membrane translocator. The same holds for the journey back to the cytoplasmic space of the two products of glucose-6P hydrolysis, that is, P_i and glucose. In the liver, glycerol, the end-product of triacylglycerol hydrolysis in fat cells (see below), is also used for the *de novo* synthesis of glucose. Hepatocytes contain a relatively high activity of glycerol kinase that catalyzes the conversion of glycerol to glycerol-3P. After conversion of glycerol-3P to glyceraldehyde-3P by glycerol-3P dehydrogenase, the latter serves as precursor for the synthesis of fructose-1,6biP and eventually glucose.

The rate of the gluconeogenetic pathway is regulated primarily by the enzymes that bypass the irreversible steps in glycolytic degradation of glucose to pyruvate.

In general, circumstances favorable for glycolysis are unfavorable for gluconeogenesis and *vice versa*. From a hormonal point of view, glucagon and insulin both affect the rate of gluconeogenesis. Glucagon enhances the gluconeogenetic activity and insulin exerts the opposite effect.

Storage of Glucose in Glycogen

Glycogen is quantitatively the major storage form of glucose in the human body. A special feature of glycogen is the fast rate by which glucose, required for energy production, is liberated from the carbohydrate polymer. Liver glycogen is mobilized when the blood glucose level drops. Glycogen reserves in the hepatocytes are restored after a carbohydrate containing meal. Muscle glycogen is used during prolonged-moderate and strenuous exercise and its intracellular level is restored after cessation of the exercise. Under fed conditions, one molecule of glycogen consists of approximately 100,000 glucose residues. The glycogen macromolecule shows a typically branched pattern. This pattern is caused by the presence of $\alpha[1\rightarrow6]$ glucosidic bonds in addition to $\alpha[1\rightarrow4]$ glucosidic linkages and ensures a fast release of glucose moieties from the polymer.

The synthesis of glycogen starts with the conversion of glucose to glucose-6P. Instead of acting as a precursor in the glycolytic pathway, glucose-6P is converted to glucose-1P by phosphoglucomutase (Figure 1). Glucose-1P reacts with UTP to yield UDP-glucose and pyrophosphate (PP_i). This reaction type is catalyzed by glucose-1P uridylyltransferase and is irreversible, since PP_i is hydrolyzed to 2 mol inorganic phosphate by pyrophosphatase (Figure 1). Subsequently, the glucosyl moiety of UDP-glucose is transferred to a glucosyl residue at the nonreducing end of the existing glycogen molecule in an $\alpha(1 \rightarrow 4)$ linkage. When 11 new glucosyl moieties are linked to the growing glycogen chain, "branching" of the newly formed chain occurs by action of the 1,4-α-branching enzyme. This enzyme removes a strand of seven-glucosyl residues of the growing chain and attaches this oligosaccharide to an earlier made chain in the glycogen molecule via an $\alpha[1 \rightarrow 6]$ linkage. In this way, a highly branched macromolecule is constructed containing at least 100,000 glucosyl moieties, but with a very small effect on the osmolarity of the cell. Accumulation of the same amount of free glucose molecules would be impossible without creating insurmountable problems for the cell to keep its osmotic pressure within physiological limits.

It should be noted that glycogen synthesis cannot start from "nothing", that is, from one single glucose molecule. Glycogen formation most likely starts with linking the glucosyl moiety of UDP-glucose to one of the tyrosine residues present in the amino acid chain of glycogenin, a protein that functions as a glycogen primer (Devlin, 1992).

The first step in the liberation of glucose from glycogen is phosphorolysis of a glucosyl residue at one of the terminal, nonreducing ends of the glycogen macro-

molecule. The cleavage of the α[1 → 4] linkage is catalyzed by glycogen phosphorylase. In this reaction step, 1 mol inorganic phosphate is consumed to yield 1 mol glucose-1P (Figure 1). The catalytic action of phosphoglucomutase results in the conversion of glucose-1P to glucose-6P. In the hepatocyte, glucose-6P is normally hydrolyzed to glucose, while in muscle cells glucose-6P serves as precursor in the glycolytic pathway.

Complete hydrolysis of branched glycogen structures requires the activity of a debranching enzyme since the above mentioned glycogen phosphorylase acts only on α[1 → 4] linkages and becomes ineffective four-glucosyl residues from an α[1 → 6] branch point. Now a debranching enzyme comes into play to transfer a block of three-glucosyl residues to a hydroxyl group at the C-4 position of an end-standing glucosyl moiety. This action of the debranching enzyme is commonly referred to as 4-α-D-glucanotransferase activity. The debranching enzyme is also capable of hydrolyzing the fourth-glucosyl residue left behind in an α[1 → 6] bond after removing the strand of three-neighboring glucosyl residues. The amylo-α-[1,6] glucosidase activity of the debranching enzyme results in the release of a molecule of free glucose instead of glucose-1P.

Regulation of Glycogen Synthesis and Mobilization

The rate of glycogen formation and degradation is controlled by the activity of glycogen synthase and glycogen phosphorylase, respectively. Both allosteric effectors and covalent modification, that is, protein phosphorylation/dephosphorylation, regulate the activity of the glycogen synthesizing and degrading enzyme.

Glycogen phosphorylase exists in the cell in an active "a" form and an inactive "b" form (Figure 2). The activity of glycogen phosphorylase a is inhibited by ATP and glucose. Under conditions where the energy state of the cell is high, or glucose supply from other sources is sufficient, endogenous glycogen degradation is blocked. High intracellular levels of AMP, reflecting enhanced energy consumption, activate the inactive b form of glucagon phosphorylase. In addition to this allosteric type of regulation, the inactive glycogen phosphorylase b can be converted into the active a form by phosphorylation of the enzyme protein. Phosphorylation is achieved by an activated phosphorylase kinase a. Activation of the latter enzyme occurs by phosphorylation of the inactive b form by a cyclic AMP-dependent protein kinase. It is worthy to note that high cytoplasmic concentrations of Ca^{2+} increase the activity of both phosphorylase kinases a and b (Figure 2).

Inactivation of the enzyme phosphorylase kinase a is accomplished by dephosphorylation by phosphoprotein phosphatase. A similar phosphoprotein phosphatase is required to convert the active form glycogen phosphorylase a to the inactive glycogen phosphorylase b. It is obvious that a maximal effect of cyclic AMP-stimulated glycogen degradation is obtained when phosphorylation of phosphorylase kinase and glycogen phosphorylase is accompanied by a concomitant

inactivation of phosphoprotein phosphatase. This is accomplished by activation of a cytoplasmic protein called inhibitor-1. This protein inhibits phosphoprotein phosphatase and is present in an inactive inhibitor-1b form and an active inhibitor-1a form. The inactive b form is converted to the active inhibitor-1a form by a cyclic AMP-dependent protein kinase.

Binding of glucagon to the glucagon receptor on the plasma membrane of liver cells results in an increase in cytoplasmic cyclic AMP content. A similar effect is evoked by binding of epinephrine to β-adrenoceptors on the liver plasma membrane. Cyclic AMP turns on the activity of glycogen phosphorylase in the bicyclic fashion as described above. The advantage of this bicyclic-regulatory system is that it provides a very effective amplification mechanism. In combination with the

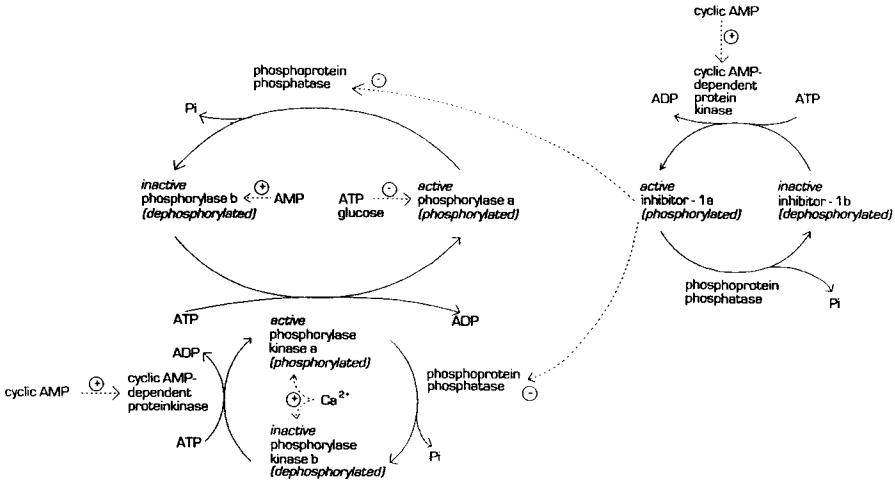

Figure 2. Regulation of glycogen phosphorylase by covalent modification and allosteric effectors. The signs (+) and (–) indicate stimulation or inhibition, respectively, of the enzyme under consideration; a and b refer to the active and inactive forms of the enzyme, respectively. In general, glycogen phosphorylase b (the inactive form) is activated by phosphorylation of the enzyme, that is, a covalent modification. Phosphorylation is achieved by phosphorylase kinase a (the active form). The latter enzyme is activated by phosphorylation, catalyzed by a cyclic AMP-dependent protein kinase. Inactivation of both phosphorylase kinase and glycogen phosphorylase occurs by dephosphorylation of the respective enzymes. The activity of the enzyme responsible for this process, phosphoprotein phosphatase, is regulated by an inhibitor protein present in either an active (inhibitor-1a) or inactive (inhibitor-1b) form. Allosteric activation of phosphorylase b occurs by AMP (low-energy state of the cell). Inhibition of the active phosphorylase a is achieved by ATP and glucose (high-energy state of the cell or sufficient supply of glucose from other sources). Ca^{2+} allosterically stimulates both the inactive and active form of phosphorylase kinase. Details of the regulation of glycogen breakdown are given in the text.

highly branched structure of glycogen, that is, a multitude of end-standing glucosyl residues, the amplification mechanism enables the cell to mobilize the total glycogen pool within a couple of minutes.

Skeletal muscle cells lack glucagon receptors. Hormonal control of glycogen degradation is achieved by epinephrine via β-adrenergic activation of adenylate cyclase, resulting in enhanced cytoplasmic cyclic AMP levels. Neural activation of skeletal muscle cells considerably increases the cytoplasmic Ca^{2+} level. Cyclic AMP and Ca^{2+} act in a synergistic fashion to fully express the activity of glycogen phosphorylase in the process described above (Devlin, 1992).

The activity of glycogen synthase, which controls the rate-limiting step in glycogen synthesis, is also regulated by allosteric effectors and by covalent modifications of the enzyme protein. Glycogen synthase is present in an inactive glycogen synthase b form and an active a form. The activity of glycogen synthase b is enhanced by high cytoplasmic levels of glucose-6P, a typical example of feed-forward activation. Glycogen synthase b can be converted to the active form by dephosphorylation of the enzyme protein by phosphoprotein phosphatase. In turn, glycogen synthase a can be inactivated by phosphorylation of the enzyme, yielding glycogen synthase b. Phosphorylation is achieved by a variety of kinases. Inside the cell, a cyclic AMP-dependent kinase is present which phosphorylates glycogen synthase. In addition, a calmodulin-dependent kinase (stimulated by cytoplasmic Ca^{2+}), a protein kinase (activated by diacylglycerol), glycogen synthase kinase-3, and casein kinases I and II are also able to convert the active glycogen synthase a to the inactive, phosphorylated b form. Glycogen synthase b is converted to the a form by the hydrolytic action of phosphoprotein phosphatase. The latter enzyme is inhibited by the active form of inhibitor-1, that is, the phosphorylated a form. The inactive inhibitor-1b is converted to the active phosphorylated inhibitor-1a by a cyclic AMP-dependent protein kinase. Therefore, cyclic AMP has two functions in the regulation of glycogen synthesis. First, inactivation of glycogen synthase by phosphorylation of the enzyme protein, and second, prevention of dephosphorylation, that is, reactivation of glycogen synthase, by activation of inhibitor-1.

High blood plasma glucose levels stimulate the synthesis of glycogen in liver cells via insulin. Insulin promotes activation of glycogen synthase by dephosphorylation of the enzyme. Glucagon, the levels of which will rise in blood when extrahepatic tissues are in need of glucose, shuts off the hepatic incorporation of glucose into glycogen by promoting phosphorylation and, hence, inactivation of glycogen synthase by a cyclic AMP-dependent mechanism. It can be concluded that conditions favorable for enhanced glycogen degradation (e.g., increased cytoplasmic levels of cyclic AMP and Ca^{2+}) will block glycogen synthesis and *vice versa*. The tight coupling of the regulation of anabolic and catabolic processes is of the utmost importance to prevent excessive futile (ATP consuming) cycling of glucose substrates in mammalian cells.

Lactate as Substrate

Lactate, either as a salt or in its undissociated form (lactic acid), is present in minor quantities in our food. However, inside our body this monocarboxylic acid plays an important role in the exchange of substrates between organs. In this regard, lactate cannot be considered as a dead-end product of the anaerobic glycolytic pathway, but represents a substantial source of energy stored in carbohydrate during exercise and after a carbohydrate-rich meal (Brooks, 1991).

Lactate is produced by reducing pyruvate in the last step of the anaerobic glycolytic pathway (Figure 1). This step is catalyzed by lactate dehydrogenase, requires NADH as cofactor, and is fully reversible (see above). In red blood cells, lactate is the sole end-product of glucose degradation due to their lack of mitochondria. Lactate is produced in the liver in the postprandial phase when hepatic glycogen stores are fully restored and gluconeogenesis is not operative. Furthermore, lactate is released from muscles. The amount of lactate produced depends strongly on the type of muscle under consideration, the level and duration of the exercise, and the blood concentration of the stress hormone, epinephrine. Net release of lactate is relatively high during the initial phase of strenuous exercise. Thereafter, net lactate release declines. This is partly caused by metabolic adaptation inside the muscle fiber and a decline in cellular glycogen content. Net release from the total muscle can also decrease by increased uptake of lactate by adjacent muscle fibers. This is particularly true for human muscles, which are largely mixed muscles, that is, a combination of fast-twitch white muscle cells, prone to produce lactate, and slow-twitch red fibers, capable of oxidizing lactate under aerobic conditions. Epinephrine increases net production of muscle lactate, most likely by activating glycogenolysis and glycolysis. When pyruvate dehydrogenase activity and citric acid cycle turnover do not keep pace with the enhanced intracellular supply of pyruvate, the excess pyruvate will be converted to lactate which is released by the cell into the interstitial compartment. In addition to red muscle fibers, the heart consumes considerable amounts of lactate. When arterial lactate levels are sufficiently high, lactate is the preferred substrate of the heart (Drake et al., 1980). Lactate is converted to pyruvate and subsequently oxidized by the concerted action of enzymes of the citric acid cycle and respiratory chain.

Liver cells remove lactate from the blood in substantial amounts, especially when the body is in need of glucose. After extraction from the blood, lactate is converted to pyruvate, which is then used as a precursor in the gluconeogenic pathway (see above) (Figure 1).

The total turnover of lactate in a normal human being at rest is on the average 200 g per day. During exercise this value can easily be doubled. Approximately 40% and 75% of the total amount of lactate produced is used for oxidative production of energy in the resting and exercising body, respectively. On the order of 10 g lactate is consumed by the heart of a sedentary person per day. The

remaining lactate is utilized for the *de novo* synthesis of glucose (the so-called Cori cycle). Hardly any lactate is lost via sweat and urine (Brooks, 1991).

In general, transport of lactate is not rate-limiting in the overall process of cellular production and consumption. Lactate is transported across the plasma membrane of red blood cells and parenchymal cells, such as hepatocytes and muscle cells, at a relatively high rate. This is accomplished by at least three different mechanisms. At either high-circulating or enhanced-intracellular lactate levels (> 10mM) the majority of lactate crosses the cellular membrane by free diffusion in its undissociated form (i.e., lactic acid, a compound bearing no electrical charge). At concentrations present under physiological circumstances (1 to 10mM) lactate is transported across the plasmalemma by carrier systems. Mammalian cell membranes contain a monocarboxylate carrier, capable of cotransporting lactate and H^+ (Poole and Halestrap, 1993). Lactate can also pass the cell membrane in exchange for such anions as phosphate, a process catalyzed by a membrane protein called the band 3-exchanger. Distinct Na^+-monocarboxylate cotransporters are present at the luminal surface of intestinal and renal epithelial cells for resorption of lactate and other monocarboxylates from digested food in the lumen of the gut and from renal filtrate, respectively.

LIPIDS

In the Western world, lipids constitute a considerable proportion of the daily diet. In addition, the capacity of mammals to synthesize lipids from other sources, such as carbohydrates, is remarkable. Liver cells and adipocytes play important roles in the conversion of carbohydrates to lipids. Neutral lipids like triacylglycerol are mainly stored in adipocytes, forming fat tissue at various locations in the mammalian body. Storage of lipids has several advantages over that of carbohydrates, such as glycogen, when the absorption of nutrients from the intestinal tract exceeds the need for substrates for energy production. Due to their hydrophobic nature, triacylglycerols do not retain water, and per gram, the energy content of lipids is about twice the energy content of carbohydrates. In general, the content of fat (triacylglycerol) and carbohydrates (glycogen) in a 80 kg adult man is 12 kg and 0.3 kg, respectively (Guyton, 1986). The corresponding energy content of the fuel stores is on the order of 450,000 kJ and 5000 kJ, respectively. These values underline the quantitative importance of fat as fuel store in the human body.

Lipoproteins and Lipoprotein Lipase

In our diets, lipids consist mainly of triacylglycerols, phospholipids, and cholesterol. After digestion of food, the hydrolytic products, that is, fatty acids, monoacyl glycerol, and cholesterol, are taken up by the intestinal cells. After resynthesis of triacylglycerol from fatty acids and monoacyl glycerol, lipids are

transported from the intestines to other organs almost exclusively as chylomicrons. Chylomicrons are a member of the lipoprotein family. Chylomicrons are transported via the lymphatic system (thoracic duct) to the superior caval vein and via the blood to all organs. In the extrahepatic organs, the triacylglycerol core of the chylomicrons is hydrolyzed in the microvascular compartment. To this end, proteins with lipolytic properties ("lipoprotein lipases") are attached to the luminal surface of the endothelial cells, lining the blood capillaries (Figure 3). In addition to chylomicrons, very low density lipoproteins (VLDL), produced by hepatocytes (see below), are substrates for lipoprotein lipase. The enzyme is activated by specific proteins present in the membrane enclosing the triacylglycerol core of the lipoproteins, indicating that the fat particle stimulates its own digestion.

The lipoprotein lipase molecules (about 55 kDa in weight) are synthesized in the parenchymal cells of the target organs. This means that cells in need of the fatty acyl moieties present in the triacylglycerol core of circulating chylomicrons and VLDL produce the enzyme that renders the fatty acids available for their intracellular consumption. The presence of this enzyme has been positively assessed at the luminal side of endothelium in a variety of tissues, including adipose tissue, lactating mammary glands, red skeletal muscle, heart, adrenals, small intestine, lung, kidney, and neonatal liver.

The regulation of the biosynthesis and intraorgan translocation of lipoprotein lipases has been extensively studied in adipose and heart tissue (Braun and Severson, 1992). Synthesis of lipoprotein lipase involves transcription of the gene coding for the protein, and transportation of the mRNA to the ribosomes at the rough endoplasmic reticulum. In the endoplasmic reticulum, the newly synthesized proteins are posttranslationally modified by attachment of carbohydrate chains, consisting of glucosyl- and mannosyl-residues, to the protein. After transportation to the Golgi system, the oligosaccharide chain is further modified. Finally, the active enzyme is transported in vesicles from the Golgi system to the extracellular space. Transport through the endothelial cells is the following step, after which the enzyme is attached to the glycocalyx, that is, a layer of glycoproteins at the luminal site of the endothelium, through its oligosaccharide chain.

When fatty acids are released from the triacylglycerol core of chylomicrons and VLDL by the hydrolytic action of lipoprotein lipase, the bulk is taken up by the parenchymal cells. A relatively small proportion of fatty acids escapes to the vascular compartment.

In addition to chylomicrons and VLDL as sources of fatty acids, fatty acids complexed to albumin are also supplied to the organs. The latter fatty acids are almost exclusively released by adipocytes when the body is in need of oxidizable and energy delivering substrates. Albumin displays a high affinity for fatty acids and one molecule of albumin can carry up to 6–8 fatty acid molecules.

Cellular Uptake and Transport of Fatty Acids

The mechanism of uptake of fatty acids by the cell is not completely understood. Transport of fatty acids across the endothelial cells, the interstitial space, and the plasmalemma of the parenchymal cells is most likely a diffusion-like process (Van der Vusse et al., 1992). Recent experimental findings indicate that (1) specific membrane proteins are involved in transmembrane transport and (2) the uptake of fatty acids by brain is limited by the blood–brain barrier.

Transport of fatty acids inside the parenchymal cells, that is, from the plasmalemma to the intracellular site of conversion, is most likely mediated by fatty acid-binding proteins (FABPs). At least seven different isoforms of this low molecular weight protein (15 kDa) have been described, among which there is the liver-type and muscle-type FABP (Glatz and Van der Vusse, 1990). FABPs facilitate intracellular transport of fatty acids by increasing their solubility in the aqueous environment.

Intracellular Consumption of Fatty Acids

Before intracellular utilization, the relatively unreactive carboxylic head-group of the fatty acid molecule has to be converted to the more reactive CoA derivative (Van der Vusse et al., 1992) (Figure 3). For long-chain fatty acids, this step is catalyzed by acyl-CoA synthetase, located at the outer surface of the mitochondrial outer membranes. In some organs, enzyme activity is also present in the endoplas-

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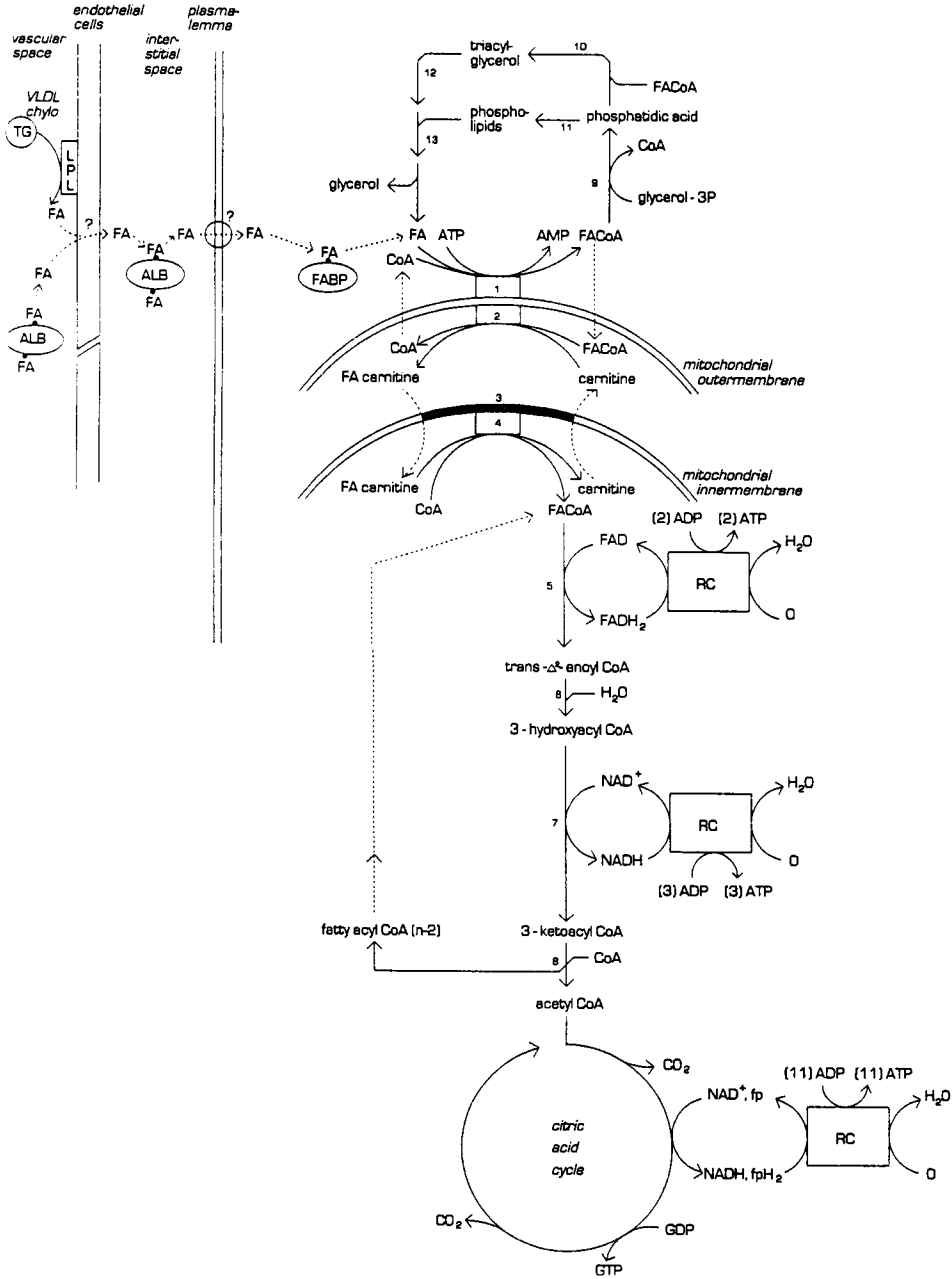
Figure 3. Schematic representation of fatty acid degradation and incorporation into triacylglycerol and phospholipids in a mammalian cell. Note that primarily the metabolic pathways in the muscle cells are described. Solid and broken arrow lines refer to metabolic conversions and transport routes, respectively. ALB refers to albumin, VLDL to very low density lipoproteins, chylo to chylomicrons, LPL to lipoprotein lipase, TG to triacylglycerol in the core of chylomicrons and VLDL, RC to respiratory chain, FA to fatty acid or fatty acyl, FABP to fatty acid-binding protein, CoA to coenzyme A, fp to flavoprotein, FAD to flavine adenine dinucleotide, GDP to guanosine diphosphate, GTP to guanosine triphosphate, O to molecular oxygen. The numbers in brackets refer to the number of moles of ATP produced. Other numbers refer to enzymes and transport proteins; 1, fatty acyl-CoA synthetase; 2, carnitine–acylcarnitine transferase I (CAT-I); 3, carnitine–acylcarnitine translocase; 4, carnitine–acylcarnitine transferase II (CAT-II); 5, fatty acyl-CoA dehydrogenase; 6, enoyl-CoA hydratase; 7, 3-hydroxyacyl-CoA dehydrogenase; 8, 3-ketothiolase; 9, acyltransferases I and II; 10, phosphatidate phosphatase and acyltransferase III; 11, phospholipid biosynthetic pathway; 12, triacylglycerol, diacylglycerol, and monoacylglycerol lipase; 13, phospholipases.


endothelial cells
plasma-lemma
vascular space
inter-stitial space
VLDL chylo
TG
LPL
FA
ALB
FABP
triacyl-glycerol
phospho-lipids
phosphatidic acid
FACoA
CoA
glycerol
glycerol - 3P
ATP
AMP
FA carnitine
carnitine
mitochondrial outermembrane
mitochondrial innermembrane
(2) ADP
(2) ATP
FAD
FADH2
RC
H2O
trans -Δ2- enoyl CoA
3 - hydroxyacyl CoA
NAD+
NADH
(3) ADP
(3) ATP
fatty acyl CoA (n-2)
3 - ketoacyl CoA
acetyl CoA
CO2
citric acid cycle
(11) ADP
(11) ATP
NAD+, fp
NADH, fpH2
GDP
GTP

mic reticulum. For medium-chain and short-chain fatty acids, the conversion to acyl-CoA takes place inside the mitochondrial matrix.

Long-chain fatty acyl-CoA is at a crossroad of various metabolic pathways. Inside the mitochondrial matrix, fatty acyl-CoA is converted to acetyl-CoA, a compound that serves as substrate for aerobic energy production by concerted action of enzymes of the citric acid cycle and respiratory chain, and as precursor for ketone-body formation. The latter process is confined to the liver. The fatty acyl residue of acyl-CoA can also be incorporated into triacylglycerols and phospholipids (Figure 3). These anabolic processes occur in the extramitochondrial cytoplasmic space (see below).

The fact that the mitochondrial inner membrane is virtually impermeable to long-chain fatty acyl-CoA, while the fatty acid oxidative machinery is located inside the mitochondrial matrix, a space enclosed by the inner membrane, might create a serious problem for cellular energy production. The problem is solved by the development of a transmembrane carnitine-dependent transport system for the long-chain acyl residue of acyl-CoA. Catalyzed by carnitine acyltransferase I (CAT-I), which is attached to the inner surface of the mitochondrial outer membrane, fatty acyl-CoA is converted to fatty acyl-carnitine by replacing the CoA residue with carnitine (Figure 3). Fatty acyl-carnitine is transported across the mitochondrial inner membrane in exchange for a molecule of free carnitine by carnitine–acylcarnitine translocase. After arrival in the mitochondrial matrix, fatty acyl-carnitine is converted back to acyl-CoA by carnitine acyltransferase II (CAT-II), an enzyme located on the inner surface of the mitochondrial inner membrane.

Because medium- and short-chain fatty acids can freely diffuse across the mitochondrial inner membrane and conversion to their respective CoA esters takes place in the mitochondrial matrix, no carnitine-dependent translocase is required to shuttle these substrates from the cytoplasm to the mitochondrial matrix space (Guzman and Geelen, 1993).

Inside the mitochondrial matrix, fatty acyl-CoA is subject to further degradation by a set of three enzymes in a process called β-oxidation. The first step is oxidation of the fatty acyl-chain of acyl-CoA by removing a hydrogen atom from carbon atoms 3 and 4. Carbon atom 3 is located at the β-position with respect to the carboxylic acid group. Removal of the hydrogen atoms creates a double bond between the two carbon atoms. This oxidation step is catalyzed by acyl-CoA dehydrogenase. The two hydrogen atoms are taken up by a flavoprotein. The next step is hydration of the double bond to 3-hydroxyacyl-CoA by enoyl-CoA hydratase. The catalytic action of 3-hydroxyacyl-CoA dehydrogenase converts 3-hydroxyacyl-CoA to 3-ketoacyl-CoA. NAD^+ serves as acceptor of the two hydrogen atoms removed from 3-hydroxyacyl-CoA. The final step is catalyzed by 3-ketothiolase. The end-standing 2-carbon fragment at the CoA ester side is cleaved from the acyl-CoA molecule, while a second CoA group is esterified to the carbon atom originally at carbon position 3 (Figure 3). By this procedure, fatty acyl-CoA is

shortened by two carbon atoms producing one mol of acetyl-CoA. The shortened acyl-CoA is then again subject to β-oxidation starting with the catalytic action of acyl-CoA dehydrogenase.

The rate of fatty acid oxidation is most likely regulated by the carnitine-dependent transport of acyl residues across the mitochondrial inner membrane, the supply of fatty acids to the cell, and the concentration of cofactors such as CoA and carnitine. Moreover, feedback inhibition is exerted by NADH on 3-hydroxyacyl-CoA dehydrogenase and by acetyl-CoA on 3-ketothiolase. Interestingly, malonyl-CoA, an intermediate in the fatty acid biosynthetic pathway, is a strong inhibitor of CAT-I.

Recent studies have indicated that in addition to mitochondria, peroxisomes are capable of removing acetyl-CoA moieties from long-chain fatty acids. The peroxisomal β-oxidative pathway is different in two major respects from the mitochondrial system. First, the hydrogen atoms removed are oxidized to water via H_2O_2. This process does not generate energy in the form of ATP. Second, peroxisomal β-oxidation stops when octanoyl-CoA (C8-CoA) is produced.

Fatty Acid Synthesis in Mammalian Cells

Besides uptake of fatty acids from lipids in our daily diets, our body is capable of producing significant amounts of these important substrates. *De novo* synthesis of long-chain fatty acids primarily occurs in liver and fat cells. The precursor of fatty acid biosynthesis is acetyl-CoA. The acetyl residue of acetyl-CoA is derived from pyruvate which, in turn, is either produced in the glycolytic pathway or generated from amino acids (Figure 1). Especially after a carbohydrate-rich meal, excess sugars are degraded to acetyl-CoA for storage in the form of fat. Fatty acids produced in liver cells are incorporated into triacylglycerols that are packed together as the core of VLDL and secreted into the bloodstream to provide extrahepatic tissue with fatty acid moieties. In the fat cells, *de novo* synthesized fatty acids are stored in the intracellular lipid droplets in the form of triacyglycerols. When the body is in need of fatty acids, the adipocyte lipid stores are hydrolyzed and fatty acids are released into the extracellular compartment and transported in the blood by the fatty acid carrier, albumin.

The first step in *de novo* fatty acid synthesis is the production of malonyl-CoA from acetyl-CoA and bicarbonate. This committed step is catalyzed by acetyl-CoA carboxylase present in the cytoplasm of liver cells and adipocytes. After replacement of the CoA residue in acetyl-CoA by ACP (acyl carrier protein), malonyl-ACP is used to convert acetyl-ACP to butyryl-ACP by the fatty acid synthase complex. In this multistep reaction, NADPH is used as donor of hydrogen atoms and CO_2 is produced. Butyryl-ACP is subsequently elongated to hexanoyl-ACP by a similar process in which malonyl-ACP serves as donor of two carbon atoms required for lengthening of the growing acyl chain. This process is repeated until palmitic acid

(C-16) is formed. By elongation and desaturation, palmitic acid can be used as precursor for the production of most natural fatty acids in the human body. Humans lack enzymes to synthesize linoleic and linolenic acid. Hence, these two fatty acids are "essential" and must be supplied to the body in the diet.

Overall, *de novo* fatty acid synthesis is primarily regulated at the committed step, that is, the production of malonyl-CoA from acetyl-CoA. Citrate activates acetyl-CoA carboxylase by promoting the aggregation of the inactive form of the enzyme to an active enzyme complex. Feedback inhibition is exerted by the end product of the biosynthetic pathway, palmitoyl-CoA. The activity of acetyl-CoA carboxylase is also under hormonal control. Short-term effects are accomplished by a cyclic AMP-dependent phosphorylation–dephosphorylation mechanism. As can be expected, phosphorylation of the enzyme renders the enzyme less active than the unphosphorylated form. Phosphorylation and, hence, inactivation is most likely brought about by glucagon, while insulin keeps the enzyme active by preventing phosphorylation. Long-term feeding on carbohydrate-rich, fat-free diets promotes the synthesis of acyl-CoA carboxylase. The opposite is achieved when a person is fasting or consuming high-fat food.

Storage of Fatty Acids in Triacylglycerol

When the supply or the *de novo* synthesis of fatty acids exceeds the actual need for these substances, the fatty-acyl moieties are stored as triacylglcyerols in adipocytes and to a lesser extent in skeletal muscle cells and cardiomyocytes. Triacylglycerol synthesis in hepatocytes is used primarily for the formation of VLDL. As discussed earlier, the first step in storing fatty acids in triacylglycerols is the activation of fatty acids to fatty acyl-CoA by acyl-CoA synthetase (Figure 3). Subsequently, the acyl residue of the newly formed acyl-CoA is esterified to the hydroxyl group at the C-1 position of either glycerol-3P or dihydroxyacetone phosphate. The latter two compounds are produced from glucose (Figure 1). The liver can also use glycerol for glycerol-3P synthesis since it contains the enzyme glycerokinase. The above mentioned acylation step, which is catalyzed by acyl-transferase, results in the production of lysophosphatidic acid. This intermediate is converted to phosphatidic acid when the hydroxyl group at the C-2 position is esterified to a fatty-acyl residue of a second acyl-CoA. Phosphatidic acid is at a crossroad of various anabolic routes. It serves as a precursor for triacylglycerol synthesis and it can be used for the synthesis of complex lipids such as phospholipids. To produce triacylglycerol, the phosphate group at the C-3 position of phosphatidic acid is removed by phosphatidate phosphatase yielding diacylglycerol. The free hydroxyl group at position C-3 of this intermediate is esterified with the fatty-acyl residue of a third acyl-CoA molecule, giving rise to the formation of triacylglycerol. The latter step is catalyzed by acyl-transferase.

The rate of triacylglycerol formation is most likely governed by the cytoplasmic concentration of glycerol-3P, which is increased when the glycolytic conversion of glucose to the 3-carbon intermediates, dihydroxyacetone phosphate and glyceraldehyde-3P, is promoted. This production prevails at increased levels of circulating insulin. Excess supply of fatty acids, either from extracellular sources or from intracellular biosynthesis, also enhances the formation of triacylglycerol.

Mobilization of Fatty Acids from Intracellular Triacylglycerol Depots

Fatty acids are mobilized from the intracellular triacylglycerol pool by cleavage of the ester bonds between glycerol and the fatty-acyl residues. The initial event in this hydrolytic pathway is the removal of the fatty-acyl residue at position C-1. This reaction step is catalyzed by triacylglycerol lipase. The second fatty-acyl moiety is removed by diacylglycerol lipase and the remaining fatty-acyl residue by monoacylglycerol lipase. In the overall reaction, triacylglycerol is converted to glycerol and three fatty acids. The first step is rate-limiting.

In adipose tissue, the activity of triacylglycerol lipase is regulated by phosphorylation/dephosphorylation of the enzyme by a cyclic AMP-dependent protein kinase. Phosphorylation of the lipase enhances its activity, while dephosphorylation exerts the opposite effect. Such hormones as (nor)epinephrine, glucagon, and ACTH stimulate lipolysis by increasing the cytoplasmic concentration of cyclic AMP. Insulin displays an antilipolytic effect. The regulation of tissue triacylglycerol lipase in cells other than adipocytes is less clear. Lysosomes may be involved, whereas the stimulating effect of cyclic AMP-dependent protein phosphorylation can not be excluded (Van der Vusse et al., 1992). Suggestions have been made that fatty acids exert a feedback inhibition on the lipolytic activity.

Ketone-bodies as Alternative Sources of Energy

It is noteworthy that ketone-bodies are an important lipid-based fuel when impairment of energy production due to a severe shortage of glucose is imminent. Under conditions such as prolonged fasting and insulin defects as in severe diabetes, fatty acids are converted via acetyl-CoA to acetoacetate and 3-hydroxybutyrate. At least four different steps are involved to produce hydroxybutyrate from acetyl-CoA. First, the condensation of two molecules acetyl-CoA to acetoacetyl-CoA by 3-ketothiolase; second, the incorporation of a third molecule of acetyl-CoA into acetoacetyl-CoA to yield 3-hydroxymethyl glutaryl-CoA by hydroxymethyl glutaryl-CoA synthetase; third, the cleavage of the latter intermediate to acetyl-CoA and acetoacetate by hydroxymethylglutaryl-CoA cleavage enzyme; and fourth, the reduction of acetoacetate to 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase. Ketone-bodies are released from their site of origin, the hepatocytes, into the vascular compartment and transported by the blood to brain and nerve tissue as

alternative energy sources. Other organs, such as heart and skeletal muscles, are also capable of using ketone-bodies as fuel.

AMINO ACIDS

The bulk of amino acids in mammalian cells is present in proteins, that is, macromolecules consisting of a multitude of amino acid residues linked together in the so-called peptide bond. Twenty different amino acids are incorporated in the protein pool. Eleven of them are nonessential, meaning they can be produced in the human body from carbohydrate precursors. The remaining nine essential amino acids have to be supplied to the body in the diet. The nonessential amino acids are alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine. Histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are the essential amino acids.

In the western world, the daily intake of protein and, hence, amino acids is on the order of 70 g per day for an adult person. This amount is more than sufficient to fulfill the daily requirement of amino acids, provided all essential amino acids are present in the right quantity in the diet. Proteins are digested to amino acids and dipeptides by a concerted action of enzymes present in the lumen of the stomach and the small intestines. The products of protein digestion are transported into the epithelial cells of the small intestines by specific transmembrane transporters. The basic mechanism is cotransport with Na^+. The driving force is the concentration gradient of Na^+ across the epithelial luminal membrane. At present, at least five different transport systems have been identified. One for the neutral amino acids alanine, serine and threonine; a second for the neutral amino acids isoleucine, leucine, methionine, phenylalanine, tyrosine and valine; a third transporter for the imino acids hydroxyproline and proline; a fourth transporter for cystine and the basic amino acids arginine and lysine; and a fifth transport protein for the dicarboxylic amino acids aspartate and glutamate. The amino acids are transported via the blood in the hepatic portal vein to the liver. The majority of the amino acids are extracted by the liver from the portal blood. The remaining amino acids are rapidly taken up by other organs in need of these important substrates.

Uptake and Intracellular Consumption of Amino Acids

Specific transport systems are most likely present in the plasmalemma of the mammalian cell to efficiently channel circulating amino acids into the cell interior. Inside the cell, amino acids are involved in a variety of metabolic processes. A substantial part of the amino acids is used for the protein synthesis that takes place in all mammalian cells with the exception of red blood cells. It is important to realize that protein synthesis only occurs when all amino acid precursors, that is, both essential and nonessential amino acids, are present in adequate amounts. When one

or more essential amino acids are lacking, protein synthesis is hampered and the remaining amino acids will be deaminated (see below).

Amino acids can also be used for energy production and for the synthesis of glucose and ketone bodies. In regard to amino acid metabolism, only the main pathways will be discussed in this chapter.

Over 50% of the amino acids absorbed by the small intestines are extracted by the liver from the blood in the hepatic portal vein. The amino acids that are not used for protein synthesis in the hepatocytes are subjected to removal of the NH_2-group. This is accomplished by the concerted action of deaminating and transaminating enzymes. Hepatocytes contain glutamate dehydrogenase (Figure 4). This enzyme catalyzes the reversible deamination of glutamate in the following reaction:

$$\text{glutamate} + \text{NAD(P)}^+ \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NAD(P)H} + NH_4^+$$

Other amino acids cannot be directly deaminated. The NH_2-group is first transferred to glutamate in the following reaction step catalyzed by aminotransferases.

$$\text{amino acid} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{ketoacid} + \text{glutamate}$$

Note that the overall reaction is:

$$\text{amino acid} + \text{NAD(P)P}^+ \rightleftharpoons \text{ketoacid} + \text{NAD(P)H} + NH_4^+$$

The newly formed ketoacids are used either for the production of glucose (gluconeogenesis) and ketone-bodies (ketogenesis), or for the generation of ATP in the oxidative pathway to fulfill the energy requirements of the hepatocytes. When glucose or ketone-bodies are formed, these compounds are released into the blood and serve as fuel in extrahepatic tissues. Alanine, glycine, cysteine, serine, threonine, asparagine, aspartate, methionine, valine, glutamate, glutamine, histidine, proline, and arginine are glucogenic amino acids. Leucine and lysine are ketogenic, while isoleucine, tryptophan, tyrosine, and phenylalanine are both glucogenic and ketogenic (Stryer, 1988).

The ammonia (NH_4^+) molecules produced by deamination of amino acids are removed from the hepatocytes mainly in the form of urea or glutamine (Figure 4). Urea is synthesized in the urea cycle, released into blood and subsequently disposed from the body by the kidneys via urine. In the liver, NH_4^+ can also be coupled to glutamate yielding glutamine. The reaction step is catalyzed by the enzyme glutamine synthetase. Glutamine is an important intermediate as it represents one of the twenty amino acids used for protein synthesis. In addition, glutamine functions to carry ammonia as a nontoxic amide from the liver to the kidney. After extraction of glutamine by the kidney, NH_3 is released from glutamine by glutaminase and is used to regulate urinary pH (Devlin, 1992). Glutamine is also avidly extracted by the cells of the small intestine as it provides a major source of energy for this organ (Souba, 1991). The ammonia produced during processing of glutamine in the

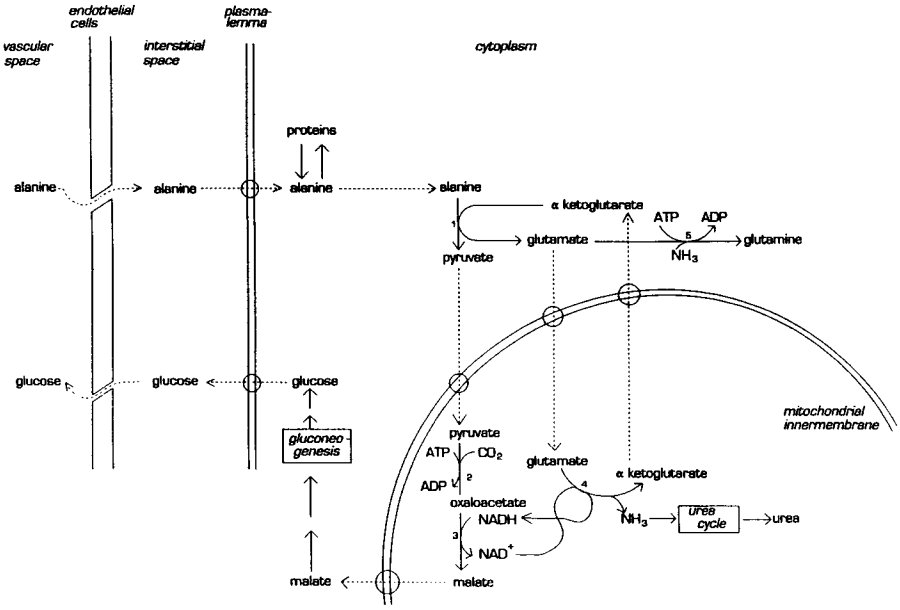


Figure 4. Schematic representation of the metabolic fate of alanine in hepatocytes. Note that striking differences may exist between mammalian cell types on the one hand and individual amino acids on the other (see text). Solid and broken arrow lines refer to metabolic conversions and transport routes, respectively, and circles in membranes refer to specific transporters. Numbers refer to enzymes involved in alanine metabolism; 1, alanine transaminase; 2, pyruvate carboxylase; 3, malate dehydrogenase; 4, glutamate dehydrogenase; 5, glutamine synthetase.

enterocytes is carried by the blood in the hepatic portal vein to the liver, where it is taken up and converted either to urea or glutamine, or used for amino acid synthesis by the concerted action of glutamine dehydrogenase and aminotransferase (see above)

During prolonged fasting, skeletal muscle releases substantial amounts of amino acids as fuel for other organs. Protein hydrolysis is promoted by a low insulin/glucagon ratio in the blood. Amino acids mobilized from the intramuscular protein pool are mainly converted to glutamate and alanine by muscle aminotransferase. Glutamate is metabolized to glutamine, a substrate for cells with a high proliferation rate, e.g., enterocytes and macrophages (Souba, 1991). It is interesting to note that under normal conditions, muscle cells contain appreciable amounts of glutamine. Alanine derived from muscle proteolysis is extracted by the liver and used in the gluconeogenic pathway for *de novo* glucose synthesis.

Branched-chain Amino Acids

A special group of amino acids are the branched-chain amino acids—valine, leucine and isoleucine. Although these compounds belong to the group of essential amino acids, their intake from normal diets exceeds the daily requirement for protein synthesis. They share with other amino acids the common feature of transamination. Branched-chain amino acid (BCAA) aminotransferase is present in mammalian cells. The branched-chain ketoacids (BCKA) formed in the transamination process are irreversibly decarboxylated by branched-chain ketoacid dehydrogenase. BCKA dehydrogenase is a multienzyme complex that is attached to the inner surface of the mitochondrial inner membrane. The activity of this enzyme is regulated by a phosphorylation/dephosphorylation mechanism. The phosphorylated form of the enzyme displays the lowest catalytic activity.

The activity of BCAA aminotransferase is low in liver and relatively high in skeletal muscle. The opposite is true of the activity of BCKA dehydrogenase. This difference in enzyme activity is most likely the cause of the small extraction of BCAA by the liver. The majority of BCAA is taken up by skeletal muscles and converted to their respective branched-chain keto acids, which are partly consumed in the muscle cells. The remainder of BCKA is released into the blood. Part of the circulating BCKA is extracted by the liver. Inside the hepatocytes, BCKA is either degraded in the oxidative metabolic pathway or reaminated to preserve the total body content of BCAA (Harper et al., 1984).

INTERORGAN RELATIONSHIP

In mammals, organs like the gut, liver, pancreas, brain, adipose tissue, and muscles play a critical role in the overall carbohydrate, fat, and amino acid homeostasis. In Figures 5 and 6, the metabolic relationships are summarized for the fed and starving states in a simplified manner.

Glucose and amino acids in digested food are taken up by enterocytes and transported to the liver via the hepatic portal vein (Figure 5). Insulin secretion by the pancreas is increased, among other things, as a response to elevated levels of glucose. In the hepatocytes, glucose is used for repletion of glycogen stores and synthesis of fatty acids. Amino acids extracted by liver cells are utilized for protein synthesis and production of fatty acids via pyruvate. The fatty-acyl moieties are released into the hepatic veins as triacylglycerol, the fat core of very low density lipoproteins (VLDL).

Glucose and amino acids that escape extraction by the liver are metabolized in extrahepatic tissues. Red blood cells and brain rely completely on glucose for ATP production. In adipocytes, glucose is also used for the synthesis of fat that is stored in the cell in lipid droplets. Muscle cells use glucose for repletion of the glycogen store and for the production of energy via aerobic and anaerobic glycolysis.

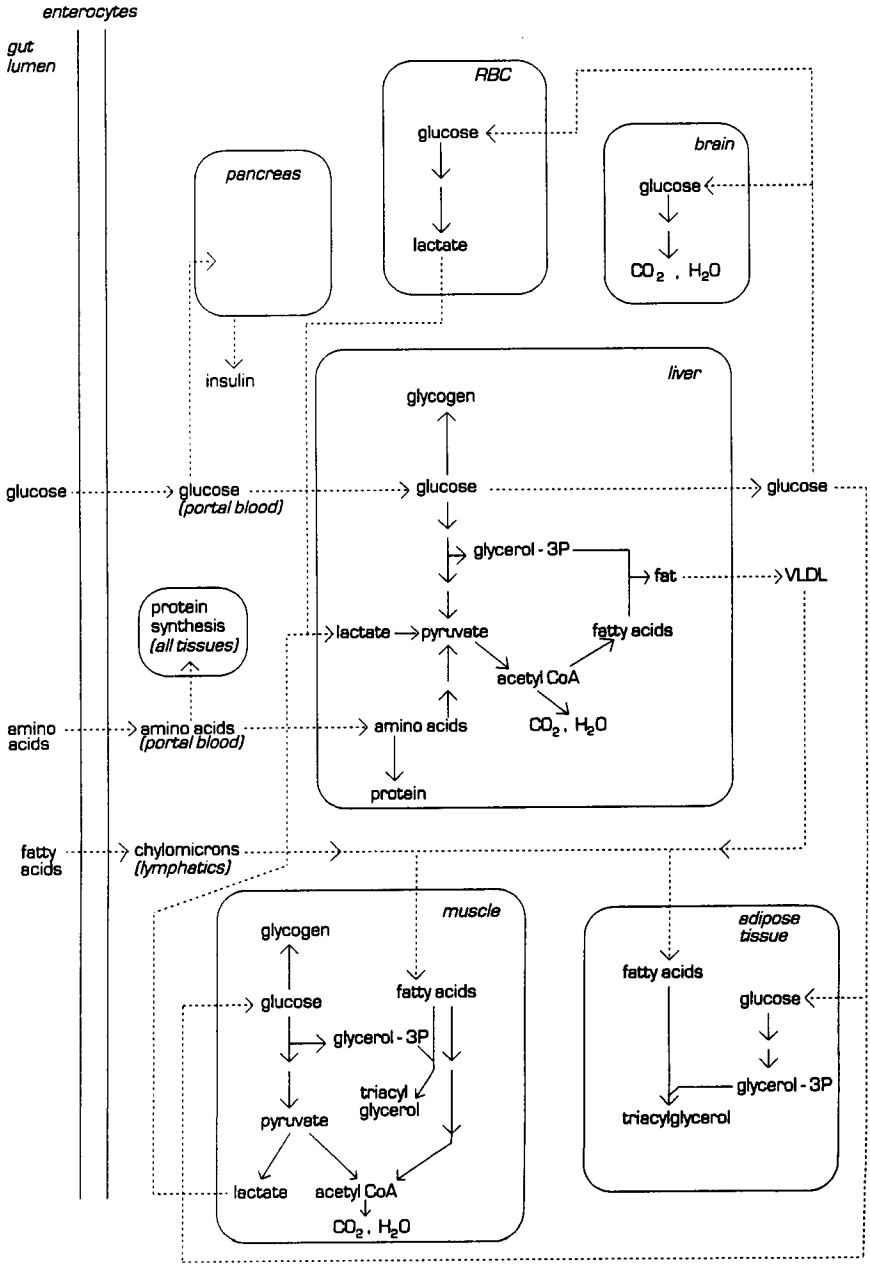


Figure 5. Interrelationships of carbohydrate, fat, and amino acid metabolism between various organs in mammals in the fed state. Note that this scheme is highly simplified. RBC refers to red blood cells and VLDL to very low density lipoproteins.

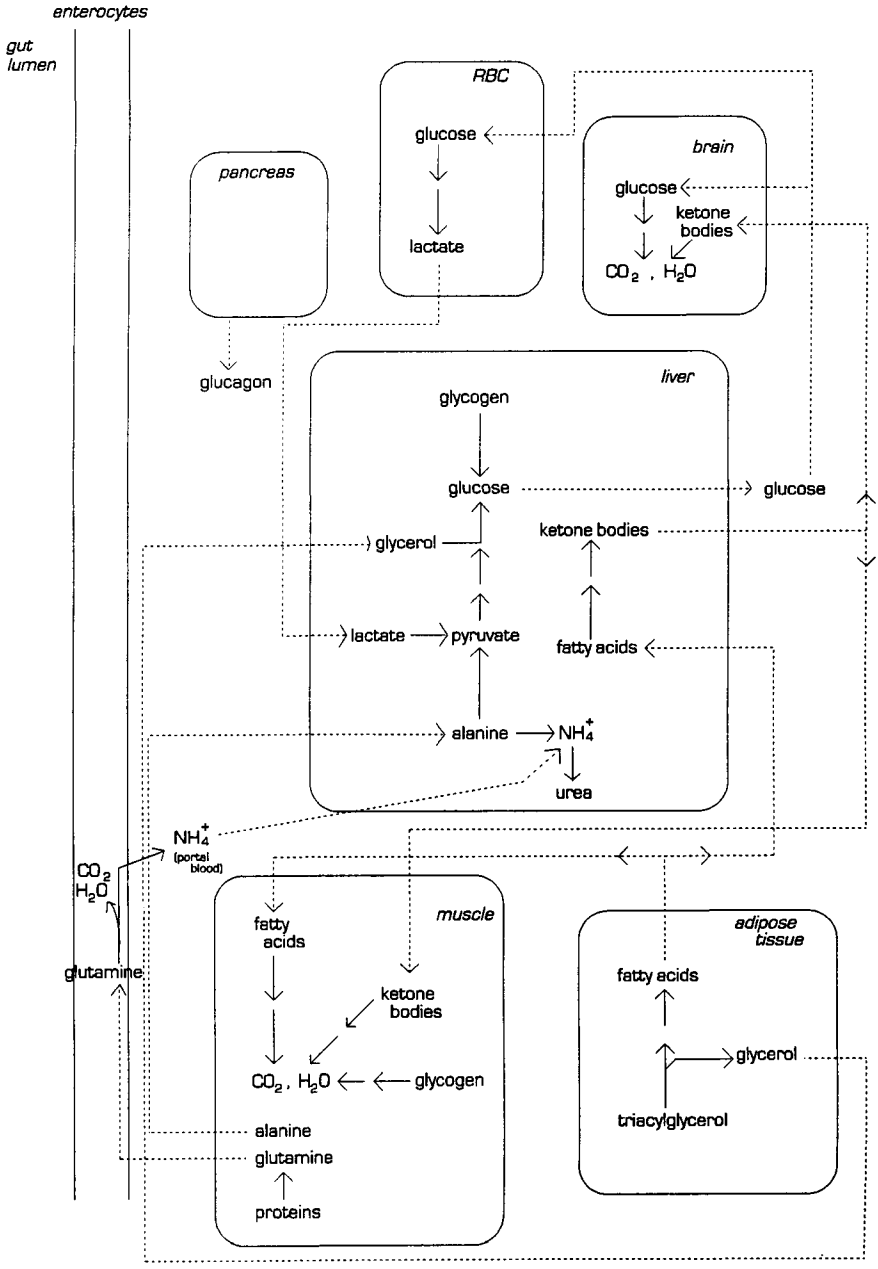


Figure 6. Interrelationships of carbohydrate, fat, and amino acid metabolism between various organs in fasting mammals.

Triacylglycerols, either in the form of chylomicrons that are released by enterocytes into the lymphatic system or in the form of VLDL, are hydrolyzed to fatty acids by lipoprotein lipase at the luminal side of endothelial cells lining the microvasculature of muscle and adipose tissue. Inside the cell, fatty acids are used for synthesis of triacylglycerol (adipose tissue, muscle) or oxidized to generate ATP.

During fasting, the metabolic interrelationships change appreciably (Figure 6). Blood glucose levels tend to fall. Insulin release from the pancreas is strongly reduced and glucagon release is enhanced. The latter hormone promotes the release of glucose from liver cells by activating the conversion of glycogen to glucose, the *de novo* synthesis of glucose from lactate, glycerol, and amino acids (e.g., alanine) released from muscle cells as the consequence of increased protein hydrolysis. Fat stores in adipocytes are mobilized, and fatty acids are transported to the liver and muscle cells. Fatty acids are used as preferential fuel in exercising muscles. Glucose utilization is depressed by the enhanced supply of fatty acids to muscle cells (Randle cycle). In the liver, fatty acids are converted to ketone-bodies that serve as fuel for brain and muscle tissue. Glutamine, stored in relatively high quantities in muscle, is released into the vascular compartment and used by enterocytes and other proliferating cells as a source of energy (Figure 6).

SUMMARY

In the present overview, the uptake and metabolic conversion of glucose, fatty acids, and amino acids in mammalian cells have been discussed. Only the main metabolic routes and mechanisms underlying the regulation of enzymes and transport proteins controlling the rate-limiting steps are described. It should be noted that at some points, details are left out and metabolic interrelationships are simplified for the sake of clarity. For details the reader is referred to the original publications and modern textbooks of biochemistry.

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REFERENCES

Beitner, R. (1993). Control of glycolytic enzymes through binding to cell structures and by glucose-1,6-biphosphate under different conditions. The role of Ca^{2+} and calmodulin. Int. J. Biochem. 252, 297–305.

Braun, J. E. A. & Severson, D. K. (1992). Regulation of the synthesis, processing and translocation of lipoprotein lipase. Biochem. J. 287, 337–347.

Brooks, G. A. (1991). Current concepts in lactate exchange. Med. Sci. Sports Exerc. 23, 895–906.

Devlin, T. M. (ed.). (1992). Textbook of Biochemistry, Wiley-Liss, New York.

Drake, A. J., Haines, J. R., & Noble, M. I. M. (1980). Preferential uptake of lactate by the normal myocardium in dogs. Cardiovasc. Res. 14, 65–72.

Glatz, J. F. C. & Van der Vusse, G. J. (1990). Cellular fatty acid-binding proteins: current concepts and future directions. Mol. Cell. Biochem. 98, 247–251.

Guyton, A. C. (1986). Textbook of Medical Physiology, Saunders, Philadelphia.

Guzman, M. & Geelen, M. J. H. (1993). Regulation of fatty acid oxidation in mammalian liver. Biochim. Biophys. Acta 1167, 227–241.

Harper, A. E., Miller, R. H., & Block, K. P. (1984). Branched-chain amino acid metabolism. Ann. Rev. Nutr. 4, 409–454.

Lienhard, G. E., Slot, J. W., James, D. E., & Mueckler, M. M. (1992). How cells absorb glucose. Sci. Amer. 1, 34–39.

Poole, R. C. & Halestrap, A. P. (1993). Transport of lactate and other monocarboxylates across mammalian plasma membrane. Am. J. Physiol. 264, C761–C781.

Souba, W. W. (1991). Glutamine; a key substrate for the splanchnic bed. Ann. Rev. Nutr. 11, 285–308.

Stryer, L. (1988). Biochemistry, Freeman, New York.

Van der Vusse, G. J., Glatz, J. F. C., Stam, H. C. G., & Reneman, R. S. (1992). Fatty acid homeostasis in the normoxic and ischemic heart. Physiol. Rev. 72, 881–940.

Widdas, W. F. (1988). Old and new concepts of the membrane transport for glucose in cells. Biochim. Biophys. Acta 947, 385–404.

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Chapter 4

The Organization of Metabolic Pathways *In Vivo*

G. RICKEY WELCH

INTRODUCTION

The notion of "organization," as a defining characteristic of life, is fundamental to all levels of the biological hierarchy (Welch, 1987). At the cellular level, it is important to comprehend how the structural organization of enzyme-catalyzed

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reactions affects metabolic processes. Though advances in the science of cytology have provided an increasingly intricate picture of the substructure of the cell, the view of cellular metabolism continues to be dominated by an air of bulk-phase homogeneity and isotropy, in accord with artificial *in vivo* conditions. Biochemists, in their research laboratories and in their textbooks, have become all too accustomed to regarding the living cell as a dilute, test-tube "solution"—uniformly dissolved with enzymes, metabolites, and weak electrolytes.

Notwithstanding, there is increasing evidence for widespread metabolic organization *in vivo*, engendering a variety of structural motifs, as well as local reaction–diffusion microenvironments and metabolic "channeling" *in situ*. It is now apparent that a full understanding of cellular metabolism requires that we couch biochemistry in the physico-chemical context of such a structure.

THE ENZYME-ORGANIZATIONAL SUBSTRATUM

Protein–Organelle

The molecular complexity of cellular microenvironments varies with their size and nature. Other than the cytosol itself, the largest compartment (~1 micron) would be an organelle, such as the mitochondrion or chloroplast. Obviously, the design of metabolism in terms of such organelles serves as a kind of gross segregation of groups of generally related metabolic processes. Even at this relatively large scale though, the situation is far from an aqueous "bag" homogeneously dispersed with enzyme proteins and metabolites. The protein density within the mitochondrial inner matrix, for example, is so high as to create a gel-like (or quasi-crystalline) state (Sitte, 1980; Srere, 1987). Moreover, the *water* in this kind of microenvironment has an associative, vicinal character (Clegg, 1984). Many of the intermediate substrates therein exist at concentrations comparable to that of their cognate enzymes (Srere, 1987). Even at this level, biochemical reactions differ significantly from the character of a dilute, test-tube "solution."

Protein–Membrane

For enzyme systems adsorbed to cytomembranes, the microenvironment may be of diverse form. Here, too, the proteins often exhibit crystal-like density (Sitte, 1980). Overlaying these reacting systems are layers of adsorbed ions and "structured" water molecules (Clegg, 1984).

A consequence of enzyme–membrane association is that metabolic events can be confined to a two-dimensional subspace. While some membrane-bound enzymes catalyze transport reactions, the functional significance of many others so localized is not immediately obvious. Is the membrane serving simply as a passive anchor-site for colocalization of metabolically-related enzymes, or do membranous

processes function more "actively" in local metabolic events? Attachment to cellular membranes, potentially, provides energetic contact of an enzyme reaction with major intracellular power supplies. Many biomembranes are free-energy capacitors. Proton pumps, driven by membrane-associated redox reactions, generate an electrochemical potential difference for protons, or "proton-motive force" ($\Delta\mu_{H^+}$), across the membrane. The $\Delta\mu_{H^+}$ consists of a pH difference and an electrical potential difference ($\Delta\psi$). This "force" drives ATP synthesis, transport of ions and metabolites, flagellar motion, *inter alia.* Its potential involvement in certain enzyme reactions of intermediary metabolism is a matter of serious speculation (Welch and Kell, 1986).

Protein–Cytoskeleton

In the last decade or so, electron microscopy has revealed a complex and richly diverse particulate infrastructure in eukaryotic cells—at a level below that of the constituent organelles. The hyaloplasmic space (erstwhile "ground substance" [from the Greek *hyalo-*, meaning "glassy," "transparent"]) is now known to be laced with a dense array of various cytoskeletal elements (e.g., microtubules, microfilaments, intermediate filaments) and an ephemeral, interlocking, microtrabecular lattice (Porter, 1984). A variety of experimental analyses have revealed that the interstitial voids within this cytomatrix are virtually devoid of macromolecules (Clegg, 1984; Luby-Phelps et al., 1988). Increasingly, reports have appeared in the literature, attesting to the colocalization of metabolically-related enzymes on various elements of the cytomatrix (Clegg, 1984, 1988; Welch and Clegg, 1987; Stadtman and Chock, 1992). The microenvironment therein may be likened, in some respects, to that at the surface of membrane-adsorbed systems.

Multienzyme Assemblages

The ultimate in enzyme organization exists when the metabolically-sequential enzymes themselves form a physical aggregate (Srere, 1987). Such organization is most commonly in the form of "multienzyme complexes," wherein the individual enzymic polypeptides are conjoined noncovalently; the strength of association varies from system to system. There are also many examples (particularly in eukaryotes) of "multifunctional enzyme proteins," whereby multiple enzymatic activities are associated (as separate structural domains) with a single polypeptide chain; phylogenetic analysis indicates that such systems are generated by evolutionary gene-fusion events (Welch and Gaertner, 1980). For the multienzyme assemblages, the metabolic microenvironment approaches molecular dimension. In the extreme case, the intermediary metabolites are passed directly from active site to active site within the proteinaceous organized state (Figure 1).

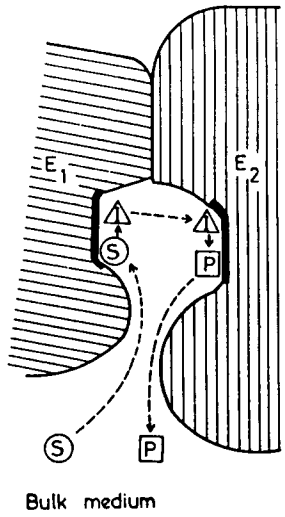

Figure 1. Scheme of metabolite "channeling" in a stable two-enzyme complex. The substrate, S, of the first enzyme, E_1, can enter the microcompartment, where it is bound and transformed to I at the active site of E_1. This intermediate, after release from E_1, will be picked up by the active site of E_2 rather than diffuse out of the channel, and be transformed to P, which can leave the channel. The size of the microcompartment, relative to the size of metabolites, may be markedly smaller than depicted here, bordering the case when the facilitated diffusion of I from E_1 to E_2 becomes an intracomplex transfer effected by the concerted action of functional groups from both enzymes. (From Friedrich, 1984).

The General Organizational Picture

Examination of the literature reveals evidence for enzyme organization in virtually all of the major metabolic pathways (see Table 1). The organization, in particular cases, may entail static assemblages or weakly interacting protein–protein (or protein–cytomatrix) arrays—the latter usually being dependent on extrinsic factors (e.g., pH, ionic strength) and/or regulatory effectors (Figure 2). Examples of the former are seen in the interaction of glycolytic enzymes with muscle and neuronal cytofilaments and in the interaction of Krebs cycle enzymes with the inner mitochondrial membrane (Srere, 1987; Welch and Clegg, 1987; Stadtman and Chock, 1992). Examples of the latter are found in some of the amino acid biosynthetic pathways (Welch and Gaertner, 1980; Srere, 1987). The intermediate substrates may subsist as local pools (*viz.*, in the weakly interacting systems) or as protein-bound forms (*viz.*, in the static aggregates). Hence, the idea of a metabolic

Table 1. Complexes of Sequential Metabolic Enzymes[a]

Metabolic Pathway	*Evidence*[b]
DNA biosynthesis	A,B,C,E,F
RNA biosynthesis	A,B,C,E,F
Protein biosynthesis	A,B,C,D,F
Glycogen biosynthesis	B,E
Purine biosynthesis	A,E
Pyrimidine biosynthesis	A,B,D,E,F
Amino acid metabolism	A,B,D,H
Lipid biosynthesis	B,C,F,H
Steroid biosynthesis	A,C,E
Glycolysis	A,B,C,D,E,I
Tricarboxylic acid cycle	B,C,D,G
Fatty acid oxidation	A,B,C,D
Electron transport	C,I
Antibiotic biosynthesis	A,E
Urea cycle	B,D
Cyclic AMP degradation	A,D,E

Notes: [a]Source: Srere 1987.
[b]A. channeling; B. specific protein–protein interactions; C. specific protein–membrane interactions; D. kinetic effects; E. isolation of complexes or multifunctional proteins. F. genetic evidence; G. model systems; H. existence of multifunctional or multienzyme proteins; I. physical chemical evidence.

"channel" does not always connote a molecular-size microenvironment. The fundamental point is that a fraction of the metabolic flow is somehow sequestered from the bulk cytosol and maintained at a localized site.

Judging from the experimental evidence accumulated thus far, there seems to be a correlation in the nature of the organizational state and the functionality of the metabolic process. The weakly interacting systems are found almost exclusively in *amphibolic/catabolic pathways* (*viz.*, glycolysis, hexose–monophosphate shunt, Krebs cycle), which by nature, have numerous flow-bifurcations along the way; whereas, the static systems are seen in *anabolic pathways* (*viz.*, amino acid synthesis, nucleotide synthesis), which usually entail a distinct initial substrate and a final product. In the case of amphibolic pathways, metabolites must be shared by alternate metabolic routes; and static "channels" may not allow, as freely, the exchange there between. Notwithstanding, the cell must be capable of selectively "channeling" an adequate fraction of the metabolic flow in specific directions—especially under stringent conditions. This appears to be achievable via modulation of weak, pairwise enzyme–enzyme interaction (and weak enzyme–cytomatrix association) at metabolic branchpoints by the binding of key regulatory substances (Westerhoff and Welch, 1992). This operational scheme has been well-documented, for example, in the case of the interaction of glycolytic enzymes with filamentous

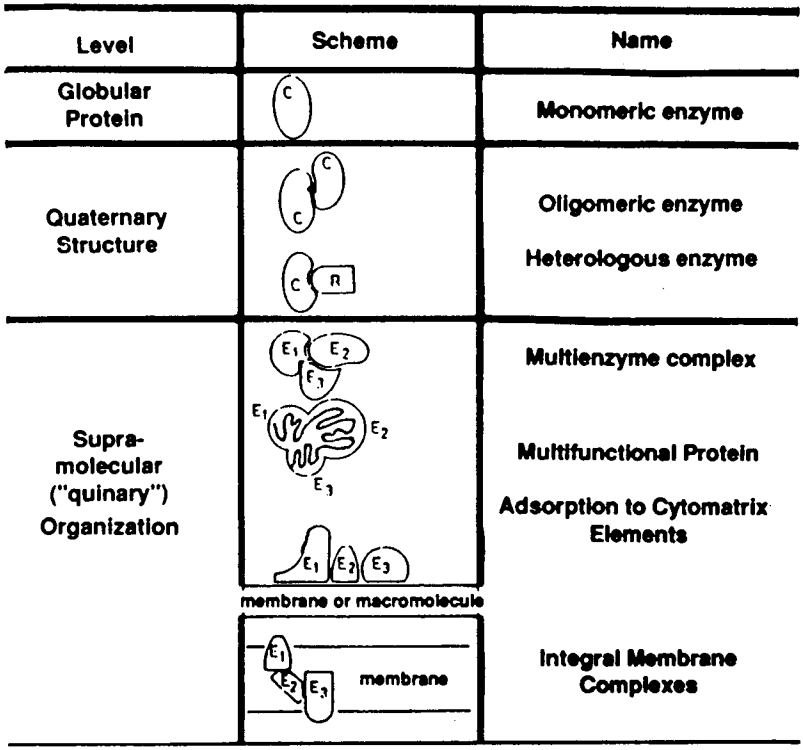

Figure 2. The hierarchy of enzyme structure. Individual contours correspond to globular entities, usually composed of not more than one polypeptide chain. C and R denote catalytic and regulatory subunits, respectively. E_1, E_2, and E_3 are enzymes catalyzing three sequential steps in a pathway. In the "multifunctional protein," the continuity of polypeptide chain over $E_1 \rightarrow E_3$ is indicated. (See text for discussion). (Adapted from Friedrich, 1984).

structures in skeletal muscle (Masters, 1981). Enzymes (e.g., in glycolysis) known to partition reversibly between the cytomatrix and the cytosol, depending on physiological conditions, have been termed "ambiquitous" (Wilson, 1978).

THE QUESTION OF PHYSIOLOGY

The potential roles of enzyme organization are multiple and have been reviewed extensively in the literature (Welch, 1977, 1985a; Friedrich, 1984; Srere, 1987; Welch and Clegg, 1987). Below are listed the most commonly discussed functions, for which there are numerous examples to be found in the biochemistry research journals:

1. *Metabolite "channeling".* Compartmentation of metabolic flow in organized states leads to local sequestration of competing pathways, protection of potentially unstable compounds, and maintenance of high local concentrations of intermediate substrates. This feature endows the larger, intricate eukaryotic cells with a metabolic topography not attainable for a homogeneous, bulk-phase solution of unorganized enzymes. Such concern is heightened for the anabolic pathways and for branchpoints in the catabolic/amphibolic pathways.
2. *Reduction metabolic transition time.* This relates to the temporality of the lag phase during the transition of a metabolic process from one steady-state to another (see Figure 3). It is a function of metabolite diffusion, enzyme density, and kinetic parameters. Close spatial proximity of sequentially-acting enzymes in organized microcompartments can sharply reduce the temporality associated with reaction–diffusion events for sequences of enzyme reactions in dilute solution. Accordingly, the flux condition in metabolic pathways can rapidly switch in response to external stimuli.
3. *Conservation of solvent capacity.* Localization of the multitudinous biochemical reactions in microcompartments expedites the extensive network of metabolic processes with minimal taxation of the "carrying capacity" of the aqueous cytosol. Although subtle in its interpretation, this factor may have played an important role in the early evolution of cellular metabolism (Atkinson, 1977). There is a naïve tendency—whether it be biochemists viewing the dumping of metabolic products into the cell's aqueous bulk *or*

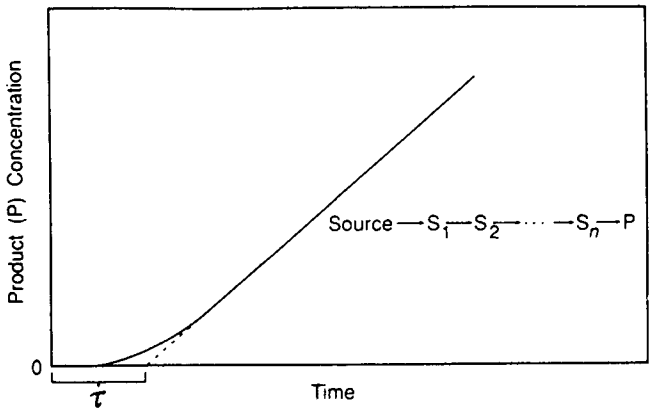

Figure 3. Graphical definition of the metabolic transition time, τ. The concentration of some metabolic product, P, is plotted as a function of time after the pathway is switched on (e.g., by addition of the initial substrate), with the system being devoid of metabolic intermediates initially. The extrapolation to the time axis, of the linear portion of the product accumulation curve is denoted as the transition time (see Welch and Easterby, 1994).

ecologists viewing the dumping of human products into the earth's aqueous bulk—to assume an infinite biological carrying capacity of water.

4. *Conservation of free energy.* Various modes of Gibbs free energy transduction are feasible in organized enzyme systems, when insulated from the thermalizing bulk phase, leading to greater economy of metabolic flux. Part of the realization thereof has come from the deepening understanding of the active, dynamic role of the macromolecular matrix of the enzyme–protein in energy-exchange with the bound chemical subsystem. The classical view of the globular enzyme–protein as a static, background scaffolding for the active site is now outmoded (Welch, 1986). With enzymes, the large protein molecule can assume, transiently, some of the thermodynamic–kinetic properties associated with the familiar function of the stable *coenzymes* (e.g., vitamins) often found in metabolic reactions. Whereby, in some multienzyme aggregates, protein-bound substrate/product species can be passed from active site to active site in energetic states differing from the condition when those intermediate substances are free to equilibrate with the surrounding aqueous medium.
5. *Coordinate regulation.* Metabolic regulation is made more efficient via coordinated control of a multienzyme aggregate via the binding of single effectors. This idea simply represents an extension of the conventional principles of *allosterism* to the heteroenzyme level and was portended in the classical work of Monod, Wyman, and Changeux (see Welch, 1977).

In asking *why* enzyme sequences are organized in the living cell, we enter the bailiwick of physiology—the most central science pertaining to both biology and medicine. The *Oxford English Dictionary* defines physiology as "the science of the normal functions and phenomena of living things." In contemporary usage, physiology basically relates the parts to the whole (or *vice versa*) in the body proper. Dating particularly to the work of the patriarchal 19th-century physiologist, Claude Bernard (1878), the same principles of "physiological determinism" (as Bernard called it) are realizable at all levels of complexity in the biological hierarchy—with the term "body" carrying a different connotation at each scale. Notably, Bernard stressed the primacy of the cellular, *protoplasmic* level in infusing common analogical precepts into the dialectical materialism spanning the hierarchy of the living world.

In viewing the functions of enzyme organization listed above, we see the analogical/metaphorical basis for comparison with physiological concerns at higher organismic levels (Miller, 1978). Indeed, we gain a profound, holistic appreciation of the *cytosociology* (Welch and Keleti, 1981) and the *cytoecology* (Welch, 1987) of the living cell—a picture which is sadly lacking (or incompletely presented) in today's biochemistry textbooks.

IMPLICATIONS FOR CYTOGENETICS

The single cell would seem to present the observer with a simple scale for comprehension of the phenotypic expression of the genome, as regards the manifestation of cellular metabolism. The pioneering work of Beadle and Tatum yielded the notion of "one gene—one protein," with the implication of a direct one-to-one correspondence between the gene and the individual, enzyme-catalyzed metabolic reaction. However, the original Beadle–Tatum dictum did not last long. For, it was soon realized that many proteins are multimeric, thereby necessitating a modification to say "one gene—one polypeptide." The individuality of gene action was further complicated with the elucidation of *allosterism*, which sometimes entails heterologous (and heterotropic) polypeptide interaction in the enzymological character of cellular metabolism. Obviously, the high-order organization of enzymes in the cell obscures the genetic identity even more.

An awareness of the supramolecular, interactive nature of enzyme action *in vivo* answers, in part, the question of why enzymes are so large. (For most enzymes, the active-site region occupies a relatively small part of the total volume of the globular protein.) The size of enzymes must, to some extent, be prescribed by the requirement for sufficient surface area to contain "social" binding sites, for localization on cytomatrix elements (e.g., membranes, cytoskeleton), or for association with other enzymes (Welch and Keleti, 1981; Srere, 1984). Comparative study indicates that such "social sites" on proteins have been conserved during the course of biological evolution (McConkey, 1982). It seems very appropriate to designate this higher-order organization as "quinary-protein structure," to distinguish it from the four structural scales commonly applied to individual protein molecules in the biochemistry lexicon and textbooks

Enzyme organization carries a number of implications in the realm of cytogenetics. Some time ago (Munkres and Woodward, 1966), the concept of "genetics of locational specificity" was suggested to describe the multiplicity of genetic loci that influence enzyme superstructure *in vivo*. Accordingly, there would be two classes of enzyme mutation, structural and integrational—the latter referring to amino acid residues that affect the organizational state. The significance of interallelic (intercistronic) complementation must be reassessed as regards heterologous protein–protein interactions. Mutational pleiotropy, observed in some of the more stable multienzyme aggregates, is an example of genetic anomaly related to the organization (Welch and Gaertner, 1980). Such effect is likely more widespread than heretofore revealed by the customary analytical methodologies (e.g., nutritional auxotrophy). Abandoning the "one-to-one correspondence" basis of genetic action, the experimenter may have to construct stringent conditions that allow the "integrational" mutant to be distinguished in a competitive situation from the wild type (e.g., Srere, 1992).

THE KINETIC NATURE OF BIOCHEMICAL REACTIONS IN STRUCTURED MICROENVIRONMENTS

The familiar kinetic laws, which allow us to predict the behavior of chemical processes, dictate that the rate (velocity) of a reaction in bulk solution is given as the mathematical product of the reactant concentration(s) and a unitary rate constant. It is usual to think of the term "concentration" very simplistically, as a quantity reflecting the random statistical distribution of solutes in the bulk phase. Based on a long established tradition of *in vitro* analysis, the general assumption has been that the biochemical reactions occur in a well-stirred solution that is homogeneous throughout the cytoplasm (or, for some restricted pathways, an organelloplasm). Consequently, the *in vivo* concentrations are "measured" by gross counting of the total number of metabolite (or enzyme) molecules (e.g., by freeze–clamp analysis) in large cellular populations and extrapolating to the whole volume of the individual cell (or organelle). Sometimes the flow of radioisotopically-labeled precursors is followed in metabolizing cells, and elaborate computer programs are constructed to simulate the system and to sort the data. If the results do not fit a preconceived idea as to how metabolism *should* behave, based on *in vitro* reasoning, we all too often malleate the data.

Such a macroscopic conceptualization of "concentration" breaks down for microheterogeneous enzyme systems in organized states, wherein the kinetically competent value of "concentration" for the intermediary metabolites takes on a local, anisotropic character that is part-and-parcel of the organization itself. For structured systems that tightly "channel" the intermediates on a one-by-one basis, the notion of "concentration" may not even apply, kinetically speaking, except for the initial substrate and the final product. Commensurate with this picture, the measured "concentrations" of many metabolites are found to be of the same order of magnitude as that of their cognate enzymes (Srere, 1987).

The second major kinetic factor determining the flux of a chemical reaction is the rate constant itself. An enzyme reaction may be represented, in the simplest case, as follows (ignoring the back reaction of product):

$$\mathrm{E} + \mathrm{S} \underset{k_{-s}}{\overset{k_{+s}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P}$$

with the usual Michaelis–Menten form for the forward velocity:

$$v_f = \frac{V_{\max}[S]}{K_m + [S]} \tag{1}$$

and the definitions:

$$V_{\max} = k_{cat}[E]_T \tag{2}$$

and

$$K_m = \frac{k_{-s} + k_{cat}}{k_{+s}} \tag{3}$$

where $[E]_T$ is the total enzyme concentration.

Advances in analytical methodologies over the years have provided a quantitative handle on the unitary rate constants for many enzymes. Knowledge thereof has afforded a window into the physical chemistry of binding and catalytic events at the active site. Of course, this information has been of great value to the sciences of enzymology and biochemistry. Unfortunately though, the inference from the analytical results has been that these rate constants are unmalleable quantities, with uniquely defined values that apply both *in vitro* and *in vivo*. A more perspicacious analysis of enzyme action shows the rate constants to be influenced by the local microenvironment—which, for organized enzyme systems *in vivo*, can differ significantly from the *in vitro* situation (Westerhoff and Welch, 1992). Such conclusion has been aided and abetted by studies on artificially immobilized enzyme systems—which provide macroscopic *in vitro* models for the kind of dense, heterogeneous, polyelectrolytic conditions that exist within typical cellular microenvironments (Siegbahn et al., 1985). Moreover, the very binding of enzymes to cytomatrix structures (or to other enzymes) can modify the rate constants. (Note, again, the analogy to cooperative allosteric systems.)

A rigorous theoretical treatment of the subject of reaction kinetics, as applied to real cellular conditions, is far more complex mathematically than the elementary view presented in the standard biochemistry textbooks (Welch, 1977). It is the opinion of a growing number of concerned biochemists that much of the numerology of contemporary biochemical kinetics has fallen ill to human contrivance, spawned by a myopia that prevents us from seeing the enzyme reaction beyond the test-tube existence.

THE THERMODYNAMICS OF BIOCHEMICAL REACTIONS IN STRUCTURED SYSTEMS

The kinetic description of enzyme reactions gives much information on the flow properties and the regulation of cell metabolism, but it does not provide an adequate representation of the *driving force* on chemical processes. One must turn to thermodynamics here. Gibbs free energy is a time-honored thermodynamic state function used in biochemistry to grasp the essence of driving forces, directionality, efficiency, *inter alia*, in the operation of cell metabolism. For macroscopic systems, there are definite ideas on the transduction of free energy from one degree of freedom to another; it is due to the spatial displacement of things by prescribed forces. To store energy as "potential energy," one must battle an elastic force in the case of a spring, a coulombic force in the case of a capacitor, etc.

In metabolic systems, the approach to "forces" (thermodynamics) and "flows" (kinetics) is less apparent. The two are interconnected in subtle, yet important,

ways. For example, in the simple Michaelis–Menten reaction scheme above, the famous *Haldane relation* stipulates that

$$K_{eq} = \frac{V^f_{\max} K^r_m}{V^r_{\max} K^f_m} \tag{4}$$

with

$$\Delta G^\circ = -RT \ln K_{eq} \tag{5}$$

where K_{eq} and ΔG° are the equilibrium constant and the standard-state Gibbs free energy change, respectively, for the reaction $S \leftrightarrow P$; "*f*" and "*r*" denote the forward and reverse directions, respectively; R is the gas constant and T is the absolute temperature. The enzyme interweaves thermodynamics and kinetics; the enzyme–protein is, basically, a molecular machine for transducing a "force" into a "flow" (Welch and Kell, 1986).

For the case of bulk-phase ("pool") metabolism, where there is no enzyme organization, free energy transduction is limited to a single type of modality: stoichiometric coupling in (electro)chemical reactions. One enzymatic process generates substances at a certain concentration, or chemical potential (and, where appropriate, at a given electrical potential); while the next enzymatic process utilizes the substance at that (electro)chemical potential. In many cases, the transfer of free energy between two processes is inextricably linked to the transfer of chemical elements. In some situations, however, the transfer reduces to free energy parceled on "carriers." Such is the case where coenzyme couples mediate between (or shift the thermodynamic equilibrium of) different processes in the cell; the paragon thereof is the chemical "force" embodied in the ATP/ADP couple (Atkinson, 1977). Another important example is the proton-motive "force" ($\Delta\mu_{H^+}$) across energy-transducing (e.g., mitochondrial) membranes; electron-transfer driven proton pumps generate a difference in electric potential and in pH.

The foregoing examples represent indirect free energy transduction; the enzymes, between which free energy is transduced, do not make direct contact with each other. The contact is through a diffusing intermediate species. With organized enzyme systems, the interacting enzymes may directly transduce free energy among themselves, in principle, through a variety of possible intrinsic and extrinsic modes. As noted above, intrinsically, the bound chemical subsystem (insulated from the thermalizing bulk phase) in "channeling" complexes may engage in a fluid exchange of free energy with the macromolecular (protein) subsystem. Extrinsically, enzymes organized in conjunction with energy-transducing membrane systems may transduce free energy from local electric fields and protonic sources directly to their respective bound chemical subsystems (Welch and Kell, 1986; Westerhoff and Welch, 1992). Not surprisingly, such holistic modalities are difficult to probe experimentally, with the usual techniques of "solution chemistry." Notwithstanding, the empirical evidence attesting thereto is now approaching credibility.

The comprehension of (electro)chemical "forces" is based on the macroscopic notion of a (electro)chemical potential difference (ΔG). As seen in biochemistry textbooks, ΔG is composed of two mathematical parts: a "standard-state" part (ΔG°) and a concentration term. The standard-state part (which depends, ultimately, on the local energetic interaction of a particle with its surroundings) of metabolites in "channeled" systems can be vastly different from that in the bulk solution. For microheterogeneous systems, the *local* thermodynamic functions cannot be defined by macroscopic rules, and even the Second Law of Thermodynamics may "apparently" be violated therein (Westerhoff and Welch, 1992).

Many theoretical and empirical questions remain, pertaining to the thermodynamics (as well as the kinetics) of metabolic processes in organized states. Nevertheless, there is an ominous shadow of doubt on much of our current quantitative knowledge of flow–force relationships for biochemical reactions in the living cell (Welch, 1985b).

DEVELOPMENTS IN THE THEORY OF METABOLIC CONTROL

An understanding of the control of cellular metabolism as a whole is perhaps the most central issue in the science of cell physiology, as it draws on an edifice of knowledge from such fields as biochemistry, enzymology, cytology, and cytogenetics. Despite the accumulation of a vast amount of empirical information during the ongoing molecular biology era, the elucidation of the principles of metabolic control still looms as a key, unfinished pursuit. The task seems almost hopeless, in view of the enormous complexity of the living cell. The approach must, necessarily, be one of successive approximations. In this context, the reductionistic paradigm of "solution chemistry" has served as an essential stepping stone. Along the way, reductionistic attempts to rationalize the quantitative aspects of cellular metabolism have relied on the kinetics of individual enzymes in a homogeneous bulk phase (Atkinson, 1977).

Aside from the inordinately dominant light of molecular genetics, the new wave in biochemistry today is, what has come to be called, "metabolic control analysis" (MCA) (Cornish-Bowden and Cárdenas, 1990). The impetus behind this wave is the desire to achieve a holistic view of the control of metabolic systems, with emphasis on the notion of "system." The classical, singular focus on individual, feedback-modulated (e.g., allosteric), "rate-limiting" enzymes entails a naïve and myopic view of metabolic regulation. It has become increasingly evident that control of metabolic pathways is *distributive*, rather than localized to one reaction. MCA places a given enzyme reaction into the kinetic context of the network of substrate–product connections, effector relationships, etc., as supposedly exist *in situ*; it shows that control of fluxes, metabolite concentrations, *inter alia*, is a systemic function and not an inherent property of individual enzymes. Such

contention may seem obvious; yet, the full, quantitative appreciation thereof has heretofore been lacking in the conceptualization of metabolic control.

MCA is designed, in principle, to enable experimental measurement of this "distributive control" of the metabolic system, under defined environmental conditions. It is geared toward the parametrization of enzyme action according to the complete spectrum of metabolic factors relevant to a given flow process. The theoretical framework of MCA, although highly sophisticated mathematically, is readily interpretable in terms of simple Michaelis–Menten concepts. One key mathematical construct in the MCA is the "flux control coefficient," which is assigned to each biochemical reaction in a metabolic pathway; it measures to what quantitative extent a given enzymic step controls the overall pathway flux. This coefficient is defined in the (normalized) differential form, $d\ln(J)/d\ln(e)$, where J is the pathway flux and e is some adjustable enzymic property (e.g., V_{max}) for the given reaction step; it is actually measured *in vivo* by altering individual enzyme activities (e.g., by titrating with exogenous enzyme inhibitors or by altering the genetic dosage of the enzyme–protein). A set of theorems is in place for deciphering the control design of a whole pathway, based on the composite character of such measurable quantities as the "flux control coefficients." One such theorem is the *Flux Summation Theorem*, which stipulates that the "flux control coefficients" (which are fractional quantities) for the individual enzymes therein must sum to unity.

The control patterns of several metabolic pathways have been studied on the basis of MCA (Cornish-Bowden and Cárdenas, 1990). Thus far, the following unorthodox themes have emerged:

1. the flux-control tends to be distributed over more than one (though usually not all) enzymes in the pathway,
2. the "flux control coefficients" are not always the same, but vary with metabolic conditions, and
3. the largest portion of the flux control does not always reside in the enzyme reaction that is farthest from equilibrium. In addition, the tenets of MCA have been extended to the realm of *organized multienzyme systems* which "channel" metabolites, with some novel predictions for the control strategy in such cases (Westerhoff and Welch, 1992).

The MCA theory has infused present-day biochemistry with a holistic spirit that is exceeding the confines of basic science and bearing fruits in such applied disciplines as biotechnology and pharmacology (Cornish-Bowden and Cárdenas, 1990). Furthermore, this is an area where biochemistry can be expected to have a great impact on the practice of medicine in the very near future.

CONCLUDING REMARKS

Biology seems afflicted today with the same *fin-de-siècle* euphoria which is evident in the science of physics. It is being widely hailed that the "end of physics" is in

sight, what with the apparent explanatory power of relativity theory, quantum theory, "string" theory, etc.; indeed, many prominent physicists are speaking openly nowadays of a "Theory of Everything." Likewise, the discovery of the molecular basis of genetics in the 1950's (what many biologists call the "Secret of Life") has cast an air of finality on the study of living systems. As the 20th-century comes to a close, the subject of "metabolism" has become quite *passé*. The great focus is now on the isolation, cloning, sequencing, and cutting/splicing of genetic elements. As we enter the 21st-century, the USA government (with some contribution from other countries) stands poised to spend billions of dollars on the singular mission of identifying all the loci within the human genome.

It may be argued that the science of biology has lost its philosophical view of life as a "process," in favor of the perspective of "substance." Life is not a *thing*; rather, life is a *thing that happens*. Biology must regain its hierarchical buoyancy by moving from "molecular biocentrism" back to "physiological biocentrism" (Welch, 1987). At its most fundamental level—*protoplasm*, the process of life is based on that montage of chemical reactions that we call "*metabolism*." The subject of metabolism is far from being "solved"—particularly regarding the organizational aspects thereof. Looking at the spectrum of life, from the level of biomolecules to that of socio-ecosystems, we realize that the living world consists of *hierarchically-ordered* designs; whereby, we must seek to analyze the "parts" within the context of the "whole." It is only in this way that an understanding of the emergent, functional properties at each level of organization is attainable. In today's utilitarian science, the "understanding" of Nature has come to mean the "control" of Nature. In this vein, we may conclude that the organizational properties of the cellular metabolic machinery will prove crucial to (our understanding of) its control.

REFERENCES

Atkinson, D. E. (1977). Cellular Energy Metabolism and Its Regulation. Academic Press, New York.

Bernard, C. (1878). Leçons sur les Phénomènes de la Vie Communs aux Animaux et aux Végétaux. (Translated from the French, 1974, by H. E. Hoff, R. Guillemin, and L. Guillemin), Charles C. Thomas Publisher, Springfield, Illinois.

Clegg, J. S. (1984). Properties and metabolism of the aqueous cytoplasm and its boundaries. Amer. J. Physiol. 246, R133–R151.

Clegg, J. S. (1988). On the internal environment of animal cells. In: Microcompartmentation (Jones, D. P., ed.), pp. 1–16, CRC Press, Boca Raton, Florida.

Cornish-Bowden, A. & Cárdenas, M. L. (eds.) (1990). Control of Metabolic Processes, Plenum Press, New York.

Friedrich, P. (1984). Supramolecular Enzyme Organization, Pergamon Press, New York/Oxford.

Luby-Phelps, K., Lanni, F., & Taylor, D. L. (1988). The submicroscopic properties of cytoplasm as a determinant of cellular function. Ann. Rev. Biophys. Biophys. Chem. 17, 369–396.

Masters, C. J. (1981). Interactions between soluble enzymes and subcellular structure. CRC Crit. Rev. Biochem. 11, 105–143.

McConkey, E. H. (1982). Molecular evolution, intracellular organization, and the quinary structure of proteins. Proc. Nat. Acad Sci. USA 79, 3236–3240.

Miller, J. G. (1978). The Living System, McGraw-Hill, New York.

Munkres, K. D. & Woodward, D. O. (1966). On the genetics of enzyme locational specificity. Proc. Nat. Acad. Sci. USA 55, 1217–1224.

Porter, K. R. (ed.) (1984). The cytomatrix. J. Cell Biol. (Supplement) 99, 1s–248s.

Siegbahn, N., Mosbach, K., & Welch, G. R. (1985). Models of organized multienzyme systems: use in microenvironmental characterization and in practical application. In: Organized Multienzyme Systems (Welch, G. R., ed.), pp. 271–301, Academic Press, New York.

Sitte, P. (1980). General principles of cellular compartmentation. In: Cell Compartmentation and Metabolic Channelling (Nover, L., Lynen, F., & Mothes, K., eds.), pp. 17–32, Elsevier/North-Holland, New York.

Srere, P. A. (1984). Why are enzymes so big? Trends Biochem. Sci. 9, 387–391.

Srere, P. A. (1987). Complexes of sequential metabolic enzymes. Ann. Rev. Biochem. 56, 89–124.

Srere, P. A. (1992). The molecular physiology of citrate. In: Current Topics in Cellular Regulation, Vol. 33 (Stadtman, E. R. & Chock, P. B., eds.), pp. 261–275, Academic Press, New York.

Stadtman, E. R. & Chock, P. B. (eds.) (1992). From Metabolite, To Metabolism, To Metabolon (Current Topics in Cellular Regulation, Vol. 33), Academic Press, New York.

Welch, G. R. (1977). On the role of organized multienzyme systems in cellular metabolism: a general synthesis. Prog. Biophys. Mol. Biol. 32, 103–191.

Welch, G. R. (ed.) (1985a). Organized Multienzyme Systems, Academic Press, New York.

Welch, G. R. (1985b). Some problems in the usage of Gibbs free energy in biochemistry. J. Theor. Biol. 114, 433–446.

Welch, G. R. (ed.) (1986). The Fluctuating Enzyme, Wiley, New York.

Welch, G. R. (1987). The living cell as an ecosystem: hierarchical analogy and symmetry. Trends Ecol. Evol. 2, 305–309.

Welch, G. R. & Clegg, J. S. (eds.) (1987). The Organization of Cell Metabolism, Plenum Press, New York.

Welch, G. R. & Easterby, J. S. (1994). Metabolite channeling versus free diffusion: Transition-time analysis. Trends Biochem. Sci. 19, 193–197.

Welch, G. R. & Gaertner, F. H. (1980). Enzyme organization in the polyaromatic pathway. In: Current Topics in Cellular Regulation, Vol. 16 (Horecker, B. L. & Stadtman, E. R., eds.), pp. 113–162, Academic Press, New York.

Welch, G. R. & Keleti, T. (1981). On the "cytosociology" of enzyme action *in vivo*: a novel thermodynamic correlate of biological evolution. J. Theor. Biol. 93, 701–735.

Welch, G. R. & Kell, D. B. (1986). Not just catalysts—molecular machines in bioenergetics. In: The Fluctuating Enzyme (Welch, G. R., ed.), pp. 451–492, Wiley, New York.

Westerhoff, H. V. & Welch, G. R. (1992). Enzyme organization and the direction of metabolic flow: physicochemical considerations. In: Current Topics in Cellular Regulation, Vol. 33 (Stadtman, E. R. and Chock, P. B., eds.), pp. 361–390, Academic Press, New York.

Wilson, J. E. (1978). Ambiquitous enzymes: variation in intracellular distribution as a regulatory mechanism. Trends Biochem. Sci. 3, 124–127.

RECOMMENDED READINGS

Clegg, J. S. (1984). Properties and metabolism of the aqueous cytoplasm and its boundaries. Amer. J. Physiol. 246, R133–R151.

Porter, K. R. & Tucker, J. R. (1981). The ground substance of the living cell. Sci. Amer. 244, 56–67.

Srere, P. A. (1987). Complexes of sequential metabolic enzymes. Ann. Rev. Biochem. 56, 89–124.

Welch, G. R. (1987). The living cell as an ecosystem: hierarchical analogy and symmetry. Trends Ecol. Evol. 2, 305–309.

Chapter 5

Enzyme Kinetics *In Vitro* and *In Vivo*: Michaelis–Menten Revisited

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INTRODUCTION

Nothing so characterizes the living state as the ubiquitousness of chemical change—not chaotic flux, but integrated systematic change. Autotrophic organisms utilize solar energy and simple minerals to synthesize the complex organic molecules required for their maintenance and reproduction. Heterotrophic organisms degrade such complex molecules to simpler compounds and then utilize these compounds, as well as the energy released in the degradation process, for their biosynthetic needs. Despite extreme differences in structure and function among these major groups of organisms, there is a common chemical basis for all forms of life as we know it.

Although organisms must deal with many forms of energy, the basic unit of exchange is chemical. All other types of energy are interconvertible with the chemical form by means of specialized energy transduction processes. Electromagnetic energy is converted to chemical energy in photosynthesis, whereas chemical energy is converted to mechanical energy in muscular contraction. Aside from such specialized energy conversions, the overwhelming majority of cellular functions are of a strictly chemical nature.

The reactions occurring in the cell are difficult for the organic chemist to reproduce, even under extreme conditions of temperature and acidity not available to the cell. This is because nearly all these reactions normally proceed by themselves at a very slow rate. However, organisms have numerous catalysts that increase the speed of chemical reactions and do so in a highly specific fashion. No matter what cellular function is considered, systematic chemical changes occur and these changes are catalyzed by specific enzymes.

This view of the living organism as a complex network of enzyme-catalyzed reactions derives in large part from the well established analytical tradition in biochemistry known as enzymology.

Role of the Enzymologist's Test Tube

The enzymologist's test tube is an indispensable tool for the daily practice of biomedical science. Students are made familiar with this common piece of laboratory apparatus in their earliest studies. At the same time, the test tube is a symbol that points to the larger framework of assumptions within which the practice of biomedical science is carried out. Indeed, it has become one of the more familiar

symbols of modern science, frequently incorporated into the logos of chemical supply houses, elementary science texts, and weekly newspaper columns. It symbolizes careful observation and controlled study of a limited aspect of nature in isolation from the host of other interactions that otherwise make understanding difficult.

Role of Mathematics

The introduction of mathematics at this point may appear to shift the focus away from the biological system we wish to understand. However, it will become clear in the following that mathematics is the only language available that is sufficiently systematic and precise to deal with the distinctive characteristics of integrated biological systems. For example, two prominent advantages of mathematics in this regard are its ability to deal with large numbers of variables and its ability to relate qualitatively distinct behavior at the system level to subtle quantitative characteristics at the molecular level.

Large numbers of variables in and of themselves pose an enormous book-keeping problem, and some systematic way of keeping track of all their values is required. Because of the numbers involved in organizationally complex systems, the only suitable language is mathematics. When systems become even moderately large, it often will be necessary to implement the methodology on a computer, and this is possible only when the problem can be formulated with mathematical precision.

The richness of interactions and their nonlinear character lead to critical quantitative relationships that characterize systemic behavior. Only a quantitatively precise language like mathematics has the power to elucidate these design principles and the structure to represent them in an efficient manner. Mathematical methods can be employed to make rigorous comparisons, which in turn form the basis for predictive theories of alternative design.

Some of the necessary mathematical concepts and tools can be adopted from other fields and applied to biological systems. Others must be fashioned specifically to deal with novel aspects of biological complexity. The development of a general formalism for the characterization and analysis of organizationally complex biological systems must begin with an appropriate mathematical description for their component parts and associative processes. We shall return to these issues below.

Purpose of This Chapter

In this chapter I shall examine some of the underlying assumptions and practical implications of enzymology as practiced *in vitro*, and contrast this approach with other complementary approaches for dealing with integrated biochemical systems *in vivo*. There is always a certain tension between these two approaches. This can

be seen most clearly in the practice of medicine, which is based on knowledge of the biochemical infrastructure, as well as knowledge in other specialized disciplines, but by its very nature must ultimately deal with the whole organism.

We shall be concerned with one of the more important methodologies for studying these two complementary approaches to the nature of biological systems; namely enzyme kinetics. First, I shall distinguish kinetics as the study of molecular mechanism from kinetics as the study of system dynamics. Then, I will show the relevance of the Power-Law Formalism for representing intact biochemical systems, describe methods for relating their molecular and systemic behavior within this formalism, and discuss some of the implications of power-law kinetics. I shall finish with a general summary. A large portion of the material in this chapter previously appeared as part of a lengthier account entitled "Critique of the Enzymologist's Test Tube" (Savageau, 1992).

There are numerous excellent texts that deal at length with many of the topics considered in this chapter. I shall not attempt to duplicate these, but give a sufficient number of references that the interested reader may explore topics in greater depth. In particular, there are numerous texts that consider the limitations of enzyme kinetics as practiced *in vitro* for the purpose of elucidating the mechanism of an isolated reaction. These are often considered in conjunction with the "canons of good enzymological practice". I shall refer to many of these points, but the focus of this article will be on issues more specifically related to the relevance of kinetics *in vitro* to the understanding of integrated biochemical systems as they function *in vivo*.

KINETICS IN THE TEST TUBE

Chemical change can be described in a variety of ways. For example, qualitative descriptions might include (a) numbers and types of atoms or functional groups undergoing change, (b) alterations in geometry or steric configuration of the reacting molecules, (c) number and nature of the bonds that are made and broken, or (d) at a more fundamental level, modifications in the quantum-mechanical wave functions. Each of these descriptions provides an important way of viewing chemical change at the molecular level. However, unless one is specifically interested in the molecular mechanism of a particular reaction, these descriptions are of limited value because there is no way of efficiently incorporating this information into a meaningful description of biochemical systems containing a large number of reactions.

Alternatively, chemical change can be described by its quantitative aspects: to what extent a reaction normally takes place, and how fast it proceeds. These thermodynamic and kinetic aspects have had extensive mathematical development. In this section we shall consider the kinetics of homogeneous enzyme-catalyzed reactions in a closed reaction vessel at constant pressure and temperature. Not only do these techniques provide useful information about the mechanism of individual

reactions, but this information is in such a form that it can be incorporated into an appropriate description of systems containing many such reactions. Hence, in the search for a formalism that is appropriate for the analysis of organizationally complex systems one naturally looks to kinetic approaches.

Chemical Kinetic Background

The chemical kineticists of the previous century developed a phenomenological understanding of chemical change that provided an accurate description of many chemical reactions *in vitro*. This description is known as the *Law of Mass Action* and is now rationalized in terms of certain probability considerations, which in turn can be represented by empirically determined rate constants and the concentrations of reactants. Let us begin with two elementary cases.

Monomolecular Reactions

Intramolecular rearrangements or cleavages of a molecule, the only types of reactions that involve a single substrate, are called monomolecular. These can be represented schematically as:

$$X_1 \rightarrow X_2$$

The probability that a given molecule in the X_1 population will have sufficient thermal energy to make a transition to X_2 in a given time interval is proportional to the Boltzmann factor exp $(-E_A/RT)$, where E_A is the activation energy, T is the absolute temperature, and R is the gas constant. The number of molecules undergoing conversion per unit of time is given by the transition probability per molecule times the number of molecules, N_1.

$$dN_2/dt \propto [\exp(-E_A/RT)]N_1$$

or

$$dN_2/dt = kN_1 \tag{1}$$

where dN_2/dt represents the increase in the number of X_2 molecules per unit of time, and k is the constant of proportionality which includes the Boltzmann factor. The conventional rate equation in terms of concentration is obtained by dividing both sides of Eqn. (1) by the volume of the reacting solution. Thus,

$$dX_2/dt = kX_1 = -dX_1/dt \tag{2}$$

The symbols X and X will be used henceforth to represent the name of a substance and its concentration; it also will be clear from the context which of the two meanings is intended. The proportionality constant k is called the *rate constant*, and for monomolecular reactions it has the units of reciprocal time.

Bimolecular Reactions

Reactions that require the collision of two molecules are called *bimolecular*, and they are schematically represented as follows:

$$X_1 + X_2 \rightarrow X_3$$

The probability of reaction is proportional to two factors. The first, as in the monomolecular case, has to do with substrate molecules possessing the required amount of thermal energy to yield a reaction. The second is the probability of a fruitful collision between an X_1 and an X_2 molecule, which may be expressed roughly as the joint probability of their being at the same place at the same time with the appropriate orientation.

The probability of finding a given X_1 molecule at a given time in a given position within a volume V is

$$p_1 \propto \sigma_1 / V \tag{3}$$

where σ_1 is the effective volume or "cross section" for the X_1 molecule and includes orientation effects, and V is the volume of the reacting solution. Similarly, the expression for a given X_2 molecule is

$$p_2 \propto \sigma_2 / V \tag{4}$$

Therefore, the net probability of the $X_1 \cdot X_2$ pair undergoing a conversion to X_3 is proportional to the product of the Boltzmann factor and the two probabilities in Eqns. (3) and (4).

$$p_{12} \propto \exp(-E_A/RT)[(\sigma_1/V)x(\sigma_2/V)] \tag{5}$$

This probability can be written as

$$p_{12} = k/V^2 \tag{6}$$

if the temperature and shape factors are grouped into one constant of proportionality. The total number of $X_1 \cdot X_2$ pairs making the conversion to X_3 per unit volume per unit time is given by the transition probability p_{12} times the number of different $X_1 \cdot X_2$ pairs, $N_1 \, x \, N_2$. Thus,

$$(1/V)(dN_3/dt) = (k/V^2)N_1N_2 \tag{7}$$

where N_3 represents the number of X_3 molecules formed. Rewriting this expression in terms of concentrations yields the familiar form for the rate equation of a bimolecular reaction:

$$dX_3/dt = kX_1X_2 = -dX_1/dt = -dX_2/dt \tag{8}$$

The rate constant k for this type of reaction has the units of reciprocal concentration–reciprocal time.

Basic Concepts

These two simple examples illustrate a number of fundamental concepts in chemical kinetics. The expression giving the rate of the reaction as a function of the concentrations of the reactants is referred to as the *rate law* for the reaction. It corresponds to an instantaneous rate, and, thus, it is customary to measure *initial rates* of reactions *in vitro* following the addition of known concentrations of reactants. If the rates are determined over sufficiently short times, then few reactant molecules will have undergone conversion and their concentration will not have changed appreciably during the course of the initial rate measurement. The number of molecules entering into the reaction is the *molecularity* of the reaction. This is an integer—1, 2, 3—for monomolecular, bimolecular, trimolecular reactions. The power to which the concentration of a given reactant is raised in the rate law is the *kinetic order* of the reaction with respect to that reactant. In the simple examples given above, the reactions were first order with respect to each reactant, i.e., all the exponents in the rate laws were unity. This is not always the case. For example, if the reactant molecules in the bimolecular reaction were identical ($X_1 = X_2$), then the rate law in Eqn. (8) would be written

$$dX_3/dt = kX_1^2 = -\tfrac{1}{2}\, dX_1/dt \tag{9}$$

and the reaction would be second order with respect to the reactant X_1. As noted above, the proportionality constant in the rate laws of chemical kinetics is the *rate constant* of the reaction.

Estimation of Parameter Values

In general, the values of the kinetic orders and the rate constant are unknown and must be determined from experimental measurements. The methods for doing this follow directly from the mathematical form of the rate law. The rate law for each of the elementary reactions considered thus far is in the form of a product of power-law functions. A more general example is the following:

$$dX_4/dt = kX_1^2X_2 \tag{10}$$

Such a relation is transformed easily into a sum of linear terms by taking logarithms:

$$\ln(dX_4/dt) = \ln k + 2 \ln X_1 + \ln X_2 \tag{11}$$

Consider X_2 as a parametric concentration variable that is temporarily held constant. The experimentally determined rate then can be plotted against the concentration X_1 in a log–log coordinate system for various values of X_1 (Figure

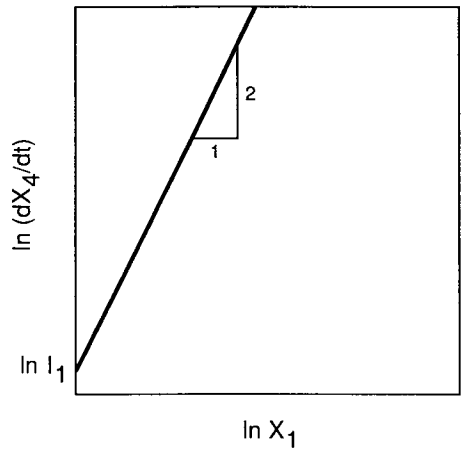


Figure 1. Log–log plot of initial rate *versus* concentration $X_1 \cdot X_2$ is the parametric concentration held constant in this case at a value of X_2'. $\ln I_1$ is the value of the intercept, and the slope represents the kinetic order of the reaction with respect to X_1. The value of the slope in this case is 2.

1). The kinetic order with respect to X_1 is given by the slope of the straight line in such a plot. Similarly, if X_1 is the parametric concentration and the logarithm of the rate is plotted against the logarithm of X_2, a straight line with a slope equal to the kinetic order of the reaction with respect to X_2 is obtained (Figure 2).

The value of the rate constant k can be determined from the intercept of either curve when the value of the parametric concentration and the kinetic order with respect to the corresponding reactant are known. For instance, the value of the intercept in Figure 1 is given by

$$\ln I_1 = \ln k + g \ln X_2' \tag{12}$$

where g is the unknown kinetic order with respect to X_2 and X_2' is the particular value of the parametric concentration. From Figure 2 one determines the kinetic order with respect to X_2; its value is $g = 1$. Therefore,

$$k = \exp(\ln I_1 - g \ln X_2')$$

$$k = \exp[\ln(I_1/X_2')]$$

$$= I_1/X_2' \tag{13}$$

These methods can be generalized to handle the case with n different concentrations appearing in the rate law. In this way, the kinetic parameters of elementary reactions can be estimated from data involving the experimental determination of initial rates.

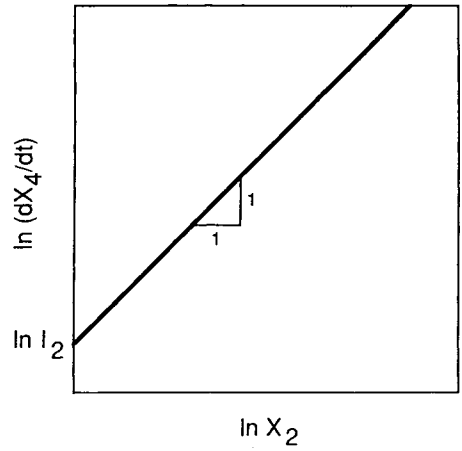


Figure 2. Log–log plot of initial rate versus concentration X_2. X_1 is the parametric concentration held constant at a value of X_1'. LnI_2 is the value of the intercept, and the slope has a value of unity.

For further information on the mathematical treatment of chemical kinetics, see the thoughtful monograph by Erdi and Toth (1989).

Enzyme Kinetic Fundamentals

By the turn of the century it was clear that many reactions of biological interest could not be adequately described by the methods developed for chemical kinetics. Sometimes the initial rate was a constant independent of substrate concentration (zero order with respect to substrate), sometimes it was linearly dependent on substrate concentration (first order with respect to substrate), and sometimes it was something in between. In 1902, Brown interpreted such data in terms of a mechanism involving the formation of a compound from substrate and enzyme that slowly decomposes into product and enzyme. In the following year, Henri (1903) formalized the description of this mechanism in terms of elementary chemical reactions and showed that such a mechanism could, in fact, explain the experimental observations. Michaelis and Menten (1913) elaborated on the work of Brown and Henri, provided more definitive experimental support, and developed methods for experimentally determining the values of kinetic parameters for enzyme-catalyzed reactions. The work of these pioneers laid the foundation for classical enzyme kinetics, which for another fifty years was to be the paradigm for rigorous description of enzyme-catalyzed chemical change. The reader is referred to the classic paper of Harold Segal (1959) for a more detailed account of the early development of chemical and enzyme kinetics.

Postulates

Six postulates typically are assumed in the derivation of classical Michaelis–Menten kinetics.

1. Reactions occur in dilute homogeneous systems.
2. The total reactant concentration is much greater than the total enzyme concentration, which is constant.
3. The intermediate complexes are in steady-state with respect to the rate of the overall reaction. This is sometimes called the quasi-steady state assumption, since not all molecular concentrations are required to be in steady-state. In many cases, intermediate complexes are treated as being in equilibrium with respect to the rate of the overall reaction. This is referred to as the quasi-equilibrium assumption, which is generally more restrictive.
4. The reactants and the corresponding enzyme form intermediate complexes, each containing a single enzyme molecule.
5. Enzyme-catalyzed reactions are composed of a system of elementary chemical reactions governed by traditional chemical kinetics.
6. The mechanism is assumed to operate far from thermodynamic equilibrium, thus allowing one to neglect the reverse reaction in the final step (see below).

Derivation of the Michaelis–Menten Rate Law

The conventional notation that represents the simplest version of the Michaelis–Menten mechanism is the following:

$$\mathrm{S} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\mathrm{ES}) \xrightarrow{k_2} \mathrm{E} + \mathrm{P}$$

where k_1 is the rate constant for the bimolecular reaction, k_2 is the rate constant for the monomolecular reaction in the forward direction, k_{-1} is the rate constant for the monomolecular reaction in the reverse direction, E is the free enzyme, S is the free substrate, P is the free product, and (ES) is the enzyme–substrate complex.

The total enzyme concentration, E_t remains constant. The free and bound forms may vary, but the constraint

$$E_t = E + (ES) = constant \tag{14}$$

must always be satisfied. In contrast, the total substrate concentration

$$S_t = S + (ES) \tag{15}$$

is a variable that decreases with time as substrate is converted to product. However, S_t can be considered approximately the same as S because (ES) can be no larger

than E_t, which by assumption is much less that S_t. Hence, of the four variables in this mechanism (E, S, ES, and P), two are fixed experimentally (S and $E_t = E + ES$), leaving two dependent variables (ES and P). E can be determined subsequently from the solution for (ES) by using Eqn. (14), or E could be chosen as the dependent variable rather than (ES).

The dynamic behavior of the two dependent variables is constrained by the conservation of mass, which can be formulated as follows:

$$dP/dt = k_2(ES) \tag{16}$$

$$d(ES)/dt = k_1 S\, E - (k_{-1} + k_2)(ES) \tag{17}$$

The first equation describes the rate of accumulation of product that results from the monomolecular breakdown of intermediate complex; the second states that the difference between the rate of production of intermediate complex by the bimolecular reaction of free enzyme and substrate, and the rate of removal of intermediate complex by the forward and reverse monomolecular reactions, must appear as a change in the concentration of the intermediate complex. These equations are also referred to as mass balance equations, or Kirchhoff's flux equations.

In general, such nonlinear differential equations are difficult to solve exactly. Therefore, Michaelis and Menten (1913) made the simplifying assumption that the intermediate complex is in equilibrium with the free enzyme and substrate (the "quasi-equilibrium" postulate). While this is not strictly true, it is nearly so when k_2 is much less than k_{-1} or k_1, as was the case for Michaelis and Menten, who were concerned with the enzyme invertase. More often valid is the "quasi-steady state" postulate, which was first developed in detail by Briggs and Haldane (1925).

To solve for dP/dt in terms of total enzyme concentration, substrate concentration, and the kinetic parameters of the mechanism, we must eliminate E and (ES) from Eqns. (16) and (17). The steady-state equation for the intermediate complex is obtained from Eqn. (17) by setting the time derivative equal to zero. This algebraic equation, together with the constraint equation for E_t (Eqn. 14), can be written as

$$0 = k_1 S\, E - (k_{-1} + k_2)(ES) \tag{18}$$

$$E_t = E + (ES) \tag{19}$$

This is now a set of linear algebraic equations in two variables—E and (ES). The solution of these equations for (ES) in terms of E_t, S, and the kinetic parameters can be easily obtained by a variety of methods from linear algebra. Thus,

$$ES = \frac{k_1 E_t S}{k_1 S + (k_{-1} + k_2)} \tag{20}$$

Substitution of this result into Eqn. (16) yields

$$dP/dt = \frac{k_2 k_1 E_t S}{k_1 S + (k_{-1} + k_2)} \tag{21}$$

If one substitutes the usual definitions

$$v_P = dP/dt$$

$$V_m = k_2 E_t$$

and

$$K_M = (k_{-1} + k_2)k_1$$

then one obtains the Michaelis–Menten equation in the conventional form

$$v_P = \frac{V_m S}{S + K_M} \tag{22}$$

where V_m is the maximal velocity of the reaction and K_M is the Michaelis constant. The stoichiometric relationship between the substrate and the product implies that v_P also is equal to $-dS/dt$ or $-v_S$. Thus, Eqn. (22) is the rate law that describes the reaction throughout the period when the intermediate complex is in quasi-steady state with respect to the rate of the overall reaction.

Estimation of Parameter Values

A number of methods have been developed to estimate the parameters in the Michaelis–Menten rate law. In each instance, the data consist of initial rate determinations for different values of the reactant concentrations. In one method, the rate is plotted as a function of substrate concentration in rectangular coordinates. In this case the parameter V_m is the asymptotic value of the rate for high substrate concentrations. The parameter K_M is given by the concentration of substrate that produces a reaction rate equal to $V_m/2$. This method is illustrated in Figure 3. An accurate estimation of the high-substrate asymptote for this type of curve often is difficult to obtain; as a result, other methods of estimating the parameters have been developed.

Simple inversion of Eqn. (22) gives

$$\frac{1}{v_P} = \frac{1}{V_m} + \frac{K_M}{V_m}\frac{1}{S} \tag{23}$$

which has the form of a linear relationship if one plots the reciprocal of the substrate concentration versus the reciprocal of the velocity. In such a plot $1/V_m$ is obtained by extrapolating the line to give the intercept on the $1/v_P$ axis. The parameter K_M

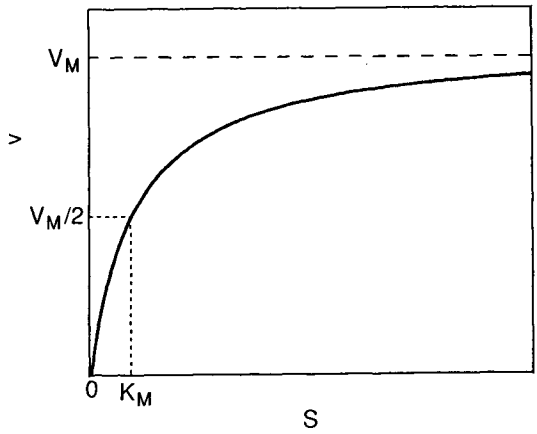


Figure 3. Michaelis–Menten rate law expressed in Cartesian coordinates. The maximum velocity of the reaction V_m is the asymptotic value of the rate v at high concentrations of the substrate S. The parameter K_m is given by the value of S that yields half the maximum velocity, or $V_m/2$.

is determined from the slope of the line or, by further extrapolation, from the negative intercept on the *1/S* axis. This double-reciprocal plot, attributed to Lineweaver and Burk (1934), is graphically depicted in Figure 4. By performing assays with approximately 10 different substrate concentrations and using standard statistical methods for (weighted) linear regression, one can obtain estimates of the

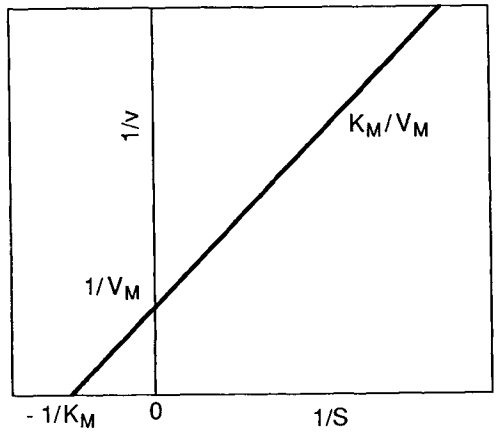


Figure 4. Michaelis–Menten rate law expressed in double-reciprocal coordinates. In this plot, which is attributed to Lineweaver–Burk, $1/V_m$ is given by the intercept on the *1/v* axis. The parameter K_m can be obtained from the slope of the straight line or the intercept on the negative *1/S* axis.

two parameters in the Michaelis–Menten rate law. There are a number of variations on this theme of representing the rate law in the form of a linear equation. The advantages and disadvantages of each of these methods and the statistical considerations that are important for the analysis of experimental data have been discussed by Wilkinson (1961), Cleland (1967a), and others, and reviewed by Wong (1975).

Extensions of the Classical Michaelis–Menten Formalism

Many of the subsequent developments in enzyme kinetics share the same basic postulates of Michaelis–Menten kinetics. Although the mechanisms and equations may be different in detail, they all lead to rate laws that are linear functions of enzyme concentration and rational functions of the reactant and modifier concentrations. Hence, all these developments are based upon the same underlying formalism, which I shall refer to as the *Michaelis–Menten Formalism.*

Multiple Intermediates, Substrates, and Products

Some of the natural extensions of this classical approach include the treatment of mechanisms with multiple intermediate complexes and near-equilibrium conditions (e.g., Peller and Alberty, 1959). Enzyme-catalyzed reactions that involve two substrates and two products are among the most common mechanisms found in biochemistry (about 90% of all enzymatic reactions according to Webb, 1963). It is not surprising, then, that this class of mechanisms also has received a great deal of attention (e.g., Dalziel, 1957, 1969; Peller and Alberty, 1959; Bloomfield et al., 1962a,b; Cleland, 1963a,b,c). This class includes mechanisms in which reactant molecules enter and exit a single pathway in fixed order and mechanisms with parallel pathways in which reactant molecules enter and exit in a random order (Cleland, 1970).

Classical Modulation of Enzyme Activity

The kinetic description of enzyme-catalyzed reactions *in vitro* has allowed their rates to be expressed as a function of reactant and enzyme concentrations, and a set of empirically defined kinetic parameters. Enzymologists also have sought to modify this basic relationship in systematic ways so as to reveal something about the mechanism by which the enzyme acts. Traditionally, in such studies the enzymologist has been concerned primarily with the enzyme, its proper substrates and products, and closely related chemical analogs of these proper reactants (Jencks, 1969).

Kinetic techniques have played an important role in such mechanistic studies. In fact, kinetic studies have been associated so intimately with the investigation of mechanism that for many people, kinetics and mechanism are almost synonymous.

Not surprisingly, one of the principal definitions of kinetics given by Webster is the mechanism by which chemical change is effected. The principal advantage associated with this use of kinetics is the ability to reject putative mechanisms that are inconsistent with existing data. However, different mechanisms often can produce the same kinetic behavior, and, therefore, kinetics alone cannot be used to identify a mechanism unambiguously. These advantages and disadvantages have been clearly recognized by kineticists from the very beginning (Henri, 1903). A large literature has grown up around this approach, which is referred to as classical enzyme inhibition (Cleland, 1963b, 1967b).

Allosteric Modulation of Enzyme Activity

According to classical enzyme kinetics, molecules sterically related to the reactants of an enzyme were useful in probing its mechanism, but were not expected to be present and affect the activity of the enzyme under physiological conditions. The exquisite specificity of enzymes was presumed to guard against interference by unrelated or distantly related molecules, and in this way the numerous cellular reactions could proceed at the same time in the same space without resulting in chaos. By the 1950's there were experiments using radioactive isotopes in intact cells that implicated a much more profound kind of integration in the organism than was provided by simple specificity and mass action (Roberts et al., 1955). In 1956, Umbarger, and Yates and Pardee discovered biochemical feedback at the molecular level. They showed that the end product of a sequence of enzyme-catalyzed reactions could modify the activity of the first enzyme in the sequence. The first product in such a sequence is sterically similar to the initial substrate, and the second product is similar to its immediate precursor, etc., because the transformation effected in each step is small. Nevertheless, after a long sequence of such small transformations, the end product no longer is significantly related to the original substrate. Thus, distantly related molecules can indeed modify enzymatic activity in the cell, at least the activity of certain key enzymes.

It is important to emphasize the change in perspective that occurred with this discovery. As indicated in the preceding paragraph, modification of enzymatic activity was viewed previously as something done by the investigator to probe the mechanism of enzyme action. Following this discovery, modification of enzymatic activity was considered within the realm of normal cellular function; i.e., something done by the cell. After the discovery of feedback inhibition at the molecular level, a great deal of investigation was initiated to answer the question "how do these key enzymes catalyze the transformation of one type of molecule and modulate this activity in response to another, sterically unrelated molecule?" This aspect of enzyme kinetics has become known as allosteric modulation and the principal kinetic models are those developed by Monod et al. (1965), and Koshland et al.

(1966). See also the reviews by Wyman (1972), Hammes (1982), and Kurganov (1982).

General Properties of the Rate Law

We shall conclude this section by emphasizing some general properties of the rate law in the Michaelis–Menten Formalism (Wong and Hanes, 1962; Savageau, 1969a).

- Rate laws based on the Michaelis–Menten Formalism are functions only of kinetic parameters and concentration variables.
- The form of these functions is always a ratio of polynomials involving variables for reactant and modifier concentrations.
- The highest power to which these variables are raised in the denominator is always greater than or equal to the corresponding power in the numerator.
- The rate is directly proportional to the concentration of total enzyme.
- All the kinetic parameters in the unidirectional rate law are positive.

Because rate laws based on the Michaelis–Menten Formalism have this particular mathematical form, they can be analyzed with a general method developed for such functions by Bode (1945). For details see Savageau (1976).

Canons of Good Enzymological Practice

The *goal* of the kinetic approach is to establish the rate law for an isolated enzyme-catalyzed reaction under clearly specified conditions. This provides a quantitative characterization of reaction rate as a function of the variables that affect it and kinetic parameters that are independent of these variables. The primary use of this information is the elucidation of reaction mechanisms.

The Michaelis–Menten Formalism provides the mathematical framework within which most of the kinetic theory of biochemical reactions has developed. The common steps in the application of this approach are:

1. *Postulate* a mechanism, or preferably two, to account for the behavior of the enzymatic reaction in question.
2. *Analyze* the putative mechanisms to uncover distinctive kinetic consequences of each. This involves determination of the rate law for some portion of the mechanism, or in the most complete analysis, for the entire mechanism.
3. *Test* the distinctive predictions by performing critical experiments that discriminate between the alternative mechanisms. At this step, one or both of the putative mechanisms will be eliminated from consideration.

This is of course a specific instance of the iterative process we associate with the scientific method. The postulated mechanisms surviving this process can be considered consistent with the experimental data. Kinetics provides a powerful method for eliminating putative reaction mechanisms, but kinetic methods alone can never establish a mechanism unambiguously. Other chemical and physical methods can be of help in this regard, but it must be acknowledged that all our models, at some level, are tentative and subject to revision. In practice, one must accept a certain amount of ambiguity, but for many, if not most, applications this is not crucial.

A number of practical prescriptions have been developed over the years that are designed in large part to ensure that the postulates of the Michaelis–Menten Formalism are satisfied.

Laboratory Practice

Under this heading one often finds basic issues such as selection and maintenance of equipment and reagents, washing of glassware, making of buffers and other solutions, sterilization, use of pipettes, etc. These are perhaps the most obvious prescriptions. They are fundamental to any study in biochemistry, and thus one finds them emphasized in beginning laboratory courses.

Initial Rates

The rate of a reaction is a function of the reactant and modifier concentrations. Determination of this relationship is greatly facilitated if one measures *initial rates*. By establishing the concentrations of all the reactants and modifiers in the test tube, and then measuring the rate of the reaction under conditions where these concentrations do not change appreciably during the time needed to determine the rate, one can use the concentrations established in the test tube without having to measure them directly. This condition can be verified by determining the amount of substrate converted to product over the time period used to measure the rate. If this amount is less than 5% to 10% of the initial substrate, then to within this degree of accuracy one can consider the substrate concentration constant and equal to the initial value. If the amount converted is too large, then one must make measurements over shorter periods of time or slow the reaction to ensure that one is accurately approximating initial rates.

Purify Enzymes

Although kinetic studies can be done with complex systems if the elements do not interact, it is difficult to ensure that this condition is satisfied. The only way to guard against confusion resulting from such interaction is to work with purified enzyme preparations. One test to ensure that one is working with a sufficiently pure

preparation of enzyme is to determine the rate of the reaction with different concentrations of enzyme preparation. If the rate is curvilinear when plotted as a function of concentration of enzyme preparation, then further purification is required. When one obtains a linear relationship, the preparation is sufficiently pure and one has the basis for beginning a good enzyme-kinetic study.

Dilute Enzyme Solutions

There are several reasons to work with dilute solutions of enzyme. First, there is the obvious practical issue of conserving what is often a precious supply of enzyme that has been obtained with some labor and cost. Second, dilution can aid in eliminating unwanted interactions, thereby linearizing the rate *vs* enzyme curve as described above. Finally, it may be difficult to make measurements of initial rates in steady state unless the enzyme preparation is sufficiently dilute. If too much substrate is converted in the time required to make the measurement, then one must slow the reaction, and this is typically done by reducing the amount of enzyme in the assay. Transient kinetic methods typically require the use of concentrated enzyme solutions. Hence, these methods are seldom used until after basic understanding of the reaction mechanism has been obtained through steady-state kinetic methods, and critical tests can be designed to elucidate further the mechanism by transient kinetic methods.

Vary Conditions Over a Wide Range

By exploring a wide range of conditions, including extreme conditions that an enzyme might never experience *in vivo*, one is more likely to uncover unique or unexpected features of the reaction mechanism. Examples of extreme conditions include low temperatures to stabilize certain forms of an enzyme, high ionic strength to eliminate certain interactions, and low pH to emphasize specific reactive groups. These conditions accentuate certain parts of the mechanism, and rate laws that characterize these parts are often much simpler than the rate laws that characterize the reaction as a whole. Quite complicated mechanisms often can be broken down into smaller, more manageable, pieces by means of such techniques. The advantages in dealing with complex enzyme-catalyzed reactions are obvious. The risks involve the generation of artifacts that have little to do with the actual behavior of the enzyme under normal physiological conditions. The risk is fairly great if one is focusing on quantitative behavior; it can be much less if one is concerned with qualitative features of the mechanism. A good example of the judicious use of these techniques is provided by the work of Fersht (1985), in which he traps specific intermediates, and thereby measures the rates of specific elementary steps within the mechanism. The judicious use of such partial rate laws allows one to establish a number of important qualitative features of the mechanism such as the order in

which certain steps occur and the relative rates at which they occur. Once the essential qualitative features of a mechanism are clearly established, one can attempt to characterize the overall reaction quantitatively under more physiological conditions.

Optimized Activity

It is expected that a thorough study exploring a diversity of conditions will lead to the establishment of conditions that produce optimal activity *in vitro*. These conditions involve specification of all relevant variables, including temperature, pH, ionic strength, metal concentration, and cofactor concentration. It is important to optimize activity for practical reasons. By using these conditions one tends to obtain more accurate and reliable results with a minimum expenditure of materials. Establishing conditions that optimize activity *in vitro* also is important for what these tell us about the physiological conditions under which the enzyme normally operates. However, it is important to note that full rate laws under physiological conditions are almost never developed in this way.

Conclusions

The Michaelis–Menten Formalism has been remarkably successful in elucidating the mechanisms of isolated reactions in the test tube. There are numerous treatments of this use of kinetics, and many of these provide a thoughtful critique of the potential pit falls. In short, reliable results can be obtained with steady-state methods if one is careful to follow the canons and if one remembers that several mechanisms may yield the same kinetic behavior. Isotope exchange, pre-steady state, and other transient or relaxation kinetic techniques, as well as various chemical and physical methods, also have been applied in conjunction with steady-state kinetic methods to dissect the elementary reactions within an enzyme-catalyzed reaction and to distinguish between various models (e.g., see Cleland, 1970; Kirschner, 1971; Segel, 1975; Hammes, 1982; Fersht, 1985).

Finally, it should be noted that while the Michaelis–Menten Formalism may be appropriate for many isolated enzymes *in vitro*, this does not imply that the resulting rate law for the reaction will be the classical Michaelis–Menten rate law [Eqn. (22)]. Hill et al. (1977) have made a careful assessment of this issue and, on the basis of their results, have come to question whether the simple Michaelis–Menten rate law fits any enzyme that is examined with sufficient care. The tendency to ignore inconsistencies, and continue to treat rate laws as if they were the classical case, indicates that the grip of the conventional Michaelis–Menten paradigm is very strong. We shall examine this point from another perspective in the following section.

KINETICS IN THE LIVING ORGANISM

A more serious critique applies when the Michaelis–Menten Formalism is used to characterize the behavior of enzymes within integrated biochemical systems under conditions *in vivo*. The general reasons are two. First, kinetic analysis of integrated biochemical systems, which has not been central in biochemistry, is only now being seen as an important activity in need of fundamental development. Consequently, the field has not had the years of careful scrutiny that one associates with other more developed areas of biochemistry. Second, the underlying assumptions typically have been taken over uncritically from those used for the elucidation of mechanisms, even though they do not always apply. The canons of good practice developed for the study of kinetics *in vitro* are appropriate for the study of isolated reaction mechanisms, but they are often highly inappropriate for the study of integrated biochemical systems *in vivo*.

In this section I shall point out problems that obstruct understanding of the kinetic behavior of intact biochemical systems. Later we shall focus on alternative approaches that circumvent some of these difficulties.

Identifying Relevant Interactions

Enzymes operate within an extremely complex milieu *in vivo*, and one rarely knows all the relevant molecules with which they normally interact. This of course does not prevent one from establishing important features of the reaction mechanism by focusing upon the interactions with known reactants. Much of our knowledge of enzyme catalysis has been generated in such studies (see above). The features that are more likely to be missed are catalysis of alternative reactions and regulatory interactions, which are often quite subtle.

There is no panacea for coming up with the correct model for a complex network of reactions and regulatory interactions. Suggestions for putative models have come from many sources; the disciplines of biochemistry, genetics, physics, physiology, and mathematics all have contributed valid suggestions. However, once a specific reaction or regulatory interaction has been suggested, kinetic studies with purified components *in vitro* provide a powerful method for verifying the molecular basis of the interaction. A few examples will illustrate this point.

The structural studies of Pauling (1957) led to the prediction that enzymes like isoleucyl-tRNA synthetase, which catalyzes the ligation of isoleucine and $tRNA^{ile}$, also should catalyze the ligation of valine and $tRNA^{ile}$. Subsequently, this reaction and related proofreading reactions were verified and the mechanisms established by kinetic studies *in vitro* (see Fersht, 1985). In another case, the physiological studies of Roberts et al. (1955) suggested specific regulatory mechanisms affecting amino acid metabolism in whole cells. This suggestion was verified in the case of isoleucine by the kinetic studies *in vitro* reported by Umbarger (1956). In a third case, mathematical analysis of feed-forward inhibition in amino acid biosynthetic

pathways showed that the stability and temporal responsiveness of such systems might be improved if the first intermediate in the pathway inhibited the aminoacyl-tRNA synthetase and if this enzyme were spatially associated in a macromolecular complex with the first enzyme of the pathway (Savageau and Jacknow, 1979). These specific predictions were subsequently verified in the case of the isoleucine pathway by kinetic studies *in vitro* (Singer et al., 1984).

Specifying a relevant network of reactions and regulatory interactions requires at least qualitative kinetic information, but this specification establishes only the skeleton of the model. While certain qualitative results that are independent of specific parameter values can be obtained by mathematical analysis at this stage, more detailed quantitative predictions require that appropriate rate laws be specified for the reactions of the system.

Obtaining Appropriate Rate Laws

To assess the relative importance of the various interactions that constitute the intact system one needs an accurate representation of the rate laws under the appropriate conditions *in vivo*. This is easy to say—determine the rate laws for the enzymes under the conditions that exist *in vivo*! However, in most cases it is difficult to determine what these conditions are.

In general, full rate laws for the various reactions of an intact system have not been determined under physiological conditions. This is true of the more complex enzymes even under standard experimental conditions *in vitro* because of limitations in the classical methods for estimating the value of kinetic parameters and the large number of assays required for such estimations.

Methods for Estimating Parameter Values

Cleland (1963b) has described fairly general methods for extracting parameter values by kinetic means. These methods cover the more complex types of inhibition, and mechanisms with more than two substrates and products, provided the reciprocal plots are linear. Methods applicable in certain specific cases involving nonlinear reciprocal plots also have been described (Cleland, 1967b). A number of other *ad hoc* methods have been reported; however, at the present time there is no generally recognized method of kinetic analysis that applies to the wide variety of nonlinear rate laws that are possible for enzyme-catalyzed reactions. Furthermore, the inevitable presence of experimental error makes it difficult to distinguish reliably between different putative forms for the rate law (Bardsley and McGinlay, 1989).

Bode analysis (Bode, 1945) in principle provides a general method for estimating the parameter values in rational functions of the type that characterize rate laws based on the Michaelis–Menten Formalism. The usefulness of this method, which

was originally developed for another purpose, has long been established in a variety of fields (e.g., see Truxal, 1955). However, it is not widely known among enzyme kineticists, even though some aspects of it have been used to characterize the effects of pH on reaction rate (Dixon and Webb, 1964). The basic features of Bode analysis in the context of enzyme kinetics are given by Savageau (1976). Rate laws involving several concentration variables are handled in the same fashion. For example, if there are two variables, one can be fixed as a parametric concentration while the other is varied. The primary plots of rate *vs* concentration are treated by Bode analysis to yield a set of kinetic parameters. Repeating the analysis for different fixed values of the parametric concentration generates the data for secondary plots of the kinetic parameters as functions of the parametric concentration. These secondary plots have all the same properties as the primary plots and are analyzed in the same fashion to yield the final set of kinetic parameters, which are independent of either concentration variable. If there were three variables, the analysis would involve tertiary plots. In principle, the method can be applied to an arbitrary rational function in n variables. However in practice, this method, like all others, suffers from a limited ability to discriminate between different rate laws on the basis of experimental data with realistic error and from a combinatorial explosion in the amount of experimental data necessary to characterize kinetically the rate law for complex enzymes involving many reactants and modifiers.

Number of Assays Required for Parameter Estimation

Determination of the rate law for a particular enzyme by kinetic means requires an amount of data that increases as a power function of n, where n is the number of reactants and modifiers associated with the enzyme. In general, one must determine initial rates of the reaction for all combinations of reactant and modifier concentrations of interest. This can be seen even in the simpler cases where the highest power for each reactant and modifier in the rate law is unity, so that the classical double-reciprocal plots can in principle be used to estimate the parameter values. Analysis of a typical bisubstrate reaction with its product concentrations initially equal to zero requires a family of 6 to 10 initial-rate curves having 6 to 10 points per curve, which represents approximately 10^2 assays (e.g., Raval and Wolfe, 1962). When this approach is extrapolated to the case of n reactants and modifiers, the number of assays required increases with the power n. Thus, when the kinetics of more complex regulatory enzymes are considered, the difficulties in collecting the required experimental data become immense. For example, the enzyme glutamine synthetase is known to be affected by at least eight reactants and modifiers (Woolfolk and Stadtman, 1967). As many as 10^8 assays might be necessary to establish the rate law for such an enzyme by kinetic means.

The Use of Approximate Rate Laws Obtained In Vitro

Given the limitations described above, it is easy to understand why full rate laws for all the enzymes of an intact biochemical system are unlikely to become available in the near future. Current practice typically involves the use of approximate rate laws obtained for other purposes and under experimental conditions *in vitro* that tend to differ somewhat for each enzyme. Since the appropriate physiological conditions are seldom known, adjustments are often made in the rate law for each enzyme to reflect a common set of conditions that is thought to exist *in vivo*. This involves a number of assumptions and *ad hoc* adjustments, but this is often the only course of action if one is to utilize existing information on the rate laws for the enzymes of the system. It has to be recognized that the practice of using rate laws obtained under conditions *in vitro* is potentially a serious source of error that must be properly evaluated. Hence, let us turn to a critical re-examination of the Michaelis–Menten Formalism with an emphasis on its appropriateness for kinetics *in situ*.

Critique of the Michaelis–Menten Formalism

This section will focus on the fundamental postulates and the canons of good practice that are associated with the kinetic characterization of enzyme mechanisms *in vitro* (see above).

Dilute Homogeneous Systems

If systems were homogeneous, then the rates of reactions would increase in direct proportion to the volume of the system. While this may be true in the enzymologist's test tube with a purified enzyme in dilute solution, it is not the case in many, if not most, instances *in vivo*. For example, it has long been known that metabolic rate in related cells of different sizes, or organisms of different sizes, does not increase as the volume of the system. Rather, the relationship is typically a power-law function with a fractional exponent. These types of relationships are known as allometric relationships and are widespread in biology (Huxley, 1932; Bertalanffy, 1960; Savageau, 1979b). Needham (1950) recognized very early that the pervasiveness and regularity of these allometric relationships at a biochemical level indicates a common chemical ground plan for all organisms, and this is now well established as a result of advances in molecular biology (see also, Needham, 1968).

This common chemical ground plan is not based on homogeneous systems. Indeed, the study of catalysis has shown that essentially all catalysis is associated with surfaces. There is abundant evidence that enzymes in the cell are organized in multi-enzyme complexes as in the well known cases of the pyruvate dehydrogenase

and the fatty-acid synthetase complexes (Reed and Cox, 1966). They also are found along membrane surfaces, and within channels, all of which make for decidedly nonhomogeneous systems (see also below). These forms of association lead to interactions between enzymes, and between enzymes and structural elements within the cell. Two types of rationale have been advanced for such spatial organization *in vivo*: catalytic efficiency and regulatory effectiveness.

When enzymes carry out a sequence of reactions, complexes among consecutive enzymes can promote the catalytic efficiency of the sequence. Bulk diffusion is minimized and local concentrations are enhanced by channeling intermediate metabolites from one enzyme surface to the next (for a sample of relevant reviews, see Davis, 1967; Ginsburg and Stadtman, 1970; Srere and Mosbach, 1974; Friedrich, 1984; Clegg, 1984; Srivastava and Bernhard, 1986; Srere et al., 1989; Ovadi, 1991). Channeling of metabolites also can be promoted among enzymes when they are bound near each other on structural elements, as observed *in vitro* with catalysts bound to carriers (Weisz, 1973; Barker and Somers, 1978; Shimizui and Lenhoff, 1978) and as has been proposed for the tight coupling *in vivo* between ATP produced by glycolysis and ion-specific gates in cardiac muscle cells (Weiss and Lamp, 1987). The rationale of catalytic efficiency provides an appropriate explanation for complexes that have been observed among enzymes that carry out consecutive reactions.

When enzymes catalyze key reactions (typically) at the beginnings and ends of unbranched pathways, complexes among such *non*consecutive enzymes can enhance the regulation of the entire pathway or system. Dysfunctional responses in branched pathways are avoided when complexes among such regulatory enzymes provide a balanced response among the several enzymes affected by a common regulatory molecule (Savageau, 1972, 1976). Complexes among nonconsecutive reactions also can transmit important regulatory information via "short circuits" that effectively bypass the cause–effect sequence dictated by the intervening reactions (Savageau and Jacknow, 1979; Singer et al., 1984). The rationale of regulatory effectiveness provides an appropriate explanation for complexes that have been observed among key enzymes that carry out nonconsecutive reactions.

The demonstration of functional advantages by rigorous analysis has gone hand-in-hand with experimental documentation of the widespread occurrence of enzyme–enzyme organization in cells. Far from being exceptional, one should expect a high degree of such organization to characterize the cytoplasm of all cells, and any formalism proposed for representing realistic biochemical systems *in situ* must be capable of dealing with this class of phenomena.

Thus, rate laws determined *in vitro* with purified enzymes in dilute homogeneous solutions in many cases will not reflect the enzyme–enzyme interactions that are important *in vivo*. Purification to eliminate any nonlinearity in the rate *vs* enzyme concentration curve also will eliminate any small molecular weight modifiers that may have been important *in vivo*. Some of these may be discovered by

testing specific metabolites with the purified enzyme, but it is impractical to test even a small fraction of the possible metabolites in various combinations.

Reactant Concentration Much Greater Than Enzyme Concentration

This need not be true *in vivo* where the concentrations of reactants and their enzymes in some cases are nearly comparable. Under these conditions, the nominal concentration of substrate could be significantly greater than the level of unbound substrate, and the reaction rate calculated with nominal concentrations inserted into the rate law clearly would overestimate the rate observed *in vivo* (Wright et al., 1992; Shiraishii and Savageau, 1993). This condition does not alter the basic chemical kinetic equations that describe the mechanism, but it does mean that the quasi-steady state assumption (e.g., see Peller and Alberty, 1959; Segel and Slemrod, 1989) may be inappropriate when reaction rates change with time *in vivo*.

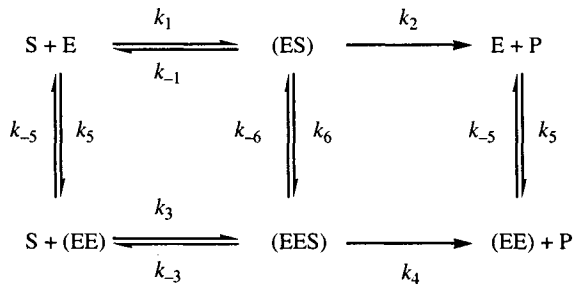
Intermediate Complexes In Steady-state

This remains true *in vivo* for systems that are in steady-state. However, it is not true in general when reaction rates change with time. The conditions (see above) that can make it a valid assumption *in vitro* do not necessarily pertain to the situation *in vivo*.

Intermediate Complexes Contain A Single Enzyme Molecule

The Michaelis–Menten Formalism did not anticipate the type of enzyme–enzyme organization described above. One of its fundamental assumptions has been that complexes do *not* occur between different forms of an enzyme or between different enzymes (Segal, 1959; Webb, 1963; Cleland, 1970; Segel, 1975; Wong, 1975). From the derivation of the classical Michaelis–Menten rate law, it can be seen that such complexes must be excluded or they will destroy the linear structure of the kinetic equations.

For example, consider the following mechanism with enzyme–enzyme complexes.



In this case, the constraint on the total concentration of enzyme and the steady-state equations involving the different forms of enzyme become

$$E_t = E + 2(EE) + (ES) + 2(EES) \tag{24}$$

$$d(ES)/dt = 0 = k_1 S\, E + k_{-6}(EES) - (k_{-1} + k_2)(ES) - k_6 E\,(ES) \tag{25}$$

$$d(EES)/dt = 0 = k_6 E\,(ES) + k_3 S\,(EE) - (k_{-3} + k_{-6} + k_4)(EES) \tag{26}$$

$$d(EE)/dt = 0 = k_5 E^2 + (k_{-3} + k_4)(EES) - k_{-5}(EE) - k_3 S\,(EE) \tag{27}$$

Because there are terms involving products of the dependent variables—e.g., $E\,(ES)$ and E^2—this system of equations is no longer linear and the resulting rate law is no longer a rational function. These are examples of mass action equations, for which there is no known general solution (for further discussion, see Sorribas and Savageau, 1989b).

Evidence indicating the presence of enzyme–enzyme interactions might never arise *in vitro* where dilute concentrations of enzyme typically are used. At low concentrations of enzyme very few enzyme–enzyme complexes would form, and the mechanism would be viewed as a conventional Michaelis–Menten mechanism. If the concentration of enzyme could be made sufficiently high, so that the vast majority of enzyme molecules were in the form of enzyme–enzyme complexes, then the mechanism also would approximate a conventional Michaelis–Menten mechanism in which the functional form of the enzyme is a dimer.

Although in recent years specific cases of enzyme–enzyme interaction have been approximated in various ways and treated by various modifications of the Michaelis–Menten Formalism, no general method for dealing with this class of mechanisms has developed from this approach. In any case, the rate laws that result from such interactions can be quite different in mathematical form from the rational functions characteristic of the Michaelis–Menten Formalism.

Elementary Reactions Governed By Traditional Chemical Kinetics

In traditional chemical kinetics, where reactions are assumed to occur in dilute homogeneous solutions, the kinetic orders are typically integer values that add up to the molecularity of the reaction. The concepts of kinetic order and molecularity are distinct, although they often are confused and considered to be equivalent because of this association in traditional chemical kinetics. However, even within traditional chemical kinetics, it has long been known that reactions involving free radicals can have kinetic orders with noninteger value (Benson, 1960).

Modern treatments of chemical kinetics (e.g., see, Kopelman, 1986; Newhouse and Kopelman, 1986) have shown that the kinetic order of a reaction with respect

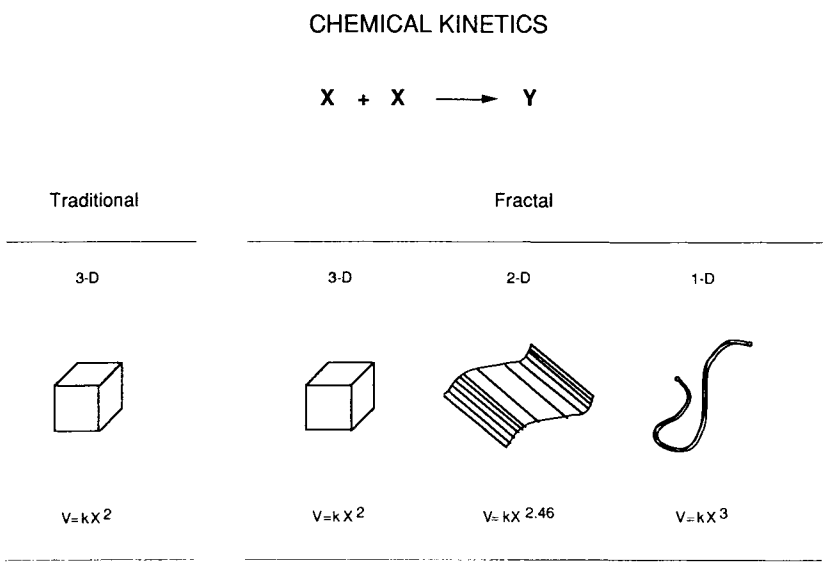


Figure 5. The kinetic order of a bimolecular reaction increases as the degree of dimensional restriction increases. In traditional chemical kinetics the kinetic order with respect to a given reactant is equal to the number of molecules of the reactant that participate in the reaction.

to a given reactant is a function of the geometry within which the reaction takes place. In the simplest case of reactions occurring in a homogeneous solution within a three-dimensional volume, the kinetic order is identical to the number of molecules entering into the reaction. When reactions occur on a two-dimensional surface, the kinetic order is larger, and when they are restricted to a one-dimensional channel, it becomes even larger still (Figure 5). For example, a bimolecular reaction in homogeneous solution has a kinetic order of 2, but the same reaction in a one-dimensional channel has a kinetic order of 3 (Kopelman, 1986).

In vivo, where reactions often occur on membranes or in channels, the kinetic order can be expected to exhibit noninteger values that are larger than the number of molecules entering into the reaction, and the forms of the rate laws will be different from those obtained by assuming homogeneous conditions.

Far From Thermodynamic Equilibrium

This is not a fundamental assumption, but rather one that is often made to simplify the characterization of an enzyme *in vitro*. However, any careful study of an enzymatic mechanism must include kinetic measurements done in the presence of all reactants and modifiers, including the products of the reaction. In such studies the reverse reactions cannot be ignored (Haldane, 1930; Alberty, 1959; Cleland,

1970). In practice, this requires a marked increase in the amount of experimental data necessary to characterize an enzyme kinetically (see above). There also may be increased difficulty in interpreting the kinetic analysis (see above). While many reactions may operate far from thermodynamic equilibrium *in vivo*, there also are examples of reactions that operate near equilibrium and actually reverse direction under physiological conditions. Thus, one generally cannot assume rate laws for irreversible reactions.

Relevance of Extreme Conditions

Extreme conditions are often used to reveal most clearly the distinctive qualitative features of a mechanism such as the order of reactions or their relative magnitudes. However, the quantitative behavior of the mechanism under such conditions may be quite different from the actual behavior under physiological conditions.

For the purposes of characterizing the rate of a reaction *in vivo* it would be much more useful to have kinetic measurements concentrated over the range of normal operation, and perhaps much of the relevant pathological range as well. Although systematically collected data relevant to this point are few, there are abundant data that characterize many biochemical and cellular variables in humans. These variables include biochemical concentrations and fluxes, cellular concentrations and turnover rates, and concentrations of therapeutic agents. The range of variation seen in a few major hospitals has been tabulated for each of these variables, e.g., see Orland and Saltman (1986) and Wallach (1986). For a wide variety of metabolites, the range can be small. For example, sodium and fasting glucose variations are no greater than 7% and 69%, respectively. Examples of metabolites that exhibit a larger range are aldosterone (16-fold) and 17-hydroxyprogesterone (30-fold). On average, the range is 3.9-fold for 160 variables. Of course, all these ranges may be considerably greater than one would find in the normal population, since they are biased toward the extremes in a clinical setting. More detailed data, including dynamic responses, from clinical studies gives the same general picture (Bondy and Rosenberg, 1980). Many metabolites have ranges around 2-fold, with hormones tending to have the highest normal ranges (typically 5-fold, but may be as high as 10- to 100-fold) as well as the highest pathological ranges (up to 10,000-fold for some tumors). The average range over a wide variety of metabolites is about 3- to 5-fold.

Although these data leave much to be desired, they are probably the best indication available of what normal and pathological ranges are likely to be. The data suggest that the appropriate range of variation to mimic steady-state kinetic behavior *in vivo* is typically an order of magnitude or less, and that kinetic behavior exhibited *in vitro*, when concentration variables are varied over more extreme

ranges, may not be relevant for characterizing the behavior of intact biochemical systems.

Optimal Conditions In Vitro and In Vivo

It is commonly assumed that enzymes are designed by natural selection for maximum molecular activity, and thus, that determining the conditions for maximal activity of an isolated enzyme *in vitro* will identify physiological values for the variables that affect it. However, this is not necessarily the case. The criteria for optimization of an isolated reaction (Albery and Knowles, 1977; Fersht, 1985; Kraut, 1988) are different from the criteria for optimization of the integrated biochemical system. Optimal behavior for the intact system may mean nonoptimal behavior for the isolated enzyme, and *vice versa.*

For example, stability of a simple biosynthetic pathway subject to end-product inhibition requires that a small number of reactions in the sequence have lower activities than the others, and those with lower activity determine the temporal behavior of the pathway. If the activity of these "temporally dominant" reactions is increased so that the kinetic properties of all the reactions in the sequence become more nearly comparable, then the system can oscillate in a dysfunctional manner—starving the organism for the end product in one phase and overproducing and wastefully excreting it in the next (Savageau, 1976). Hence, maximizing the activity of these temporally dominant reactions does not lead to optimization of the intact system, but just the reverse.

Similarly, the binding of a regulatory protein to its modulator site on the DNA is not designed to be maximum *in vivo*. Elevated binding of mutant *lac* repressor molecules to *lac* operator DNA *in vitro* was expected to be associated with lower basal expression of the *lac* operon *in vivo*. However, in some cases the behavior *in vivo* was just the opposite—partially constitutive expression. As von Hippel et al. (1974) showed, binding of the two elements in isolation is not the relevant event. *In vivo*, there are numerous other sites on the DNA to which the *lac* repressor binds, and the physiological behavior is determined in large part by the binding of *lac* operator DNA relative to that of nonspecific DNA. If a mutant repressor molecule has increased nonspecific binding to DNA, then the larger number of nonspecific sites will titrate the limited number of protein molecules away from the operator site and constitutive expression results.

There are numerous other examples where an increase in the activity of a single enzyme leads to degradation in the performance of the intact system—to the point of death in some cases. These examples make it clear that enzymes *in situ* operate with activities that are less than the maximum they can achieve by common mutations, or by nonphysiological conditions *in vitro*. Thus, the conditions that lead to maximal activity of an isolated enzyme *in vitro* may fortuitously identify the

physiological values for some variables that affect it, but one cannot expect this to be true in general.

Conclusions

From the results presented in this section, we conclude that the postulates of the Michaelis–Menten Formalism and the canons of good enzymological practice *in vitro* are not appropriate for characterizing the behavior of integrated biochemical systems. The very conditions that may have made it possible to identify important qualitative features of an enzymatic mechanism and produce a rate law *in vitro* tend to make the quantitative characterization of the reaction rate *in vivo* by this rate law invalid.

The limitations of the Michaelis–Menten Formalism encountered in this section arose in the context of what might be called a "bottom-up" approach. That is, in the reconstructionist phase of the reductionist program (Savageau, 1991a) one begins by putting back together the elements that have been characterized in isolation and attempting to reconstruct the behavior of the intact system. At this stage it becomes apparent that the conditions used to characterize the isolated elements may not correspond to the conditions that actually exist *in vivo*. In the next section we will approach the problem from another perspective, which might be termed the "top-down" approach, and introduce an alternative formalism. It will be seen that regular quantitative phenomena typical of the intact system are consistent with the alternative formalism, but not in general with the Michaelis–Menten Formalism.

THE POWER-LAW FORMALISM: A REPRESENTATION FOR INTACT BIOCHEMICAL SYSTEMS

Since there is little reliable data on the mathematical form of rate laws *in vivo*, we must look elsewhere for information that will guide us to an appropriate formalism for characterizing intact biochemical systems. The natural place to look is the behavior of the intact system itself. There is a large body of classical observations concerning growth and development that has revealed regular, quantitative relationships among the systemic variables of organizationally complex systems. These relationships consist of various growth laws and allometric properties (see below) among the constituents of the developing organism. Because no convincing explanation of these systemic relationships in terms of the underlying molecular elements was provided, these more physiological observations were not incorporated into the mainstream of modern biology, and with the ascendancy of molecular biology the focus became characterization of the molecular elements themselves. The time has now come to relate these complementary aspects of biology in a way that will give deeper meaning to both.

Systemic Behavior That Must be Accounted For

Growth laws and allometric relationships are among the most regular features of the systemic behavior of intact biochemical systems. Any formalism that is to be considered appropriate for understanding organizationally complex biochemical systems must be able to account for these regular, quantitative features. Awareness of and interest in these two manifestations of organizationally complex biological systems probably go back to the early hunters and gatherers who observed changes in the plant and animal populations upon which their existence critically depended.

Growth Laws

Data pertaining to growth can be found in the earliest recorded history. Exponential growth was recorded in tablets of baked clay about 4000 years ago by the early Babylonians. Inevitably, the accumulation of data led to the search for simple "laws of growth"—mathematical formulas that would provide a useful summary of large amounts of data and, by extrapolation, predictions concerning future growth. Often noted examples include linear, exponential, monomolecular, logistic, Bertalanffy, logarithmic, power law, hyperbolic, Weibull, stochastic, Gompertz, Monod, Teissier, Contois, and Lotka–Volterra growth laws. (For reviews and references to the original literature see Bertalanffy, 1960; Brody, 1964; Laird, 1969; Richards, 1969; Savageau, 1979a; Savageau and Voit, 1982.)

Allometric Morphogenesis

The development of form or pattern by differential growth among the component parts of a complex biological system appears to follow relatively simple rules in spite of the diversity of growth laws exhibited by intact systems. When one variable in a growing organism is plotted in a log–log plot against another, or against the total weight of the growing organism, one obtains a straight-line relationship. This is called an allometric relationship and the phenomenon is referred to as allometric growth (Huxley, 1932; Bertalanffy, 1960; Reiss, 1989). The astonishing fact is not that allometric relationships are found in many cases, but that a wide variety of phenomena is described by this simple law. Numerous examples can be found in the original work of Huxley (1924, 1932) and Teissier (1931, 1937), and since that time additional examples have been found among all the major groups of animals (Bertalanffy, 1951) and higher plants (Sinnott, 1963; Richards, 1969). Allometry has been found in studies of morphology, physiology, pharmacology, biochemistry, cytology, and evolution (see Bertalanffy, 1960). It also has been found in the etiology of certain diseases; e.g., coronary disease is related allometrically to the concentration of serum cholesterol (Keys et al., 1963). Needham (1950) has emphasized the widespread occurrence of allometry at the

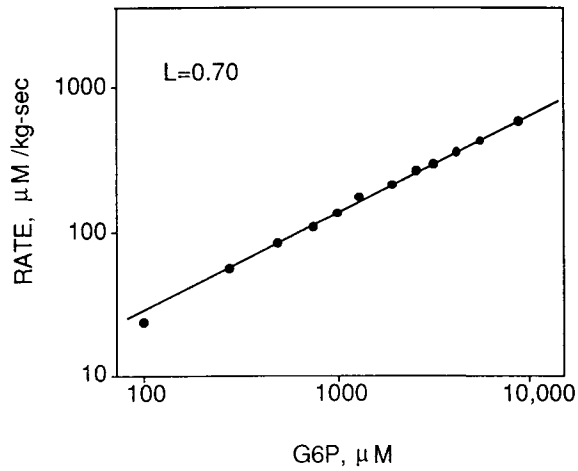


Figure 6. Rate of the glucose-6-phosphate dehydrogenase reaction as a function of the concentration of the substrate glucose-6-phosphate expressed in log–log coordinates. An isolated ascites cell (EL2) was impaled with a microelectrode, which then was used to electrophoretically inject known concentrations of the substrate into a localized region of the cell. The oxidation/reduction state of the cofactor to which the reaction is coupled was monitored by microspectrophotometric methods to determine the rate of the corresponding reaction *in situ*. (Replotted from the data of Kohen et al., 1973.)

chemical and biochemical levels. Some examples that illustrate the diversity of allometric relationships at various levels of organization in biological systems include activity of an individual enzyme (Figure 6), regulation of a single-gene circuit (Figure 7), signal transduction within an organ (Figure 8), and metabolite storage within an intact organism (Figure 9).

A Representation That Accounts for Systemic Behavior

The simplest representation of integrated biochemical systems known to account for the growth laws and allometric relationships is one that falls within the Power-Law Formalism (Savageau, 1979a,b). In this representation, the rates of formation and removal of each elemental component of the system are described by a product of power-law functions, one power function for each variable affecting the rate process in question (Savageau, 1969b, 1976). For example, the rate of synthesis of X_i *in vivo*, given by V_i, might be dependent upon the three variables X_1, X_2, X_3. The corresponding rate law would then be represented as follows:

$$V_i = \alpha_i X_1^{g_{i1}} X_2^{g_{i2}} X_3^{g_{i3}} \tag{28}$$

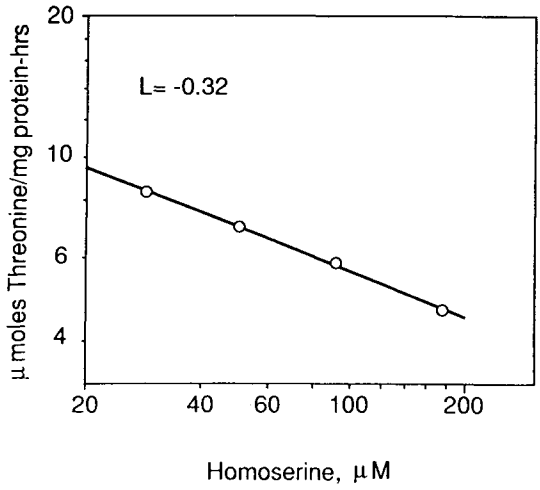


Figure 7. Logarithmic gain for the repressible threonine biosynthetic operon of *Escherichia coli.* An auxotrophic mutant of *E. coli* B unable to synthesize homoserine was grown for more than four generations in glucose minimal medium supplemented with various concentrations of homoserine (Savageau and Steward, 1970). At low concentrations of homoserine, a correspondingly low amount of threonyl-tRNA is produced, which leads to derepression of the threonine biosynthetic operon; at high concentrations homoserine (and threonyl-tRNA) the operon is repressed (Savageau, unpublished data).

Given this representation for the individual rate processes leading to the formation and removal of the system constituents, one can demonstrate that all the well known growth laws and allometric relationships follow by deduction (Savageau, 1979a,b). Thus, it has been proved that this representation is consistent with known systemic behavior.

This is not the case for the other two formalisms commonly used in biochemistry—the Linear Formalism and the Michaelis–Menten Formalism. The Linear Formalism implies linear relationships among the constituents of a system in quasi-steady state, which is inconsistent with the wealth of experimental evidence showing that these relationships are nonlinear in most cases. The case of the Michaelis–Menten Formalism is more problematic. An arbitrary system of reactions described by rational functions of the type associated with the Michaelis–Menten Formalism has no known solution in terms of elementary mathematical functions, so it is difficult to determine whether or not this formalism is consistent with the experimentally observed data. It is possible to deduce the systemic behavior of simple specific systems involving a few rational functions and find examples in which the elements do not exhibit allometric relationships. So, in

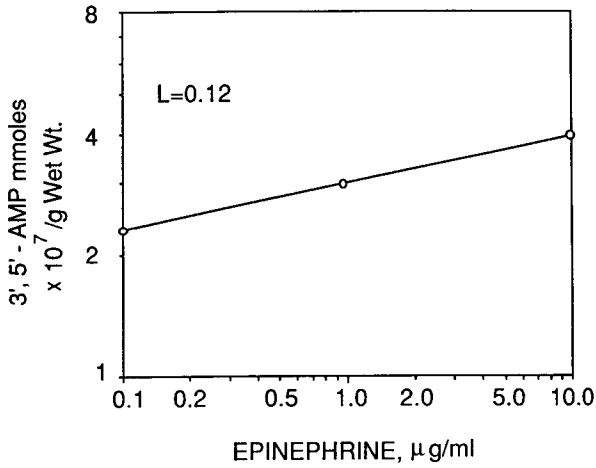


Figure 8. Logarithmic gain for the second messenger cAMP in the fat pad of the rat responding to perfusion with various concentrations of the hormone epinephrine (replotted from the data of Butcher et al., 1965).

general, the Michaelis–Menten Formalism is not consistent with these experimental data. As indicated above, there are other reasons to suspect that the Michaelis–Menten Formalism is inappropriate for representing the kinetics of reactions *in vivo*.

There may be other formalisms, yet to be proposed, that will prove to be consistent with the systemic behavior of integrated biochemical systems. However,

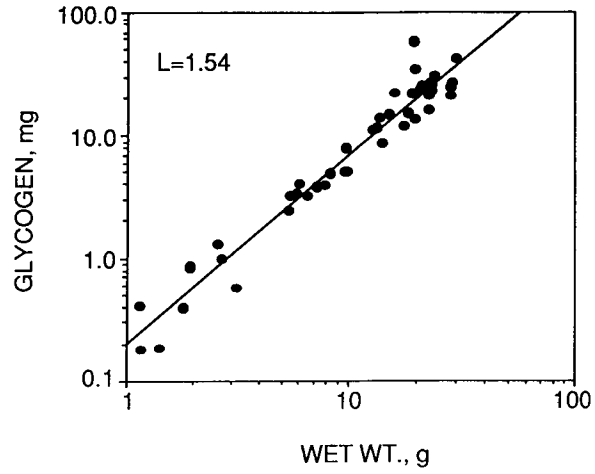


Figure 9. Allometric relationship between glycogen accumulation and growth of the chicken (replotted form the data of Wladimirow and Danilina, 1930).

at the present time, the Power-Law Formalism is the only one for which this has been demonstrated.

Characterization of Molecular Elements

The relatively simple form of the rate law in the Power-Law Formalism has several important implications for the characterization of the molecular elements of the system. In particular, there are known methods for estimating the kinetic parameters and the amount of data required for the estimation is minimal.

Methods of Parameter Estimation

As is clear from Eqn. (28), there are two kinds of parameters in the Power-Law Formalism: multiplicative and exponential. These are familiar from chemical and biochemical kinetics and are referred to as rate constants and kinetic orders, respectively.

Kinetic analysis of rate laws in the Power-Law Formalism is particularly simple and straightforward. By taking the logarithms of both sides of Eqn. (28) one can convert the product of power laws into a sum of terms involving logarithms of the variables.

$$\ln V_i = \ln \alpha_i + g_{i1} \ln X_1 + g_{i2} \ln X_2 + g_{i3} \ln X_3 \tag{29}$$

The kinetic parameters in this form of the rate law can be identified with the slopes (g_{i1}, g_{i2}, and g_{i3}) and intercept ($\ln \alpha_i$) in a linear coordinate system relating the logarithm of V_i to the logarithms of the X_i. Estimating the values for these kinetic parameters from appropriate experimental data is a solvable problem in linear-regression (see above). This is in sharp contrast to most other nonlinear formalisms for which there are no general methods that are practical for extracting kinetic parameters from experimental data (see above).

Amount of Data Required

A plot of $\ln V_i$ as a function of $\ln X_1$, while all other X's are held constant at their nominal values *in situ*, yields a straight line whose slope determines the exponential parameter g_{i1}. Given that there will always be a certain amount of experimental error associated with each assay, it will in general require about 10 data points to obtain a reasonably good estimate of the slope. Thus, we can conclude that $10n$ assays will be needed to estimate the kinetic parameters of the rate law in the Power-Law Formalism when there are n variables that influence the rate law under consideration.

This is substantially less data than are required in the more general situation involving nonlinear estimation of parameter values (see above). The difference is

$10n$ *vs* 10^n, which for $n = 8$, as in the case of glutamine synthetase, represents 80 assays *vs* 100 million assays. It might be possible to perform 80 assays in a day, but at the same rate it would require 3424 years to perform the 100 million.

Characterization of the Intact System

In the Power-Law Formalism each rate law is represented as a product of power-law functions [e.g., Eqn. (28)]. The fundamental equations governing the behavior of the intact biochemical system are Kirchhoff's flux equations, which are obtained by combining the rate laws for synthesis and degradation of each molecular constituent. There are a number of general strategies for combining the individual rate laws to obtain Kirchhoff's flux equations (see below); the simplest of these strategies allows one to write a local representation as

$$dX_i/dt = \alpha_i \prod_{j=1}^{n+m} X_j^{g_{ij}} - \beta_i \prod_{j=1}^{n+m} X_j^{h_{ij}} \qquad i = 1, \ldots, n \tag{30}$$

where n is the number of dependent concentration variables, m is the number of independent concentration variables, α_i and β_i are the rate constants, and g_{ij} and h_{ij} are the kinetic orders of biochemical kinetics. The parameters α_i and g_{ij} are associated with the rate law for aggregate synthesis of X_i, while β_i and h_{ij} are associated with the rate law for aggregate degradation of X_i. The system behavior, which corresponds to the solution of these differential equations, is represented by the variation of the dependent variables as a function of the independent variables and parameters of the system.

Steady-State Behavior

Concentrations and fluxes are constant in a steady-state, that is, their time derivatives are equal to zero. The nonlinear algebraic equations that result when the time derivatives are set equal to zero are transformed into a set of linear algebraic equations by taking logarithms (Savageau, 1969b). The explicit steady-state solution of these algebraic equations is obtained by standard methods, which give the values of each dependent concentration and flux in terms of the independent concentrations and parameters of the system (Savageau, 1971, 1976). This explicit solution provides a complete characterization of the steady-state behavior about any operating point of a biochemical system (e.g., see Sorribas and Savageau, 1989a,b,c). Numerical results also can be obtained by a single command from within ESSYNS, an integrated software package for the evaluation and simulation of synergistic systems (Voit et al., 1989).

Although the explicit steady-state solution provides all the available information about the steady-state behavior of the system, it also is useful to characterize the

system in terms of standard factors that relate its global properties to its underlying molecular determinants; namely, gain or amplification factors (Bode, 1945; Mason, 1953; Truxal, 1955; Dorf, 1974), and parameter sensitivities (Bode, 1945; Mason, 1953; Truxal, 1955; Cruz, 1973). These factors that characterize the systemic response to change in specific independent concentrations or parameters can be determined readily from the explicit steady-state solution (see below).

Existence

The existence of a steady-state is governed by a simple determinant condition among the kinetic orders of the system (Savageau, 1969b; Savageau et al., 1987). If the determinant of kinetic orders is zero there is no steady-state solution; if it is non-zero then a steady-state solution exists. In addition to simple existence, the steady-state solution obtained mathematically must be consistent with the corresponding state of the system that is experimentally measured. When both these conditions are met, one can proceed to additional tests of local equivalence and consistency.

Local Stability

The local stability of a steady-state can be ascertained by an examination of the eigen values for the local representation of the system (Savageau, 1976); these eigen values also can be obtained by a single command from within ESSYNS (Voit et al., 1989). If the real parts of all the eigen values are negative, then the steady-state is locally stable, and the system will return to the steady-state following small perturbations. This condition also is required for the consistency of any model that represents a stable-steady state.

Robustness

System sensitivity theory (Bode, 1945; Cruz, 1973) provides important methods for characterizing the quality of a model. *In situ*, the effect of a small change in any parameter will propagate through the system, with gain or attenuation determined by the kinetic orders (Savageau, 1971, 1976; Sorribas and Savageau, 1989c), and in principle will influence all the dependent variables. In a good model, these changes will not be amplified, i.e., the sensitivities typically will be small, and the model will continue to exhibit essentially the same structure and behavior.

The relative values of the parameter sensitivities allow one to identify those parameters that must be scrutinized most carefully in experimental studies. A relatively high sensitivity to certain parameters indicates that error in the experimental determination of these parameters will influence the accuracy of the

representation to a greater extent than comparable error in the determination of other parameters in the system [e.g., see Okamoto and Savageau (1984)].

There are two types of fundamental parameters in the Power-Law Formalism, rate constants and kinetic orders; the definitions of sensitivity with respect to changes in these parameters are summarized below. A full discussion of the relationships among these sensitivities is given in Savageau and Sorribas (1989).

Rate-Constant Sensitivities—The ratio of relative change in a dependent concentration X_i to relative change in a rate-constant parameter, be it an increase in a β_j or a decrease in an α_j, can be determined by differentiation of the explicit steady-state solution. In other words,

$$S(X_i,\beta_j) = (\partial X_i/\partial\beta_j)(\beta_j/X_i) = \partial(\ln X_i)/\partial(\ln \beta_j) \tag{31}$$

$$S(X_i,\alpha_j) = (\partial X_i/\partial\alpha_j)(\alpha_j/X_i) = \partial(\ln X_i)/\partial(\ln \alpha_j) \tag{32}$$

Similarly, the ratio of relative change in a dependent flux to relative change in a rate-constant parameter is defined as:

$$S(V_i,\beta_j) = (\partial V_i/\partial\beta_j)(\beta_j/V_i) = \partial(\ln V_i)/\partial(\ln \beta_j) \tag{33}$$

$$S(V_i,\alpha_j) = (\partial V_i/\partial\alpha_j)(\alpha_j/V_i) = \partial(\ln V_i)/\partial(\ln \alpha_j) \tag{34}$$

These sensitivities are readily obtained by applying the chain rule of differentiation to the explicit steady-state solution. Note that aggregate influx and aggregate effflux are equal in steady-state, so only the sensitivities with respect to aggregate influx need be given.

Kinetic-Order Sensitivities—The ratio of relative change in a dependent concentration X_i to relative change in a kinetic-order parameter, g_{jk} or h_{jk}, can be determined by differentiation of the explicit solution with respect to the parameter in question.

$$S(X_i,h_{jk}) = (\partial X_i/\partial h_{jk})(h_{jk}/X_i) = \partial(\ln X_i)/\partial(\ln h_{jk}) \tag{35}$$

$$S(X_i,g_{jk}) = (\partial X_i/\partial g_{jk})(g_{jk}/X_i) = \partial(\ln X_i)/\partial(\ln g_{jk}) \tag{36}$$

The ratio of relative change in a dependent flux to relative change in a kinetic order is defined in a similar fashion.

$$S(V_i,h_{jk}) = (\partial V_i/\partial h_{jk})(h_{jk}/V_i) = \partial(\ln V_i)/\partial(\ln h_{jk}) \tag{37}$$

$$S(V_i,g_{jk}) = (\partial V_i/\partial g_{jk})(g_{jk}/V_i) = \partial(\ln V_i)/\partial(\ln g_{jk}) \tag{38}$$

Again, these sensitivities are obtained by appropriate differentiation, and only the influxes need to be considered in steady-state.

A sensitivity with a magnitude greater than one implies amplification of the original alteration; a magnitude less than one indicates attenuation. A positive sign for the sensitivity indicates that the changes are in the same direction, both increase in value or both decrease. A negative sign indicates that the changes are in the opposite direction.

Responsiveness

When environmental constituents (independent variables) are changed in concentration, these changes are detected and propagated throughout the cell. Various cellular processes (dependent variables) are influenced to one degree or another. The extent to which such signals are amplified or attenuated as they are propagated throughout the cell is conventionally represented by a set of gain factors. In the Power-Law Formalism the variables are more naturally represented as the logarithms of concentrations, and thus these gain factors are defined as *logarithmic gain*. These are the principal systemic properties in the steady-state that can be measured experimentally.

The logarithmic gain factors are characterized by the percentage change of a dependent concentration X_i in going from one steady-state to another as the result of a 1% increase in an independent concentration X_k, while all other independent concentrations and parameters are held constant. These factors also can be determined mathematically by differentiation of the explicit steady-state solution.

$$L(X_i,X_j) = (\partial X_i/\partial X_j)(X_j/X_i) = \partial(\ln X_i)/\partial(\ln X_j) = \partial y_i/\partial y_j = L_{ij}$$

$$i = 1, \ldots, n; \quad j = n+1, \ldots, n+m \tag{39}$$

They are analogous to conventional gain or amplification factors in established network theories (Savageau and Sorribas, 1989).

In a similar fashion, one can obtain the logarithmic gains in the dependent fluxes through the pools of the system.

$$L(V_i,X_j) = (\partial V_i/\partial X_j)(X_j/V_i) = \partial(\ln V_i)/\partial(\ln X_j)$$

$$i = 1, \ldots, n; \quad j = n+1, \ldots, n+m \tag{40}$$

A logarithmic gain with a magnitude greater than one implies amplification of the original signal; a magnitude less than one indicates attenuation. A positive sign for the logarithmic gain indicates that the changes are in the same direction, both increase in value or both decrease. A negative sign indicates that the changes are in the opposite direction.

The logarithmic gain between a given independent and dependent variable is not determined by any one component, or even by a small number of components, in the system. Rather it is a systemic property that in general depends upon all of the components and their specific organization or design. These systemic factors,

which can be determined directly from physiological measurements on the intact system, are functions only of the kinetic orders, which characterize the molecular elements of the system; hence, they are useful for relating molecular and systemic behavior (Savageau, 1971).

Dynamic Behavior

The dynamic behavior of the intact system is characterized by the solution of Eqn. (30). In some cases this can be obtained as an explicit solution in terms of elementary mathematical functions (e.g., Voit and Savageau, 1984). However, in most instances there is no solution of this type, and one must rely on computer-generated solutions in which particular numbers for rate constants, kinetic orders, and initial values of the concentration variables must be specified. Even if some of these numbers are unknown for a particular system, one can explore the potential repertoire of dynamic behavior by systematically varying the values of the parameters and solving the resulting equations for the system (e.g., see Irvine and Savageau, 1985a,b).

By solving the differential equations [Eqn. (30)] one readily can predict the dynamic consequences at the system level for a particular constellation of independent variables, rate constants, and kinetic orders (Savageau, 1970, 1976). However, one cannot in general do the inverse and predict kinetic orders and rate constants that correspond to a given set of dynamic behaviors in the intact system. In any case, there now are efficient numerical methods to examine the dynamic behavior of biochemical systems (Voit et al., 1989; Irvine and Savageau, 1990), and these have been used in a variety of applications (briefly reviewed in Sorribas and Savageau, 1989a). For more information, the interested reader can consult the references given above.

TCA Cycle in *Dictyostelium*: A Case Study

A recent investigation of the current model for the tricarboxylic acid cycle in *Dictyostelium discoideum* may be viewed as a case study that illustrates the uses of local representation within the Power-Law Formalism (Shiraishi and Savageau, 1992a,b,c,d; 1993). First, it demonstrates that systemic analysis is a powerful tool to evaluate the quality of biochemical models especially those representing the function of a complex system *in vivo*. Second, it demonstrates that systemic analysis allows one to diagnose deficiencies and to predict modifications that are likely to improve the model.

It should be emphasized that a complete and accurate model is not required to begin this type of analysis. For the purposes of this case study, it is irrelevant that the model chosen (Wright et al., 1992) might not be the best available model. Indeed, all models are approximations and they will invariably contain errors or

inaccurate representations. The more important issue is whether or not these errors or inaccuracies have a significant influence on the predicted behavior of the intact system. Some will have negligible influence and these need not overly concern us; others will have major effects and these must be corrected if we are to improve our understanding and develop better models.

Let us now turn to the discussion of the specific results. Evaluation of consistency and robustness (Shiraishi and Savageau, 1992b) demonstrates that the current model of the tricarboxylic acid cycle (Wright et al., 1992) is self consistent and possesses a steady-state that is in agreement with experimental evidence. However, the results also suggest that this model is not very robust. High sensitivities for the parameters influencing pyruvate metabolism indicate that the experimental characterization of these reactions might be fruitfully re-examined. These high sensitivities led us to predict that this model of the tricarboxylic acid cycle should be accurate only over a very narrow range in variation of the independent variables.

Analysis of steady-state and dynamic behavior (Shiraishi and Savageau, 1992c) revealed additional anomalies in the current model. The logarithmic gains, which characterize the propagation of biochemical signals through the network, show that changes in certain independent variables are amplified to an unusually high degree. For example, the concentrations of malic enzyme, malate dehydrogenase, and NAD^+ have the greatest influence on pyruvate metabolism. The tricarboxylic acid cycle, according to the current model, was found to be poised on a knife edge with its behavior rigidly determined; any alteration of the system's components leads to nonviable behavior, as exemplified by loss of steady-state and an explosive accumulation of pyruvate in response to a minute change in the level of malate dehydrogenase. The dynamic analysis, which suggested an excessively long response time for the tricarboxylic acid cycle, provided another indication that this model may be inadequate. The turn-over times for the pools of the model pointed to alanine, glutamate, and aspartate as the likely contributors to the long response time. The analysis in this paper led us to propose that the model for the tricarboxylic acid cycle in *Dictyostelium* should be experimentally re-examined in three areas. (1) The distribution of flux at the malate branch point is critical, and thus the kinetics of the enzymes at the branch point should be carefully examined. (2) The pool sizes for alanine, glutamate, and aspartate seem to be anomalously large relative to the flux through them. This may be due to the neglect of amino acid reutilization for protein synthesis. (3) The cofactors, particularly NAD^+, have a strong influence on the behavior of the system, so their values should not be arbitrarily fixed. Allowing these metabolites to be dependent variables would enhance the homeostatic character of the model and make it more realistic.

In an attempt to remedy these problems, we considered a minimal modification in the previous model; namely, the inclusion of reactions for the reutilization of amino acids for protein synthesis (Shiraishi and Savageau, 1993). This modification has the potential to alleviate several of the problems, and there is a sound physi-

ological basis for recommending it. These reactions add flexibility to the distribution of flux at the malate branch point, allow additional routes for the escape of excess material from the pyruvate branch, diminish the turnover times for alanine, glutamate and aspartate, and, of course, account for the reutilization of amino acids for protein synthesis during development of the organism. The results from analysis of the modified model showed that these expectations were fulfilled to a large extent. The robustness of the modified model was considerably improved over that of the previous model; parameter sensitivities declined by approximately two orders of magnitude. Also, the locus of the most sensitive parameters became more dispersed throughout the model, which indicates that the problems previously centered on the metabolism of pyruvate were at least partially alleviated. The results of the steady-state analysis led to the same conclusion; the distribution of flux at the malate branch-point, and indeed throughout the model, is no longer rigidly fixed. The results of the dynamic analysis showed that the turnover times for the pools of alanine, glutamate, and aspartate are reduced by a factor of 107 and made more physiologically realistic. Thus, minimal modification of the previous model led to several improvements that are appropriate for a model of the tricarboxylic acid cycle in *Dictyostelium*.

Generality of the Power-Law Formalism

To what degree do actual systems in nature conform to the Power-Law Formalism? We can consider this issue from three different perspectives: *local representation*, *fundamental representation*, and *recast representation* (Savageau, 1995).

Local Representation

When the Power-Law Formalism is used to generate a local representation, the result is guaranteed to be accurate for small variations about the nominal operating values of the concentration variables. Among the several alternatives that exist within this approach, the Synergistic- or S-system representation [Eqn. (30)] generally exhibits the widest range of accurate representation (Sorribas and Savageau, 1989c; Savageau, 1991b). The simplicity of the local S-system representation has led to the most extensive development of theory, methodology, and applications within the Power-Law Formalism (Voit, 1991). Indeed, the S-system representation allows the derivation of important systemic properties that would be difficult if not impossible to deduce by other means. For these reasons, we have emphasized the local representation in the previous sections.

Over how wide a range of variation in the system variables does the power-law representation and the behavior of the observed system agree within experimental error? If the representation is to be useful, the range over which there is agreement

should be comparable to the range of variation actually exhibited by the variables of the system *in vivo*. As discussed above, there are clinical data for humans suggesting that the average range of variation for concentrations and fluxes *in vivo* is between 3- and 5-fold. It would be desirable to develop power-law representations for these systems and to determine the range of variation over which there is agreement with the actual data. This has not been done, nor is it likely to be done for some time because of the technical difficulties. However, there is other evidence suggesting that the Power-Law Formalism is sufficiently accurate to provide an appropriate representation for the integrated behavior of complex biochemical systems.

A variety of model systems has been constructed with specific mathematical functions for their rate laws. When the behavior of these model systems is compared with the behavior of the corresponding power-law representation one finds agreement over a wide range of variation in the independent variables. Examination of isolated processes has shown that the minimum range of accurate representation is about two-fold (Savageau, 1969b; Roels, 1983; Voit and Savageau, 1987). Comparisons involving the integrated behavior of more complex systems are even more relevant. For example, consider the model system involving enzyme–enzyme interactions that was analyzed in detail by Sorribas and Savageau (1989a). The range of accurate representation in this case varied from a minimum of about two-fold to a maximum greater than 90-fold, with an average range of 20-fold.

A more direct way to address the question of accuracy is to measure logarithmic gains experimentally over a wide range of variation in steady-states. Since the Power-Law Formalism implies that these logarithmic gains will be straight-line relationships in a log–log plot, one can take the points of departure from the straight-line relationship at the high- and low-end to mark the extremes for the valid range of representation. By this measure, evidence from intact systems that have been driven experimentally beyond their physiological range often shows an even wider range, as large as 100- to 1000-fold, with accurate representation by the Power-Law Formalism (see Figures 6–9). Reasons for the increased accuracy within intact systems are discussed in Savageau (1976; 1985) and Voit and Savageau (1987). This degree of accuracy with power-law representations is considerably greater than that with linear representations, which is typically measured in percentage rather than fold variation.

The local representation is usually the simplest, and in many cases it is entirely sufficient. However, should it prove a limitation in any particular case, one can develop either a *fundamental representation* or a *recast representation*. This will make for a more complex description at the system level, but, by doing so, one can improve the accuracy of representation.

Fundamental Representation

There are a number of representations that are considered fundamental descriptions of the basic entities in various fields. The Mass-Action representation and the Michaelis–Menten representation provide two common examples. It has been demonstrated (Savageau, 1995) that these are, in fact, restricted special cases of the Generalized-Mass-Action (GMA) representation,

$$dX_i/dt = \sum_{k=1}^{r} \alpha_{ik} \prod_{j=1}^{n+m} X_j^{g_{ijk}} - \sum_{k=1}^{r} \beta_{ik} \prod_{j=1}^{n+m} X_j^{h_{ijk}} \qquad i = 1, \ldots, n \tag{41}$$

which is one of the most common representations within the general framework of the Power-Law Formalism (Savageau, 1991b).

The Mass-Action representation is clearly a special case of the GMA representation in which all exponents are positive integers. The Michaelis–Menten representation is, in turn, a special case of the traditional Mass-Action representation in which two important restrictions have been imposed (Savageau, 1992). First, it is assumed that the mechanism is in quasi-steady state. The derivatives of the dependent state variables in the Mass-Action Formalism can then be set to zero, thereby reducing the description from differential equations to algebraic equations. Second, it is assumed that complexes do not occur between different forms of an enzyme or between different enzymes. The algebraic equations will then be linear in the concentrations of the various enzyme forms, and one can derive the rational function that is the representation of the rate law within the Michaelis–Menten Formalism.

Each of the formalisms considered in this subsection—Mass-Action and Michaelis–Menten—is able to serve as the foundation for representing diverse phenomena, but each also has known limitations. The Power-Law Formalism may be considered more fundamental than either of these because it includes them as special cases and is not subject to their limitations.

Recast Representation

Although the Power-Law Formalism was originally developed from the notion of a local representation in logarithmic space (Savageau, 1969b), it was subsequently discovered that nearly any nonlinear function or set of differential equations can be transformed *exactly* into the Power-Law Formalism (Savageau and Voit, 1987). Thus, this formalism provides a canonical nonlinear representation for most nonlinear functions. An example will make this clear.

Consider the nonlinear differential equations that describe growth of bacteria in a chemostat:

$$dS/dt = D\,S_0 - D\,S - (\mu/Y)\,B\,S/(S + K) \tag{42}$$

$$dB/dt = \mu\, B\, S/(S + K) - D\, B \tag{43}$$

S_0 is the constant concentration of growth substrate that is added at flow rate D to the chemostat. S and B are the concentrations of growth substrate and bacteria in the chemostat, respectively. K is the Monod constant, which is formally identical to the Michaelis constant of enzyme kinetics, μ is the maximal growth rate constant, and Y is the amount of bacterial cells produced from a given amount of substrate (yield).

First, define new variables as follows: $X_1 = S$, $X_2 = B$, $X_3 = S + K$, $X_4 = S_0 - S$. Then differentiate these variables and rewrite Eqns. (42) and (43) to obtain

$$dX_1/dt = \alpha_1 X_4 - \beta_1 X_1 X_2 X_3^{-1} \tag{44}$$

$$dX_2/dt = \alpha_2 X_1 X_2 X_3^{-1} - \alpha_1 X_2 \tag{45}$$

$$dX_3/dt = \alpha_1 X_4 - \beta_1 X_1 X_2 X_3^{-1} \tag{46}$$

$$dX_4/dt = \beta_1 X_1 X_2 X_3^{-1} - \alpha_1 X_4 \tag{47}$$

where the initial conditions are given by $X_1(0) = S(0)$, $X_2(0) = B(0)$, $X_3(0) = S(0) + K$, and $X_4(0) = S_0 - S(0)$. These equations are now in the S-system representation within the Power-Law Formalism. They are an exact recasting of Eqns. (42) and (43), and their solution produces exactly the same results as the solution of the equations in their original form.

Recasting produces equations with nonlinear forms that are simpler than those of the original equations. Furthermore, the simpler form is canonical, which implies that methods developed to efficiently solve equations having this form will be applicable to a wide class of phenomena. This provides a powerful stimulus to search for such methods. An example of what can be done along these lines is the efficient algorithm developed for solving differential equations represented in the canonical Power-Law form (Irvine and Savageau, 1990). This algorithm, when combined with recasting, can be used to obtain solutions for rather arbitrary nonlinear differential equations, and this canonical approach yields solutions in shorter time, with greater accuracy and with greater reliability than is typically possible with other methods.

Conclusions

As a local representation, the S-system variant within the Power-Law Formalism is more accurate than the GMA variant, which is generally more accurate than the conventional Linear Formalism. As a fundamental representation, the Power-Law Formalism includes as special cases the Mass-Action, Michaelis–Menten, and Linear Formalisms that are considered to accurately represent natural phenomena

within certain domains. As a recast representation, the Power-Law Formalism is capable of representing in an *exact* global fashion nearly any nonlinear function or system of equations. Thus, from a variety of perspectives, the Power-Law Formalism can be considered a canonical nonlinear formalism that is perhaps uniquely suited for the analysis of biological systems.

SUMMARY

The success of the enzymologist's test tube as both symbol and tool of modern biology is unquestioned. If it were not for the reductionist approach that it symbolizes, our understanding of living organisms would not be what it is today; it would have remained a much more superficial and descriptive kind of understanding without the knowledge of underlying mechanisms that this tool has provided. However, there is evidence in nearly every field that the reductionist approach alone is not sufficient to develop a deep understanding of all relevant phenomena. This is perhaps most evident when one considers organizationally complex biochemical systems. There is need of a rigorous integrative approach that will unify our knowledge of the molecular elements and extend our understanding of the intact system.

Enzyme kinetics, which is arguably the most quantitative methodology associated with the use of the test tube, provides the common ground where reductionist and integrative approaches meet. This chapter has examined in some detail these two complementary uses of kinetics.

The traditional and most successful use to date has been the elucidation of mechanisms for isolated reactions, and for this the Michaelis–Menten Formalism is the accepted paradigm. The major postulates and corresponding practices that have evolved to ensure the success of this program were described above. The purpose was to make explicit what is often tacitly assumed by textbook writers and practicing kineticists. Although there are numerous abuses of classical enzyme kinetics that can be documented when these issues are overlooked, it is sufficient for this part of the critique to note that when the canons of good practice are followed, one tends to obtain valid results. The more problematic implications of these classical assumptions are manifested when one attempts to transfer knowledge from the context of the test tube to that of the living system.

The use of kinetics to characterize the behavior of integrated biochemical systems is a more recent and less developed practice. One of the more important issues in this integrative context is the selection of an appropriate formal representation. The most common approach is simply to adopt the Michaelis–Menten Formalism that has served so well for the elucidation of isolated reaction mechanisms. However, as the discussion above showed, there are difficulties in estimating the parameters of this formalism in general, and there is a combinatorial explosion in the amount of data required to characterize the rate law by kinetic means. Thus, even if the Michaelis–Menten Formalism were appropriate in principle, there

would be severe practical difficulties with using it to characterize the integrated behavior of complex biochemical systems.

This problem is analogous to describing the behavior of a gas using Newton's laws of motion for the individual molecules and keeping track of all their trajectories (i.e., a microscopic approach). It can be conceptualized, but in practice it is impossible. It is much more fruitful to characterize such a system in terms of the simple gas laws of thermodynamics (i.e., a macroscopic approach). The key issue is selection of an appropriate representation for the system.

At some level all of our representations in science are approximations. It is important to acknowledge this fact, to seek appropriate approximations for the conditions of interest, and to recognize the limits of our approximations. The characterization of enzyme-catalyzed reactions within the Michaelis–Menten Formalism should be seen in this light. Indeed, some authors have clearly recognized this point. As Webb (1963) has noted, "Many important enzyme reactions are being found to be more complex than those systems upon which the [Michaelis–Menten] theory was based, and the complications ... make it evident that the basic Michaelis–Menten equation has only a limited range of applicability." However, there are several reasons why Michaelis–Menten kinetics are taken by many to be reality itself, despite evidence suggesting that few, if any, enzymes actually fit it well when examined carefully (Hill et al., 1977). In large part this has to do with the traditional approach to teaching enzyme kinetics. In most textbooks the subject is presented in an oversimplified dogmatic fashion, often justified by decrying the lack of mathematical ability on the part of the students and appealing to pedagogical requirements for simplification of the subject matter. As an antidote to this approach, which he considers responsible for numerous errors and misconceptions found in chemistry (and biochemistry) textbooks, Ross (1988) quotes Einstein: "We should make things as simple as possible, but not simpler".

If the limitations of the Michaelis–Menten Formalism were based only on these practical issues, one might argue that it could be used at least for those enzymes (and there are many) that it seems to fit reasonably well *in vitro*, while leaving the others to be dealt with on an *ad hoc* basis. However, there is a more fundamental critique (see above) demonstrating that the postulates of the Michaelis–Menten Formalism and the canons of good enzymological practice *in vitro*, which serve so well for the elucidation of isolated reaction mechanisms, are not appropriate for characterizing the behavior of integrated biochemical systems. Formalisms other than the Michaelis–Menten must be considered for this integrative purpose.

The Power-Law Formalism described above provides an attractive alternative. In this formalism, the rates of formation and removal of each elemental component of the system are described by a product of power-law functions, one power function for each variable affecting the rate process in question. These elemental rate laws can be combined according to several general strategies to yield a description of the intact system. The strategy leading to the local S-system repre-

sentation has received the most attention to date. Given this representation, it has been demonstrated that all the well known growth laws and allometric relationships follow by deduction, and, thus, that the Power-Law Formalism is consistent with known systemic behavior.

This is not the case for the other two formalisms commonly used in biochemistry—the Linear Formalism and the Michaelis–Menten Formalism. The Linear Formalism implies linear relationships among the constituents of a system in quasi-steady state, which is inconsistent with the wealth of experimental evidence showing that these relationships are nonlinear in most cases. The Michaelis–Menten Formalism has no known solution in terms of elementary mathematical functions, so it is difficult to determine the extent to which this formalism is consistent with the experimentally observed data. However, it is possible to deduce the systemic behavior of simple specific systems involving a few rational functions and find examples in which the elements do not exhibit allometric relationships.

The Power-Law Formalism possesses a number of advantages that recommend it for the analysis of integrated biochemical systems. As discussed above, we saw that estimation of the kinetic parameters that characterize the molecular elements of a system in this representation reduces to the straightforward task of linear regression. Furthermore, the experimental data necessary for this estimation increase only as the number of interactions, not as an exponential function of the number of interactions, as is the case in other formalisms. The mathematical tractability of the local S-system representation is evident in the characterization of the intact system and in the ease with which the systemic behavior can be related to the underlying molecular determinants of the system (see above). Indeed, the mathematical tractability of this representation is the very feature that allowed proof of its consistency with experimentally observed growth laws and allometric relationships. It also allowed the diagnoses of deficiencies in the current model of the TCA cycle in *Dictostelium* and the prediction of modifications that led to an improved model (see above).

These considerations demonstrate that the local S-system representation is an appropriate means to characterize integrated biochemical systems. However, if we are to use this representation intelligently, we must be aware of its valid range of application and of the signs that indicate when this range has been exceeded. This issue is inextricably linked to the quantitative question of accuracy that was considered earlier. The range of accurate representation for well-defined model systems varies from a minimum of about two-fold to a maximum greater than 90-fold, with an average range of 20-fold. Similar results have been obtained from direct measurement of logarithmic gains in the intact system, but in this case ranges as large as 1000-fold have been observed.

The range of accurate representation provided by the local S-system representation is broad enough to encompass the typical physiological range of variation seen in humans, and perhaps much of the relevant pathological range as well (see

above). Many metabolites have ranges of variation around 2-fold, with hormones tending to have the highest normal ranges (typically 5-fold, but may be as high as 10- to 100-fold) as well as the highest pathological ranges (up to 10,000-fold for some tumors). The average range of variation over a wide variety of metabolites is about 3- to 5-fold. It can be concluded that the actual ranges of variation and the ranges of accurate representation are roughly the same.

The Power-Law Formalism provides additional strategies for representing systems that require a higher degree of accuracy (see above). The fundamental representation within the Power-Law Formalism employs products of power-law functions to represent elemental chemical reactions as the basic components of the system. The result is a more general representation that includes Mass-Action and Michaelis–Menten representations as special cases and, hence, is at least as accurate as these. The recast representation within the Power-Law Formalism is more general still and can describe in a mathematically exact fashion essentially any nonlinear function of interest. This representation is produced through a series of well defined transformations in which the original function is systematically recast into an equivalent expression in the Power-Law Formalism. The result is a canonical nonlinear form that facilitates the development and application of efficient methods for mathematical analysis.

Critical studies of intact biochemical systems will require the formulation of realistic models, the measurement of relevant variables *in situ*, and the ability to relate these by means of an appropriate mathematical formalism. A variety of existing approaches provide information relevant to the formulation of biochemical models. The enzymologist's test tube will continue to provide an indispensable tool in this regard. New tools that will facilitate the measurement of biochemical variables *in situ*, in a nondestructive fashion, will become increasingly important for characterizing systemic behavior. However, the key to understanding the integrated temporal, spatial and functional behavior of complex biochemical systems will be the development of mathematical formalisms that allow us to relate the molecular and systemic behavior in a deep quantitative manner. The new symbol to represent this emerging paradigm and the indispensable tool for its elaboration will undoubtedly be the integrative biologist's computer. Let us hope that it will serve as long and as well as the enzymologist's test tube.

REFERENCES

Alberty, R. A. (1959). The rate equation for an enzymatic reaction. In: The Enzymes (Boyer, P. D., Lardy, H., & Myrback, K., eds.), Vol. I, 2nd Ed., pp. 143–155, Academic Press, New York.

Albery, W. J. & Knowles, J. R. (1977). Efficiency and evolution of enzyme catalysis. Angew. Chem. Int. Ed. Engl. 16, 285–293.

Bardsley, W. G. & McGinlay, P. B. (1989). Optimal design for model discrimination using the F-test with non-linear biochemical models. Criteria for choosing the number and spacing of experimental points. J. Theoret. Biol. 139, 85–102.

Barker, S. A. & Somers, P. J. (1978). Biotechnology of immobilized multienzyme systems. Adv. Biochem. Eng. 10, 27–49.

Benson, S. W. (1960). The Foundations of Chemical Kinetics, p. 25, McGraw-Hill, N.Y.

Bertalanffy, L. von (1951). Theoretische Biologie, 2nd Ed., Francke, Bern, Switzerland.

Benalanffy, L. von (1960). Principles and theory of growth. In: Fundamental Aspects of Normal and Malignant Growth (Nowinski, W. W., ed.), pp. 137–259, Elsevier, N.Y.

Bloomfield, V., Peller, L., & Alberty, R. A. (1962a). Multiple intermediates in steady-state enzyme kinetics II. Systems involving two reactants and two products. J. Amer. Chem. Soc. 84, 4367–4374.

Bloomfield, V., Peller, L., & Alberty, R. A. (1962b). Multiple intermediates in steady-state enzyme kinetics III. Analysis of the kinetics of some reactions catalyzed by dehydrogenases. J. Amer. Chem. Soc. 84, 4375–4384.

Bode, H. W. (1945). Network Analysis and Feedback Amplifier Design, Van Nostrand, Princeton, New Jersey.

Bondy, P. K. & Rosenberg, L. E. (1980). Metabolic Control and Disease, 8th ed., Saunders, Philadelphia.

Briggs, G. E. & Haldane, J. B. S. (1925). A note on the kinetics of enzyme action. Biochem. J. 19, 338–339.

Brody, S. (1964). Bioenergetics and Growth, Hafner, N.Y.

Brown, A. J. (1902). Enzyme-action. J. Chem. Soc. 81, 373–388.

Butcher, R. W., Ho, R. J., Meng, H. C., & Sutherland, E. W. (1965). Adenosine 3′,5′-monophosphate in biological materials. II. The measurement of adenosine 3′,5′-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine. J. Biol. Chem. 240, 4515–4523.

Clegg, J. S. (1984). Properties and metabolism of the aqueous cytoplasm and its boundaries. Am. J. Physiol. 246, R133–R151.

Cleland, W. W. (1963a). The kinetics of enzyme-catalyzed reactions with two or more substrates or products I. Nomenclature and rate equations. Biochim. Biophys. Acta 67, 104–137.

Cleland, W. W. (1963b). The kinetics of enzyme-catalyzed reactions with two or more substrates or products II. Inhibition: nomenclature and theory. Biochim. Biophys. Acta 67, 173–187.

Cleland, W. W. (1963c). The kinetics of enzyme-catalyzed reactions with two or more substrates or products III. Prediction of initial velocity and inhibition patterns by inspection. Biochim. Biophys. Acta 67, 188–196.

Cleland, W. W. (1967a). The statistical analysis of enzyme kinetic data. Advan. Enzymol. 29, 1–32.

Cleland, W. W. (1967b). Enzyme kinetics. Ann. Rev. Biochem. 36, 77–112.

Cleland, W. W. (1970). Steady state kinetics. In: The Enzymes (Boyer, P. D., ed.), Vol II, 3rd Ed., pp. 1–65, Academic Press, New York.

Cruz, J. B., ed. (1973). System Sensitivity Analysis, Dowden, Hutchinson and Ross, Stroudsburg, Pennsylvania.

Dalziel, K. (1957). The initial steady state velocities in the evaluation of enzyme–coenzyme–substrate reaction mechanisms. Acta Chem. Scand. 11, 1706–1723.

Dalziel, K. (1969). The interpretation of kinetic data for enzyme-catalyzed reactions involving three substrates. Biochem. J. 114, 547–556.

Davis, R. H. (1967). Channeling in *Neurospora* metabolism. In: Organizational Biosynthesis (Vogel, H. J., Lampen, J. O., & Bryson, V., eds.), pp. 303–322, Academic Press, New York.

Dixon, M. & Webb, E. C. (1964). Enzymes, Academic Press, New York.

Dorf, R. C. (1974). Modern Control Systems, 2nd ed., Addison–Wesley, Reading, Mass.

Erdi, P. & Toth, J. (1989). Mathematical Models of Chemical Reactions, Princeton University Press, Princeton, N.J.

Fersht, A. (1985). Enzyme Structure and Mechanism, 2nd ed., W. H. Freeman and Company, San Francisco.

Friedrich, P. (1984). Supramolecular Enzyme Organization, Pergamon, Oxford.

Ginsburg, A. & Stadtman, E. R. (1970). Multienzyme systems. Ann. Rev. Biochem. 39, 429–472.

Haldane, J. B. S. (1930). Enzymes, Longmans and Green, London.

Hammes, G. G. (1982). Enzyme Catalysis and Regulation, Academic Press, New York.

Henri, V. (1903). Lois generales de l'action des diastases, Hermann, Paris.

Hill, C. M., Waight, R. D., & Bardsley, W. G. (1977). Does any enzyme follow the Michaelis–Menten equation? Mol. Cell. Biochem. 15, 173–178.

Hippel, P. H. von, Revzin, A., Gross, C. A., & Wang, A. C. (1974). Non-specific DNA binding of genome regulating proteins as a biological control mechanism: I. The *lac* operon: Equilibrium aspects. Proc. Natl. Acad. Sci. USA 71, 4808–4812.

Huxley, J. S. (1924). Constant differential growth-ratios and their significance. Nature 114, 895–896.

Huxley, J. S. (1932). Problems in Relative Growth, Dial, N.Y.

Irvine, D. H. & Savageau, M. A. (1985a). Network regulation of the immune response: alternative control points for suppressor modulation of effector lymphocytes. J. Immunol. 134, 2100–2116.

Irvine, D. H. & Savageau, M. A. (1985b). Network regulation of the immune response: modulation of suppressor lymphocytes by alternative signals including contrasuppression. J. Immunol. 134, 2117–2130.

Irvine, D. H. & Savageau, M. A. (1990). Efficient solution of nonlinear ordinary differential equations expressed in S-system canonical form. SIAM J. Numerical Anal. 27, 704–735.

Jencks, W. P. (1969). Catalysis in Chemistry and Enzymology, McGraw-Hill, New York.

Keys, A., Taylor, H. L., Blackburn, H., Brozek, J., Anderson, J. T., & Simonson, E. (1963). Coronary heart disease among Minnesota business and professional men followed fifteen years. Circulation 28, 381–395.

Kirschner, K. (1971). Kinetic analysis of allosteric enzymes. Curr. Top. Cell. Reg. 4, 167–210.

Kohen, E., Kohen, C., Thorell, B., & Wagener, G. (1973). Quantitative aspects of rapid microfluorometry for the study of enzyme reactions and transport mechanisms in single living cells. In: Fluorescence Techniques in Cell Biology (Thaer, A. A. & Sernetz, M., eds.), pp. 207–218, Springer-Verlag, N.Y.

Kopelman, R. (1986). Rate processes on fractals: theory, simulations, and experiments. J. Stat. Physics 42, 185–200.

Koshland, D. E., Nemethy, G., & Filmer, D. (1966). Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochem. 5, 365–385.

Kraut, J. (1988). How do enzymes work? Science 242, 533–540.

Kurganov, B. I. (1982). Allosteric Enzymes, John Wiley & Sons, New York.

Laird, A. K. (1969). Dynamics of growth in tumors and in normal organisms. Nat. Cancer Inst. Monog. 30, 15–27.

Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56, 658–666.

Mason, S. J. (1953). Feedback theory—some properties of signal flow graphs. Proc. I.R.E 41, 1144–1156.

Michaelis, L. & Menten, M. L. (1913). Die Kinetik der Invertinwirkung. Biochem. Z. 49, 333–369.

Monod, J., Wyman, J., & Changeux, J.-P. (1965). On the nature of allosteric transitions: a plausible model. J. Mol. Biol. 12, 88–118.

Needham, J. (1950). Biochemistry and Morphogenesis, University Press, Cambridge, England.

Needham, J. (1968). Order and Life, The M.I.T. Press, Cambridge, Mass.

Newhouse, J. S. & Kopelman, R. (1986). Reaction kinetics on clusters and islands. J. Chem. Phys. 85, 6804–6806.

Okamoto, M. & Savageau, M. A. (1984). Integrated function of a kinetic proofreading mechanism: steady-state analysis testing internal consistency of data obtained *in vivo* and *in vitro* and predicting parameter values. Biochemistry 23, 1701–1709.

Orland, M. J. & Saltman, R. J. (1986). Manual of Medical Therapeutics, 25th ed, Little, Brown & Co., Boston.

Ovadi, J. (1991). Physiological significance of metabolic channelling. J. Theoret. Biol. 152, 1–22.

Pauling, L. (1957). The probability of errors in the process of synthesis of protein molecules. In: Festschr. Arthur Stoll, pp. 597–602, Birkhauser, A. G., Basel, Switzerland.

Peller, L. & Alberty, R. A. (1959). Multiple intermediates in steady state enzyme kinetics I. The mechanism involving a single substrate and product. J. Amer. Chem. Soc. 81, 5907–5914.

Raval, D. N. & Wolfe, R. G. (1962). Malic dehydrogenase II. Kinetic studies of the reaction mechanism. Biochemistry 1, 263–269.

Reed, L. J. & Cox, D. J. (1966). Macromolecular organization of enzyme systems. Annu. Rev. Biochem. 35, 57–84.

Reiss, M. J. (1989). The Allometry of Growth and Reproduction, Cambridge University Press, N.Y.

Richards, F. J. (1969). The quantitative analysis of growth. In: Plant Physiology (Steward, F. C., ed.), Vol. VA, pp. 3–76, Academic Press, N.Y.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., & Britten, R. J. (1955). Studies of biosynthesis in *E. coli*. Carnegie Inst. Wash., Publ. 607.

Roels, J. A. (1983). Energetics and Kinetics in Biotechnology, Elsevier, Amsterdam.

Ross, J. (1988). Stability and constraints. Am. Scientist 76, 232–233.

Savageau, M. A. (1969a). Biochemical systems analysis I. Some mathematical properties of the rate law for the component enzymatic reactions. J. Theoret. Biol. 25, 365–369.

Savageau, M. A. (1969b). Biochemical systems analysis II. The steady state solutions for an n-pool system using a power-law approximation. J. Theoret. Biol. 25, 370–379.

Savageau, M. A. (1970). Biochemical systems analysis III. Dynamic solutions using a power-law approximation. J. Theoret. Biol. 26, 215–226.

Savageau, M. A. (1971). Concepts relating the behavior of biochemical systems to their underlying molecular properties. Arch. Biochem. Biophys. 145, 612–621.

Savageau, M. A. (1972). The behavior of intact biochemical control systems. Curr. Top. Cell. Reg. 6, 63–130.

Savageau, M. A. (1976). Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology, Addison-Wesley, Reading, Mass.

Savageau, M. A. (1979a). Growth of complex systems can be related to the properties of their underlying determinants. Proc. Nat. Acad. Sci. USA 76, 5413–5417.

Savageau, M. A. (1979b). Allometric morphogenesis of complex systems: derivation of the basic equations from first principles. Proc. Nat. Acad. Sci. USA 76, 6023–6025.

Savageau, M. A. (1985). Mathematics of organizationally complex systems. Biomed. Biochim. Acta 44, 839–844.

Savageau, M. A. (1991a). Reconstructionist molecular biology. New Biologist 3, 190–197.

Savageau, M. A. (1991b). Biochemical systems theory: operational differences among variant representations and their significance. J. Theoret. Biol. 151, 509–530.

Savageau, M. A. (1992). A critique of the enzymologist's test tube. In: Fundamentals of Medical Cell Biology, Vol. 3A, (Bittar, E. E., ed.), pp. 45–108, JAI Press Inc., Greenwich, Connecticut.

Savageau, M. A. (1995). Power-law formalism: A canonical nonlinear approach to modeling and analysis. Proc. First World Congress of Nonlinear Analysts, Walter de Gruyter, Berlin.

Savageau, M. A. & Steward, J. P. (1970). Repression of the threonine synthetase system in *Escherichia coli*. Arch. Biochem. Biophys. 137, 181–184.

Savageau, M. A. & Jacknow, G. (1979). Feedforward inhibition in biosynthetic pathways: Inhibition of the aminoacyl-tRNA synthetase by intermediates of the pathway. J. Theoret. Biol. 77, 405–425.

Savageau, M. A. & Voit, E. O. (1982). Power-law approach to modeling biological systems I. Theory. J. Ferment. Technol 60, 221–228.

Savageau, M. A. & Voit, E. O. (1987). Recasting nonlinear differential equations as S-systems: A canonical nonlinear form. Math. Biosci. 87, 83–115.

Savageau, M. A. & Sorribas, A. (1989). Constraints among molecular and systemic properties: implications for physiological genetics. J. Theoret. Biol. 141, 93–115.

Savageau, M. A., Voit, E. O., & Irvine, D. H. (1987a). Biochemical systems theory and metabolic control theory 1. Fundamental similarities and differences. Math. Biosci. 86, 127–145.

Segal, H. L. (1959). The development of enzyme kinetics. In: The Enzymes (Boyer, P. D., Lardy, H., & Myrback, K., eds.), Vol I, 2nd Ed., pp. 1–48, Academic Press, New York.

Segel, I. H. (1975). Enzyme Kinetics, Wiley, New York.

Segel, L. A. & Slemrod, M. (1989). The quasi-steady state assumption: a case study in perturbation. SIAM Review 31, 446–477.

Shimizu, S. Y. & Lenhoff, H. M. (1978). Efficiency of two immobilized enzymes acting in sequence. Enzyme Eng. 3, 155–161.

Shiraishi, F. & Savageau, M. A. (1992a). The tricarboxylic acid cycle in *Dictyostelium discoideum* I. Formulation of alternative kinetic representations. J. Biol. Chem. 267, 22912–22918.

Shiraishi, F. & Savageau, M. A. (1992b). The tricarboxylic acid cycle in *Dictyostelium discoideum* II. Evaluation of model consistency and robustness. J. Biol. Chem. 267, 22919–22925.

Shiraishi, F. & Savageau, M. A. (1992c). The tricarboxylic acid cycle in *Dictyostelium discoideum* III. Analysis of steady state and dynamic behavior. J. Biol. Chem. 267, 22926–22933.

Shiraishi, F. & Savageau, M. A. (1992d). The tricarboxylic acid cycle in *Dictyostelium discoideum* IV. Resolution of discrepancies between alternative methods of analysis. J. Biol. Chem. 267, 22934–22943.

Shiraishi, F. & Savageau, M. A. (1993). The tricarboxylic acid cycle in *Dictyostelium discoideum*: Systemic effects of including protein turnover in the current model. J. Biol. Chem. 268, 16917–16928.

Singer, P. A., Levinthal, M., & Williams, L. S. (1984). Synthesis of the isoleucyl- and valyl-tRNA synthetases and the isoleucine–valine biosynthetic enzymes in a threonine deaminase regulatory mutant of *Escherichia coli* K-12. J. Mol. Biol. 175, 39–55.

Sinnott, E. W. (1963). The Problem of Organic Form, Yale University Press, New Haven.

Sorribas, A. & Savageau, M. A. (1989a). A comparison of variant theories of intact biochemical systems I: Enzyme–enzyme interactions and biochemical systems theory. Math. Biosci. 94, 161–193.

Sorribas, A. & Savageau, M. A. (1989b). A comparison of variant theories of intact biochemical systems 2: Flux oriented and metabolic control theories. Math. Biosci. 94, 195–238.

Sorribas, A. & Savageau, M. A. (1989c). Strategies for representing metabolic pathways within biochemical systems theory: reversible pathways. Math. Biosci. 94, 239–269.

Srere, P. A. & Mosbach, K. (1974). Metabolic compartmentation: symbiotic, organellar, multienzymic, and microenvironmental. Ann. Rev. Microbiol. 28, 61–83.

Srere, P. A., Jones, M. E., & Matthews, C. (1989). Structural and Organizational Aspects of Metabolic Regulation, Alan R. Liss, New York.

Srivastava, D. K. & Bernhard, S. A. (1986). Metabolite transfer via enzyme–enzyme complexes. Science 234, 1081–1086.

Teissier, G. (1931). Recherches morphologiques et physiologiques sur la croissance des insectes. Trav. de la Stat. Biol. Roscoff 9, 27–238.

Teissier, G. (1937). Les Lois Quantitatives de la Croissance, Hermann, Paris.

Truxal, J. G. (1955). Automatic Feedback Control System Synthesis, McGraw-Hill, New York.

Umbarger, H. E. (1956). Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine. Science 123, 848.

Voit, E. O. (1991). Canonical Nonlinear Modeling: S-System Approach to Understanding Complexity, Van Nostrand Reinhold, New York.

Voit, E. O. & Savageau, M. A. (1984). Analytical solutions to a generalized growth equation. J. Math. Analysis Applic. 103, 380–386.

Voit, E. O. & Savageau, M. A. (1987). Accuracy of alternative representations for integrated biochemical systems. Biochem. 26, 6869–6880.

Voit, E. O., Irvine, D. H., & Savageau, M. A. (1989). The User's Guide to ESSYNS. Medical University of South Carolina Press, Charleston.

Wallach, J. (1986). Interpretation of Diagnostic Tests, 4th ed, Little, Brown, & Co., Boston.

Webb, J. L. (1963). Enzyme and Metabolic Inhibitors, Vol I, p. 29, Academic Press, New York.

Weiss, J. N. & Lamp, S. T. (1987). Glycolysis preferentially inhibits ATP-sensitive K^+ channels in isolated guinea pig cardiac myocytes. Science 238, 67–69.

Weisz, P. B. (1973). Diffusion and chemical transformation. Science 179, 433–440.

Wilkinson, G. N. (1961). Statistical estimations in enzyme kinetics. Biochem. J. 80, 324–332.

Wladimirow, G. E. & Danilina, M. J. (1930). Beitrage zur Embryochemie und Embryophysiologie. IV. Mitteilung: Die Anhaufung des Glykogens im Korper des sich entwickelnden Huhnerembryos. Biochem. Z. 224, 69–78.

Woolfolk, C. A. & Stadtman, E. R. (1967). Regulation of glutamine synthetase III. Cumulative feedback inhibition of glutamine synthetase from *Escherichia coli*. Arch. Biochem. Biophys. 118, 736–755.

Wong, J. T.-F. (1975). Kinetics of Enzyme Mechanisms, Academic Press, New York.

Wong, J. T.-F. & Hanes, C. S. (1962). Kinetic formulations for enzymatic reactions involving two substrates. Can. J. Biochem. Physiol. 40, 763–804.

Wright, B. E., Butler, M. H., & Albe, K. R. (1992). Systems analysis of the tricarboxylic acid cycle in *Dictyostelium discoideum*. J. Biol. Chem. 267, 3101–3105.

Wyman, J. (1972). On allosteric models. Curr. Top. Cell. Reg. 6, 209–226.

Yates, R. A. & Pardee, A. B. (1956). Control of pyrimidine biosynthesis in *Escherichia coli* by a feed-back mechanism. J. Biol. Chem. 221, 757–770.

RECOMMENDED READINGS

Bertalanffy, L. von (1968). General Systems Theory, George Braziller, New York.

Erdi, P. & Toth, J. (1989). Mathematical Models of Chemical Reactions, Princeton University Press, Princeton, N.J.

Fersht, A. (1985). Enzyme Structure and Mechanism, 2nd ed., W. H. Freeman & Company, San Francisco.

Hammes, G. G. (1982). Enzyme Catalysis and Regulation, Academic Press, New York.

Neidhardt, F. C., Ed. (1987). *Escherichia coli* and *Salmonella typhimurium*, Vols. 1 and 2, American Society for Microbiology, Washington, DC.

Savageau, M. A. (1976). Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology, Addison-Wesley, Reading, Mass.

Voit, E. O. (1991). Canonical Nonlinear Modeling: S-System Approach to Understanding Complexity, Van Nostrand Reinhold, New York.

Chapter 6

The Basis of Enzymatic Adaptation

KENNETH B. STOREY and STEPHEN P.J. BROOKS

INTRODUCTION

Life on earth has radiated to exploit virtually every conceivable habitat and lifestyle. Life persists at the extremes of environmental temperature (from –50°C to > 90°C) and in the depths of the ocean (at hydrostatic pressures of up to 500 atm). Desert

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species survive extreme desiccation, in some cases drying to the point where no free water and no metabolic activity can be detected. Freeze-tolerant organisms survive for weeks with no vital signs and up to 65% of total body water existing as extracellular ice. Varying environmental oxygen supply has produced obligate aerobes, facultative anaerobes, and obligate anaerobes as well as species that have replaced their dependence on oxygen with the use of sulfur compounds as terminal electron acceptors.

Biochemical adaptation is an integral part of animal diversity for it molds molecular structure, metabolic function, and metabolic regulation to serve two purposes: (1) compensation—the preservation of basic cellular processes in the face of adverse environmental stresses, and (2) exploitation—the development of new metabolic capacities and/or regulatory mechanisms to support survival in a new environment or lifestyle.

The present chapter focuses on the effects of environmental stress on enzyme structure and function and the various mechanisms that respond to these stresses to alter enzyme function (and metabolic pathways). Many examples will be drawn from animals that live at environmental extremes, for it is here that we find the principles of enzyme structure and function most clearly illustrated. Because this subject is complex and covers a wide variety of different fields, the present chapter will highlight key principles using selected enzymes as examples. The reader is referred to other sources for more exhaustive treatments of individual topics in comparative biochemistry and enzyme adaptation (Crowe and Clegg, 1978; Hochachka and Somero, 1984; Gilles, 1985; Bowler and Fuller, 1987; Storey, 1988; Storey and Storey, 1988).

DEMANDS ON ENZYME FUNCTION

It is possible to broadly classify the types of environmental or physiological stresses on enzymes into three categories.

Physical Stresses

Changes in environmental temperature and hydrostatic pressure have inescapable effects on all biological molecules and metabolic reactions. Temperature change alters metabolic rates (on average by 2-fold for each 10°C change, but Q_{10} values ranging from 1 to 4 are not uncommon for different reactions) and disrupts the weak-bond interactions that determine protein secondary, tertiary, and quaternary structures as well as enzyme–ligand binding (Hochachka and Somero, 1984). Obviously then, temperature change has the potential to destroy the integrated functioning of diverse metabolic processes. Indeed, the damaging and often lethal effects of hypothermia on homeotherms such as man, attest to this (Hochachka, 1986). Ectothermic animals must have mechanisms, therefore, that adapt enzyme function to permit metabolic regulation to be maintained over a wide temperature

range. It is interesting to note that human biochemistry is actually a case where metabolism has adapted to function only within a very restricted temperature range and has lost the flexibility of structure/function with respect to temperature change that occurs even in other types of mammals (Malan and Canguilhem, 1989).

High hydrostatic pressure alters any process that proceeds with a volume change. This affects enzyme catalysis because both substrate binding and associated protein conformational changes proceed with changes in the hydration state of amino acid residues that alter the total volume of the water–protein–ligand system (Low and Somero, 1975). Therefore, animals adapted to the deep sea require changes to the amino acid composition of enzymes that create pressure-insensitive kinetic parameters and pressure-resistant structures.

Chemical Composition of Cells

All enzymes function within a hydrating shell of vicinal (or bound) water that is key to stabilizing enzyme conformation. All cellular reactions take place within an aqueous medium through which reactants move by diffusion (Clegg, 1981). Most enzymatic reactions have also evolved to function optimally within a narrow pH range (around neutral pH) and, for animal cells, within a narrow window of inorganic ion concentrations (typically 100–200 mM K^+ and < 100 mM Na^+). Various environmental stresses can seriously disrupt the water, ion, and pH balance of the cell with potentially damaging effects for enzyme function and metabolic regulation. Desiccation, exposure to a hyperosmotic external medium, or extracellular freezing all dehydrate and/or raise ion concentrations in cells with negative effects on enzyme structure and function (Crowe and Clegg, 1978; Storey and Storey, 1988). Adaptations for survival of these environmental stresses must include molecular strategies to counter these effects.

Changing Metabolic Demands

A change in an animal's activity level demands a corresponding change in metabolism which is brought about by an alteration in enzyme regulatory mechanisms. Extreme environmental stress has frequently resulted in the development of metabolic arrest strategies that permit animals to extend survival by entering a dormant state (Hochachka and Guppy, 1987). Hibernation, estivation, diapause, and anaerobiosis all require mechanisms of enzyme and pathway regulation that allow a coordinated depression of metabolic rate to 10% or less of the normal basal metabolic rate (Storey, 1988). Anoxia-tolerant animals frequently complement metabolic rate depression with the addition of alternative enzymatic pathways for fermentative ATP production (Hochachka and Somero, 1984; Storey, 1985a).

MOLECULAR MECHANISMS OF ENZYME ADAPTATION: HOW ENZYMES REGULATE METABOLISM

All biological processes are chemical reactions and as such, must obey the fundamental rules governing chemical processes. These rules determine how fast a reaction happens and how far a reaction proceeds toward completion. A fundamental understanding of what governs chemical processes is essential toward the understanding of how enzymes control cellular processes since chemical processes and enzyme action are intimately linked. It is only through the understanding of how enzymes function that one can comprehend the mechanisms which act to regulate enzyme action.

Kinetic Considerations

Processes Not Under Enzyme Control

The degree to which a particular reaction proceeds is determined only by the nature of the starting material and products in any chemical reaction. The degree to which a reaction proceeds is defined by the equilibrium constant:

$$K_{eq} = [\text{Product}]/[\text{Starting material}] \quad (1)$$

and is measured when product and starting material concentrations are no longer changing (equilibrium condition). The K_{eq} is independent of the path of molecular transformation and the time required to reach equilibrium: how far a reaction proceeds depends only on the energy of the starting material, the products, and the system. It is important to remember, therefore, that the equilibrium concentrations of a reaction depend only on the properties of the reactants themselves: it is not possible to alter the equilibrium concentrations of any reaction by manipulating enzyme activity. We will devote the rest of this chapter to investigating factors that are under enzyme control.

Processes Under Enzyme Control—Reaction Rates

All enzymes are catalysts which act to increase reaction rates. In fact, in most cases, the difference between enzyme catalyzed and noncatalyzed reactions is so great that only the enzyme-catalyzed reactions occur to any significant extent *in vivo*. Exactly how enzymes participate in this process has been detailed earlier in this book and will not be reviewed here. However, in order to understand enzyme adaptation, it is essential that a few basics of enzyme kinetics be reviewed. The basic enzyme mechanism describes the case where a single substrate binds to an enzyme before being chemically converted to a product. Although the majority of enzyme-catalyzed reactions are two-substrate reactions, several can be adequately described by this mechanism and an understanding of this mechanism is essential

to an understanding of enzyme kinetics. When free enzyme (E_{free}) binds a single substrate:

$$\text{Substrate} + E_{free} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E_{free} + \text{Product} \tag{2}$$

the reaction scheme can be described by Equation 3 (the Michaelis–Menten equation):

$$v = V_{max}[S]/(K_m + [S]) \tag{3}$$

where v is the observed rate of reaction (v = change in product over time) and V_{max} is the maximal rate of the reaction measured at infinite substrate concentrations. The K_m value represents the concentration of substrate producing half maximal velocity and is a measure of the dissociation constant of the substrate: the greater the K_m value, the lower the enzyme affinity for substrate.

Although Equation 3 can be used to describe many cellular enzyme reactions, it is not adequate for others. This is because many regulatory enzymes are multisubunit structures containing several copies of a unique polypeptide chain (subunit) bound together in a higher-order structure. Often, binding of substrate to one of these subunits influences substrate binding at another subunit within the structure. If binding of one substrate molecule increases the enzyme affinity for subsequent substrate molecules, a situation will arise where the K_m value of Equation 3 is not a unique value but changes with differing substrate concentrations. We will call such enzymes cooperative enzymes. This type of mechanism is extremely complex but can be approximated by the Hill equation:

$$v = V_{max}[S]^h/(K_m^h + [S]^h) \tag{4}$$

where h is the Hill coefficient. The Hill coefficient has no physical meaning but can be used to measure relative changes in the subunit interactions. Note that Equation 4 is virtually identical to Equation 3, with the exception that the K_m and [S] terms are raised to the power of the Hill coefficient.

It is apparent from Equations 3 and 4 that enzyme activity can easily be influenced by changes in the concentration of substrate ([S] in Equations 3 and 4). However, concentrations of enzyme substrates and products do not, in general, change substantially even when metabolic rates change dramatically. This means that it is not possible to regulate enzyme activity without the existence of other mechanisms. These considerations are especially important to "controlling" enzymes found at the beginning of metabolic pathways; overall flux through pathways is often regulated by exerting control at a single enzyme locus. Enzyme rates are regulated by two different processes: (1) changes in the total amount of enzyme present (changes in V_{max}) or (2) changes in one or more of the enzyme kinetic constants (V_{max} or K_m) by reversible inhibition, activation, or covalent modification. Inhibition refers to a change in enzyme activity brought about through binding

of a substance (either a metabolite or another protein) that reduces an enzyme's affinity for its substrate or decreases its catalytic efficiency. Inhibition may result from: (1) binding of a substance to the enzyme active site directly, in which case the inhibitor competes directly with substrate (competitive inhibitor), or (2) binding of a substance to a site different from the enzyme active site, in which case the inhibitor causes conformational changes in the enzyme three-dimensional structure (allosteric inhibitor). Activation, the opposite of inhibition, can also be brought about through binding of an allosteric activator. Covalent modification is the chemical altering of enzyme molecules (for example, the attachment of phosphate or acetyl groups) that causes either activation or inhibition of enzyme activity. Very often, inhibitors and activators are compounds which serve as monitors of the energy state of the cell (such as ATP or AMP), or are products or substrates of the enzymes themselves. These compounds serve to directly link enzyme activity to the energy state of the cell and to the demand for substrate or product.

As shown in Figure 1, a much greater controlling effect is observed in cooperative enzymes for a two fold change in the K_m value when compared with the change observed in Michaelis–Menten enzymes. Thus, with cooperative enzymes, activators can promote enzyme function by lowering enzyme–substrate affinity into the

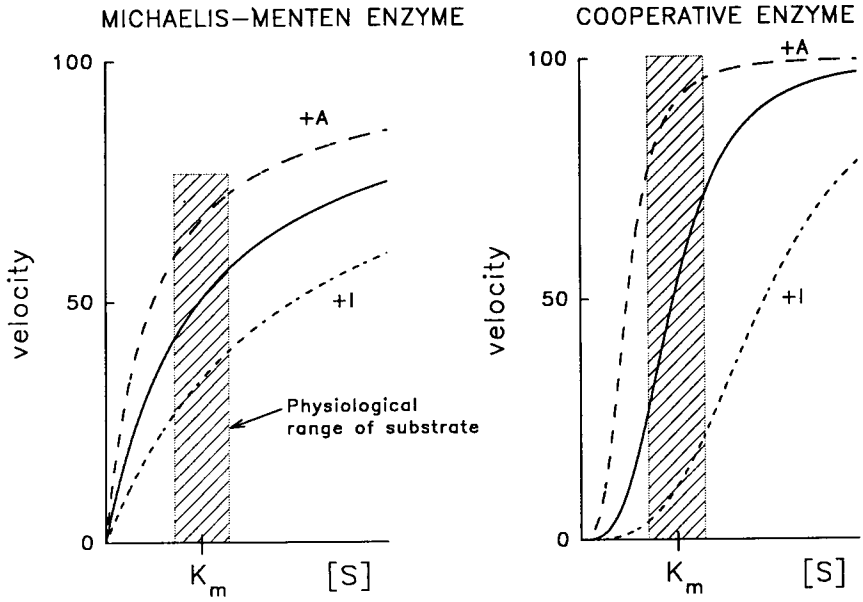


Figure 1. Effect of increasing substrate concentration on the velocity of Michaelis–Menten (left) and cooperative (right) enzymes. The shaded area represents the typical range of substrate concentrations *in vivo*. The effect of activators and inhibitors on enzyme–substrate affinity is also shown.

range of physiological substrate concentrations and inhibitors can decrease activity by increasing the K_m value so that it is no longer within the physiological range.

Changes in the Amount of Enzyme

In essence, changing the amount of enzyme is equivalent to changing the V_{max} of the enzyme because $V_{max} = k_{cat}$[Enzyme]. Thus, the flux through any particular step can be regulated by altering the enzyme concentration with a direct effect on the observed rate (Figure 2). By and large, this mechanism is used for long-term (rather than short-term) control of enzyme activity because of the enormous energy cost of synthesizing enzymes *de novo*.

Changes in the amounts of enzyme proteins in the cell may reflect both functional differences between tissue types, as well as differences in kinetic capacity between homologous tissues from different species. An examination of the levels of glycolytic and mitochondrial enzymes in vertebrate skeletal muscles provides a good example of this strategy (Table 1). White muscle fibers are designed for brief bursts of high-powered contraction and fermentative ATP production is supported by high activities of glycolytic enzymes. The white skeletal muscle of tuna has developed this strategy to perhaps its fullest extent; high-speed swimming by this predatory fish is powered by muscles with the highest amounts of glycolytic enzymes ever measured. Red muscle, by contrast, supports sustained-

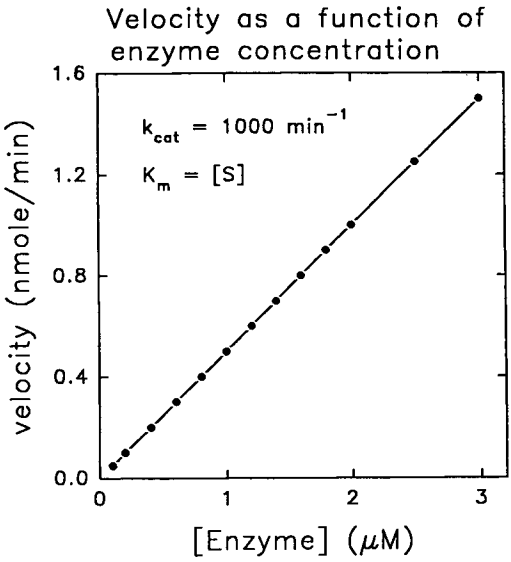


Figure 2. Effect of changing enzyme concentration on enzyme velocity. Velocity calculated according to Equation 3 with $V_{max} = k_{cat}$[Enzyme] and K_m = [S].

Table 1. Activities of Some Glycolytic and Mitochondrial Enzymes in Vertebrate Skeletal Muscles[a,b]

	Rat Quadriceps		*Hummingbird Pectoral Red Muscle*	*Tuna White Muscle*
	Red Fibers	*White Fibers*		
Glycogen phosphorylase	105	215	31	106
Hexokinase	1.5	0.6	9.2	0.8
Phosphofructokinase	72	96	110	—
Pyruvate kinase	279	473	672	1295
Lactate dehydrogenase	486	773	230	5492
Citrate synthase	—	—	343	2
Glutamate–oxaloacetate transaminase	—	—	1388	43

Notes: [a]Data from Baldwin et al. (1973), Guppy et al. (1979), and Suarez et al. (1986).
[b]Activities are expressed in units per gram wet weight.

aerobic exercise and contains large numbers of mitochondria and high levels of tricarboxylic acid cycle enzymes. Hummingbird flight muscles exemplify this strategy with an enzymatic profile optimized for the aerobic oxidation of dietary sugars (high hexokinase) or stored lipids as fuels.

Changes in the amounts of enzymes can also occur within the same tissue to allow individual organisms to adapt to changing environmental or functional demands. For example, endurance training can increase the contents of mitochondrial oxidative enzymes in skeletal muscle by 50–200% (Holloszy et al., 1970). Changes in the fat and carbohydrate content of diets result in the increase and decrease of enzymes responsible for metabolism of these compounds (Goodridge, 1987). Cold acclimation in fish proceeds with an increase in the contents of mitochondrial enzymes and cytochromes in muscle that helps to maintain power output for the swimming musculature at low temperature and compensates for the negative effects of low temperature on enzyme rates (Sidell, 1983). Interestingly, these temperature-induced effects are at least partially self-adjusting, for rates of protein degradation in fish muscle are more strongly depressed by low temperature than are rates of protein synthesis. The net effect, therefore, is an increase in enzyme concentration in the cold (Sidell, 1983).

Changes in the Form of Enzyme Expressed

Isozymes are enzyme variants that catalyze the same reaction but are structurally different (they are coded by separate gene loci). Allozymes are variants that arise due to polymorphism at a single gene locus. Isozymes can vary enormously in their properties (e.g., substrate affinities, responses to allosteric effectors, phosphorylation by protein kinases) to support the different functional needs for a particular reaction in different organs or different compartments of the cell (e.g., mitochon-

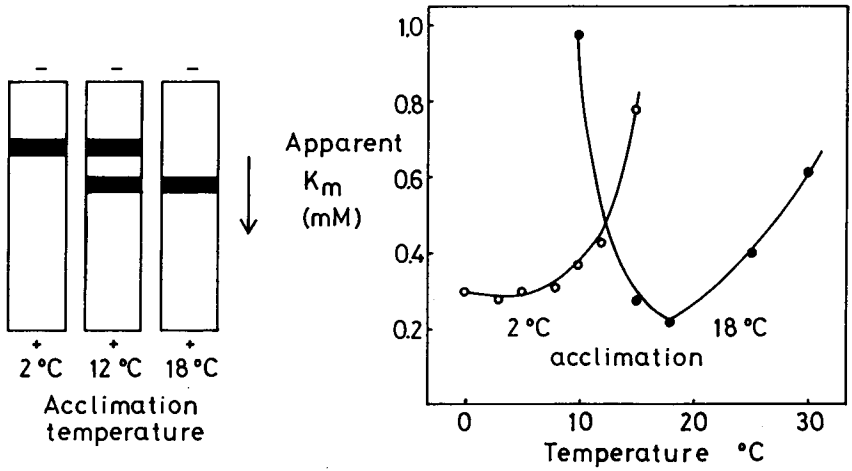


Figure 3. Acetylcholinesterase in trout brain: changes in isozyme pattern at different acclimation temperatures and temperature effects on the K_m for acetylcholine. After Baldwin and Hochachka (1970).

drial versus cytosolic isozymes). For example, the M (muscle) and H (heart) forms of lactate dehydrogenase have properties tailored for different physiological functions: lactate production during burst muscle work in muscle versus lactate oxidation which serves as an aerobic fuel in heart (Everse and Kaplan, 1973). The properties of isozymes as well as their organ distribution can also be molded and changed between species to suit the particular requirements of the animal.

As a strategy for rapid adaptive change by an individual, changing the expression of isozymic or allelic forms of enzymes occurs infrequently, but some examples are known. For example, acetylcholinesterase from trout brain occurs in warm versus cold isoforms, each occurring singly when fish are acclimated to 18°C or 2°C, but with a mixture of isoforms occurring at intermediate acclimation temperatures (Baldwin and Hochachka, 1970). The reason for the change in isozyme expression with acclimation is obvious from an examination of Figure 3. The K_m (See Equation 3) for acetylcholine is highly temperature-sensitive and a consistent enzyme–substrate affinity (permitting consistent enzyme function in neurotransmitter degradation) can only be maintained by shifting the proportions of the two isoforms with changing temperature.

Other studies have shown that the maintenance of a mixture of isozymes or allozymes at all times, each differing in their temperature-profiles for key kinetic properties, provides the mechanism for instantaneous compensation of enzyme performance with changing temperature (Moon and Hochachka, 1971; Place and Powers, 1979).

Changes in Enzyme Function by Posttranslational Modification

The most powerful mechanism for controlling enzyme activity is the tailoring of enzyme kinetic and regulatory properties brought about through interactions with other proteins, with cellular metabolites, or through covalent modification. As discussed above, both the K_m and V_{max} values may be affected to alter enzyme activity. The advantage of posttranslational modification of the enzyme protein is that it confers pseudo-stable kinetic changes to enzymes that can be rapidly reversed. Several types of posttranslational modifications are known: phosphorylation, adenylation, glucosylation, or limited proteolysis (Cohen, 1976). Probably the most important of these is phosphorylation because proteins can be easily, rapidly, and specifically phosphorylated and dephosphorylated by a family of protein kinases and phosphatases, respectively. Phosphorylation control is typically applied to: (1) regulatory enzymes that gate entry into major metabolic pathways, (2) important branchpoints in pathways, and (3) control points in bidirectional pathways (Cohen, 1980; Boyer and Krebs, 1987). Frequently, such control provides virtually on–off regulation of pathway flux. In addition, because protein kinases and phosphatases may have many different enzyme proteins as substrates, reversible phosphorylation has evolved as a major mechanism for coordinating control

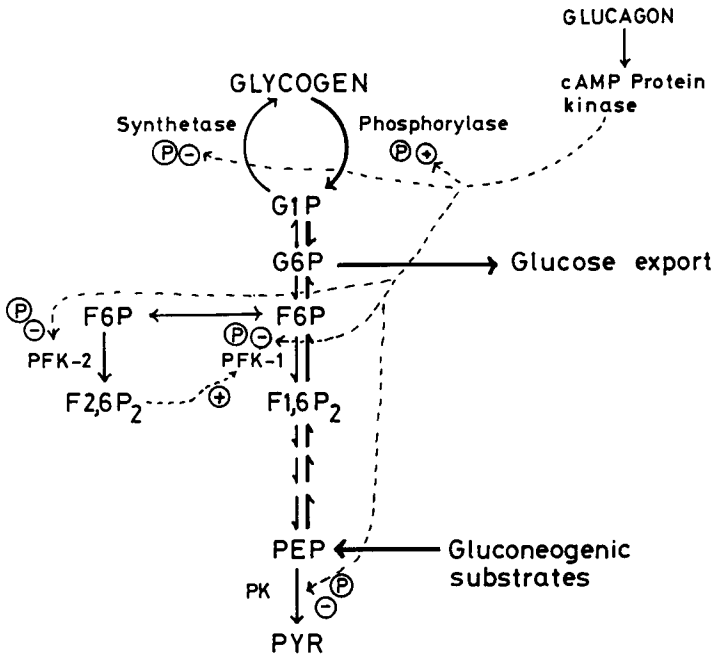


Figure 4. Coordinated control by reversible protein phosphorylation over the enzymes of carbohydrate metabolism in mammalian liver.

over multiple enzymes and/or pathways to achieve an integrated response to an extracellular signal. For example, glucagon action on mammalian liver, mediated via cAMP-dependent protein kinase, results in phosphorylation and activation of glycogen phosphorylase and phosphorylation and inactivation of glycogen synthase, 6-phosphofructo-1-kinase (PFK-1), 6-phosphofructo-2-kinase (PFK-2), pyruvate kinase (PK), and pyruvate dehydrogenase (Figure 4) (Cohen, 1980; Sakakibara and Uyeda, 1983; Boyer and Krebs, 1987). These changes act to stimulate glucose output from the liver through an activation of glycogenolysis and gluconeogenesis while at the same time inhibiting glycogen synthesis and carbohydrate catabolism.

Type of Protein Kinase or Triggering Signal

Enzyme phosphorylation is coordinated by the action of cellular protein kinases, of which the four most commonly studied are cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), Ca^{2+}-calmodulin protein kinase, and Ca^{2+}- and phospholipid-dependent protein kinase (PKC). Interestingly, a single enzyme may be acted on by more than one protein kinase with differing kinetic consequences (Kitamura et al., 1988). The extracellular triggers stimulating enzyme phosphorylation also change between organs and species. Mammalian liver PK is phosphorylated by PKA in response to glucagon stimulation (Engstrom et al., 1987). PK in whelk organs, by contrast, is phosphorylated by PKG during anoxia (Brooks and Storey, 1990). Phosphorylation of glycogen phosphorylase activates the enzyme in response to glucagon-mediated cAMP-dependent mechanisms in liver and Ca^{2+}/calmodulin-dependent mechanisms in skeletal muscle (Cohen, 1980). In cold-hardy insects, glycogen phosphorylase activation is directly triggered by exposure to low temperature: the mechanism bypasses second messengers and occurs due to the differential effects of low temperature on phosphorylase kinase versus phosphorylase phosphatase that strongly favor kinase action at low temperature (Hayakawa, 1985).

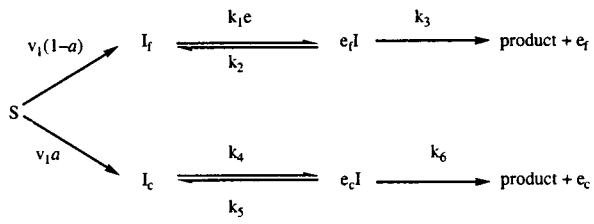
Metabolic Function of Coordinated Enzyme Phosphorylation

Enzyme control via reversible protein phosphorylation is obviously an excellent way to generate a coordinated effect on multiple enzymes spreading from a single extracellular signal. As discussed above, in application to vertebrate liver, coordinated protein phosphorylation in response to glucagon stimulation activates glycogenolysis and gluconeogenesis while inhibiting glycogen synthesis and glycolysis (Figure 4) (Hers and Hue, 1983; Boyer and Krebs, 1987). Many other responses can be coordinated by the same protein phosphorylation mechanism and even applied to the same enzymes; however, the functional result can be different in different organs or animals. For example, epinephrine stimulation of mammalian

heart leads to phosphorylation and activation of glycogen phosphorylase, PFK-1, and PFK-2 to produce an activation of glycolytic rate as part of the stimulation of muscle work by catecholamines (Narabayashi et al., 1985; Kitamura et al., 1988). For facultative anaerobes, the key response to oxygen-lack is a steep depression of metabolic rate that includes a glycolytic rate depression (i.e., there is no Pasteur effect in these animals). For marine mollusks, this occurs in all organs and is coordinated by anoxia-stimulated phosphorylation of phosphorylase, PFK-1, PFK-2, and PK, in all cases inactivating the enzymes (Storey, 1988). The same protein-phosphorylation mechanism underlies anoxia tolerance in a vertebrate animal (the goldfish) and is also being identified in other forms of metabolic depression. Hibernating small mammals show phosphorylation-mediated inactivation of key enzymes with the transition from the euthermic to the hibernating state. Since the hibernating state is an aerobic one, this control also reaches out to include mitochondrial enzymes such as pyruvate dehydrogenase (e.g., the percentage of PDH in the active form drops from 80% in heart of euthermic ground squirrels to 6% in hibernating ground squirrels; Brooks and Storey, 1992). Control may also extend to the regulation of membrane ion transport activities (ion channels versus ATP-driven ion pumps) that must be coordinated in order to reestablish homeostasis at the reduced body temperature of the hibernator (Hochachka, 1986).

Enzyme Association with Macromolecular Structural Components of the Cell: a Control Mechanism?

The cytosol is no longer viewed as a "soup" in which enzymes and low molecular weight solutes are randomly distributed. Although the final picture is still unclear, it is certain that the cell is more highly structured than previously believed. In some cases, one can directly show that enzymes and pathways are organized into microcompartments through their associations with macromolecular structures. For example, several glycolytic enzymes from smooth muscle are organized on the plasma membrane (Paul, 1986), labeling experiments suggest that the enzymes of the mitochondrion are organized into supramolecular complexes (Srere, 1987), and enzymes which make and degrade glycogen are found bound to the glycogen particle (Masters et al., 1987). Theoretically, formation of pathway associations is thought to be highly beneficial to metabolic regulation. These complexes are envisioned as consisting of sequential enzymes in a metabolic pathway (sequential enzymes use the product of the previous reaction). Ideally, supramolecular complexes should optimize the channeling of substrates and products between sequential enzymes. Such a tight coupling between sequential enzymes would lower the apparent K_m value of the second enzyme because the substrate would not have to be diluted in the surrounding medium: the second enzyme would see a much higher substrate concentration (Brooks et al., 1988). This type of reaction can be modeled as shown below:



where a represents the fraction of flux through the first enzyme (v_1) which is channeled directly to the second enzyme. Solution of this type of system gives an equation of the form:

$$\tau = \frac{[e_{2,T}](1 + aK_m^c)}{V_{max}} + \frac{(1-a)K_m^f}{V_{max}(1-b) - v_1(1-a)} \quad (5)$$

where the superscript c refers to the enzyme in a complex and the superscript f refers to the free enzyme. The value b is the fraction of the second enzyme in the complex. Figure 5 shows the kinetic effect of forming a channeling complex (shown by increasing the fraction of the second enzyme in the complex) when the first enzyme is limiting. The transition time (time taken to reach steady-state) decreases dramatically as more product is channeled through the second enzyme, but the overall flux through the enzyme pair does not increase. Models such as these show that channeling may enable the pathway to respond more quickly to changing energy

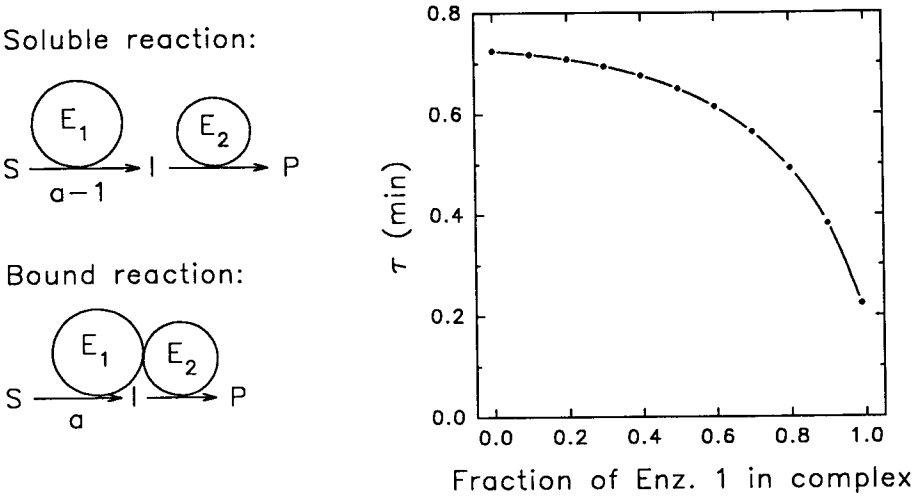


Figure 5. Relationship between the reaction transition time (τ) and the fraction of the first enzyme in the complex (a) as defined by Equation 5. Variables were set at: $[e_{2,T}]$ = 1 μM, v_1 = 2 μM/min, v_2 = 5 μM/min, K_m^c = 1 μM, K_m^f = 10 μM, and V_{max}=10 μM·min^{-1}. The ratio of b/a = 0.8.

needs, but would not increase pathway flux. This would be especially useful when metabolic complexes are physically positioned at the site where product is needed. For example, creatine phosphokinase is found associated with contractile proteins in muscle cells so that the ATP generated by creatine phosphate hydrolysis is produced in the vicinity of the myosin ATPase.

Regulation of the formation or disruption of enzyme-binding associations with macromolecular structural components of the cell is obviously another level at which both enzyme regulation and enzyme adaptation can be affected. Studies have shown, for example, that conditions that increase glycolytic rate in vertebrate skeletal muscle (e.g., burst exercise, tetanic contraction, ischemia) lead to an increased binding of some of the glycolytic enzymes to the particulate fraction of the cell (Clarke et al., 1984; Brooks and Storey, 1988a). Conversely, in anoxia-tolerant animals, the metabolic rate depression that underlies prolonged survival during anaerobiosis is accompanied by large decreases in glycolytic enzyme binding to the particulate fraction (Plaxton and Storey, 1986).

One must, however, be careful when interpreting data on enzyme binding. For example, although much *in vitro* evidence has been presented in favor of a muscle glycolytic complex bound to f-actin, the evidence is not definitive (Brooks and Storey, 1991a). In particular, studies to determine the cellular signal that mediates enzyme binding have shown that binding is not regulated by changes in cellular pH, the concentration of metabolites, the degree of enzyme phosphorylation, or Ca^{2+} concentration (Brooks and Storey, 1991b, 1993). The absence of a determined cellular signal argues for an artifact rather than a definite cellular control mechanism. Furthermore, the majority of glycolytic enzymes are less active when bound, suggesting that binding acts to inhibit activity, a view clearly opposite to the *in vivo* binding data discussed above. Finally, only a small percentage of many of the glycolytic enzymes are bound to a specific site, suggesting that glycolytic complexes may not play an important role in muscle glycolysis (Brooks and Storey, 1991a).

On the other hand, reversible binding of phosphofructokinase (PFK-1) to muscle f-actin may regulate this enzyme's activity. Evidence in favor of this hypothesis comes from data which shows that: (1) PFK-1 maintains a high binding to the muscle particulate fraction under physiological concentrations of ions and other metabolites—conditions which solubilize other so-called bound enzymes (Brooks and Storey, 1988b), (2) binding of PFK-1 to f-actin allosterically activates the enzyme (Luther and Lee, 1986), and (3) although the phosphorylation of muscle PFK-1 has relatively little effect on enzyme kinetic properties (Foe and Kemp, 1982), it strongly enhances enzyme binding to F-actin (Luther and Lee, 1986). Since conditions that stimulate muscle contraction are also those that stimulate PFK-1 phosphorylation, this would serve to physically move the key regulatory enzyme of glycolysis into close proximity with the ATP-utilizing contractile apparatus at the time when glycolytic ATP output is needed to support intense muscle work.

Changes in the Kinetic Properties of the Enzyme: Pyruvate Kinase as a Model Enzyme

The enzyme pyruvate kinase (PK), one of the sites of ADP phosphorylation in glycolysis, provides a good example of adaptation via alteration of enzyme properties. This is because it is modified by the binding of allosteric effectors, it is phosphorylated in the liver and it binds regulatory proteins in the muscle. The reaction catalyzed by PK is as follows:

$$\text{PEP} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP}$$

This reaction is found at the end of the glycolytic pathway (the pathway that converts glucose to energy) and is a branch-point which determines the future fate of glucose metabolites (see Figure 6). In vertebrates, the typical kinetic pattern for PK includes at least two organ-specific isozymes: a noncooperative muscle form that responds to changes in glycolytic flux during muscle work, and a cooperative liver form that is controlled in response to the demand for glycolytic versus gluconeogenic (reconversion of pyruvate to glucose) carbon flow.

Allosteric Modification of Activity

As Table 2 shows, mammalian skeletal muscle PK has a low K_m value (a high affinity) for its substrate, phosphoenolpyruvate (PEP). Because the K_m value is within the physiological concentration of PEP, flux through the PK locus will be high at all times. The muscle enzyme is not affected by allosteric effectors. The liver enzyme, by contrast, is sensitive to several allosteric effectors including fructose 1,6-bisphosphate (fructose 1,6-P_2), ATP, and alanine. These compounds serve to link PK activity to the energy state of the cell so that it may respond to differing needs. For example, during periods of high-fat oxidation, ATP levels are

Table 2. Kinetic and Regulatory Properties of Pyruvate Kinases[a]

	Mammalian Muscle	*Squid Muscle*	*Mammalian Liver*		*Whelk Muscle*[b]	
			Dephos	*Phos*	*Dephos*	*Phos*
K_m PEP, mM	0.02	0.15	0.3	0.8	0.07	0.85
Hill coefficient	1.00	—	1.0	1.5	1.0	2.5
K_m ADP, mM	0.30	0.40	0.25	0.25	0.27	0.25
K_a fructose-1,6-P_2, μM	N.E.	N.E.	0.06	0.13	0.05	1.3
K_i L-alanine, mM	N.E.	N.E.	0.70	0.35	24.5	0.05
K_i citrate, mM	N.E.	2.5	—	—	N.E.	

Notes: [a]Data from Storey and Hochachka (1975), Bergland and Humble (1979), Engstrom et al. (1987), and Plaxton and Storey (1984).

[b]For mammalian liver and whelk muscle pyruvate kinases, data for both the dephosphorylated (Dephos) and phosphorylated (Phos) enzyme forms are given, representing for the whelk enzymes pyruvate kinase in aerobic versus anoxic tissue, respectively.

high and PK activity is inhibited. PK activity is also inhibited during periods of prolonged fasting when amino acid concentrations are high (the cell is oxidizing amino acids for fuel or using amino acids to make glucose). In contrast, when glycolytic rates are high, fructose 1,6-P_2 concentrations build up in the initial part of the glycolytic pathway (Figure 6) and activate PK. This latter condition occurs when glucose concentrations are high (just after a meal) and the animal is converting glucose to fatty acids for long-term energy storage. Thus, high flux through the liver PK locus occurs under the influence of feed-forward activation by the product of phosphofructokinase, fructose 1,6-P_2. Under gluconeogenic conditions, when ATP and amino acid concentrations (such as L-alanine) are high, PK activity is inhibited to help push carbon units up the glycolytic chain to form glucose. Changing concentrations of these compounds can effectively regulate PK activity to control carbon flux at this important branch-point.

Posttranslational Control of Enzyme Activity

Liver PK also exists in two forms: a low-K_m form and a high-K_m form. The low-K_m form is more active at physiological PEP concentrations and is more sensitive to the activator, fructose-1,6-bisphosphate. The high-K_m form is less

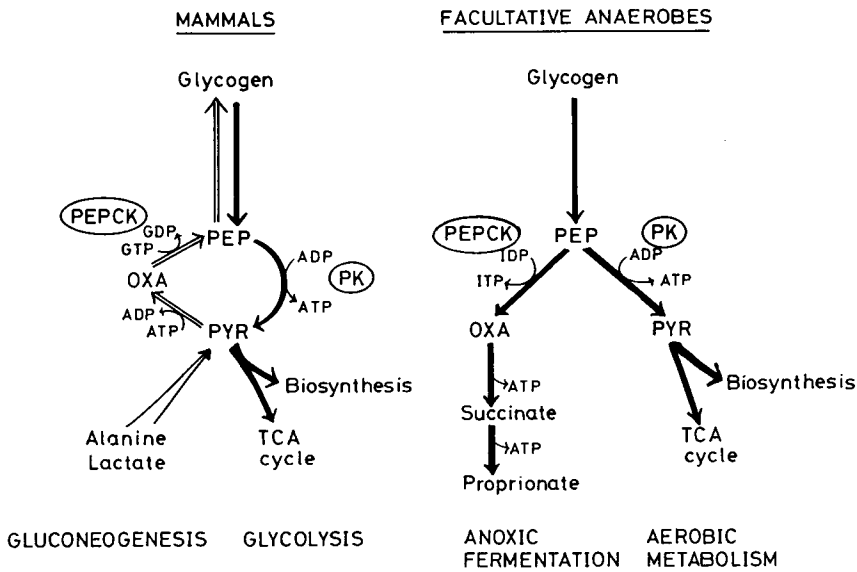


Figure 6. Variations on phosphoenolpyruvate (PEP) and pyruvate (PYR) metabolism in animals. In mammalian liver, pyruvate kinase (PK) and PEP carboxykinase (PEPCK) function in opposite directions to support glycolysis versus gluconeogenesis. In anoxia-tolerant mollusks, PEP is routed via PK when oxygen is present and via PEPCK in anoxia. Note that PEPCK is adapted for physiological function in opposite directions in the two situations.

active at physiological PEP concentrations and is more sensitive to the inhibitor, L-alanine. The two forms of PK are interconverted by reversible phosphorylation of the enzyme. Thus, enzyme phosphorylation can act to increase enzyme sensitivity to allosteric compounds and greatly increase their effectiveness. For example, PK is phosphorylated during starvation to reduce oxidation of PEP so that PEP may be used instead as a gluconeogenic precursor.

Properties of PK from Other Animals Reflect Different Metabolic Demands

Homologous pyruvate kinases from other animals have substantially different properties and, in some animals, for example, these vary in response to changing environmental oxygen. The more anoxia-tolerant the animal (e.g., diving mammals, turtles, and marine mollusks), the greater the sensitivity of PK to fructose 1,6-P_2 and L-alanine regulation, including the extension of allosteric control by these metabolites to the muscle enzyme form (Storey and Hochachka, 1974a,b; Plaxton and Storey, 1984). In the extreme, marine mollusks have developed a PK that is particularly sensitive to L-alanine, as the data for PK from whelk muscle shows (Table 2). This sensitivity is key in determining the aerobic (via PK and the tricarboxylic acid cycle) versus anoxic (via PEP carboxykinase and into succinate or propionate as fermentation end products) disposition of PEP in these facultative anaerobes (de Zwaan, 1983; Storey, 1985a). Conversely, highly aerobic animals such as squid, have muscle PKs that are completely insensitive to these modifiers, but have instead substituted inhibitory control by citrate that links PK activity to that of the tricarboxylic acid cycle (Table 2) (Storey and Hochachka, 1975; Storey, 1985b).

Binding of Pyruvate Kinase to Regulatory Proteins

Research into the control of glycolytic rate in muscle has revealed that enzyme activity may also be controlled by reversible formation of enzyme–F-actin complexes. F-actin is a polymer of actin molecules and makes up one of the two muscle filaments that participate in muscle contraction. It can be shown that enzymes, such as PK, readily bind to F-actin filaments under conditions of low ionic strength *in vitro* (Chan et al., 1986). Extrapolation of the conditions in the test tube to conditions found in cells suggests that a significant proportion of PK may be bound *in vivo* (Brooks and Storey, 1991a). In the case of PK, binding decreases the enzyme activity by increasing the K_m value for PEP. F-actin, therefore, acts like ATP and alanine in allosterically inhibiting the enzyme.

Cellular *Milieu* and Enzyme Function

Until now, we have dealt solely with changes to the enzyme molecule as the mechanism of adaptation, but enzyme function and stability can also be strongly influenced by the composition of the intracellular *milieu* in which the enzyme

operates. We will discuss several of the compounds that make up the cellular *milieu*, some that change enzyme function, and others that stabilize enzymes against injury from environmental stress.

Substrates and Allosteric Effectors

Changes in enzyme regulatory properties occur over evolutionary time, but to be of use to the individual animal, these properties must be matched to the natural concentration range of enzyme substrates and effectors in the cell. Furthermore, the levels of these metabolites must change in a meaningful way under different physiological conditions in order to produce the desired change in enzyme activity. For an example, we can again turn to the regulatory control of PK. Table 3 compares the kinetic constants of the aerobic (dephosphorylated) versus anoxic (phosphorylated) forms of whelk muscle PK with measured metabolite levels in the same tissue.

In aerobic animals, the K_m for PEP is well below the concentration of this substrate in the cell so that enzyme function is never substrate-limited. Alanine is a very poor inhibitor of the aerobic enzyme form and at physiological levels of alanine there would be very little effect of this inhibitor on the aerobic enzyme. A complete reversal occurs in the anoxic situation, however. *In vivo* levels of PEP drop and the K_m for PEP rises so that substrate concentration *in vivo* is now 17-fold lower than the K_m value. This situation greatly reduces enzyme activity *in vivo* (Figure 1). In addition, alanine inhibition of the anoxic PK form is so strong that enzyme activity is virtually undetectable at physiological alanine levels.

pH Regulation

All organisms regulate intracellular pH and typically the pH of the cytosol is kept close to 7.0 (at 25°C). The need for rigorous pH regulation stems from the importance of maintaining the charge state of ionized groups on proteins, in particular the imidazole groups of histidine, for these are critical to enzyme–ligand

Table 3. Whelk Muscle: Kinetic Constants of Pyruvate Kinase and Metabolite Levels in Aerobic Versus Anoxic Tissue[a]

	Pyruvate Kinase Kinetics			*Metabolite Concentration*	
	Kinetic Constant	*Aerobic (Dephos)*[b]	*Anoxic (Phos)*	*Aerobic*	*Anoxic*
PEP (mM)	K_m	0.07	0.85	0.33	0.05
Fructose-1,6-P_2 (μM)	K_a	0.05	1.3	60	68
L-alanine (mM)	K_i	24.5	0.05	3	6

Notes: [a]Data from Plaxton and Storey (1984) and (unpublished data).
[b]Data for dephosphorylated (Dephos) and phosphorylated (Phos) enzyme forms are given, representing whelk muscle pyruvate kinase in aerobic and anoxic tissue, respectively.

interactions, the individual catalytic steps in an enzyme reaction sequence, and structural transitions of the enzyme (Reeves, 1977; Hochachka and Somero, 1984). In homeotherms, adaptive mechanisms preserve an optimum pH for enzyme function in the face of environmental or physiological stress. To do this, animals may alter the capacity of the intracellular-buffering system to match the proton load associated with metabolic function in each organ. Intracellular buffering is supplied by imidazole groups on protein-bound histidine residues and histidine-containing dipeptides, as well as inorganic phosphate (Hochachka and Somero, 1984). Not surprisingly, then, buffering capacity is highest in white skeletal muscle which generates large amounts of H^+ along with lactate anions during burst contraction.

When cellular temperature changes (in cold-blooded animals), cellular pH is not held constant but is allowed to vary. However, the properties of the cellular-buffering substances (histidine residues, see above) are such that enzyme function is conserved with changing body temperature. This happens because the temperature coefficient of imidazole ensures a constant fractional dissociation of active site residues. Thus, the correct ionization state of reactive groups is preserved over a wide temperature range. This effect, called alphastat regulation, is illustrated for PK in Table 4. When assayed in imidazole buffer, the kinetic properties of PK are conserved when temperature drops from 20°C to 5°C if buffer pH is allowed to self-adjust with the temperature change (rising to pH 7.25 at 5°C). Kinetic constants change significantly, however, if pH is held constant at 7.0 at the lower temperature. It is obvious, then, that kinetic properties are conserved only when alphastat regulation is followed.

Stabilizing Solutes

Perturbation of enzyme structure or function by environmental stress can often be counteracted by the actions of specifically selected low molecular weight solutes whose effects on the enzyme oppose those of the perturbing stress. This type of adaptation is well developed where cells face dehydration and/or high salt concentration. Species that are desiccation tolerant and freeze tolerant (extracellular ice

Table 4. Interactions Between pH and Temperature on the Kinetic Properties of Pyruvate Kinase from Whelk Muscle[a]

	Alphastat Regulation		*pH Held Constant*
	pH 7.0 at 20°C	*pH 7.25 at 5°C*	*pH 7.0 at 5°C*
Km PEP (mM)	0.092	0.10	0.21
I_{50} Alanine (mM)	2.26	2.26	2.54
I_{50} ATP (mM)	22.0	22.8	28.6

Note: [a]Data from Michaelidis and Storey (1990).

formation draws water out of and concentrates ions in cells) face this stress in the extreme, but this is also a problem for estivating animals (e.g., lungfish) and many marine animals (Hochachka and Somero, 1984; Storey and Storey, 1988). The solution, in many cases, is the accumulation of high concentrations of polyhydroxy alcohols (glycerol is the most common) that have two functions: (1) limit the reduction of cell volume by colligative actions, and (2) stabilize macromolecular structure (Storey and Storey, 1988). Glycerol accumulates as a natural protectant in freeze-tolerant insects and frogs, desiccation-tolerant brine shrimp and nematodes, and halophytic algae. The glycerol content of cold-hardy insects, for example, may exceed 2 M or 20% of total body weight. The use of polyols is ideal for stabilizing enzyme structure and function. Polyols stabilize the native state of proteins by maintaining the hydration shell around them or, when water activity is very low, by replacing water in direct hydrogen bonding between polyols and polar residues on the proteins (Clegg et al., 1982; Fink, 1986). Polyols are also an excellent choice from the point of enzyme function, for the compounds are compatible solutes that have little or no effect on the kinetic or regulatory properties of enzymes.

It is interesting to note that polyol production itself is accomplished by employing some of the strategies of enzyme adaptation discussed above. Glycerol accumulation in insects during the autumn cold-hardening period is supported by seasonal increases in the amounts of glycogenolytic and hexose-monophosphate shunt enzymes, a structural reorganization to group these enzymes into functional complexes that channel carbon and reducing equivalents into polyol production, and a mechanism of triggering the whole process (low-temperature activation of glycogen phosphorylase) that ensures that polyols are produced only when needed (Storey and Storey, 1988).

SUMMARY

We have discussed six different types of mechanisms that are used to adapt enzyme function to allow metabolism to respond to changing physiological demands or environmental stresses. Although the present discussion has been largely limited to enzymes of glycolysis, the principles are equally applicable to virtually all aspects of metabolic function. In particular, control via reversible protein phosphorylation is proving to be widespread for the coordination of diverse cellular enzymes and proteins (Boyer and Krebs, 1987). For example, reversible phosphorylation is now well known to extend to the control of membrane ion-channel proteins, regulating both their incorporation into membranes and their activity once inserted (Reuter, 1987). Such coordinated control over a wide variety of cellular processes is proving to be critical to metabolic rate depression. Hibernating mammals, for example, retain control over membrane potential difference as body temperature falls to ambient; the same species in the summer-adapted state, however, are just as susceptible to the destructive effects of hypothermia on membrane ion regulation

as are humans. The lessons to be learned from comparative biochemistry are many. The combinations of metabolic controls that can be applied for the adaptation of enzyme function are nearly limitless and mechanisms can be found to overcome almost any environmental constraint or meet any physiological demand. Increasingly, we are applying the information gained from comparative studies and nowhere is this more true that in the development of organ preservation technology. Both the mechanisms of metabolic rate depression and of freeze tolerance that are found in animals are directly applicable to the development of mechanisms for organ transplant technology and some of the most effective therapies presently used (e.g., glycerol as a cryoprotectant; inhibitors of membrane ion transport to reduce energy demands) effectively mimic the strategies used in the natural situation.

REFERENCES

Baldwin, J. & Hochachka, P. W. (1970). Functional significance of isoenzymes in thermal acclimatization: acetylcholinesterase from trout brain. Biochem. J. 116, 883–887.

Baldwin, K. M., Winder, W. W., Terjung, R. L., & Holloszy, J. O. (1973). Glycolytic enzymes in different types of skeletal muscle: adaptation to exercise. Am. J. Physiol. 225, 962–966.

Berlund, L. & Humble, E. (1979). Kinetic properties of pyruvate kinase type A from kidney and type M from muscle. Arch. Biochem. Biophys. 195, 347–361.

Bowler, K. & Fuller, B. J. (1987). Temperature and Animal Cells. Soc. Exp. Biol. Symp. Vol. 41, Cambridge, UK.

Boyer, P. D. & Krebs, E. G., (eds.) (1987). Control by phosphorylation. In: The Enzymes, Vol. 18. Academic Press, New York.

Brooks, S. P. J. & Storey, K. B. (1988a). Subcellular enzyme binding in glycolytic control: *in vivo* studies with fish muscle. Am. J. Physiol. 255, R289–R294.

Brooks, S. P. J. & Storey, K. B. (1988b). Reevaluation of the "glycolytic complex" in muscle: a multitechnique approach using trout white muscle. Arch. Biochem. Biophys. 267, 13–22.

Brooks, S. P. J. & Storey, K. B. (1990). A cGMP stimulated protein kinase phosphorylates pyruvate kinase in an anoxia tolerant marine mollusk. J. Comp. Physiol. B 160, 309–316.

Brooks, S. P. J. & Storey, K. B. (1991a). A quantitative evaluation of the effect of enzyme complexes on the glycolytic rate *in vivo*: mathematical modeling of the glycolytic complex. J. Theor. Biol. 149, 361–368.

Brooks, S. P. J. & Storey, K. B. (1991b). Studies on the regulation of enzyme binding during anoxia in isolated tissues of *Busycon canaliculatum*. J. Exp. Biol. 156, 467–481.

Brooks, S. P. J. & Storey, K. B. (1992). Mechanisms of glycolytic control during hibernation in the ground squirrel *Spermophilus lateralis*. J. Comp. Physiol. B 162, 23–28.

Brooks, S. P. J. & Storey, K. B. (1993). Control of glycolytic enzyme binding: effect of changing enzyme substrate concentrations on *in vivo* enzyme distributions. Mol. Cell. Biochem. 122, 1–7.

Brooks, S. P. J., Suelter, C. H., & Storey, K. B. (1988). Theoretical analysis of compartmented coupling in linear enzyme systems. J. Mol. Recognition 1, 63–68.

Busa, W. B. & Nuccitelli, R. (1984). Metabolic regulation via intracellular pH. Am. J. Physiol. 246, R409–R438.

Chan, L. M., Hickmon, T., Collilns, C. J., & Davidson, Y. Y. (1986). The interaction of rabbit muscle pyruvate kinase with F-actin, Fed. Proc. 45, 1657.

Clarke, F. M., Stephan, P., Huxham, G., Hamilton, D., & Morton, D. J. (1984). Metabolic dependence of glycolytic enzyme binding in rat and sheep heart. Eur. J. Biochem. 138, 643–649.

Clegg, J. S. (1981). Intracellular water, metabolism and cellular architecture. Collect. Phenom. 3, 289–312.

Clegg, J. S., Seitz, P., Seitz, W., & Hazelwood, C. F. (1982). Cellular response to extreme water loss: the water-replacement hypothesis. Cryobiology 19, 306–316.

Cohen, P. (1976). Control of Enzyme Activity. Chapman and Hall, London.

Cohen, P. (1980). Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation. Elsevier, Amsterdam.

Crowe, J. H. & Clegg, J. S. (1978). Dry Biological Systems. Academic Press, New York.

de Zwaan, A. (1983). Carbohydrate metabolism in bivalves. In: The Mollusca (Wilbur, K. M., ed.). pp. 137–175, Academic Press, New York.

Engstrom, L., Ekman, P., Humble, E., & Zetterqvist, O. (1987). Pyruvate kinase. In: The Enzymes (Boyer, P. D. and Krebs, E.G., eds.), pp. 45–75, Academic Press, New York.

Everse, J. & Kaplan, N. O. (1973). Lactate dehydrogenase: structure and function. Adv. Enzymol. 37, 61–133.

Fink, A. L. (1986). Effects of cryoprotectants on enzyme structure. Cryobiology 23, 28–37.

Foe, L. G. & Kemp, R. G. (1982). Properties of phospho and dephospho forms of muscle phosphofructokinase. J. Biol. Chem. 257, 6368–6372.

Gilles, R. (1985). Circulation, Respiration and Metabolism. Springer-Verlag, Heidelberg.

Goodridge, A. G. (1987). Dietary regulation of gene expression: enzymes involved in carbohydrate and lipid metabolism. Ann. Rev. Nutr. 7, 157–185.

Guppy, M., Hulbert, W. C., & Hochachka, P. W. (1979). Metabolic sources of heat and power in tuna muscles. II. Enzyme and metabolite profiles. J. Exp. Biol. 82, 303–320.

Hayakawa, Y. (1985). Activation mechanism of insect fat body phosphorylase kinase by cold. Insect Biochem. 15, 123–128.

Hers, H. G. & Hue, L. (1983). Gluconeogenesis and related aspects of glycolysis. Ann. Rev. Biochem. 52, 617–653.

Hochachka, P. W. (1986). Defense strategies against hypoxia and hypothermia. Science 231, 234–241.

Hochachka, P. W. & Somero, G. N. (1984). Biochemical Adaptation. Princeton University Press, Princeton, NJ.

Hochachka, P. W. & Guppy, M. (1987). Metabolic Arrest and the Control of Biological Time. Harvard University Press, Cambridge, MA.

Holloszy, J. O., Oscai, L. B., Don, I. J., & Mole, P. A. (1970). Mitochondrial citric acid cycle and related enzymes: adaptive response to exercise. Biochem. Biophys. Res. Commun. 40, 1368–1373.

Kitamura, K., Kangawa, K., Matsuo, H., & Uyeda, K. (1988). Phosphorylation of myocardial fructose-6-phosphate,2-kinase: fructose-2,6-bisphosphatase by cAMP-dependent protein kinase and protein kinase C. J. Biol. Chem. 263, 16796–16801.

Low, P. S. & Somero, G. N. (1975). Pressure effects on enzyme structure and function *in vitro* and under simulated *in vivo* conditions. Comp. Biochem. Physiol. 52B, 67–74.

Luther, M. A. & Lee, J. C. (1986). The role of phosphorylation in the interaction of rabbit muscle phosphofructokinase with F-actin. J. Biol. Chem. 261, 1753–1759.

Malan, A. & Canguilhem, B. (1989). Living in the Cold, 2nd International Symposium. John Libbey Eurotext, Paris.

Masters, C. J., Reid, S. & Don, M. (1987). Glycolysis—new concepts in an old pathway. Mol. Cell. Biochem. 76, 3–14.

Michaelidis, B. & Storey, K. B. (1990). Influence of pH on the regulatory properties of aerobic and anoxic forms of pyruvate kinase in a marine whelk. J. Exp. Zool. 253, 245–251.

Moon, T. W. & Hochachka, P. W. (1971). Temperature and enzyme activity in poikilotherms: isocitrate dehydrogenase in rainbow trout liver. Biochem. J. 123, 695–705.

Narabayashi, H., Randolph Lawson, J. W., & Uyeda, K. (1985). Regulation of phosphofructokinase in perfused rat heart. J. Biol. Chem. 269, 9750–9758.

Paul, R. J. (1986). Smooth muscle energy metabolism: cytosolic compartmentation of metabolism and function. In: Advances in Physiological Research (McLennan, H., Ledsome, J. R., McIntosh, C. H. S. and Jones, D. R., eds.), pp. 295–304, Plenum Press, New York.

Place, A. R. & Powers, D. A. (1979). Genetic variation and relative catalytic efficiencies: lactate dehydrogenase B allozymes of *Fundulus heteroclitus*. Proc. Natl. Acad. Sci. USA 76, 2354–2358.

Plaxton, W. C. & Storey, K. B. (1984). Purification and properties of aerobic and anoxic forms of pyruvate kinase from red muscle tissue of the channeled whelk, *Busycotypus canaliculatum*. Eur. J. Biochem. 143, 257–265.

Plaxton, W. C. & Storey, K. B. (1986). Glycolytic enzyme binding and metabolic control in anaerobiosis. J. Comp. Physiol. 156, 635– 640.

Reeves, R. B. (1977). The interaction of body temperature and acid–base balance in ectothermic vertebrates. Ann. Rev. Physiol. 39, 559–586.

Reuter, H. (1987). Modulation of ion channels by phosphorylation and second messengers. News Physiol. Sci. 2, 168–171.

Sakakibara, R. & Uyeda, K. (1983). Differences in the allosteric properties of pure low and high phosphate forms of phosphofructokinase from rat liver. J. Biol. Chem. 258, 8656–8662.

Sidell, B. D. (1983). Cellular acclimatisation to environmental change by quantitative alterations in enzymes and organelles. In: Cellular Acclimatisation to Environmental Change (Cossins, A., & Sheterline, P., eds.), pp. 103–120, Cambridge University Press, Cambridge.

Srere, P. A. (1987). Complexes of sequential metabolic enzymes. Ann. Rev. Biochem. 56, 21–56.

Storey, K. B. (1985a). A re-evaluation of the Pasteur effect: new mechanisms in anaerobic metabolism. Mol. Physiol. 8, 439–461.

Storey, K. B. (1985b). Kinetic and regulatory properties of pyruvate kinase isozymes from flight muscle and fat body of the cockroach, *Periplaneta americana*. J. Comp. Physiol. 155, 339–345.

Storey, K. B. (1988). Suspended animation: the molecular basis of metabolic rate depression. Can. J. Zool. 66, 124–132.

Storey, K. B. & Hochachka, P. W. (1974a). Enzymes of energy metabolism in a vertebrate facultative anaerobe, *Pseudemys scripta*. J. Biol. Chem. 249, 1423–1427.

Storey, K. B. & Hochachka, P. W. (1974b). Glycolytic enzymes in muscle of the Pacific dolphin: role of pyruvate kinase in aerobic–anaerobic transition during diving. Comp. Biochem. Physiol. 49B, 119–128.

Storey, K. B. & Hochachka, P. W. (1975). Squid muscle pyruvate kinase: control properties in a tissue with an active α-GP cycle. Comp. Biochem. Physiol. 52B, 187–191.

Storey, K. B. & Storey, J. M. (1988). Freeze tolerance in animals. Physiol. Rev. 68, 27–84.

Suarez, R. K., Brown, G. S., & Hochachka, P. W. (1986). Metabolic sources of energy for hummingbird flight. Am. J. Physiol. 251, R537–R542.

RECOMMENDED READINGS

Bowler, K. & Fuller, B. J. (1987). Temperature and Animal Cells. Soc. Exp. Biol. Symp. Vol. 41, Cambridge, UK.

Hochachka, P. W. & Somero, G. N. (1984). Biochemical Adaptation. Princeton University Press, Princeton, NJ.

Malan, A. & Canguilhem, B. (1989). Living in the Cold, 2nd International Symposium. John Libbey Eurotext, Paris.

Storey, K. B. & Storey, J. M. (1988). Freeze tolerance in animals. Physiol. Rev. 68, 27–84.

Storey, K. B. & Storey, J. M. (1989). Facultative metabolic rate depression: molecular regulation and biochemical adaptation in anaerobiosis, hibernation, and estivation. Quart. Rev. Biol. 56, 145–174.

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Chapter 7

Our Aqueous Heritage:
Evidence for Vicinal Water in Cells

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INTRODUCTION

Aqueous Nature of the Cell

Life originated in an aqueous medium, and to this day every living cell carries with it this unique heritage. In the process of evolution, cells internalized the primordial aqueous environment; all of us are walking vestiges of the original sea. The mechanistic advantages of the aqueous pool are obvious: an internal fluid environment not only facilitates the transport of nutrients and metabolites but also provides the ability to deform, thus allowing responses to outside stresses and, in some cases, movement to occur. To preserve the internal aqueous *milieu*, a barrier was required—hence the cell membrane.

"All cellular processes take place in aqueous solution, and it is essential to understand the properties of water in order to understand biological processes." This statement comes from one of the most recent textbooks on molecular cell biology. Sadly, but common to such texts, what follows is but a brief description of the ordinary liquid. Its physical properties and peculiarities are described, but the reader is given no information about the nature and role of water at interfaces such as in the environment of the cell. In short, from such descriptions one must infer that water in cells is just the same as water in a beaker or a cup of tea—that water is water.

Until the last 30 or 40 years, relatively little effort had been made to elucidate in any detail the structure and function of water in cells. Fortunately, however, the situation is changing, and a growing body of evidence already suggests that at least some of the water in cells differs in its properties (such as density, viscosity, dielectric behavior, and heat capacity) from the ordinary bulk liquid. The lack of suitable measurement techniques has hampered the attainment of a more definitive description of intracellular water; nonetheless, much about it now appears within our grasp.

Nature of Bulk and Cell-Associated Water

It is probably fair to say that life reflects and depends on three chemical aspects: (1) sp^3 hybridization of carbon and oxygen (as well as nitrogen), (2) the facile way carbon atoms may bind to other carbon atoms, and (3) the unique ability of hydrogen to form H-bonds. The catenation and tetrahedral geometry of the carbon atoms are the fundamental features of all organic compounds in cells. However, the most abundant molecule in any living cell is water.

Consider a typical eukaryotic cell, for instance, a muscle cell. By weight, the cell is about 75% water. However, this estimate fails to convey the truly aqueous nature of the cell; a far more realistic description is in terms of mole ratios. Because of the low molecular weight of water, the nominal 75% water translates into a very large number of moles of water relative to the number of moles of other cell constituents. Thus, the aqueous nature of the cell is better illustrated by noting that for every 20,000 water molecules there are only about 75 lipid molecules, 100 sodium, potassium, and chloride ions (with at most a few hundred other small molecules or ions), and only one or two protein molecules. By sheer numbers water molecules totally dominate, and in this perspective life is merely some complex biochemistry in an extensive matrix of water, stabilized by a few lipids and macromolecules. The two dominating factors in cell biology are thus, simply, water molecules and interfaces.

Obviously, then, any fundamental analysis of cell functioning must be based on proper physico-chemical understanding of aqueous systems including the topics usually lumped together under the heading of surface and colloid chemistry. Thus, we take as our starting point water and aqueous solutions, and proceed to discuss how the properties of these may be affected by proximity to the various interfaces of the cell.

The purpose of this chapter and the next is two-fold: first, to gather and review some of the evidence for structural changes in water and aqueous solutions adjacent to an interface, and secondly, to illustrate how these structural effects are manifested in the functioning of cells. What is addressed is not only the structure of bulk water but also the unique aspects of water in cells or near any surfaces. We refer to water near interfaces as "vicinal water" after the Latin word for neighbor. It is the vicinal water that suggests itself as the most likely site for most of the intermediary metabolism in living cells.

THE STRUCTURE OF BULK WATER

Occurrence and Properties of Water

Water is a most unusual liquid; it is also the only inorganic liquid to occur naturally on earth and the only chemical compound on earth to occur in all of its

three phases: solid, liquid, and vapor. More importantly, the properties of water differ, sometimes dramatically, from the properties expected for a compound of such low molecular weight: its melting and boiling points are high, the heat of vaporization is high, and its heat capacity is remarkably high, as is the dielectric constant. Many other properties also differ from "classically" expected values: the density of solid water is less than that of the liquid (at the melting point), and the liquid water possesses a maximum in density near 4°C.

Hydrogen Bonds

The explanation for the unusual properties of water is found in the ability of the water molecule to form hydrogen bonds (H-bonds). These bonds are tetrahedrally disposed around the molecule, leading to a very open structure. In ice the number of nearest neighbors (n_x) is 4.0, and in liquid water this openness is essentially retained, with numbers of nearest neighbors being 4.2–4.4 over the range of temperatures of physiological interest. For most ordinary liquids n_x is around 8 to 11.

Notwithstanding nearly a century of inquiries into the structure of water, a final and complete description of the molecular architecture of water still escapes us. The reader is directed to the monograph by Eisenberg and Kauzmann (1969) as well as to a seven-volume treatise on water edited by Franks (1972–1982). A facile introduction to the current state of research on water and solutions is provided by Franks (1983) in a small monograph that provides a handy "abstract and summation" of the seven-volume treatise mentioned above.

Structure

In crystalline ice, water achieves a near-perfect tetrahedral bonding. From X-ray and neutron-diffraction patterns, such a tendency to four-coordination is inferred as well for bulk water. The tetrahedral nature is the result of the sp^3 hybridization of the electrons of the oxygen atom. [Note, however, that Finney (1982) points to the danger of carrying the concept of tetrahedrally disposed charge distributions too far.]

Most current theories of water depict the structure as a disordered array of H-bonded water molecules such that a continuous lattice of chains of water molecules can be found throughout the liquid. In all probability, only a few water molecules are "free" in the sense of not being H-bonded to at least one other molecule, although only a small fraction of water molecules have all four possible bonds intact at any moment. Even in the momentary absence of an H-bond between a water molecule and its nearest neighbor, strong interactions are still operating (dipole–dipole, quadrupole and higher interactions, London-dispersion forces, etc). The structure is mobile in the extreme; any given configuration of a molecule and its neighbors is likely to disintegrate and reform into a new configuration within a time interval of 10–100 psec.

Experimental Techniques

Our only direct information about the structure of water comes from X-ray diffraction and neutron-scattering studies. The scattering experiments yield information in the form of a radial distribution function, specifying essentially the average number of neighboring water molecules as a function of the distance from any one given molecule. Unfortunately, however, there is no direct way this information can reveal the structure. Instead, specific models have to be proposed and predictions made as to the likely position of neighbors based on the model; these predictions are then compared to the actually observed radial distribution function.

Two-State Model

Many other physical measurements may give clues to requirements that must be met by any suggested structural model for water. Infrared and Raman spectroscopy have provided a great deal of information. An interesting result from such optical studies is the suggestion that two distinct structural species may be present in liquid water! An obvious model in this case is the crystalline clathrate hydrates or (in the solid state) the various high-pressure ice polymorphs (of which about 10 different types are known). Other kinds of measurements also suggest the possible reality of a "two-state" model of water, a concept that was very popular 20 years ago. This model is at variance with nearly all current theoretical attempts to model water structure. Possible resolution of this dichotomy has been suggested by Blumberg et al. (1984) in terms of the Stanley–Teixeira model (Stanley and Teixeira, 1980a,b).

Computer Simulations

Over the past 15 years, a large number of papers have appeared dealing with computer simulations of water structure, thanks to the increased capacity and availability of fast computers. Simulations are based on accepting a reasonable expression for the pairwise interaction of water molecules, namely, the pair potential (energy) function. Much has been learned from simulation studies, and the results are valuable hints as to what the structure of liquid water may be, but not necessarily as to what the structure must be. Because of computational limitations, the typical sample of water molecules used in such analyses is about 500. If these molecules formed a small droplet, the radius would be five molecules, half of which would be from the "outside layer." Thus, one must expect some dramatic surface effects. Furthermore, as discussed below, the calculations are based on a pairwise potential energy function; this is probably a most serious limitation, as also discussed below.

Stanley and Teixeira Model

A particularly intriguing model of water has been proposed by Stanley and Teixeira (1980b), aimed at elucidating water structure at low temperature. (See also Stanley, 1979; Stanley and Teixeira, 1980a; Stanley et al., 1981; Geiger and Stanley, 1982; Blumberg et al., 1984). The model is based on the idea of percolation theory, i.e., interconnectivity on a lattice. The structure is that of an infinite H-bonded network, continuously being restructured. At any moment, some bonds are strained or broken; within the network are "patches" of lower density and lower entropy than the overall values. The spatial positions of the various types of connectedness are not randomly distributed but are correlated. In particular, the structure contains tiny patches of four-bonded molecules. The size of the patches increases with decreasing temperature. The correlated sites percolation model has been further developed by Stanley et al. (see Sciortino et al., 1990). In this model, the water is pictured as a transient gel with a random network of H-bonds linking water molecules over relatively large distances in the liquid.

Cooperativity

Some most impressive computer simulations have been made in efforts to model the structure of liquid water. Yet, because these calculations usually are based on pair additivity of the potentials for the H-bonded water molecules, the possibility exists that subtle effects may escape the theoretician, as no means are provided to incorporate the possibility of extensive cooperativity—an aspect that Henry Frank (1972) has so eloquently stressed. Very likely, this is the crux of the problem of interfacially modified water; if nothing else, the thermal anomalies (discussed below) in the properties of vicinal water strongly implicate cooperativity on a large scale—a collective behavior of water molecules that no existing potential function is able to reproduce. The cooperativity reflects nonpair additivity, and it does not seem plausible that "effective" potential energy functions can be devised that will remedy the specific lack of a detailed understanding of many-body interactions in water. Attempts to allow for cooperativity have been made by Finney, Barnes, and co-workers, notably Quinn and Nicholas (see Barnes et al., 1979).

The above gives the reader some insight into the proposed structure and the complexities of defining ordinary bulk water, and we now turn our attention to its "conceptual relative," vicinal water.

WATER NEAR INTERFACES

Interfaces and Forces

Vicinal water is defined as water the structure of which is modified by proximity to an interface but excluding chemically "bound" water directly on the surface. In

biological systems reference is often made to "bound water" and "nonfreezable" water. Such terms refer to water that is chemically strongly bonded, corresponding to energies of the order of kilocalories per mole of bound water. Vicinal water represents energies of interaction that are far less—perhaps by one to two orders of magnitude—but that exhibit cooperative effects of long range. As for bound water, there are about as many definitions as ways of measuring the water in question. More than anyone, Deryaguin and co-workers in Moscow have contributed to our current understanding of modified liquid layers at interfaces ("boundary layers"). See, for instance, Deryaguin (1964, 1980, 1987), Churaev and Deryaguin (1985), and Deryaguin and Churaev (1972, 1974, 1977, 1985, 1986, 1987).

As will be emphasized, some "interfaces" do not induce vicinal water, such as the water/air and water/saturated water vapor interfaces. Furthermore, it is beyond the scope of this chapter to discuss the structural aspects of water/immiscible liquid interfaces or water/pure metal interfaces (free of any oxides).

It is important to recognize that vicinal water is not likely to exist as a definite layer (of a given thickness) adjacent to a smooth surface. Rather, what does exist is a gradient of enhanced vicinal structuring as one approaches the surface (at least up to those few layers of water molecules that are energetically strongly influenced by the surface). The "enhancement" experienced as one approaches the surface may be merely in the fraction of four-coordinated water molecules relative to those with three or fewer H-bonds. In any case, it is still possible to speak of an "apparent thickness" of the vicinal water, for instance, the distance from the surface over which the enhanced four-coordination decreases to 1/e of its maximal extent. It should also be obvious that there must be an equilibrium between the four-coordinated water molecules and those with only three, two, one, or no H-bonds to neighboring water molecules. Additionally, this equilibrium is probably as labile as any bulk water structure, say within one or two orders of magnitude. Note, however, examples of very slow rates in cases where notable hysteresis is observed.

Surface tension measurements of water have frequently revealed the existence of anomalies in the temperature dependence of the surface tension. The structures responsible for the anomalies occur in the water near the interface, and they do not occur at the pure air/water interface. Thus, in experiments where surface tension has been measured with minimal influence of a confining solid, no thermal anomalies are observed. However, there is little doubt that the structure of the water at the water/air (or water/water vapor phase) interface is also different from that of the bulk water. Adamson (1982) argues "that a 'quiescent' liquid surface is actually in a state of violent agitation on the molecular scale with individual molecules passing rapidly back and forth between it and the bulk regions on either side." The depth of this strongly disturbed layer is probably of the order of 10 to 20 molecular diameters. [As we discuss later, the presence of a monolayer at the vapor/liquid interface appears to change the situation dramatically (Alpers and Hühnerfuss, 1983; Hühnerfuss, 1983; Hühnerfuss and Walter, 1983; and Hühnerfuss, Lange, and Walter, 1985a,b).]

Surface-Ion Water Interactions

It should be stressed again that the structure of bulk water continues to escape a satisfactory description in spite of vast efforts to overcome this problem. Computer simulations and scattering techniques yield structure determinations that are often ambiguous and inconclusive. Thus, it is hardly surprising that our knowledge of the molecular structure of water at interfaces is notably incomplete. Obviously, water near a charged surface will differ from the bulk phase because of strong hydration forces, i.e., ion/water-dipole interactions. Such forces are generally very strong and lead to solvation energies of the order of many kilocalories per mole of "water of hydration." Obviously these interactions are crucial to the structural and functional integrity of macromolecules and membranes, and are of signal importance to cell physiology, being related to such phenomena as "nonfreezable water" and "bound water" in individual cells and whole tissues.

However, ion-hydration effects are not likely to lead to extensive restructuring of the aqueous phase of the cell interior. It is probable that surface ion hydration leads to structural effects extending only over a few molecular diameters, say two or three layers or roughly 5 to 8 Å. Evidence is now accumulating that suggests that other structural effects of surfaces do occur and that these may influence the adjacent water structures over much larger distances. Some of the evidence for such vicinal water structuring is reviewed here, and in Chapter 8 we discuss how this vicinal water influences cell functioning.

Paradoxical Effect

There is considerable evidence that suggests that *vicinal water is induced by proximity to most (or all) "solid interfaces," regardless of the detailed specific chemical nature of the surface.* This phenomenon is referred to as the "paradoxical effect." The phrase "solid interface" is used in this context in a most general sense, from mica or quartz plates, mineral grains, and membranes to large macromolecules (above a certain critical molecular weight) and possibly some types of aggregates, such as micelles and liposomes. The existence of the paradoxical effect is an important point, as it is far more difficult to study the intracellular aqueous environment experimentally than to study physico-chemically well-defined model systems. If we are able to demonstrate that vicinal water occurs near *any* aqueous/solid interface, then we may conclude that vicinal water must also be induced by some (or all) of the surfaces within cells (such as membranes and cytomatrix structures). Furthermore, some direct evidence is available that indicates the existence and role of vicinal water in cellular systems. We are not concerned here with the very important, more traditional aspects of the hydration of the cell constituents. Although this aspect is, of course, of immense direct significance to osmotic properties (and cell volume regulation), the main point of this chapter is

the "vicinal hydration" that occurs relatively independently of the ionic charges in the cell, be they from free ions or from the ionized groups on any of the macromolecules present.

Part of the evidence for vicinal structuring of water at interfaces is outlined below. Of necessity, this is a relatively condensed enumeration of some of the available evidence. Far more detail can be found in papers and reviews by various authors, and the reader is encouraged to look to the reference section for appropriate sources. However, one informative review of water at solid surfaces is Clifford's (1975) classic paper examining a vast literature on aqueous interfacial phenomena. The main conclusion reached is that surface-induced changes in water structure may occur but that it is not likely that any such structures—if real—extend more than 100 Å from the surface. Clifford also speculates that one possible mechanism for the creation of interfacial water structures (different from the bulk structure) might be local, geometric constraints preventing the full development of large structured elements of bulk water. One of us (Drost-Hansen, 1976) has tentatively suggested a similar mechanism to explain the paradoxical effect: external geometric constraints might occur with any solid or nonsmooth surface and thus account for vicinal water being induced by ionic, hydrophilic, and hydrophobic surfaces, regardless of the chemical nature of the surface. Given the enormous geometric complexity of a cell, the aqueous cytoplasm would be the obvious place to find vicinal water.

SOME EXPERIMENTAL RESULTS

Etzler's Density Data

Using simple, classical pycnometry, Etzler and Fagundus (1983) have measured the density of water and some other liquids inside the pores of a silica gel with an average pore diameter of 140 Å. With acetone and methanol, the observed densities were close to the values reported for bulk liquids; however, for water a value of 0.966 g/cm^3 was obtained (compared to a bulk value of 0.997 g/cm^3 at 25°C), i.e. 3% lower than the bulk value.

Etzler (1983) has proposed a first-order theory of vicinal water based on the percolation theory treatment of bulk water developed by Stanley and Teixeira (1980a,b). Etzler assumes two distinct types of H-bond connectivity, namely, a perfect four-connected set of water molecules and the remainder, with three, two or one (or no) H-bonds. Based on the idea that two distinct populations of water molecule environments exist, Etzler calculated the increase in the amount of "ice-like" (four-coordinated) local environments and proposed that the tendency to create such more nearly tetrahedral arrangements is somehow induced by proximity to the surface. The vicinal water thus represents an enhancement of the ice-like structure. Etzler then calculated the density of the water in silica pores. This was

found to be 0.970 g/cm^3, which is in excellent agreement with his observed values. Other investigators have measured the density of interfacial water (for a discussion see Drost-Hansen, 1982), notably Low (1979), who observed a value of 0.970 to 0.980 g/cm^3 at a distance of about 50 Å from the surface of clay platelets. It is of some interest that estimates of the density of water in intact cells of *Artemia* (brine shrimp) cysts give a value of 0.979 g/cm^3, in good agreement with these model systems (Clegg, 1985).

The characteristic four-coordination of vicinal water is likely to be maximum near the interface and to decay away from the surface. Hence, the value for the density of vicinal water as reported by Etzler must be the average over the range of the pore radius of 70 Å. In more recent experiments, Etzler and Fagundus (1986) have measured the density of water in silica gels with various porediameters. From these data, it is possible to deduce the density profile as a function of distance. The observation that the average density of vicinal water is less than that for the bulk strongly suggests that the structure of vicinal water differs from that of the bulk structure. It seems probable that vicinal water must be more "open" than bulk water—most likely indicating a greater tendency to more nearly tetrahedral structuring.

Volume Contraction

Another rather simple, but striking line of evidence for the existence of vicinal water comes from the studies of Braun et al. (1987). Automatic, recording, high-precision dilatometers have been found useful for the study of small volume changes in dispersed systems. Using these dilatometers we have examined the settling of particles dispersed in water. By "classical, standard theories" this process is not expected to produce any volume changes: if there are no solvent–particle interactions, then the total volume is independent of the state of aggregation. On the other hand, if the individual particles are indeed surrounded by a "hydration hull" of vicinal water, it is then reasonable to expect that this vicinal layer may become "squeezed out" as the particles settle and are compacted into a sediment. The process of changing vicinal water ("squeezed out") into bulk water must, therefore, reflect a change in molal volumes. As mentioned earlier, the density of vicinal water is most likely near 0.97 g/cm^3 (and the specific volume thus 1.03 cm^3/g), and so the conversion of 1 mol vicinal water to bulk water will result in a volume contraction of $0.03 \times 18 = 0.54$ cm^3/mol vicinal water.

Figure 1 shows the volume contraction observed in the settling of a 35% suspension of quartz particles. Also shown is the sediment height of a column of such a suspension of quartz particles. As the sediment volume approaches a constant value (the final compaction volume), the volume contraction ceases as expected. Similar results are obtained with uniformly sized polystyrene spheres. The volume contraction effect is reversible: when the polystyrene spheres were resuspended, the total volume increased to the original value as the vicinal "hydra-

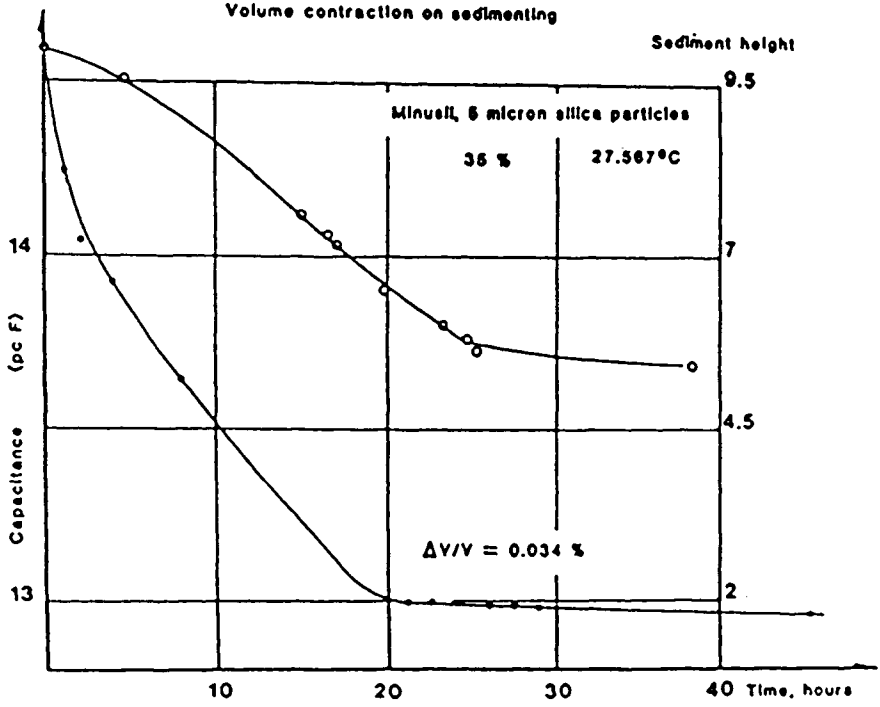

Figure 1. Change in volume on settling (small circles, left scale), expressed as a change in capacitance, as a function of time for a 35% suspension of 5-μm silica particles (Minusil). Also shown, the height of the sediment (large circles, right scale). (From Braun et al., 1987).

tion hulls" around the particles were reformed. From the volume data it is possible to calculate an equivalent "effective thickness" of the vicinal water layers. This turns out to be in the range of 0.01 to 0.04 μm (depending on the assumptions made regarding the packing density of the particles), a value in agreement with many other independent estimates (Drost-Hansen, 1982).

Specific Heat of Vicinal Water

In addition to density differences, vicinal water differs from bulk water in many other physical ways. One of the most unusual features of vicinal water is its unexpectedly high specific heat (see, for example, Etzler, 1988). In discussing the occurrence of thermal anomalies, Lumry and Rajender (1970) noted that

> in systems at constant pressure the occurrence of a sharp change in the slope of a parameter plotted as a function of temperature can occur only if there is a large entropy change; the kinks

have been attributed to the cooperative behavior of a number of water molecules. However, such behavior must reveal itself by heat-capacity spikes which have so far defied detection.

That this may indeed be the case is suggested by differential scanning calorimeter experiments, the results of which indicate the likely occurrence of heat capacity spikes (Braun and Drost-Hansen, 1976; Ling and Drost-Hansen, 1977; and Braun, 1981). Unpublished results from experiments in our lab are referred to in two papers (Etzler and Drost-Hansen, 1983; Drost-Hansen, 1985). However, more recently Etzler and Conners (1989) have succeeded in demonstrating unequivocally the presence of heat capacity spikes using water-saturated quartz gels with an average pore diameter of 242 Å. The results of the study by Etzler and Conners not only imply that most (or all) other thermodynamic properties of vicinal water must differ from the corresponding bulk properties, but (as implied in the statement by Lumry and Rajender) also assure that thermal anomalies in both van't Hoff and Arrhenius plots may indeed reflect large-scale cooperative behavior of the water molecules in vicinal water (in those cases where "extraneous" transitions may be ruled out, such as those expected in many cellular systems due to lipid phase transformations). The temperatures where the heat capacity spikes occur are near 15°, 30°, and 45°C. These are the so-called "Drost-Hansen temperatures" (T_k) discussed in a later section. They no doubt reflect structural changes in vicinal water and must therefore be expected to play a role in cellular processes. With a few exceptions all living organisms operate within the range of temperatures from about 0° to 50°C.

It is well established that malignant cells contain more bulk-like water than do normal cells. One would therefore predict lower heat capacity values for water in malignant tissues than in healthy tissues of the same type.

Surface Force Measurements

For nearly two decades Peschel and co-workers have investigated the effects of surfaces on the properties of liquids in thin films. Peschel and Adlfinger (1969, 1970a) have designed an electromechanical high-sensitivity balance with which it is possible to monitor the forces and dynamics of a highly polished plate relative to a juxtaposed, fixed, similarly polished surface (Fig. 2). The two plates are immersed in the liquid to be studied. One use of the device is for measuring the effective viscosity of the intervening liquid layers as the top plate approaches the bottom plate, thereby squeezing out the intervening fluid radially between the plates (Peschel and Adlfinger, 1970b). The effective viscosity of the intervening liquid layers may be calculated from the dynamics of the movement of the plates. Such experiments have revealed a notable increase in viscosity of the interfacial water and that this viscosity increase is shear-rate dependent, decreasing toward the bulk value for sufficiently high shear rates. In view of the reciprocal relationship between diffusion coefficients and viscosity, it seems that the rheological properties of vicinal water must appreciably affect intracellular transport processes.

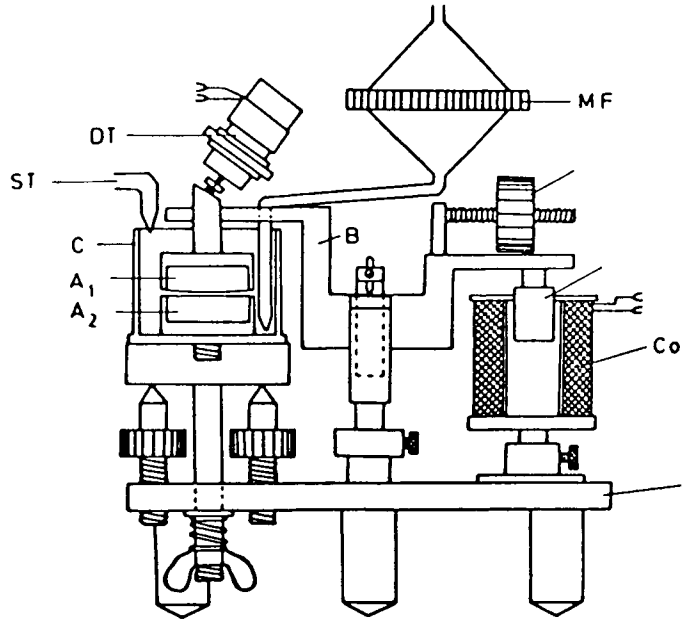

Figure 2. Surface force balance for measurements of forces between the quartz plates A_1 and A_2. (From Peschel and Adlfinger, 1970a).

In other experiments Peschel and Adlfinger (1969) measured the viscosity of the interfacial water as a function of temperature. Their results are shown in Figure 3 in the form of an Arrhenius plot. Instead of smooth, monotone curves, one observes notable peaks in the viscosity as a function of temperature for all plate separation studies (from 300 to 900 Å). Although the temperatures of these thermal anomalies were first clearly delineated some 35 years ago (Drost-Hansen and Neill, 1955; Drost-Hansen and Lavergne, 1956), it is only now that the anomalies are recognized as one of the unique characteristics of vicinal water. Peschel and Adlfinger (1970b) also measured the disjoining pressure (π) of water (and some dilute solutions) between the quartz plates as a function of temperature. Again, very pronounced thermal anomalies were observed: thus π goes through very sharp peaks at (or very near) the Drost-Hansen temperatures, i.e., 15°, 30°, 45° and 60° C.

Other studies by direct force measurements are currently being made by Israelachvili and co-workers (Israelachvili and Adams, 1971; Pashley, 1981; Pashley and Israelachvili, 1981; Israelachvili and Pashley, 1982, 1984). An ingenious alternate approach has been used by Parsegian and co-workers (LeNeveu et al., 1976a,b; Rand and Parsegian, 1982).

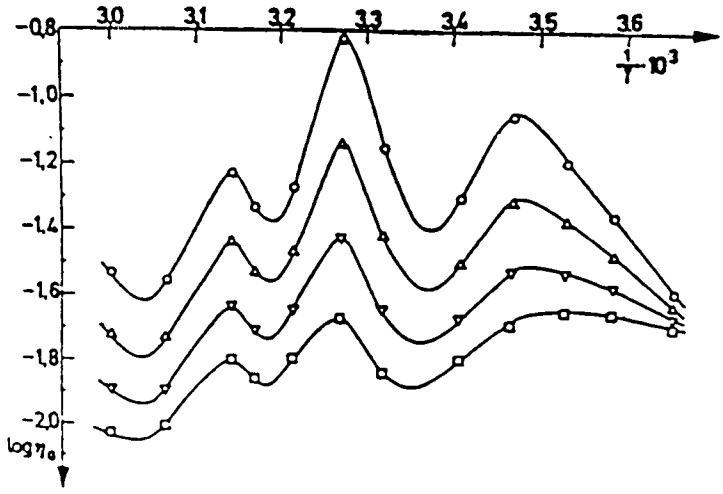

Figure 3. Logarithm of the viscosity of interfacial water between two quartz plates as a function of reciprocal absolute temperature for various plate separations, h. ○, h = 300 Å; Δ, h = 500 Å; ∇, h = 700 Å; h = 900 Å. (From Peschel and Adlfinger, 1971).

THERMAL ANOMALIES IN THE PROPERTIES OF VICINAL WATER

General Introduction

Unexpected but often abrupt changes in the properties of aqueous interfacial systems with temperature constitute one of the unique characteristics of vicinal water. Attention has already been drawn to the anomalies observed by Etzler in the specific heat values of vicinal water. Evidence of abrupt changes in the properties of both pure water and aqueous solutions have been studied in the past, but it was not until 1968 that it became clear that although unusual changes in some aqueous properties do indeed occur, they are associated only with interfacial water and not bulk water or bulk aqueous solutions (see Drost-Hansen, 1965, 1968, 1969).

Chief among the interfacial properties of aqueous systems that suggest the occurrence of thermal anomalies are the following: index of refraction, density, activation energy for ionic conductance, rates of surface reactions, surface tension, surface potentials, membrane potentials, heats of immersion, zeta potentials, rate of nucleation, viscous flow, ion activities, proton spin lattice relaxation times, optical rotation, ultrasonic velocity and absorption, sedimentation rates, coagulation rates, and dielectric properties.

There are at least four main thermal transition temperatures (T_k) that are characteristic of vicinal water. These are 15°C, 30°C, 45°C, and 60°C. The

existence of the thermal anomalies in many properties of aqueous interfacial systems is well established, yet uncertainty still surrounds the occurrence and extent of the anomalies. Thus, in some experimental model systems up to four thermal anomalies may be observed in any one study, whereas in other systems only one or a few of the anomalies are observed. Furthermore, in some systems considerable hysteresis is found: after the system is disturbed, not all the thermal anomalies may be recorded again before the recovery of the system—a process that may in some cases take minutes, hours, or even a day.

Logarithmic Rate Expressions

It is important to keep in mind that not all anomalous thermal responses in cellular systems are caused by vicinal water. Many of the lipids of cell membranes also undergo abrupt structural changes, the transition temperatures of which depend on the nature of the individual lipids. This obviously complicates the task of unraveling the underlying causes of abrupt thermal anomalies. It is well to acknowledge, however, the vast number of thermal anomalies in cellular systems that do occur near vicinal water's transition temperatures reported for entirely (nonliving) lipid-free systems. An obvious approach to the study of temperature effects on any system is to establish plots of log (parameter) versus reciprocal absolute temperature. (In the case of equilibrium quantities, such a graph is referred to as a van't Hoff plot, and for rate data it is an Arrhenius plot).

Genuine equilibria do not occur in living systems. At best, "steady-state" parameters may sometimes be measured and analyzed operationally in a useful manner in terms of the "vocabulary" of (pseudo-) equilibria, using a log (parameter) versus $1/T$ analysis. Far more often, rates are measured, and these rates are cast into the classical Arrhenius equation. Occasionally, a slightly more elaborate expression may be employed—for instance, the Eyring rate equation, where an attempt is made to separate the enthalpy and entropy effects. However, these approaches suffer from several significant limitations. For biological rate processes, the Arrhenius plot is primarily used to "straighten out" a generally "crooked" curve. This is done in order to look first for anomalous changes and/or to obtain an estimate of the energy of activation. However, the assumptions made in the derivation of the equation are rarely met. For example, use of these equations implies that the apparent energy of activation is truly temperature independent, but this is rarely (if ever) the case. Furthermore, the assumption is made that only one mechanism is involved. Such assumptions simplify the putative processes in systems to absurd proportions. For warnings about difficulties in the interpretation of anomalous Arrhenius graphs, see Drost-Hansen (1973), Solaini et al. (1984), and McElhaney (1985).

The Johnson–Eyring approach is frequently adopted because protein denaturation is commonly encountered. Taking into consideration both direct kinetic effects and the effects of temperature on proteins, Johnson and Eyring derived an equation

that embodies the general Arrhenius increase in rate with increased temperature together with a decrease in enzyme activity resulting from denaturation. See, for instance, Johnson et al. (1954) or the review by Brandts (1967).

Although it is generally agreed that the Johnson–Eyring approach is sound, we propose that superimposed on this relatively facile explanation of the effects of temperature one must also take into consideration changes in vicinal water (such as the vicinal hydration structures of the macromolecules such as enzymes). As an example, the data discussed by Johnson, Eyring and Polisar (1954) for the intensity of luminescence of a marine bacterium show a broad peak in the Arrhenius graph. Qualitatively—but not quantitatively—this agrees with the prediction from the Johnson–Eyring rate expression. However, although the broad peak in log (rate) versus $1/T$ predicted by the Eyring equation is observed, there may also be some relatively abrupt minor changes near the critical temperatures, especially 30°C and 45°C, which are superimposed on such curves (see Etzler and Drost-Hansen, 1979).

Wiggins Ion Partitioning

Wiggins (1971, 1973) has studied the distribution of ions between a bulk aqueous phase and the same solution in the pores of silica gel. Specifically she was able to show that water structure-breaking ions (K^+, Rb^+, Cs^+) tend to concentrate at the interface (in the vicinal water) while the structure-making ions (Mg^{2+}, Ca^{2+}, Na^+, Li^+) are selectively rejected from the interfacial, vicinal water layer. In a later study, Wiggins (1975) determined the temperature dependence of the partition coefficient in equimolar solutions of Na^+ and K^+. Three sets of experiments were performed with Cl^-, I^-, andSO_4^{2-}, respectively, as the common ion. The silica gel had an average pore diameter of 25 Å. As shown in Figure 4, the ion-partition coefficient, k(K,Na), is indeed larger than 1 for all three systems. Furthermore, almost quantitatively identical results were obtained for Cl^-, I^-, and SO_4^{2-}. Even more remarkable are the sharp peaks in k(K,Na) obtained at the temperatures, T_k, at which vicinal water undergoes structural changes, (i.e., 15°, 30°, and 45°C). Wiggins concludes that the unusual thermal effects reflect vicinal water contained in the silica gel pores. She also considers these effects to be consistent with the view that the distribution of ions across cell membranes and between gel water and bulk water is determined by the differences in solvent properties of interfacial and bulk-phase water.

Hurtado and Drost-Hansen (1979), who repeated some of Wiggins' experiments, obtained results that are in quantitative agreement with Wiggins' data, lending credence to the correctness of both sets of results (see also Etzler and Liles, 1986).

The temperatures at which the distribution coefficient has maxima are those of the thermal transitions of the vicinal water and do not depend on the nature of the

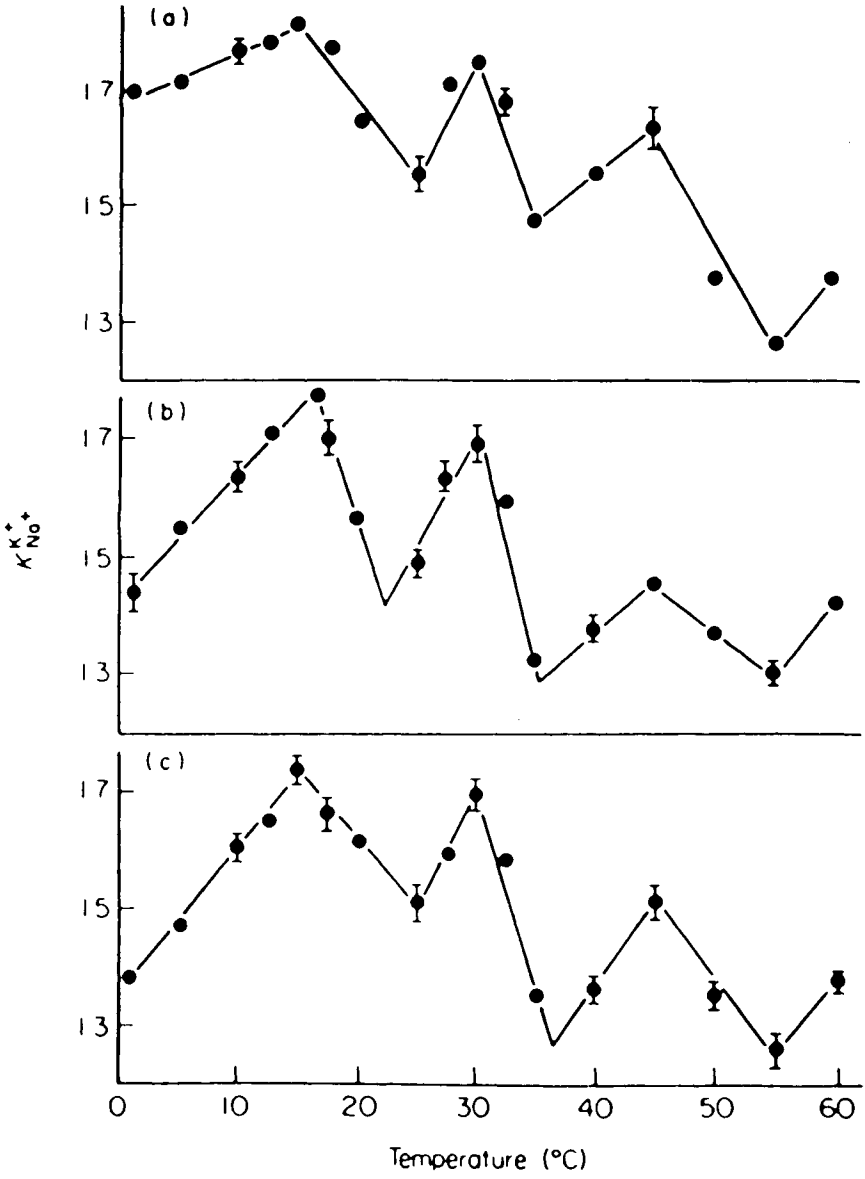

Figure 4. Variations of the selectivity coefficient, k (defined as $k = [K^+]_i[Na^+]_o/[K^+]_o[Na^+]_i$), as a function of temperature, using a 25-Å pore-diameter silica gel. The corresponding anions present are: (**a**) SO_4^{2-}, (**b**) I^-, and (**c**) Cl^-. (From Wiggins, 1975).

anions or the cations. In other words, the temperatures of the maxima reflect the intrinsic properties of the vicinal water, not the ions on the surface of the silica.

The ion selectivity data clearly indicate that very specific ion–solvent interactions take place in the vicinal water and that these bear little resemblance to expectations from bulk-phase observations. This, in turn, means that such quantities as the classical standard ion activity coefficients are not applicable, and hence osmotic coefficients must also differ from the expected values. In fact, the use of the generally accepted osmotic coefficients is simply inappropriate, and unusual osmotic behavior must be anticipated. Likewise, if the activity coefficients display anomalous behavior so must cell membrane potentials. In other words, ion distribution and the osmotic behavior of cells must be influenced by vicinal water, and models of cell volume regulation must anticipate and take into account this aspect (see also Wiggins, 1979).

PARADOXICAL EFFECT REVISITED

A remarkable feature of vicinal water is the observation that, to a first approximation, vicinal water occurs adjacent to most (or all) "solid" interfaces, regardless of the chemical nature of the surface of the solid and relatively independently of the nature and concentration of solutes in solution. This substrate independence has been termed the "paradoxical effect" for obvious reasons.

Thermal anomalies in water near surfaces as diverse as diamond, glass, quartz, clays, mica, fatty acids, chondroitin 4-sulfate, polystyrene, polyvinyl acetate, cellulose, gelatin, and other biomacromolecules (such as enzymes and other proteins) in solution are known to occur close to the critical temperatures. T_k has also been shown to be unaffected by the concentration of electrolytes in solution. The concentrations of alkali chlorides (Li^+, Na^+, K^+, Rb^+, and Cs^+) were varied by a factor of 10^4 with no detectable systematic effects on T_k (Drost-Hansen, 1985). However, the evidence for the paradoxical effect does not rely solely on the substrate independence of T_k.

Sun et al. (1986) have observed that various types of clay exhibit the same influence on the vicinal water. They concluded that a "substrate independence" exists. Packer (1977) also noted the "substrate independence" of water-structuring effects. He quotes Woessner's NMR studies showing that the ratio of the deuteron-to-proton splittings for water, oriented by proximity to a clay surface (3.75), appears independent of the type of clay and that the same ratio is found for water in oriented collagen, Li-DNA, and rayon. Thus, Packer suggests that "it is merely the presence of a 'static' surface and not its nature that matters in producing dynamic orientation of that water," and that the predominant effect is water–water interaction. The importance of the paradoxical effect lies in the prediction that vicinal water occurs at all solid interfaces and must, therefore, also occur in cellular systems—the cellular interior offers vast structural areas for the induction of vicinal water.

MACROMOLECULES IN SOLUTION

Critical Molecular Weight Range

It is clear that the evidence for vicinal hydration of macromolecules in solution may be indirect or circumstantial, but it cannot be readily dismissed. A vast literature exists on the effects of temperature on rates of enzymatic reactions. It is our view that in many cases, where sufficiently closely spaced data are available, distinct changes in the rate of enzymatic reactions occur at or very near 15°, 30°, 45°, and 60°C. It thus seems reasonable to assume that vicinal water is present and manifests its existence by affecting the rates of the reactions. For a fuller discussion of the evidence for the occurrence of kinks in enzymatic rate data, see Drost-Hansen (1971, 1973) and Etzler and Drost-Hansen (1979).

The occurrence of rather abrupt changes in the slope of the Arrhenius plot of enzyme reactions has been discussed in the literature for many decades, and several different explanations have been proposed to explain the origin of such "kinks." We have suggested that many of these anomalous temperature effects are most likely a direct reflection of changes in the vicinal water structure of the enzymes. This is based on the observation that a great many of the transition temperatures occur at or very near T_k. The evidence for this is substantial, and in view of the apparent generality of this observation, we have proposed that perhaps all (or nearly all) macromolecules in solution are vicinally hydrated. Although this then seems rather reasonable, it has yet to be proven to be valid. For the sake of argument, however, let us assume that vicinal hydration occurs in aqueous solutions containing large macromolecules and that small solutes (small inorganic ions or nonelectrolytes such as alcohol, acetone, urea, etc.) are not vicinally hydrated. The question now to be asked is whether a certain critical molecular size exists above which vicinal hydration occurs. In an attempt to test this possibility, Etzler and Drost-Hansen (1983) have called attention to some studies of solution properties reported for a series of homologous substances of widely varying molecular weights. Several examples of quantitative changes were found for properties of various solutes observed over a molecular weight range including the range from 1 to 10 kDa (for instance, the data reported by Bailey and Koleski (1976) on polyethylene oxide). This question has recently been further discussed by Drost-Hansen (1991, in press).

Perhaps the most convincing evidence for the onset of vicinal hydration for solutes with a molecular weight above the range of about 1000 to 5000 comes from a study by Nir and Stein (1971). These workers plotted the known diffusion rates of more than 30 solutes as a function of molecular weight ranging from very low to very high molecular weights. The data points fell on two distinct, different straight-line segments. The intersection of these lines is close to 1000 Da. A likely explanation is that this critical molecular weight range represents the rather sudden onset of vicinal hydration.

Cellular Implications

The possibility that vicinal hydration occurs only for molecules above a certain size is interesting, particularly in the light of the known size distribution and roles of solutes in cells. Anderson and Green (1967) analyzed the abundance of cell constituents as a function of molecular weight and found a remarkable bimodal distribution: the concentration of solutes in cells with a molecular weight between 10^3 and 10^4 is practically zero! Clegg (1979), who has speculated that this involves vicinal hydration of macromolecues, writes,

> I find it highly provocative that a distinct gap in the distribution of molecular weights of cellular molecules occurs in the region of about 1000 to 5000 Daltons, very few molecules being present in cells within this range according to the detailed compilation of Anderson and Green. This is fascinating since the lower end of this molecular weight range appears to be, albeit on indirect evidence, about the minimum size required for a molecule to generate its own vicinal water. I suggest that this somewhat puzzling hiatus in molecular size distributions could be an extremely important but overlooked feature of the intracellular environment in metabolic context. In terms of the model given here this distribution could reflect a device that cells use to partition enzymes and their substrates in such a way as to achieve favorable concentrations of each in a relatively easy fashion, while at the same time avoiding solubility (solvent) problems.

SUMMARY

On a mole basis, water is by far the most abundant molecule in any living cell. Furthermore, the internal surface area of a cell is enormous, and thus a very large fraction of cell water occurs near the interfaces.

Classical cell physiology takes into account the effects of both ionic and nonionic solutes (for instance, in calculations of osmotic properties). Only fairly recently, however, has it become clear that physico-chemical parameters based on bulk measurements fail to account for many of the properties of large surface-to-volume-ratio systems. The explanation for these differences, which can be pronounced, must be sought in the long-range effects of interfaces on the structure and properties of water and aqueous solutions.

The geometric arrangements in bulk water still defy a rigorous description, and less is known about the structure of aqueous solutions (of small solutes, both ionic and nonionic). Even less is known about the long-range geometric effects on the aqueous structure induced by proximity to an interface. The water (or solution) affected by an interface is referred to as vicinal water. Its properties often differ from the corresponding bulk properties; although the energetics of vicinal and bulk water do not differ greatly, some properties are notably different, among them the dielectric constant and relaxation properties, viscosity, density, and particularly the specific heat.

Although estimates of the extent of vicinal water vary greatly among different investigators, the thickness of modified layers is at least 10 or 20 molecular

diameters of a water molecule, and perhaps as high as 50 (to 100) molecular diameters.

One of the most characteristic features of vicinal water is the occurrence of anomalies in its properties as a function of temperature. These anomalous changes occur approximately 15°C apart, i.e., near 14–16°, 29–32°, 44–46°, and 59–62°C. The changes at these transition temperatures are quite abrupt and in many cases very pronounced. The effects of vicinal water and the thermal transitions on cell biology are frequently dramatic and surprising. The functional role of vicinal water in cell biology is the subject of the next chapter.

REFERENCES

Adamson, A. W. (1982). Physical Chemistry of Surfaces. John Wiley & Sons, New York.

Alpers, W. & Hühnerfuss, H. (1983). Molecular aspects of the system water/monomolecular surface film and the occurrence of a new anomalous dispersion regime at 1.43 GHz. J. Phys. Chem. 87, 5251–5258.

Anderson, N. G. & Green, J. B. (1967). The soluble phase of the cell. In: Enzyme Cytology (Rootyn, D. B., ed.), pp. 475–488, Academic Press, New York.

Bailey, F. E., Jr. & Koleske, J. V. (1976). Polyethylene Oxide, p. 49, Academic Press, New York.

Barnes, P., Finney, J. L., Nicholas, J. D. & Quinn, J. E. (1979). Cooperative effects in simulated water. Nature 282, 459–464.

Blumberg, R. L., Stanley, H. E., Geiger, A. & Mausbach, P. (1984). Connectivity of hydrogen bonds in liquid water. J. Chem. Phys. 80, 5230–5241.

Brandts, J. F. (1967). Heat effects on proteins and enzymes. In: Thermobiology (Rose, A. H., ed.), pp. 25–72, Academic Press, London.

Braun, C. V., Jr. (1981). Calorimetric and dilatometric studies of structural properties and relaxation of vicinal water. M.S. Thesis, Department of Chemistry, University of Miami.

Braun, C. V., Jr. & Drost-Hansen, W. (1976). A DSC study of heat capacity of vicinal water in porous materials. In: Colloid and Interface Science, Vol. III (Kerker, M., ed.), pp. 533–541, Academic Press, New York.

Braun, C. V., Jr., Hochstim, R., Crowther, G. W. & Drost-Hansen, W. (1987). Precision dilatometry on aqueous suspensions: volume contraction upon settling. In: Particulate and Multiphase Processes, Vol. 3. (Ariman, T. & Veziroglu, T. N., eds.), pp. 111–124, Hemisphere, Washington.

Churaev, N. V. & Deryaguin, B. V. (1985). Inclusion of structural forces in the theory of stability of colloids and films. J. Colloid Interface Sci. 103, 542–553.

Clegg, J. S. (1979). Metabolism and the intracellular environment: The vicinal-water network model. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S., eds.), pp. 363–413, Academic Press, New York.

Clegg, J. S. (1985). Interrelationships between water and cell metabolism in *Artemia* cysts. XI. Density measurements. Cell Biophys. 6, 153–161.

Clifford, J. (1975). Properties of water in capillaries and thin films. In: Water—A Comprehensive Treatise (Franks, F., ed.), Vol. 5, pp. 75–132, Plenum Press, New York.

Deryaguin, B. V. (1964). Recent research into the properties of water in thin films and in microcapillaries. In: The State and Movement of Water in Living Organisms: XIXth Symposium of Society for Experimental Biology, pp. 55–60, Cambridge University Press.

Deryaguin, B. V. (1980). Structural and thermo-dynamic peculiarities of the boundary layers of liquids. Pure Appl. Chem. 52, 1163–1178.

Deryaguin, B. V. (1987). Modern state of the investigation of long-range surface forces. Langmuir 3, 601–606.

Deryaguin, B. V. & Churaev, N. V. (1972). Isotherm of disjoining pressure of water films on quartz surface. DAN SSSR 207, 572–576.

Deryaguin, B. V. & Churaev, N. V. (1974). Structural component of disjoining pressure. J. Colloid Interface Sci. 49, 249–255.

Deryaguin, B. V. & Churaev, N. V. (1977). Structural component of the disjoining pressure of thin layers of liquids. Croat. Chem. Acta 50, 187–195.

Deryaguin, B. V. & Churaev, N. V. (1986). Properties of water layers adjacent to interfaces. In: Fluid Interfacial Phenomena (Croxton, C. A., ed.), pp. 663–738.

Deryaguin, B. V. & Churaev, N. V. (1987). Structure of water in thin layers. Langmuir 3, 607–612.

Drost-Hansen, W. (1965). Aqueous interfaces. Ind. Eng. Chem. 57, 18–37.

Drost-Hansen, W. (1968). Thermal anomalies in aqueous systems—manifestations of interfacial phenomena. Chem. Phys. Lett. 2, 647–652.

Drost-Hansen, W. (1969). Structure of water near solid interfaces. Ind. Eng. Chem. 61, 10–47.

Drost-Hansen, W. (1971). Structure and properties of water at biological interfaces. In: Chemistry of the Cell Interface, (Brown, H. D., ed.), Vol. 2, pp. 1–184, Academic Press, New York.

Drost-Hansen, W. (1973a). Aspects of the relationship between temperature and aquatic chemistry (with special reference to biological problems). U.S. Senate Committee on effects and methods of control of thermal discharges, Part II, Serial #93-14, November 1973, pp. 847–1140. Available from: U.S. Government Printing Office, Washington, DC.

Drost-Hansen, W. (1976). The nature and role of interfacial water in porous media. Div. Petrol. Chem. Reprints Symp. 21(2), 278–280.

Drost-Hansen, W. (1978). Water at biological interfaces—structural and functional aspects. Phys. Chem. Liq. 7, 243–348.

Drost-Hansen, W. (1982). The occurrence and extent of vicinal water. In: Biophysics of Water (Franks, F., ed.), pp. 163–169, John Wiley & Sons, New York.

Drost-Hansen, W. (1985). Anomalous volume properties of vicinal water and some recent thermodynamic (DCS) measurements relevant to cell-physiology. In: Water and Ions in Biological Systems (Pullman, A., Vasilescu, V., & Packer, L., eds.), pp. 289–294, Plenum Press, New York.

Drost-Hansen, W. & Neill, H. W. (1955). Temperature anomalies in the properties of liquid water. Phys. Rev. 100, 1600.

Drost-Hansen, W. & Lavergne, M. (1956). Discontinuities in slope of the temperature dependence of the thermal expansion of water. Naturwissenschaften 43, 511.

Eisenberg, D. & Kauzmann, W. (1969). The Structure and Properties of Water. Clarendon Press, Oxford.

Etzler, F. M. (1983). A statistical thermodynamic model of water near solid surfaces. J. Colloid Interface Sci. 92, 43–56.

Etzler, F. M. (1988). Enhancement of hydrogen bonding in vicinal water: heat capacity of water and deuterium oxide in silica pores. Langmuir 4, 878–883.

Etzler, F. M. & Drost-Hansen, W. (1979). A role for water in biological rate processes. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S., eds.), pp. 125–164, Academic Press, New York.

Etzler, F. M. & Drost-Hansen, W. (1983). Recent thermodynamic data on vicinal water and a model for their interpretation. Croat. Chem. Acta. 56, 563–592.

Etzler, F. M. & Fagundus, D. M. (1983). The density of water and some other solvents in narrow pores. J. Colloid Interface Sci. 93, 585–586.

Etzler, F. M. & Fagundus, D. M. (1986). The extent of vicinal water. J. Colloid Interface Sci. 115, 513–519.

Etzler, F. M. & Liles, T. L. (1986). Ion selectivity by solvent in narrow pores: physical and biophysical significance. Langmuir 2, 797–800.

Etzler, F. M. & Conners, J. J. (1990). Temperature dependence of the heat capacity of water in small pores. Langmuir 6, 1250–1253.

Finney, J. L. (1982). Towards a molecular picture of liquid water In: Biophysics of Water (Franks, F. & Mathias, S. eds.), pp. 73–95, John Wiley & Sons, New York.

Frank, H. S. (1972). Structural models. In: Water—a Comprehensive Treatise (Franks, F., ed.), pp. 515–543, Plenum Press, New York.

Franks, F. (ed.) (1972–1982). Water—a Comprehensive Treatise, Vols. 1–7. Plenum Press, New York.

Franks, F. (1983). Water. Royal Society of Chemistry, London.

Franks, F. & Mathias, S. (eds.) (1982). Biophysics of Water. John Wiley & Sons, New York.

Geiger, A. & Stanley, H. E. (1982). Low-density "patches" in the hydrogen-bond network of liquid water: evidence from molecular-dynamics computer calculations. Phys. Rev. Lett. 49, 1749–1752.

Hühnerfuss, H. (1983). Molecular aspects of organic surface films on marine water and the modification of water waves. Chim. Ind. 65, 97–101.

Hühnerfuss, H. & Walter, W. (1983). The thermal anomaly of relaxation effects in monomolecular surface films. J. Colloid Interface Sci. 97, 476–480.

Hühnerfuss, H., Lange, P. A. & Walter, W. (1985). Relaxation effects in monolayers and their contribution to water wave damping. I. J. Collord Interface Sci. 108, 430–441.

Hühnerfuss, H., Lange, P. A. & Walter, W. (1985). Relaxation effects in monolayers and their contribution to water wave damping. II. J. Collord Interface Sci. 108, 442–450.

Hurtado, R. M. & Drost-Hansen, W. (1979). Ionic selectives of vicinal water in the pores of a silica gel. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S. eds.) pp. 115–123, Academic Press, New York.

Israelachvili, J. & Adams, G. E. (1977). Measurement of forces between two mica surfaces in aqueous electrolyte solutions in the range 0–100 nm. J. Chem. Soc. 74, 975–1001.

Israelachvili, J. N. & Pashley, R. M. (1982). Double-layer, van der Waals and hydration forces between surfaces in electrolyte solutions. In: Biophysics of Water (Franks, F., & Mathias, S., eds.), pp. 183–194, John Wiley & Sons, New York.

Israelachvili, J. N. & Pashley, R. M. (1984). Measurement of the hydrophilic interaction between two hydrophilic surfaces in aqueous electrolyte solutions. J. Colloid Interface Sci. 98, 500–514.

Johnson, F. H., Eyring, H., & Polissar, M. J. (1954). The Kinetic Basis of Molecular Biology. John Wiley & Sons, New York.

LeNeveu, D. M., Rand, R. P., Gingell, D. & Parsegian, V. A. (1976a). Apparent modification of forces between lecithin bilayers. Science 191, 399–400.

LeNeveu, D. M., Rand, R. P. & Parsegian, V. A. (1976b). Measurement of forces between lecithin bilayers. Nature 259, 601–603.

Ling, C.-S. & Drost-Hansen, W. (1975). DTA study of water in porous glass. ACS Symp. Series Number 8: Adsorption at interfaces. ACS, Washington, DC, 129–156.

Low, P. F. (1979). Nature and properties of water in montmorillonite–water systems. Soil Sci. Soc. Am. J. 43, 651–658.

Lumry, R. & Rajender, S. (1970). Enthalpy–entropy compensation phenomena in water solutions of proteins and small molecules: A ubiquitous property of water. Biopolymers 9, 1125–1227.

McElhaney, R. N. (1985). The effect of membrane lipids on permeability and transport in prokaryotes. In: Structure and Properties of Cell Membranes (Benga, G., ed.), Vol. II. pp. 19–51. CRC Press, Boca Raton, FL.

Nir, S. & Stein, W. D. (1971). Two modes of diffusion in liquids. J. Chem. Phys. 55, 1598–1603.

Packer, K. J. (1977). The dynamics of water in heterogeneous systems. Phil. Trans. R. Soc. Lond. B.278, 59–87.

Pashley, R. M. (1981). Hydration forces between mica surfaces in aqueous electrolyte solutions. J. Colloid Interface Sci. 80, 153–162.

Pashley, R. M. & Israelachvili, J. N. (1981). A comparison of surface forces and interfacial properties of mica in purified surfactant solutions. Colloids Surfaces 2, 169–187.

Peschel, G. & Adlfinger, K. H. (1969). Temperature abhängigkeit der Viskosität sehr dünner Wasserschichten zwischen Quarzglasoberfläschen. Naturwissenschaften 56, 558.

Peschel, G. & Adlfinger, K. H. (1970a). Viscosity anomalies in liquid surface zones. III. The experimental method. Ber. Bunsen-Ges. 74, 351–357.

Peshel, G. & Adlfinger, K. H. (1970b). Viscosity anomalies in liquid surface zones. IV. The apparent viscosity of water in thin layers adjacent to hydroxylated fused silica surfaces. J. Colloid Interface Sci. 34, 505–510.

Peschel, G. & Adlfinger, K. H. (1971). Thermodynamic investigation of thin liquid layers between solid surfaces. II. Water between entirely hydroxylated fused silica surfaces. Z Naturforsch. 26a, 707–715.

Rand, R. P. & Parsegian, V. A. (1982). Phospholipid bilayer hydration—interbilayer repulsion and intrabilayer structural changes. In: Biophysics of Water (Franks, F., & Mathias, S., eds.), pp. 140–147, John Wiley & Sons, New York.

Sciortino, F., Poole, P. H., Stanley, H. E., & Havlin, S. (1990). Phys. Rev. Letters, 64, 1686–1689.

Solaini, G., Baracea, A., Parenti Castilla, G., & Lenarz, G. (1984). Temperature dependence of mitochondrial oligomycin-sensitive proton transport ATPase. J. Bioenerg. Biomembr. 16, 391–406.

Stanley, H. E. (1979). A polychromatic correlated-site percolation problem with possible relevance to the unusual behavior of supercooled H_2O and D_2O. J. Phys. A. Math. Gen. 12, L329–L337.

Stanley, H. E. & Teixeira, J. (1980a). Are concepts of percolation and gelation of possible relevance to the behavior of water at very low temperatures? Ferroelectrics 30, 213–226.

Stanley, H. E. & Teixeira, J. (1980b). Interpretation of the unusual behavior of H_2O and D_2O at low temperatures: tests of a percolation model. J. Chem. Phys. 73, 3404–3422.

Stanley, H. E., Teixeira, J., Geiger, A., & Blumberg, R. L. (1981). Interpretation of the unusual behavior of H_2O and D_2O at low temperature: are concepts of percolation relevant to the "puzzle of liquid water"? Physica 106A, 260–277.

Wiggins, P. M. (1971). Water structure as a determinant of ion distribution in living tissue. J. Theor. Biol. 32, 131–146.

Wiggins, P. M. (1973). Ionic partition between surface and bulk water in silica gel. Biophys. J. 13, 385–397.

Wiggins, P. M. (1975). Thermal anomalies in ion distribution in rat kidney slices and in a model system. Clin. Exp. Pharmacol. Physiol. 2, 171–176.

Wiggins, P. M. (1979). Metabolic control of the properties of intracellular water as a universal driving force for active transport. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S., eds.), pp. 69–114, Academic Press, New York.

RECOMMENDED READINGS

Drost-Hansen, W. (1971). Structure and properties of water at biological interfaces. In: Chemistry of the Cell Interface, Part B. (Brown, H. D., ed.), pp. 1–184, Academic Press, New York.

Drost-Hansen, W. & Clegg, J. S., (eds.) (1979). Cell-Associated Water. Academic Press, New York.

Franks, F. (1983). Water. Royal Society of Chemistry, London.

Franks, F. & Mathias, S. (eds.) (1982). Biophysics of Water. John Wiley & Sons, Chichester.

Chapter 8

Our Aqueous Heritage:
Role of Vicinal Water in Cells

W. DROST-HANSEN and J. LIN SINGLETON

INTRODUCTION

The question of the range of vicinal water structuring is of the utmost importance for biology and biochemistry. As early as 1957, Szent-Györgyi called attention to

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possible long-range ordering of water at interfaces, and Drost-Hansen (1965, 1969) elaborated on this notion and the likely implications for cellular systems. More recently, Clegg (1983a,b; 1984), as well as Etzler and Drost-Hansen (1983), proposed that most, if not all, macromolecules in aqueous solution are vicinally hydrated. In such circumstances, given the large intracellular concentrations of proteins, nucleic acids, etc., it is reasonable to hold the view that most of the water found in cells is indeed vicinal (especially if macromolecules were uniformly distributed in the cytoplasm, which is, however, not the case).

RANGE OF VICINAL WATER

It is difficult to estimate the decay length of vicinal water. However, on the basis of data from several laboratories, an acceptable estimate would be a decay length in the range of 20 to 50 molecular diameters (of a water molecule)—that is, roughly 50 to 150 Å (Drost-Hansen 1969, 1978, 1982). What this means is that vicinal water lies adjacent to most solid interfaces, representing structural changes that may be readily sensed over a distance of about 100 Å. In all probability, the structural characteristics of vicinal water are close to maximal at the interface; they decay away from the interface, possibly exponentially.

WATER IN CELLS

An Overview

A large fraction of the metabolism of the cell occurs within the aqueous compartments—the nucleoplasm, the interior of membrane-bound organelles, and the aqueous cytoplasm. In the past, studies of the composition and metabolic activities of the cytosol, based on the use of cell fractions, have traditionally been considered as reflecting the situation in intact living cells. However, it is now clear that the cytosol does not approximate the aqueous cytoplasm, though the terms are used somewhat interchangeably and indiscriminately. The aqueous cytoplasm is defined here as that volume of the cytoplasm of intact cells not already occupied by ultrastructure, including the cytoskeletal components. Therefore, conclusions drawn from cell fractionation studies are likely to be misleading or wrong when applied to metabolism in the aqueous cytoplasm.

The dominant component of the aqueous compartments in a cell is water, a fraction of which exhibits properties that are known to differ rather markedly from those of pure water. However, the exact proportion of differently structured water is not yet clear. Although the implications of such differences for metabolism and cell ultrastructure remain to be determined, they are likely to be profound. Shown in Figure 1 is a cartoon of Clegg's (1984) proposed ordering of water in the immediate surroundings of the microtrabecular lattice (MTL) of a cell.

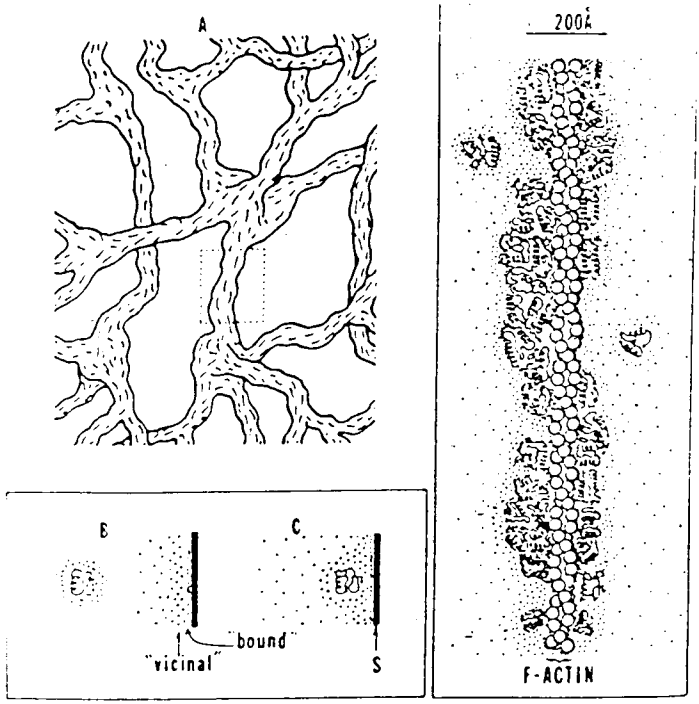

Figure 1. Schematic representations of (**A**) microtrabecular lattice. Inset at right represents a magnified view of hypothetical composition of a strand of the lattice (**B** and **C**). "Vicinal" and "bound" refer to water (dots) immediately adjacent to a surface, S, and a protein molecule (squiggly line). (From Clegg, 1984).

The properties of intracellular water have yet to be completely defined, and the subject is quite controversial. One extreme view is that all cellular water is the same as pure water or, more precisely, that the water structure of the intracellular solution is the same as that which would prevail in a bulk solution of the same solutes (and assuming that the macromolecules present did not induce vicinal water structures). Another view suggests that none of the water in cells exhibits the same properties as the bulk liquid. Methods of studying the properties of water include proton magnetic resonance (PMR), electron spin resonance (ESR), simple gel studies, dielectrics, and quasielastic neutron scattering (QNS). Although each of these techniques has built-in difficulties and limitations, the results in general show that the properties of a large fraction of cell water are indeed altered. Among the many authors who have discussed the likely state of water in cells, see Hazlewood et al. (1979, 1988), Kasturi et al. (1987), Drost-Hansen and Clegg (1979), Negendank (1986), Rorschach et al. (1988), Wiggins (1979, 1987), Clegg and Drost-Hansen (1991), and the numerous papers by Ling (1962, 1965, 1972, 1979, 1988a,b). The next step now is to examine this result as it relates to cell architecture.

The Vicinal Water Network Model

Cells are not mere "bags" of dilute solutions. Aside from the organelles, the cells possess a cytoskeleton, MTL, and various membrane systems, which provide a vast surface area. It is then almost inescapable that these surfaces interact with the neighboring water in a way that alters the physical properties of water and affects water molecules over some considerable distance. A conservative estimate of the distance is 30 Å, though estimates of up to 300–500 Å have been made. If one accepts as reasonable the 30 Å estimate, then 20–40% of the water in the aqueous cytoplasm would be directly influenced by membrane surfaces.

It is possible that the aqueous cytoplasm consists in part of more or less ordinary solution at farthest distances from membrane surfaces, but that water structure changes as these surfaces are approached. The case for altered cell water is strengthened by the following main lines of evidence:

1. **Viscosity.** ESR measurements of the spin of suitable labels in the aqueous compartments of a number of mammalian cells indicate a spin less than five times that of pure water.
2. **Motion.** From PMR experiments, three parameters can be measured: T_1 (spin–lattice relaxation), T_2 (spin–spin relaxation), and the self-diffusion coefficient (D). T_1 and T_2 have been shown to be reduced in cellular water as compared to the pure liquid. The interpretation of these data is disputed, but not the facts. Measurements of D for cells reveal reductions of two- to seven-fold.
3. **Dielectric Properties.** Cell water behaves as a poorer solvent than ordinary bulk water. Dielectric measurements have shown the average permittivity of water in *Artemia* (brine shrimp) is one-third to one-half lower than that of pure water.

Table 1 lists some of the methods and results obtained by Clegg and co-workers in attempts at elucidation of the properties of vicinal water in a cellular (and one model) system.

In light of the evidence for altered cell water, one must begin to look for possible relationships among water, enzymes, and cellular ultrastructure. It is no longer reasonable to hold that enzymes and metabolites of intermediary metabolism exist as dissolved solute species in a nonordered intracellular soup. It is known, for example, that many enzymes exist as complexes, organized beyond any degree explained by conventional theory (Schliwa et al., 1981a,b; Clegg, 1983a; Masters, 1984).

Conventional wisdom holds that substrates reach their enzymes by three-dimensional diffusion and that reaction rates are governed by simple mass action laws. As a result, metabolic studies based on a solution description would yield a grossly

Table 1. Various Methods Used for the Study of Vicinal Water

Method	*System*	*Result*	*References*
Density	*Artemia* cysts, Variable hydration	Density of cell-associated water less than that of bulk; compare Etzler and Fagundus (1983)	Clegg and Drost-Hansen (1982) Clegg (1985)
Vapor-phase adsorption	Same	Onset of different metabolic processes at various discrete hydration levels (g H_2O/100 g cyst) over range 0 to 140	Clegg (1979) Clegg (1978)
Specific heat	Same	c_p for cell-associated water differs from bulk for hydration levels less than 70. Values exceed the bulk value; compare Braun and Drost-Hansen (1976)	Clegg (1979)
NMR	Same	Diffusion coefficient (D) in cells reflects water structuring (above estimated contributions caused by presence of water cellular barriers or compartmentation); marked reduction in relaxation times (T_1 and T_2)	Seitz et al. (1980) Seitz et al. (1981)
Microwave dielectric studies (0.8–70 GHz)	Same	Only little of the cell-associated water behaves as bulk water dielectrically regardless of level of hydration. The permittivity of the cell-associated water is lower than for bulk	Clegg et al. (1982) Clegg et al. (1984)
Quasielastic neutron scattering spectra	Same; also, 20% agarose gel	Majority of cell water has reduced translational and rotational diffusion constants not related to obstructions, compartments, or exchange with minor phases	Trantham et al. (1984)

misleading portrayal of metabolism if evidence of an ordered/structured regime can be substantiated. There seems to be little doubt that some, if not most, enzymes are under tight control by the cell. Examples include the enzymes of the glycolytic pathway, now shown to exist as enzyme complexes, and aldolase, shown to be held tightly by actin filaments under certain conditions.

It is believed that cellular enzymes are partitioned in the cell, loosely associated with cellular ultrastructure and hence not free in solution. Because simple dilution is sufficient to elicit the dissociation of enzymes from ultrastructure, the term "loosely bound" is used. However, the cell retains tight control of metabolism, and these enzymes are not free to diffuse. Recall that water within 30 to perhaps 100 or more angstroms of surfaces most likely has altered properties, including altered solvent properties. Thus, it is conceivable that there is a solute concentration distribution caused by a gradient of altered solvent properties as the enzyme and metabolites approach the surfaces. (See Clegg 1986, 1988).

Water has been implicated in enzyme reactions in addition to its recognized role in protein folding and solvation. For example, water plays a part in the volume of

activation accompanying catalysis by several enzymes via changes in hydration of residues at enzyme surfaces. Moreover, water is involved in the free energy exchanges between enzymes and their microenvironments, thus allowing propagation of free energy from the protein surface to the active site. The viscosity of water has also been shown to play a key role in rate enhancement. As discussed in the preceding chapter in this volume, vicinal water is expected to exist near any interface, including the surfaces occurring in cells. Thus it is hardly surprising that there is considerable evidence for vicinal water structuring effects in cellular systems. Recall furthermore that one of the most characteristic properties of vicinal water is the occurrence of thermal anomalies in both equilibrium and rate processes at a number of discrete temperature ranges. These anomalies fall in a span of temperature which are of physiological interest—namely near 15°, 30°, 45°, and 60°C. An inspection of Table 2 will demonstrate that a vast number of biologically interesting processes do indeed exhibit anomalies at or near these vicinal water transition temperatures, strongly suggesting an important role of vicinal water in cell functioning. (The reader is reminded once again, however, that other causes of more or less abrupt transitions with temperature must be expected in cells, for instance due to lipid phase transitions—see the preceding chapter).

Table 2. Biological and Thermal Anomalies

Authors	*System and Effect*	*Temp. of Anomaly (°C)*
Crozier and Stier (1926)	Changes in log (rate of gliding motion) versus $1/T$ of thiobacterium (*Beggiatoa alba*)	16.5
Meuwis and Heuts (1957)	Abrupt changes in log (respiratory frequency) versus temp for carp (*Cyprinus carpio*)	15–18, 32–34
Maynard Smith (1958)	Abrupt change in life expectancy, *Drosophila subobscura*	31
Lipman et al. (1963)	Minimal in motor activity of albino rats	31–32
Davey et al. (1966)	Growth minima: *Pseudomonas fragi*, *streptococcus faecalis*, *Bacillus coagulans*	15 29 45
Carpenter and Graham (1967)	Abrupt change in respiration rate of bat, *A. hirsutus*	31–32
Houston and Madden (1968)	Sharp drop in plasma osmolarity in carp, *Cyprinus carpio*	30 ± 3
Wang and Gill (1970)	Rapidly increasing mortality of unfed adult stable flies (*Stomoxyus calcitrans*)	27–30
Lau (1972)	Change in log (doubling time) of 929 cells	30
Wanka and Geraedts (1972)	Minimum in DNA contents of *Chlorella*	15
Wulff et al. (1972)	Minimum in germination rate in seeds of *Jussiaea suffructicosa*	30(–35)
Heinzelmann et al. (1972)	Abrupt changes in excretion of surface active agents from *Saccaromyces cerevisiae* and *Candida curiosa*	15,27 43,61
Henizelmann et al. (1972)	Abrupt changes in growth rates of these organisms	17,27

(*continued*)

Table 2. (Continued)

Authors	*System and Effect*	*Temp. of Anomaly (°C)*
Keirns et al. (1973)	Stimulation of rat hepatic adenylate cyclase	32
Steveninck and Ledeboer (1974)	Cell survival (yeast) as function of rehydration temperature	14.7
Walmsley (1976)	Sharp increase in mating-pair formation, *E. coli*	30
McIver (1978)	Acetylcholine release from guinea pig ileum	30
Stoddart et al. (1978)	Lettuce hypocotyls response to gibberellin (GA_3) above critical temperature	13
Stoddart et al. (1978)	Lettuce hypocotyls: membrane-based nonspecific tetrazolium reduction shows sudden rate change	14
Spoor et al. (1978)	Rabbit atria: time of response as function of temperature	31–32
Cheung and Daniel (1980)	Maximal rate of rise of action potential in muscle	32
Beaudoin and Mercier (1982)	Minimum in pancreatic excretion of amylase as function of temperature (rat pancreas)	13–15
Carbineau and Côme (1980/81)	Abrupt changes in germination capacity of seeds from tropical member of *Rubiaceae* family	15, 32
Bravo and Uribe (1981a,b)	Abrupt change in cell membrane electropotentials of corn roots	15–16
Bravo and Uribe (1981b)	Change in slope of Arrhenius plot of K^+ absorption of corn roots (mechanism I)	13–17
Lóvtrup and Hansson Mild (1981)	Sharp maximum in cortical tension of amphibian eggs	16
Lóvtrup and Hansson Mild (1981)	Peak in Arrhenius graph of diffusion coefficient of water in cytoplasm of oocytes from two amphibian species	16
Hageseth (1982)	Germination of seeds; abrupt changes in rate	19, 33

The role of water in cells remains a challenging area of research. Because it is so common, and yet so difficult to study, water is frequently overlooked. However, with the growing evidence supporting the case for altered cell water, it is important to determine how cells have taken advantage of this remarkable liquid. It seems reasonable and probable that cells evolved the capacity to escape the chaos of solutions, leaving little to random processes (such as mass action and solution chemistry), and that cell water contributes to the regulation of cellular processes. The molecular organization of cells, as yet only dimly perceived, is an uncharted "universe" awaiting exploration.

Ion Selectivity

Wiggins found remarkable ion selectivity of vicinal water, which tends to enhance the concentration of structure-breaking ions and reduce the concentration

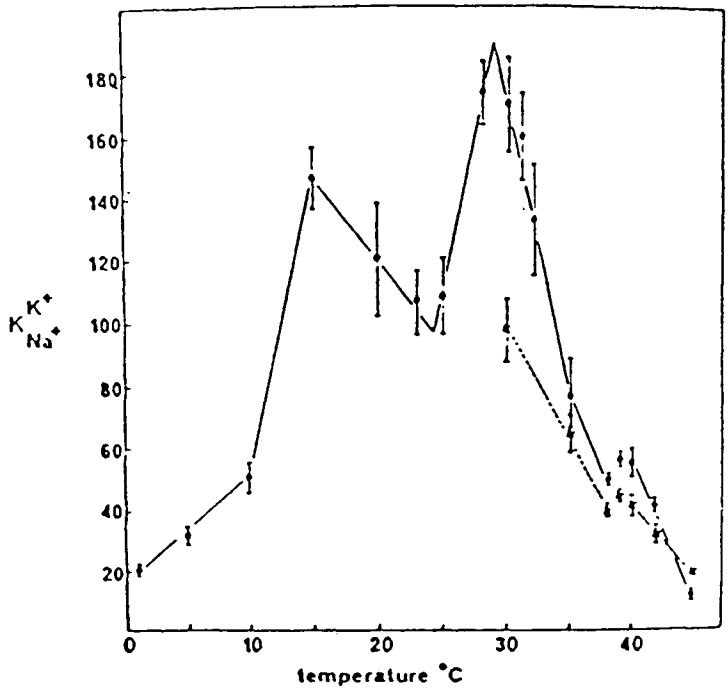

Figure 2. The selectivity coefficient, *k*(K/Na) (see legend to Figure 4 of Chapter 7 for definition) as a function of temperature in rat kidney cortex slices. Note maxima at 15° and 29°C, similar to maxima for *k*(K/Na) in silica pores. (From Wiggins, 1975).

of structure-making ions. Wiggins repeated these experiments using slices of rat kidney cortex instead of silica gel. The results obtained, as shown in Figure 2, indicate the occurrence of sharp peaks for *k*(K,Na) at 15° and 28°C. These are almost precisely the temperatures for which *k*(K,Na) reaches maximal values in the silica gel system. The data of Wiggins (see Drost-Hansen, 1976) also show a remarkable linear relationship between ln[*k*(K,Na)] for kidney tissue and ln[*k*(K,Na)] for silica gel, further suggesting that the causative factor in rat kidney cortex ion distribution is vicinal water. More recently, Wiggins (1988) discussed the question of water structure in polymer membranes in terms of a role of vicinal water in the mechanism of cation membrane pumps (see Wiggins, 1987).

Rheological Aspects

Rheological aspects must surely have a bearing on the question of rates of volume regulation by the cell, both via the viscosity (of the aqueous part of the cytoplasm) and via the effect of viscosity on diffusion coefficients. Figure 3 shows the modulus of rigidity of a minced fish paste as a function of temperature. This

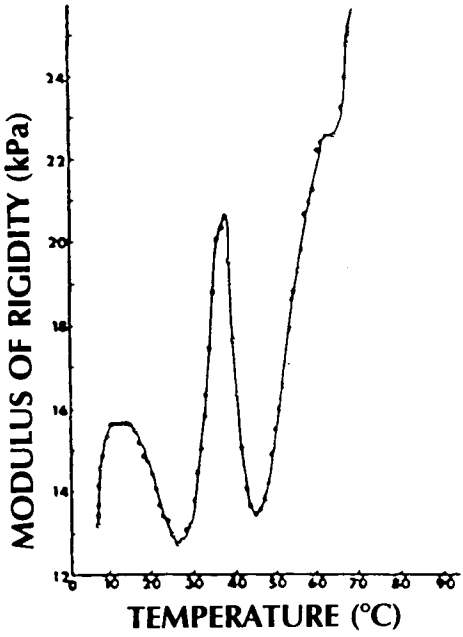

Figure 3. Modulus of rigidity during heating of fish protein concentrate as a function of temperature. Note minima at 28° and 45°C. (From Wu et al., 1985a).

was obtained at a constant rate of heating (Montejano et al., 1983). The temperature sensitivity is obviously pronounced, with minima in the modulus of rigidity near 27° and 45°C. Somewhat similar results were obtained by Wu et al. (1985b), who found pronounced minima in the viscosity of actomyosin solutions near 32° and 45–47°C. This is indicated in Figure 4. It is thus tempting to attribute these unique rheological effects to changes in the vicinal water.

Unusual viscosity anomalies were also observed as early as 1924 by Heilbrunn (1924), whose results are shown in Figure 5. Protoplasmic viscosity of *Cumingia* eggs is plotted as a function of temperature. It seems likely that abrupt changes near 15° and 31°C reflect structural changes in internal vicinal water. The important point here is the remarkable degree of abruptness, which cannot readily (if at all) be explained in terms of "classical" processes involved in viscous flow. For a general review of the role of vicinal water in transport processes of both model systems and in cell biology, see Drost-Hansen (1995).

Membrane Potentials

There is a surprising paucity of data relating to the effects of temperature on simple membrane systems. In our laboratory (Drost-Hansen, 1970), we have

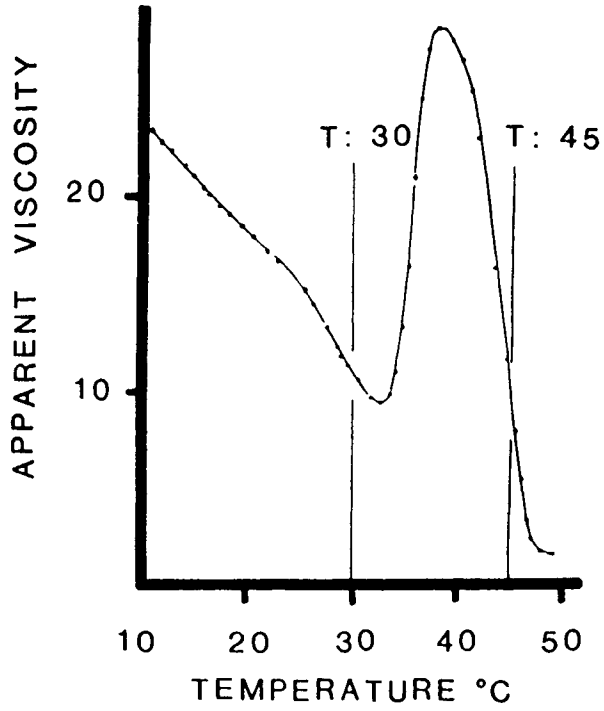

Figure 4. Apparent viscosity of 1.4% actomyosin solution during heating (1°C per minute, shear rate 1.02 sec^{-1}. (From Wu et al., 1985b).

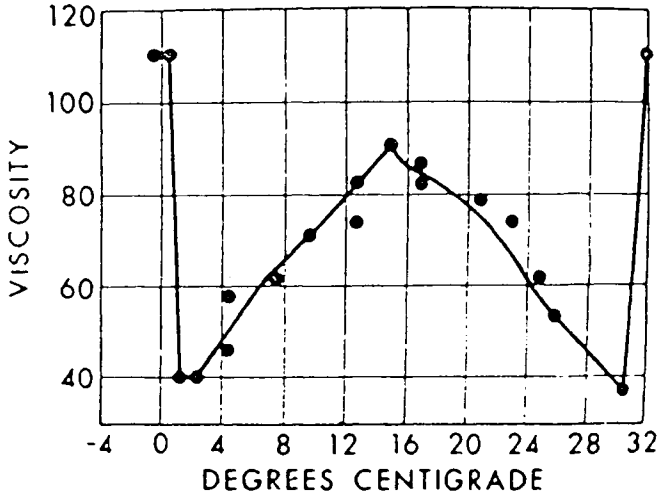

Figure 5. Apparent viscosity of protoplasm in *Cumingia* eggs as a function of temperature. Note sharp minima near 2° and 30°C and distinct maximum near 15°C. (From Heilbrunn, 1924).

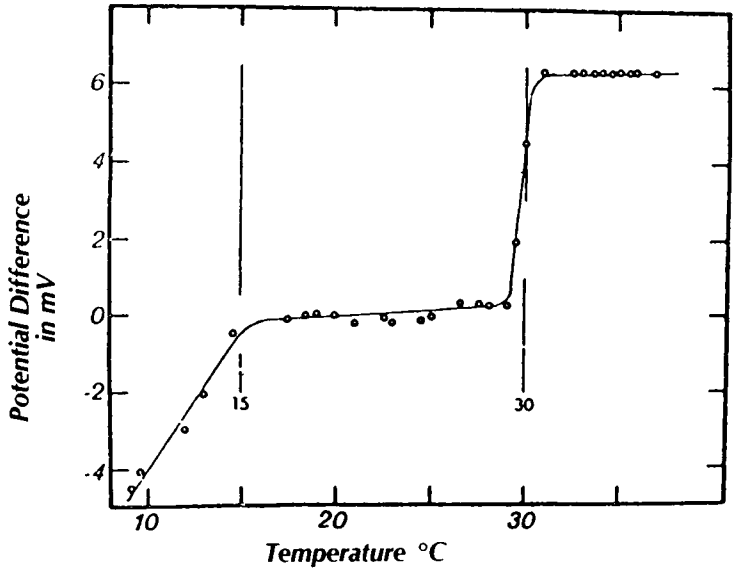

Figure 6. Potential difference across cell wall in the (large) green photosynthesizing alga *Valonia utricularis* as a function of temperature. Note abrupt changes near 15° and 29°C. (From Drost-Hansen and Thorhaug, 1967).

measured the effects of temperature on concentration potentials and biionic potentials across a very simple membrane, namely, a cellulose filter impregnated with toluene. Distinct abrupt changes were observed at 30°C. An anomaly (an inflection point) in the membrane resistance as a function of temperature was also seen at 30°C. Obviously, the membrane does not come close to simulating the normal cell membrane. However, for the one case where a biological membrane (that of the giant alga *Valonia*) has been studied in some detail, pronounced anomalies were observed near 15° and 30°C. This is illustrated in Figure 6, where abruptness of the change near the transition temperature is rather striking (Drost-Hansen and Thorhaug, 1967; Thorhaug, 1976).

Molecular Disorder at T_k

Based on surface tension measurements using the rise height of water in narrow capillaries and then obtaining the entropy term by numerical differentiation of the data, Drost-Hansen (1965) found a large peak in entropy of surface formation near 30°C. This is taken to mean that vicinal water is disorganized at 30°C (see also Drost-Hansen, 1973). Another example is provided by the data of Wershun (1967), who studied the effects of temperature on chromosome aberration rate in the broad-leaf bean *Vicia faba*. As shown in Figure 7, a notable peak occurs at 30°C.

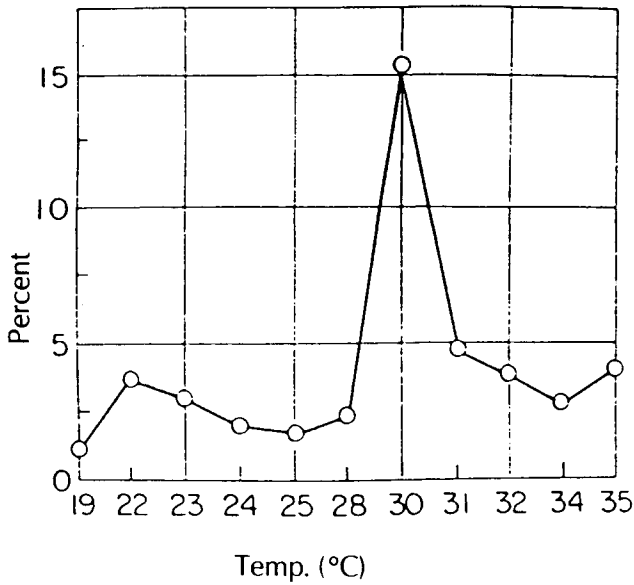

Figure 7. Rate of chromosome aberration in the broad leaf bean *Vicia faba* as a function of temperature. Note sharp peak near 30°C. (From Wersuhn, 1967; see also Drost-Hansen, 1981).

This peak is about 10-fold higher than the values found below 29° or above 31°C. We have suggested that this reflects the enhanced disorder caused by a vicinal water transition at this particular temperature. That is to say, the transition affects the stability of nucleic acids and hence affects the integrity of genetic information.

Temperature Effects on Growth

Nowhere in cell biology are the effects of vicinal water more pronounced than in the responses of growth characteristics to temperature. This is true of the effects of temperature on both single cells and whole organisms. This may seem surprising, as the energetics of vicinal water are not very different from those of bulk water. Thus, pronounced effects of vicinal water on cell functioning are not expected in what might loosely pass for "equilibrium aspects" of cellular functioning (more correctly, conditions approaching steady state). On the other hand, large effects may occur where *kinetic* aspects are involved; indeed, growth is a rate process and hence readily influenced by subtle changes in the kinetic parameters. Some illustrative examples are discussed below (see also Etzler and Drost-Hansen, 1980).

The properties of vicinal water are known frequently to change rather abruptly as a function of temperature. Figures 8 and 9 show the survival of the green macroalga *Valonia ventricosa* and the caridean shrimp *Periclimenes* as a function

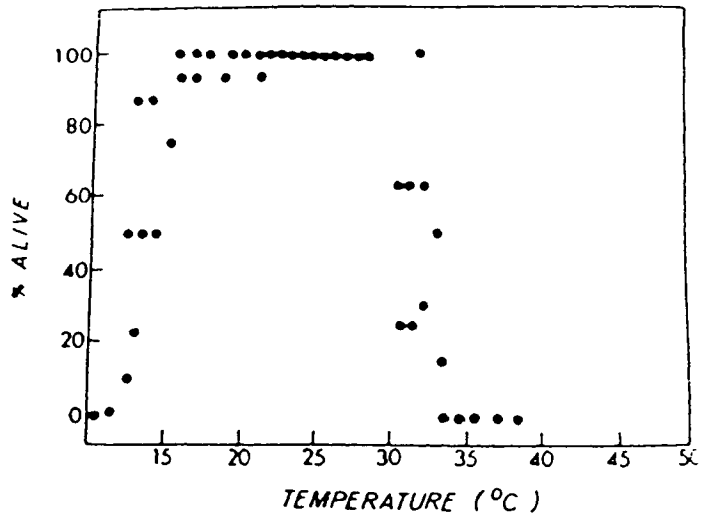

Figure 8. Percentage survival of the alga *Valonia ventricosa* after 3 days of exposure to various temperatures. Note changes near 15–16° and 31–32°C. (From Thorhaug, 1976; see also Drost-Hansen, 1981).

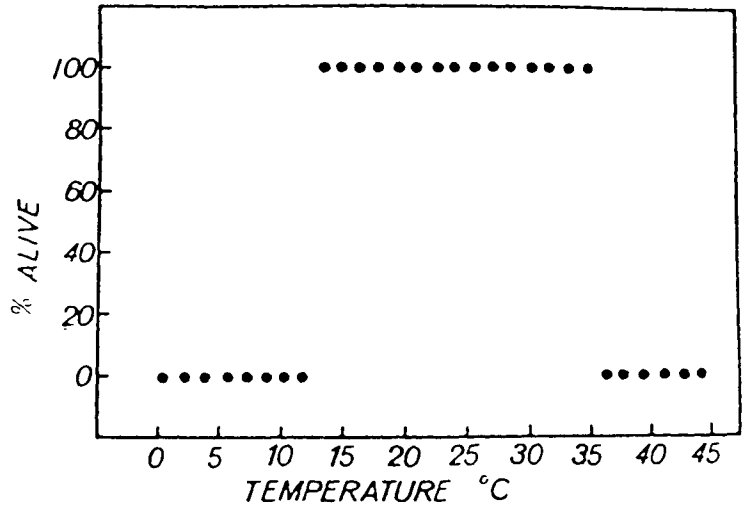

Figure 9. Percentage survival of the shrimp *Periclimenes sp.* after 168 hr of exposure to various temperatures. Note the abrupt changes near 14° and 34°C. (From Thorhaug, 1976; see also Drost-Hansen, 1981).

of temperature. The change with temperature is striking. Multiple growth maxima and minima have been observed for a wide variety of organisms, the important point being that growth minima occur at the transition temperatures for vicinal water (see Oppenheimer and Drost-Hansen, 1960). It thus seems likely that temperature minima or maxima in growth rates represent manifestations of changes in vicinal water at some interface that affect one or more rate-limiting processes. In this connection note also that thermal anomalies are frequently observed in studies of rates of seed germination. See for instance Drost-Hansen (1971, 1981), Etzler and Drost-Hansen (1980), Nishiyama (1969, 1972), Hageseth (1976), and Wulff et al. (1972). It is tempting to speculate about the possible role of vicinal water in the evolution of biological systems. Conceivably the body temperatures of mammals and birds are the result of evolutionary pressure to avoid temperatures near T_k as discussed below.

Body Temperature Selection

Homeothermy is a device developed to protect the organism from the capriciousness of climate. At the same time it must be imperative to avoid operating the system close to one of the thermal transition temperatures (see above). In fact, it must have been desirable to be as far removed from any of these transitions as possible. In other words, it would be preferable to operate a homeothermic system at the midpoint between two consecutive thermal transition temperatures. The ranges then for optimum body temperature would be near 23°, near 37°, or near 53°C. The latter temperature range obviously would correspond to an excessive temperature differential between the core of the organism and the environment under the normal climatic conditions during the geological period in which homeothermy developed. Conversely, a body temperature of 23°C would involve the risk of higher environmental temperatures during the summer (especially in subtropical and tropical climates) and, hence, a problem in heat dissipation. The least undesirable alternative would be a temperature in the region of 37°C. This happens to be the body temperature of mammals and, to a first approximation, that of birds. This interpretation is strengthened by the fact that the body temperatures of about 150 mammals fall in a remarkably narrow range of 37° to 38°C with a half-width of approximately 2°. Consistent with this notion are the observations that no mammal or bird is known to be able to withstand body temperatures in excess of 45°C and that profound physiological changes occur around 30°C as well (also in those mammals that are able to withstand cooling, namely, the hibernators).

The average body temperature of birds is somewhat higher than that of mammals, namely, 41° to 42°C. This elevated body temperature is interpreted as a concession to the requirement for rapid energy delivery for sustaining flight, which is one of the most energetically demanding physiological activities. It is noteworthy that such nonflying birds as the kiwi, ostrich, and penguin have lower body

temperatures, 37° to 39°C. Thus, when the need for rapid energy delivery for flight disappears, it is more favorable to "reset" the homeostatic thermostat to a lower set point. Birds and mammals show an upper thermal limit close to 45°C. That is, birds have chosen to live closer to the upper edge without any fundamental changes in temperature tolerances (see Drost-Hansen, 1956, 1965, 1971).

Vicinal Water and Evolution

Life, which undoubtedly developed in an aqueous environment, depends on the unusual properties of water. These properties are intimately tied to H-bonding. This hydrogen bonding is perhaps the most significant aspect of life, particularly since the stability and functioning of proteins, nucleic acids, polysaccharides, amino acids, and a vast number of other solutes depend on it. H-bonding is time invariant; thus, whatever adaptations cellular systems may have had to undergo must have taken advantage of H-bonding. Those features must surely have been the same since life began and to the present time. Specifically, therefore, whatever information we may now be able to deduce about living systems, reflecting the structural properties of water, must be generally true from protocells to the most complex of present-day organisms.

It is not implausible to propose that at a relatively early time in evolution, cells were able to devise ways of escaping the chaos of solution chemistry. One method would be the attachment of their enzymes to a framework that is under the cell's control. Whereas prokaryotic cells are small enough to allow solution-based metabolism to occur, the dimensions of eukaryotic cells are unfavorable for many random processes. Based on what is now known about the intracellular environment, one may suggest that the MTL and cytoskeletal networks might be linked in the aqueous regions to key enzymes at their surfaces and that the regions between strands of the MTL are relatively dilute with respect to macromolecules.

Eukaryotes in early evolution must have been subjected to wide variations in environmental temperature and water activity. Probably intracellular water was also involved. For example, fluxes in water across the plasmalemma would force the cell to cope with changes in water content in order to ensure that metabolism would not shut down. Metabolic stability would certainly be the selective factor.

Since mammalian cells are capable of undergoing significant shifts in water content, the question arising is whether the ability to cope with desiccation is a remnant of an earlier evolutionary mechanism regulating water loss. It is speculative, but not inconceivable, that the fundamental design of cells has been conserved from early times.

As an example of the possible role of vicinal water in determining cell functioning, consider the size of cells (Drost-Hansen, 1985). A minimum cell size appears to be about 0.2 μm. Traditionally, the existence of a minimum cell size has been related to the solution volumes of the macromolecules required in living cells. If

indeed this is so, and if this is the main or only consideration, then a minimum cell size must have been an invariant in time over the span in which the present-day known macromolecules were present in earlier forms of cells.

It is suggested here that the minimum cell size may also (or instead) reflect the need for the existence of vicinal water in the cell. In view of the suspected minimum geometric extent of vicinal water, in the range of 50–300 Å, it is hardly surprising that a minimum cell size exists. This becomes particularly likely if the detailed functioning of metabolic processes in the cell requires the presence of both vicinal and more bulk-like water.

As discussed earlier, vicinal water exhibits a certain amount of ion selectivity; thus, there exists a tendency to concentrate structure-breaking solutes over structure-making solutes. Hence, the interior milieu of the first protocell may likely have been characterized by a relatively higher K^+ to Na^+ concentration ratio than in the surrounding "sea." This enhanced ratio then constituted the "normal" interior environment, and it is interesting to surmise that active membrane transport developed as a means of insuring and/or enhancing the unequal ion distribution originally present.

SUMMARY

Most of the intermediary metabolism in cells occurs within the aqueous compartments. However, cells are not dilute bags of juice. They have evolved the capacity to exert exquisite organizational and structural control of their internal environments. It is probable that the design of present-day cells is retained from early cells' evolution of relationships among metabolism, cell architecture, and water.

It is becoming increasingly apparent that much, if not most, of the water in cells is vicinal water and therefore altered in many of its properties, including viscosity and thermodynamic properties. The implications of altered water properties for cellular processes are profound. Water affects numerous functions of the cell, for example, cell volume, ion selectivity, membrane potentials, enzyme rate enhancement, and chromosome aberrations.

The effects of temperature on biological systems have been discussed in this chapter. Because the properties of vicinal water change abruptly at T_k, cells exhibit anomalous behavior at T_k. For instance, survival and growth curves for a variety of living systems show remarkable changes at one or more of the transition temperatures. Since these changes are profound, one must speculate that vicinal water played a crucial role in cellular evolution. From cell metabolism to body temperature homeostasis, vicinal water continues to play a key role.

DEDICATION

Chapters 7 and 8 are dedicated to two "grand old men" of science: Professor Boris Deraguin, who, more than anyone else, has contributed to our understanding

of water near interfaces, and to the late Albert Szent-Györgyi, who was among the first to recognize that "water is not only the most important biological substance, it is also the most fascinating one which has unique qualities, which are deeply involved in the subtle mechanisms of life and are equally important for biology and pathology." W. D-H. is grateful to both of these scientists for their kindness, encouragement, and friendship over many, many years.

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REFERENCES

Beaudoin, A. R. & Mercier, M. (1982). Exocytosis in mammalian cells. VI. Temperature dependence of pancreatic exocrine secretion. Can. J. Physiol. Pharmacol. 58, 1509–1512.

Braun, C. V. Jr. & Drost-Hansen, W. (1976). A DSC study of heat capacity of vicinal water in porous materials. In: Colloid and Interface Science, Vol. III, (Kerker, M., ed.), pp. 517–531, Academic Press, New York.

Bravo-F. P. & Uribe, E. G. (1981a). An analysis of the relationship between respiration and the transmembrane potential in corn roots. Plant Physiol. 67, 809–814.

Bravo-F. P. & Uribe, E. G. (1981b). Temperature dependence of the concentration kinetics of absorption of phosphate and potassium in corn roots. Plant Physiol. 67, 815–819.

Carpenter, R. E. & Graham, J. B. (1967). Physiological responses to temperature in the long-nosed bat, *Liptonyeteris sanborni*. Comp. Biochem. Physiol. 22, 709–722.

Cheung, D. W. & Daniel, E. E. (1980). Imidazole inhibits a temperature-dependent component of mammalian skeletal muscle action potential. Nature 283, 485–486.

Clegg, J. S. (1978). Interrelationship between water and cellular metabolism in *Artemia* cysts. VIII. Sorption isotherms and derived thermodynamic quantities. J. Cell. Phys. 94, 123–138.

Clegg, J. S. (1979). Metabolism and the intracellular environment: the vicinal-water network model. In: Cell-Associated Water (Drost-Hansen, W. & Clegg, J. S., eds.), pp. 363–413, Academic Press, New York.

Clegg, J. S. (1982). Alternative views on the role of water in cell function. In: Biophysics of Water (Franks, F. & Mathias. S., eds.), pp. 365–383, John Wiley & Sons, New York.

Clegg, J. S. (1983a). What is the cytosol? Trends Biochem. Sci. 8, 436–437.

Clegg, J. S. (1983b). Intracellular water, metabolism and cell architecture: Part 2. In: Coherent Excitations in Biological Systems (Fröhlich, H. & Kremer, F., eds.), pp. 162–177, Springer-Verlag, Berlin.

Clegg, J. S. (1984). Intracellular water and the cytomatrix: Some methods of study and current views. J. Cell Biol. 99, 167s–171s.

Clegg, J. S. (1985). Interrelationships between water and cell metabolism in *Artemia* cysts. XI. Density measurements. Cell Biophys. 6, 153–161.

Clegg, J. S. (1986). The physical properties and metabolic status of *Artemia* cysts at low water contents: The water replacement hypothesis. In: Membrane, Metabolism and Dry Organisms (Leopold, A. C., ed.), pp. 169–187, Cornell University Press, Ithaca, NY.

Clegg, J. S. (1988). On the internal environment of animal cells. In: Microcompartmentation (Jones, D. P., ed.), CRC Press, Boca Raton, FL.

Clegg, J. S. & Drost-Hansen, W. (1982). On the density of intracellular water. J. Biol. Phys. 10, 75–84.

Clegg, J. S., Szwarnowski, S., McClean, V. E. R., Sheppard, R. J., & Grant, E. H. (1982). Interrelationships between water and cell metabolism in *Artemia* cysts. X. Microwave dielectric studies. Biochim. Biophys. Acta. 721, 458–468.

Clegg, J. S., McClean, V. E. R., Szwarnowski, S., & Sheppard, R. J. (1984). Microwave dielectric measurements (0.8–70 GHz) on *Artemia* cysts at variable water contents. Phys. Med. Biol. 29, 1409–1419.

Clegg, J. S. & Drost-Hansen, W. (1991). On the biochemistry and cell physiology of water. In: Biochemistry and Molecular Biology of Fishes. Vol 1: Phylogenetic and Biochemical Perspectives (Hochachka, P. W. & Mommsen, T. P., eds.), pp. 1–23, Elsevier, New York.

Crozier, W. J. & Stier, T. J. B. (1926). Temperature characteristics for speed of movement of thiobacteria. J. Gen. Physiol. 10, 185–193.

Davey, C. B., Miller, R. G., & Nelson, L. A. (1966). Temperature-dependent anomalies in the growth of microorganism. J. Bacteriol. 91, 1827–1830.

Drost-Hansen, W. (1956). Temperature anomalies and biological temperature optima in the process of evolution. Naturwissenschaften 43, 511.

Drost-Hansen, W. (1965). The effects on biologic systems of higher-order phase transitions in water. Ann. N.Y. Acad. Sci. 125, 471–501.

Drost-Hansen, W. (1969). On the structure of water near solid interfaces and the possible existence of long-range order. Indust. Eng. Chem. 61, 1–47.

Drost-Hansen, W. (1970). Role of water structure in various membrane systems. In: Physical Principles of Biological Membranes (Snell, Wolken, Iverson, & Lamb, eds.), pp. 243–258, Gordon and Breach, New York.

Drost-Hansen, W. (1971). Structure and properties of water and biological interfaces. In: Chemistry of the Cell Interface (Brown, H. D., ed.), Part B, pp. 1–184, Academic Press, New York.

Drost-Hansen, W. (1973). Phase transitions in biological systems: manifestations of cooperative processes in vicinal water. Ann. N.Y. Acad. Sci. 204, 100–112.

Drost-Hansen, W. (1976). Structure and functional aspects of interfacial (vicinal) water as related to membrane and cellular systems. Colloq. Int. C.N.R.S. 246, 177–186.

Drost-Hansen, W. (1978). Water at biological interfaces—structural and functional aspects. J. Phys. Chem. Liquids, 7, 243–346.

Drost-Hansen, W. (1981). Gradient device for the study of temperature effects on biological systems. J. Wash. Acad. Sci. 71, 187–201.

Drost-Hansen, W. (1982). The occurrence and extent of vicinal water. In: Biophysics of Water (Franks, F. & Mathias, S., eds.), pp. 163–169.

Drost-Hansen, W. (1985). Role of vicinal water in cellular evolution. In: Water and Ions in Biological Systems (Pullman, A., Vasilescu, V., & Packer, L., eds.), pp. 523–534, Plenum Press, New York.

Drost-Hansen, W. (1995). Role of vicinal water in some transport processes: from model systems to living cells (in preparation).

Drost-Hansen, W. & Thorhaug, A. (1967). Temperature effects in membrane phenomena. Nature 215, 506–508.

Drost-Hansen, W. & Thorhaug, A. (1974). Biologically allowable thermal pollution limits. Ecological Research Series, U.S. Environmental Protection Agency Report EPA-600/3-74-003. Available from: U.S. Government Printing Office, Washington, DC.

Drost-Hansen, W. & Clegg, J. S. (eds.) (1979). Cell-Associated Water, Academic Press, New York.

Etzler, F. M. & Drost-Hansen, W. (1980). A role for vicinal water in growth, metabolism and cellular organization. Adv. Chem. 188, 485–497.

Etzler, F. M. & Drost-Hansen, W. (1983). Recent thermodynamic data on vicinal water and a model for their interpretation. Croat. Chem. Acta. 56, 563–592.

Hageseth, G. J. (1976). Activation energy and germination times for isothermal seed germination. J. Theor. Biol. 59, 247–249.

Hazlewood, C. F. (1979). A view of the significance and understanding of the physical properties of cell-associated water. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S., eds.), pp. 165–259, Academic Press, New York.

Hazlewood, C. F. & Kellermayer, M. (1988). Ion and water retention by permeabilized cells. Scan. Microsc. 2, 267–273.

Heilbrunn, L. V. (1924). Am. J. Physiology 68, 645–648.

Heinzelmann, H., Kraepelin, G., & Bogen, H. J. (1972). Leakage-Producte von Hefen. I. Diskontinuitäten in der Oberfläschenspannung des Mediums und in Wachstum der Zellen. Arch. Mikrobiol. 82, 300–310.

Houston, A. H. & Madden, J. A. (1968). Environmental temperature and plasma electrolyte regulation in the carp, *Cyprinus carpio*. Nature 217, 969–970.

Kasturi, S. R., Hazlewood, C. F., Yamanashi, W. S., & Dennis, L. W. (1978). The nature and origin of chemical shift for intracellular water nuclei in *Artemia* cysts. Biophysics 52, 249–256.

Keirns, J. J., Kreiner, P. W., & Bitensky, M. W. (1973). An abrupt temperature-dependent change in the energy of activation of hormone-stimulated hepatic adenylate cyclase. J. Supramol. Struct. 368–378.

Lau, L. C. (1972). Effects of liquid water phase transition on the growth of 929 cells. Exp. Cell Res. 71, 475–477.

Ling, G. N. (1962). A Physical Theory of the Living State: The Association–Induction Hypothesis. Blaisdell, Waltham, MA.

Ling, G. N. (1965). The physical state of water in living cells and model systems. Ann. N.Y. Acad. Sci. 125, 401–417.

Ling, G. N. (1972). Hydration and macromolecules. In: Water and Aqueous Solutions. Structure, Thermodynamics and Transport Processes (Horne, R. A., ed.), pp. 663–699, Wiley-Interscience, New York.

Ling, G. N. (1979). The polarized multilayer theory of cell water according to the association–induction hypothesis. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S., eds.), pp. 261–269, Academic Press, New York.

Ling, G. N. (1988a). Solute exclusion by polymer and protein-dominated water. Scan. Microsc. 2, 871–884.

Ling, G. N. (1988b). A physical theory of the living state: Application to water and solute distribution. Scan. Microsc. 2, 899–913.

Lipman, V. C., Shurrager, P. S., & Abood, L. G. (1963). Effect of anticholinergic phychotomimetics on motor activity and body temperature. Arch. Int. Pharmacodyn. 146, 174–191.

Lóvtrup, S. & Hansson Mild, K. (1981). Permeation, diffusion and structure of water. In: International Cell Biology (Schweiger, H. G., ed.), pp. 889–903, Springer-Verlag, Berlin.

Masters, C. (1984). Interactions between glycolytic enzymes and components of the cytomatrix. J. Cell Biol. 99, 222S–225S.

Maynard Smith, J. (1958). Prolongation of the life of *Drosophila subobscura* by a brief exposure of adults to a high temperature. Nature 161, 496–497.

McIver, D. J. L. (1978). Thermal anomalies in acetylcholine release: Evidence for control of membrane by interfacial water. Physiol. Chem. Phys. 10, 511–516.

Meuwis, A. L. & Heuts, M. J. (1957). Temperature dependence of breathing rate in carp. Biol. Bull. 112, 97–107.

Montejano, J. G. & Lanier, T. C. (1983). Final strength and rheological changes during processing of thermally induced fish muscle gels. J. Rheol. 27, 557–579.

Negendank, W. (1986). The state of water in the cell. In: The Science of Biological Specimen Preparation (Mueller, M., Becker, R. P., Boyde, A., Waloswick, & J. J., eds.), pp. 71–75, Scanning Microscopy International, Chicago.

Nishiyama, I. (1969). Proc. Crop, Sci. Soc. Japan, 38, 554–555.

Nishiyama, I. (1970). Kagaku to Seibutsu 8, 14–20.

Nishiyama, I. (1972). Physiological kinks around 15°, 30°, and 45°C in plants. Res. Bull. No. 2, Hokkaido Nat'l. Agri. Exp. Station, 47, 126–168.

Oppenheimer, C. H. & Drost-Hansen, W. (1960). A relationship between multiple temperature optima for biological systems and the properties of water. J. Bacteriol. 80, 21–24.

Rorschach, H. E., Bearden, D. W., Hazlewood, C. F., Heidorn, D. B., & Nicklow, R. M. (1988). Quasi-elastic scattering studies of water diffusion. Scan. Microsc. 2, 2043–2049.

Schliwa, M., Euteneuer, U., Bulinski, J. C., & Izant, J. G. (1981a). Calcium liability of cytoplasmic microtubules and its modulation by microbule-associated proteins. Proc. Natl. Acad. Sci. USA 78, 1037–1041.

Schliwa, M., van Blerkom, J., & Porter, K. (1981b). Stabilization of the cytoplasmic ground substance in detergent-opened cells and a structural and biochemical analysis of its composition. Proc. Natl. Acad. Sci. USA 78, 4329–4333.

Seitz, P., Hazlewood, C. F., & Clegg, J. S. (1980). Proton magnetic resonance studies on the physical state of water in *Artemia* cysts. In: The Brine-shrimp *Artemia*. Physiology, Biochemistry and Molecular Biology (Persoone, G., Sorgeloos, P., Rolls, O., & Jaspers, E., eds.), Vol. 2. pp. 545–555, Wetteren, Universe, Belgium.

Seitz, P., Chang, D. C., Hazlewood, C. F., Rorschach, H. E., & Clegg, J. S. (1981). The self-diffusion of water in *Artemia* cysts. Arch. Biochem. Biophys. 210, 517–524.

Spoor, R. P., Walerstein, S. J., Achtellik, D. M., Levine, R. L., & Schiff, M. A. (1978). Temperature dependence in the effect of ouabain on rabbit atria. Physiol. Chem. Phys. 10, 73–78.

Steveninck, J. V. & Ledeboer, A. M. (1974). Phase transitions in the yeast cell membrane—the influence of temperature on the reconstitution of active dry yeast. Biochim. Biophys. Acta 352, 64–70.

Stoddart, J. L., Tapster, S. M., & Jones, T. W. A. (1978). Temperature dependence of the gibberellin response in lettuce hypocotyls. Planta 141, 283–288.

Szent-Györgyi, A. (1957). Bioenergetics, Academic Press, New York.

Thorhaug, A. (1976). The effect of temperature and ouabain on the membrane properties of a giant algal cell. In: Charged Gels and Membranes (Sélégny, E., ed.), Part II, pp. 63–82, Dordrecht, Netherlands.

Trantham, E. C., Rorschach, H. E., Clegg, J. S., Hazlewood, C. F., Nicklow, R. M., & Wakabayashi, N. (1984). Diffusive properties of water in *Artemia* cysts as determined from quasi-elastic neutron scattering spectra. Biophys J. 45, 927–938.

Walmsley, R. H. (1976). Temperature dependence of mating-pair formation in *Escherichia coli*. J. Bacteriol. 126, 222–224.

Wang, Y.-H. E. & Gill, G. D. (1970). Effect of temperature and relative humidity on mortality of adult stable flies. J. Econ. Entomol. 63, 1666–1668.

Wanka, F. & Geraedts, J. (1972). Effect of temperature in the regulation of DNA synthesis in synchronous cultures of *chlorella*. Exp. Cell Res. 71, 188–192.

Wersuhn, G. (1967). Temperatur als Ursache spontanes Chromosomenaberrationen. Naturwissenschaften 54, 27.

Wiggins, P. M. (1972). Thermal anomalies in ion distribution in rat kidney slices and in a model system. Clin. Exp. Pharmacol. Physiol. 2, 171–176.

Wiggins, P. M. (1979). Metabolic control of the properties of intracellular water as a universal driving force for active transport. In: Cell-Associated Water (Drost-Hansen, W., & Clegg, J. S., eds.), pp. 69–114, Academic Press, New York..

Wiggins, P. M. (1987). Two states of water found in hydrophobic clefts: Their possible contribution to mechanisms of cation pumps and other enzymes. Int. Rev. Cytology 108, 149–303.

Wiggins, P. M. (1988). Water structure in polymer membranes. Prog. Polymer Sci. 13, 1–35.

Wu, M. C., Hamann, D. C., & Lanier, T. C. (1985a). Rheological and calorimetric investigations of starch–fish protein systems during thermal processing. J. Texture Stud. 16, 53–74.

Wu, M. C., Lanier, T. C., & Hamann, D. D. (1985b). Rigidity and viscosity change of croaker actomyosin during thermal gelation. J. Food Sci. 50, 14–19.

Wulff, R., Arias, I., Ponce, M., & Munoz, V. (1972). A bimodal temperature response and effect of light intensity in the photocontrol of germination of seeds in *Jussiaea suffrutiosa*. Planta 107, 369–373.

RECOMMENDED READINGS

Clegg, J. S. (1988). On the internal environment of animal cells. In: Microcompartmentation (Jones, D. P., ed), CRC Press, Boca Raton, FL.

Drost-Hansen, W. (1971). Structure and properties of water at biological interfaces. In: Chemistry of the Cell Interface, Part B (Brown, H. D., ed.), pp. 1–184, Academic Press, New York.

Drost-Hansen, W. & Clegg, J. S. (eds). (1979). Cell-Associated Water, Academic Press, New York.

Franks, F. & Mathias, S. (eds.) (1982). Biophysics of Water, John Wiley & Sons, New York.

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Chapter 9

Intracellular Water and the Regulation of Cell Volume and pH

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INTRODUCTION

This chapter will discuss cell water, the regulation of cell volume, and the modifications produced by cell pH. These topics are housekeeping functions and do not have the glamour of genetic engineering. Nevertheless, it is quite probable that one of the first requirements for life to evolve was a guarantee of a stable fluid environment. Mechanisms had to be established that would regulate the cell content of water.

Since water makes up 75% or more of most cells and tissues of the human body, it is implicit that the regulation of cell volume is synonymous with the regulation of cell water. It is not difficult to understand how important this process is to the normal functions of the human organism. Cell growth, i.e., the enlargement of cells, requires that the cells take up more water, since larger cells have the same proportion of cell water as do smaller cells. Cell proliferation is, by definition, an increase in cell number usually of the same size. To increase their number, cells either enlarge and then divide, or divide and the daughter cells get bigger. Therefore, it is easy to see how important the regulation of cell water is to such pathological problems as organ hypertrophy and neoplasia. It is also worth remembering that water is the universal solvent of life on this planet. Macromolecules like large proteins exist in a delicate state inside cells. Shifts in cell water may determine their solubility and their ability to function properly.

IMPORTANT RULES THAT GOVERN THE REGULATION OF CELL WATER

Several mechanisms have evolved to regulate cell water, yet all these mechanisms have to follow certain fundamental rules. Our first goal will be to review these rules.

Rule W1

Water requires free energy to move into or out of cells. Water is a dipole with a negatively charged oxygen atom and a pair of positively charged hydrogen atoms. This property permits the molecules to form hydrogen bonds with each other or with solutes. The ability of the water molecule to move from one place to another will depend in part on the degree of hydrogen bonding. Under some conditions, water may be associated with macromolecules by these hydrogen bonds and be restrained. Free of macromolecules, water has a free energy or an escaping tendency defined by the existing temperature and pressure. However, since our discussion will be limited to isothermic and isobaric conditions, these factors are not significant in the regulation of cell water. However, when small solutes are introduced into the solvent, the interaction between solutes and solvent lowers the escaping tendency of water. The measure of restraint on the escaping tendency of water is defined by the osmolality of the solution. A solution which is 1 osmolar has one

mole of osmotically active solute (1 osmole) dissolved in 1000 grams of water. Pure water has an osmolality of 0. It is an irony of our definitions that the emphasis is placed on the amount of the solute doing the restraining rather than on the solvent being restrained. Pure water with an osmolality of 0 has a greater escaping tendency than a solution with an osmolality of 1 osmolar.

Rule W2

In order for cell water to change, the free energy of the water in the medium has to differ from the free energy of the water in the cell. The free energy of water inside the cell permits it to leave the cell and enter the medium. The free energy of water in the medium permits it to enter the cells. If the two free energies are the same, as much water will enter the cell as leaves it, and the content of water in the cell will not change. If the free energy of water in the medium is greater than the free energy of water in the cells, then more water will enter the cells than leaves the cells, and the content of water in the cells will increase. Similarly, if the free energy of water in the cell is greater than the free energy of water in the medium, then more water will leave the cells than enters and the content of water in the cells will decrease.

If the free energy of water is expressed in terms of the osmolality of the medium solution and of the cell solution, then cells placed in pure water will gain water and swell. Cells placed in a medium whose osmolality is greater than the osmolality of the solution in the cell will lose water and shrink. If the medium has the same osmolality as the cells, then water may enter the cells or leave the cells, but the net change will be 0.

The species of solute is not important; only its effect on the free energy of water is, i.e., its osmolality. Several terms help us compare solutions of osmotically active solutes. Given two solutions, if the free energy of water in solution 1 equals the free energy of water in solution 2, the two solutions are isosmotic. If the free energy of water in solution 1 is less than the free energy of solution 2, solution 1 is hyperosmotic to solution 2. If the free energy of water in solution 1 is more than the free energy of water in solution 2, then solution 1 is hypoosmotic to solution 2. Confusing, isn't it? The confusion is caused by the misplaced emphasis on the solute rather than the water. Think of it this way. The higher the osmolality, the lower the free energy of water. Water will always move to the hyperosmotic solution.

Rule W3

The rate at which cells will gain or lose water will depend upon two factors: the difference in free energies of water and the permeability of the barrier to water. Another way to express the difference in free energies is to use the term driving force. It emphasizes the forward push of the free energy. The larger the difference in free energies, the larger is the driving force. Admittedly, these are jargon terms

since a force is not an energy, but they add drama to the scene. The second factor cannot be discounted either. If the membrane is not permeable to water, then the driving force will have no effect, regardless of its value. Like plumbing, if the faucet is turned off, no water flows, even if there is plenty of water pressure. A simple relationship among the three components: water flow, permeability, and driving force is:

$$\text{Flow} = \text{Permeability} \times \text{Driving Force}$$

This expression emphasizes the interrelation between driving force and permeability needed to produce flow.

The discussion to this point has considered the entry or exit of water. The movements of water depend critically on the concentration of osmotically active solutes. The next section will discuss the entry and exit of solutes into and out of cells. When osmotically active solutes change, cell water will change. An analogous set of rules may be stated for solutes.

Rule S1

Solutes require free energy to move into or out of cells. If the solute is uncharged, the free energy will come from the concentration of the solute in the solvent, water. If the solute is an ion and has a charge on it, additional free energy will arise if the ion is subject to a potential difference across the cell membrane. Expressed in mathematical terms:

$$\text{Free energy} = R\,T\,\ln C \qquad \text{Concentration component}$$

$$\text{Free energy} = z\,F\,\Psi \qquad \text{Electrical component}$$

R is the universal gas constant. In this context it has the dimensions of cal/mol/°K. T is the absolute temperature in °K. C is the concentration in mol/L. The dimensions for the electrical component are in volt coulombs (VC) which is also an energy term. It may be converted to cal by the mechanical equivalent of heat (4.185 VC/cal). z is the valence in g equivalents/mol. F is Faraday's constant in c/g equivalent. Ψ is the potential and is considered positive. With this convention, if the ion is a cation and z is positive, and the potential is positive, then the ion will be repelled from the region. In effect, the ion will be given additional free energy to leave the region. If the ion is an anion, and the potential is positive, then the ion will be attracted to the region and will lose free energy.

Rule S2

Cells will gain solute from the medium if the free energy of solute in the medium is greater than its free energy in the cell. Conversely, cells will lose solute if the free energy is greater in the cell than its free energy in the medium. This rule applies

to unmediated transport. The free energy for transport comes from the solute concentration and from electrical forces also if the solute is charged. In some cases, additional free energy may be imparted to a solute when it binds specifically to a membrane transporter or carrier. Under such circumstances, the solute will be transported against its concentration and against any restraining potential. The extra free energy comes from the metabolic processes of the cell and is called active transport.

Rule S3

The net transport of the solute, or its net flux, is equal to the driving force on the solute multiplied by the permeability of the membrane barrier:

$$\text{Net flux} = \text{Permeability} \times \text{Driving Force}$$

When transport is unmediated, the driving force comes from the difference in concentrations of the solute between the cell and its medium, as well as the difference of potential across the membrane barrier, if the solute is an ion.

Permeability as used here is a generic term which characterizes a variety of membrane properties. The actual mechanism may vary and will depend upon the solute. Simple uncharged solutes, those which are soluble in water, may use aqueous channels in the intrinsic proteins which span the membrane. Others, because of lipid solubility, may dissolve in the phospholipid component of the membrane. Permeability under these circumstances may define pore size or lipid solubility. Ions may also use aqueous channels, but these channels may be highly specialized. Some may be influenced by the membrane potential across the channel. These are called voltage-gated ion channels. Some channels may allow movement only in one direction: rectifying channels. When the transport is mediated, permeability may represent the numbers of free sites on a specific transporter as well as the rate at which the transporter transfers the solute across the membrane and resets itself for another cycle of transport. The permeability coefficient, therefore, is a phenomenological parameter which assigns a quantitative value to the overall biochemical process.

RESPONSE OF CELL VOLUME TO AN ISOSMOTIC MEDIUM

Let us see how well we can use the rules to deduce the unknown osmotic properties of a cell. Figure 1a summarizes the changes in cell volume when cells were placed in three different experimental solutions. For the upper curve, cells were suspended in 0.3 osmolar sodium chloride. These cells are functionally impermeable to sodium chloride. The middle curve is the response of the cells when they were placed in 0.6 osmolar solution. The medium contained both sodium chloride, the impermeant solute, and a permeant nonelectrolyte. The lower curve was the response of the cells to 0.6 osmolar sodium chloride.

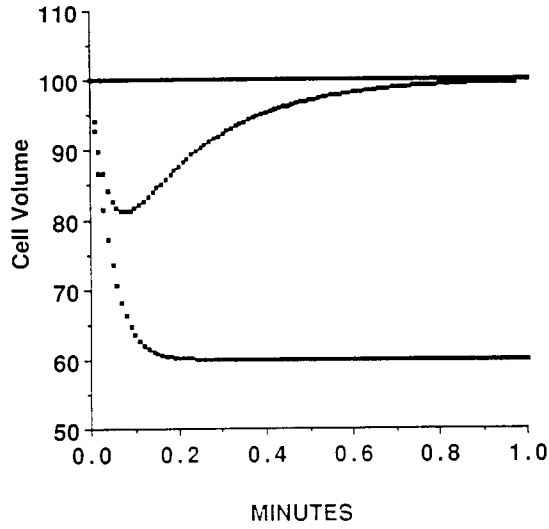

Figure 1a. The effect of hyperosmotic media on cell volume. Cells suspended in 0.3 osmolar sodium chloride (upper curve), 0.6 osmolar sodium chloride and nonelectrolyte (middle curve), 0.6 osmolar sodium chloride (lower curve).

As the red queen said to Alice, let us "begin at the beginning, go all the way to the end and stop."

1. From the upper curve, cell volume did not change. The cell volume is 100 and remains constant in 0.3 osmolar sodium chloride medium. If no net change in cell water occurred, then the free energy of water inside the cell must equal the free energy of water in the medium. Expressed in osmolalities, the osmolality of the cells must be 0.3 osmolar. The other possibility is that the cell membrane is impermeable to water. However, the middle and the lower curves rule out that possibility because the cells lose water in an isosmotic media.
2. The next question is, "How much of the cell volume is osmotically active water?" The cell volume is 100, is it all water? To get at that question, look at the lower most curve. How far did the cells shrink in 0.6 osmolar sodium chloride? They stopped shrinking when the free energy of water inside the cells equaled the free energy of water in the medium. Since the medium was very much larger in volume than the cell volume, any loss of water to the medium from the cells will not change the concentration of sodium chloride in the medium. The medium will still remain at 0.6 osmolar. Therefore, the water will stop leaving the cells when cell osmolality is 0.6 osmolar. How did the cell arrive at 0.6 osmolar? It lost water. How much water was lost

to make the cell solution 0.6 osmolar? It had to lose half its water. Following this line of reasoning, if the cell volume of 100 was all water, then the cells should shrink to a cell volume of 50. Instead they shrank to 60. Thus, not all of the 100 cell volume is water. Some of the volume is osmotically inactive and does not change in volume, but continues to occupy the same space. The following calculations may help to find the osmotically inactive material, the so-called b value.

a. Volume of cell = Cell water + b
   1a Cell volume – b = Cell water
b. After equilibration in 0.6 osmolar solution, half the cell water will be lost. Cell water after equilibration in 0.6 osmolar solution = 1/2 cell water in 0.3 osmolality.

$$60 - b = 1/2\,(100 - b)$$

$$(60-b)/(100-b) = 1/2$$

$$b = 20$$

Thus, with a little reasoning and a few calculations, we have determined the volume of osmotically inactive material and by difference, the volume of osmotically active water.

3. What is the permeability of the membrane to water? To obtain the permeability coefficient of the membrane to water, the following equation for water has to be solved:

   Change in cell water = Permeability Coefficient × Driving Force. One version was reported in 1931, but the details are not appropriate for our purposes. However, it is evident that the higher the value of the permeability coefficient, i.e., the more permeable the cell membrane is to water, the more rapidly the cell will shrink to its equilibrium volume of 60.

4. Let us now examine what is going on in the middle curve. The osmolality of the medium was also 0.6 osmolar. Why did the cells shrink less and then return to their starting volume? The answer may be found in the composition of the medium. After the cells return to their original volume, no more water is being lost or gained. The free energy of water inside the cells must equal the free energy of the water in the medium. Expressed in osmolalities, the osmolality of the cell must equal the osmolality of the medium. The osmolality of the cells under these conditions must be 0.6 osmolar. Where did the additional solute come from? It had to come from the only permeant solute in the system, the nonelectrolyte. Figure 1b summarizes the changes in cell nonelectrolyte. Starting at 0, cell nonelectrolyte rises rapidly to a value of 0.3 osmolar and then levels off at that value. At the plateau, the concentration in the cell is equal to the concentration in the medium, since the driving force was determined by the difference in the concentrations of

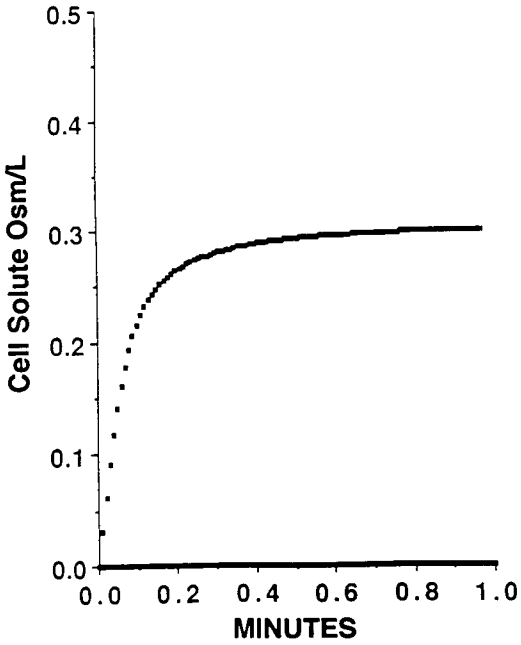

Figure 1b. The uptake of nonelectrolyte into cells suspended in 0.6 osmolar sodium chloride and nonelectrolyte (upper curve), and sodium chloride alone (lower curve).

the nonelectrolyte in the cell and in the medium. Two events were going on at the same time. When the cells were placed in the medium containing the sodium chloride and the nonelectrolyte, water left the cells according to its driving force. At the same time nonelectrolyte was entering the cells driven by its concentration gradient. Therefore, it was not necessary for the cells to lose as much water to reach 0.6 osmolar. That point was reached at the minimum point on the curve. Then, why did the water go back into the cells? If you look at the time when the minimum was reached in Figure 1a and determine how much nonelectrolyte was in the cells at that time (Figure 1b), you will note that the nonelectrolyte had not reached its equilibrium. Thus, solute would continue to enter the cells, but when it did the cell free energy for water would decrease and more water would enter from the medium. This combined process would continue until the nonelectrolyte in the cell had equilibrated with the medium.

An important concept emerges from this discussion. Cell volume will be determined by the osmolality of the impermeant solute. Even though the cells shrank and then reswelled, the final volume that was achieved was dependent upon

the osmolality of the sodium chloride, the impermeant solute. Put in the form of a rule:

Rule C1. *In mixtures of permeant and impermeant solutes, the equilibrium volume of the cells will be determined by the osmolality of the impermeant solute.*

THE EFFECT OF WEAK ACIDS AND BASES ON CELL VOLUME

Acids will dissociate into hydrogen ions, i.e., protons, and their matching anions. Bases will dissociate into a cation and a hydroxyl anion. If the acid or base is a strong acid or strong base, then the dissociation is complete. Weak acids and weak bases dissociate only partially. How much is defined by the dissociation constant of the acid or base. When expressed as the negative logarithm of the proton concentration in equivalents/liter, it is called the pK of the acid or base. Therefore, in a solution of a weak acid, three participants are present: proton, matching anion, and the undissociated parent weak acid. Similarly, a solution of a weak base contains a cation, a hydroxyl anion, and the undissociated parent base. How much of each is defined by the pH of the solution and the pK of the respective acid or base? The Henderson–Hasselbalch equation defines the relationship:

$$pH = pK + \log (\text{unprotonated form/protonated form}).$$

Three examples which will concern us in this discussion are:

$$pH = 4.87 + \log (\text{propionate anion/propionic acid})$$

$$pH = 9.19 + \log (\text{ammonia/ammonium cation})$$

$$pH = 6.1 + \log (\text{bicarbonate anion/carbon dioxide})$$

"Wait a minute", you protest, "Why isn't the denominator in the last one carbonic acid? Why is it carbon dioxide?" The explanation will be found in the choice of the pK of 6.1. Carbon dioxide produces hydrogen ion in two steps:

$$\text{Carbon dioxide} + \text{water} = \text{carbonic acid}$$

$$\text{Carbonic acid} = \text{proton} + \text{bicarbonate anion}.$$

Each step has its own pK. In the customary use of the Henderson–Hasselbalch equation for this reaction, the pK's for the two steps are combined so that the overall reaction is considered:

$$\text{Carbon dioxide} + \text{water} = \text{proton} + \text{bicarbonate anion}.$$

Small weak acids and bases such as propionic acid, ammonia and carbon dioxide are very permeant and cross cell membranes very quickly. Equilibrium with the concentration of the acid or base in the medium occurs in a few seconds. The addition of a weak acid or a weak base to the medium becomes a threat to the

homeostasis of cell pH. Indirectly, it will also have an effect on the cell water and cell volume. Two factors are important. The first is the buffering power of the intrinsic buffers of the cell and their respective pK's. The second is the pK of the permeant weak acid or weak base.

When the entering weak acid or weak base is buffered by the intrinsic buffers, salts of the weak acid or weak base are formed. These, as well as the weak acid or weak base themselves are osmotically active and will lower the free energy of the water in the cell. How much salt will be formed will depend upon the amount of the intrinsic buffers and their pK's. Their combined effect is quantitated as the buffering power of the intrinsic buffers: mM salt formed/change in pH. The amount of salt formed will also depend on the difference between the pK of the weak acid or weak base and the starting cell pH being defended. The pK's of ammonia and propionic acid are two pH units away from most cell pH values, while carbon dioxide is one pH unit away. This means that 10 to 100 or more mM/L of new solute may be added to the cells depending upon the concentration of the weak acid or weak base in the medium.

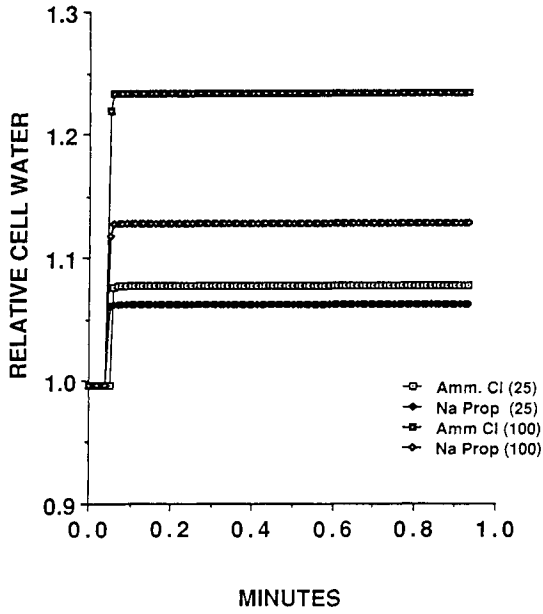

Figure 2a. Swelling of lymphocytes and erythrocytes in salts of weak acids and bases. Ion channels were **not functional**. Lymphocytes with a buffering power of 25 mM/L/pH were suspended in isosmotic solutions of ammonium chloride or sodium propionate. Erythrocytes with a buffering power of 100 mM/L/pH were suspended in similar solutions.

A third factor is not always so evident. This factor is the proton gradient which is established after buffering is complete. The smaller the buffering power of the cell or the larger the concentration of the weak acid or weak base in the medium, the greater the gradient which is developed between the new cell pH and the medium pH. The resultant proton gradient may become a driving force for the entry of other cations as we will soon see in our discussion.

Let us use a set of figures to illustrate the concepts that we just discussed. Figure 2a illustrates the relative increase in cell water that lymphocytes or erythrocytes would undergo if they were put into isosmotic solutions of sodium propionate or ammonium chloride at pH 7.4. The starting cell pH was 7.05. The buffering power was set at 25 mM/L/change in pH for lymphocytes or 100 mM/L/change in pH for erythrocytes. In this illustration, we will prevent any other solute movements except the entry of the weak acid or weak base. We will open other ion channels a little later in the discussion.

Cells gain water immediately. At 25 mM/L/change in cell pH, it is much smaller than at a buffering power of 100 mM/L/change in pH. Values this high are found in erythrocytes because of the large amounts of hemoglobin. The cause of the gain in cell water may be found in Figure 2b. Cells gain propionate from propionic acid and ammonium ion from ammonia. Much more solute is produced at the higher buffering power. Note also that more ammonium ion is produced at each buffering power than is propionate. This difference comes from the pH of the external

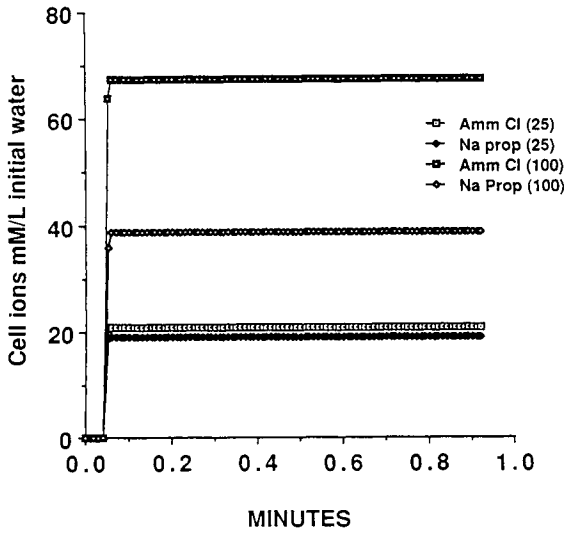

Figure 2b. Accumulation of ammonium ion (square) or propionate ion (diamond) by lymphocytes or erythrocytes suspended in isosmotic solutions of ammonium chloride or sodium propionate.

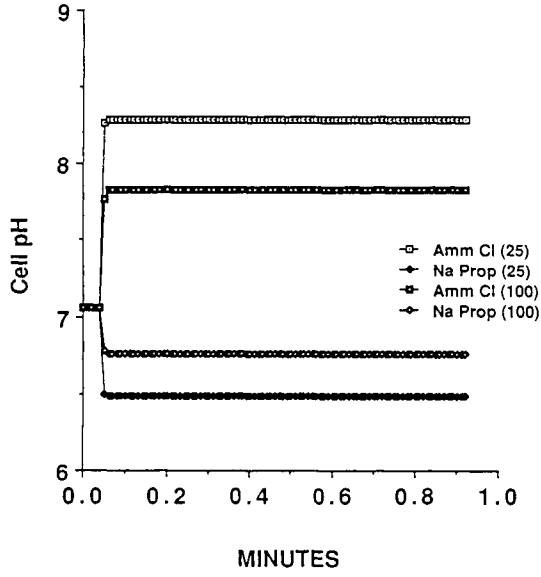

Figure 2c. Changes in cell pH when lymphocytes or erythrocytes were suspended in isosmotic solutions of ammonium chloride (square) or sodium propionate (diamond).

medium. At pH 7.4, an isosmotic solution of 149 mM/L ammonium chloride will produce 2.4 mM/L of permeant ammonia. At an external pH of 7.4 and an isosmotic concentration of sodium propionate, only 0.43 mM/L of permeant propionic acid are formed. Therefore, more buffering of ammonia is required than propionic acid. Nevertheless, note from Figure 2c how alkaline the cell pH becomes when the cells are in ammonium chloride, and how acid when placed in sodium propionate. In each case, a considerable proton gradient is established. For the propionate case, the gradient is directed outward; for the ammonium chloride medium, the proton gradient is directed inward.

ION CHANNELS AND THEIR EFFECT ON CELL WATER

As promised, let us now remove the constraints on the permeability of the membrane to other solutes. For clarity, however, let us do so in a stepwise fashion. Figures 3a–d show the effect on erythrocytes when the cells are placed in isosmotic ammonium chloride or sodium propionate and chloride anion is allowed to exchange for bicarbonate anion. The erythrocyte membrane has an intrinsic protein called Band 3 (after its position in gel electrophoresis) which permits the rapid exchange of chloride anion for bicarbonate anion. In the presence of physiological concentrations of medium bicarbonate (24 mM/L) the half-time for exchange is

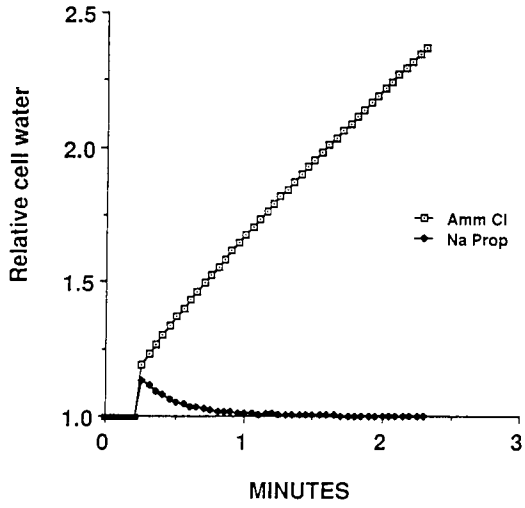

Figure 3a. Changes in cell water when erythrocytes were suspended in isosmotic solutions of ammonium chloride or sodium propionate. Chloride/anion exchangers were **functional**.

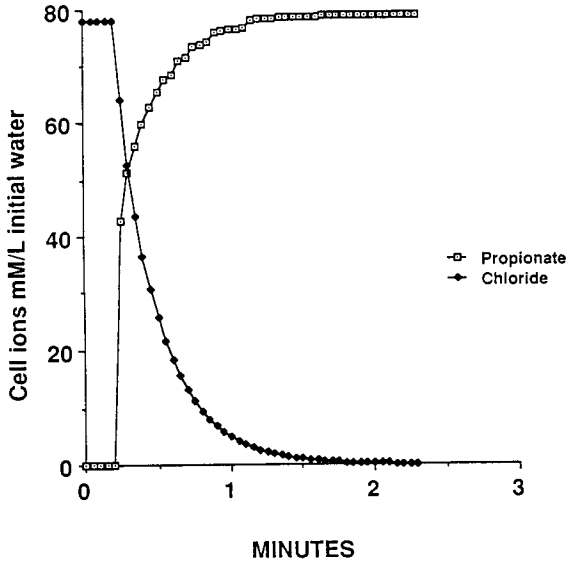

Figure 3b. Changes in cell propionate (square) or cell chloride (diamond) when erythrocytes were suspended in isosmotic sodium propionate. Chloride/anion exchangers were **functional**.

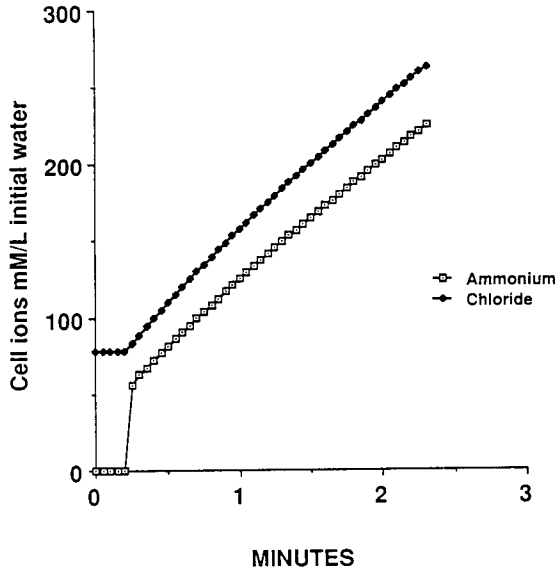

Figure 3c. Changes in cell ammonium (square) or chloride (diamond) when erythrocytes were suspended in isosmotic ammonium chloride. Chloride/anion exchangers were **functional.**

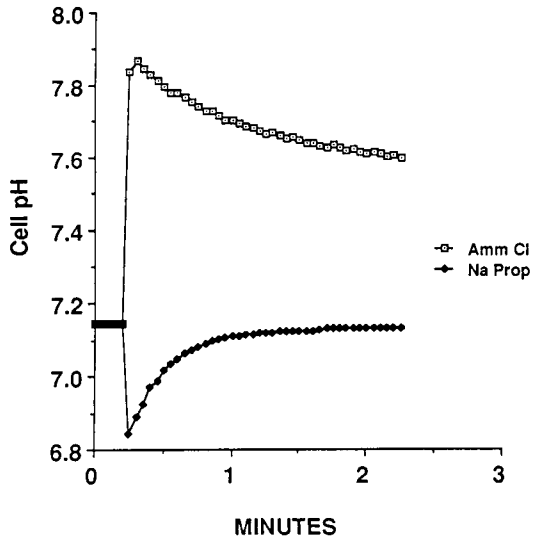

Figure 3d. Changes in cell pH after resuspension of erythrocytes in isosmotic ammonium chloride or sodium propionate.

measured in msec. In our example, to slow the process, the medium is free of added bicarbonate. The only source of bicarbonate in the cells or the medium comes from the ambient carbon dioxide in room air. This value is estimated at about 0.1 mM/L water. At this concentration of bicarbonate, the exchange rate is slowed to seconds. Under these conditions, volume changes may be followed more accurately.

Figure 3a summarizes the changes in relative cell water. In isosmotic sodium propionate, the cells gained water initially as expected from our discussion (see Figure 2a). However, instead of remaining at the new increased volume, water leaves the cells and the cells return to their starting level. Whatever solute was gained initially was lost subsequently. In contrast, when the cells were placed in isosmotic ammonium chloride, the cells also gained water very quickly, as predicted from our discussion (Figure 2a), and then continue to gain more water. Actually they gained so much water that the cells hemolyzed. The explanation for these events is found in Figure 3b. At the outset, propionate is formed as the propionic acid that entered the cell is buffered. However, Figure 2c had illustrated how the cell pH had fallen because of the entry of propionic acid in spite of intracellular buffering. The concentration of cell hydroxyl and bicarbonate also falls. This sets up a gradient for the entry of bicarbonate from the medium. Moreover, we have an even greater gradient for the exit of cell chloride, since the medium concentration of chloride is very low. The result is a loss of chloride in proportion to the gain of cell propionate. No net gain of solute occurs. Only the species of solute has changed. The rate at which these events occurs will depend upon the gradient and the permeability of the membrane, as defined by the operation of the Band 3 anion exchanger.

The results are different with ammonium chloride. Let us follow the events. Figure 2b had shown how ammonium was formed in the cells as the entering ammonia was buffered. Cell pH will rise and the concentration of hydroxyl and bicarbonate will also rise. We now have a gradient for the outward movement of bicarbonate. In addition, the concentration of chloride in the medium is greater than its concentration in the cells (149 mM/L in the medium; 78 mM/L in the cells). Consequently, chloride will enter the cells. The net result is a gain of ammonium chloride, albeit by separate mechanisms (refer to Figure 3c). The gain in solute upsets the osmotic balance, water enters, and the cells swell to hemolysis.

Figure 3d describes the changes in cell pH. In a medium of sodium propionate, cell pH drops as it did in Figure 2c. However, as cell chloride leaves in exchange for medium bicarbonate, the gain in alkaline bicarbonate restores the cell pH. When the medium is ammonium chloride, cell pH rises at the start, as already indicated in Figure 2c, but the pH decreases as medium chloride exchanges for cell bicarbonate.

Hemolysis of erythrocytes in isosmotic ammonium chloride has been used to isolate leukocytes from peripheral blood. After separation of leukocytes from blood by centrifugation through density gradients, a considerable number of contaminat-

ing erythrocytes remain. The erythrocytes may be removed by suspending the mixture of leukocytes and contaminating erythrocytes in isosmotic ammonium chloride. Since leukocytes exchange chloride for bicarbonate at a very slow rate, while this exchange occurs in seconds in erythrocytes, erythrocytes will hemolyze while the leukocytes will only swell slightly because of intracellular buffering. Gentle centrifugation will move the leukocytes to the bottom of the tube, leaving the erythrocyte ghosts in the supernatant. After removal of the supernatant, the leukocytes are resuspended in fresh medium for study.

One very important physiological process does depend upon the chloride/anion exchanger. It is the buffering of carbon dioxide by erythrocytes in the microcirculation. Let us review what happens to erythrocytes under these physiological conditions. In the capillaries, oxygen is leaving the hemoglobin of the erythrocytes and entering the tissues. At the same time, carbon dioxide is leaving the tissues and entering erythrocytes. What happens to erythrocyte volume? Figure 4a plots the change in cell water. The lower curve describes the gain in cell water if the cells had been in arterial plasma, and then entered a region where the carbon dioxide had increased from 40 mm Hg to 46 mm Hg. Note that the volume change is small and occurs in a fraction of a second. The pK of the hemoglobin was set at 6.8. Compare this curve with the upper curve. The only difference was that the pK was set at 6.9. Let us review what caused the swelling and why there was such a difference when the pK of hemoglobin was increased.

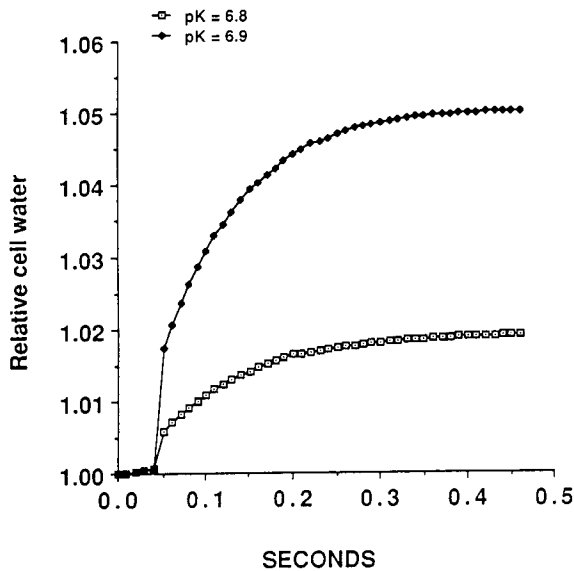

Figure 4a. Predicted changes in cell water when model erythrocytes suspended in arterial plasma were exposed to carbon dioxide at a partial pressure of 46 mm Hg.

In this particular example, the arterial plasma contained 24 mM/L of bicarbonate, 110 mM/L chloride, and the pH was 7.4. The carbon dioxide concentration was 1.2 mM/L. Inside the erythrocyte, bicarbonate was 18 mM/L cell water, chloride was 81 mM/L, and the pH was 7.27. These values were calculated to reflect the fact that bicarbonate, chloride, and hydrogen ions are in electrochemical equilibrium. "Wait a minute", you protest again, "There is a greater concentration of chloride and bicarbonate in the medium than in the cell and there is a greater hydrogen ion concentration in the cell than in the plasma water." Remember, however, that the sodium/potassium pump which keeps the high sodium of the medium out of the cell and the high potassium inside the cell, is electrogenic. In this example, the membrane potential produced by the pump was chosen at 7.5 mV, inside negative. Therefore, the anions are repelled from the cells and the hydrogen ions are attracted into the cells.

When the cells enter the region where tissues are producing carbon dioxide from metabolism, the carbon dioxide in the plasma rises. In this example, we chose a rise to a partial pressure of 46 mm Hg. The pH of the plasma dropped to 7.34, carbon dioxide equilibrated rapidly with the cell water, cell pH dropped, hemoglobin buffered the additional protons, more bicarbonate was formed, and some of the bicarbonate formed exchanged with medium chloride. The end

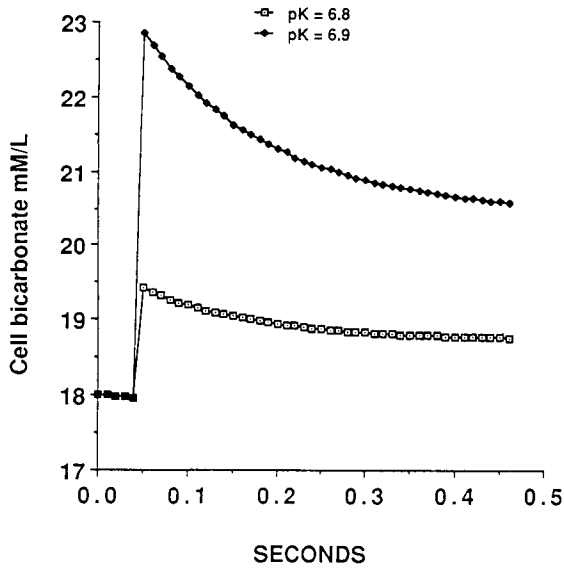

Figure 4b. Predicted changes in cell bicarbonate when model erythrocytes suspended in arterial plasma were exposed to carbon dioxide at a partial pressure of 46 mm Hg.

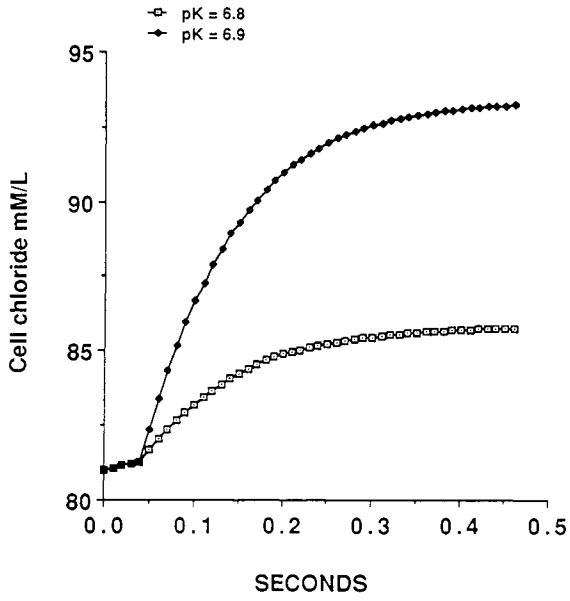

Figure 4c. Predicted changes in cell chloride when model erythrocytes suspended in arterial plasma were exposed to carbon dioxide at a partial pressure of 46 mm Hg.

result was an increase in cell chloride and bicarbonate. The osmotic balance was upset and water entered the cells. Figures 4b–c illustrate the changes in bicarbonate and chloride. The lower curves illustrate the changes when the pK of hemoglobin was 6.8.

We omitted one important physiological event which bears on our discussion. When hemoglobin loses oxygen to the tissues, its properties as a buffer improve. It becomes a weaker weak acid. Its pK increases. For that reason, the upper curve was generated to illustrate that when the pK increases, hemoglobin can buffer more carbon dioxide, and as a consequence, more water enters the cells. Since the physiological events are not as abrupt as shown in our illustration, the changes in cell volume with time generate a curve moving between the two curves plotted, as the cells pass through the microcirculation.

When the erythrocytes reach the lungs and the carbon dioxide concentration decreases as the carbon dioxide leaves the lungs, the whole process reverses. Since the passage of blood through the alveoli of the lungs may take less than a second, it is fortunate that the chloride/bicarbonate exchange occurs in less than a second. Otherwise, not all the bicarbonate formed in the tissues would be eliminated as carbon dioxide when the cells return to the lungs.

THE SODIUM/PROTON EXCHANGER

Let us continue with our stepwise analysis and deal with the sodium/proton exchanger. This ion channel exchanges sodium ion moving down its concentration gradient with hydrogen ion (proton) moving down its concentration gradient. The proton gradient between cells and medium may be accentuated if the cell pH falls while the medium pH remains constant. Let us suspend lymphocytes in isosmotic sodium propionate at pH 7.2. The normal cell pH for lymphocytes is 7.05. Recall that propionic acid will enter the lymphocytes rapidly and lower cell pH. Intrinsic cell buffers will buffer the entering propionic acid and form propionate. The pH will fall to a new lower level depending upon the buffering power of the intrinsic buffer. The cells will also gain water because of the addition of propionate to the cell. We now have two sizeable ion gradients: the sodium in the medium is 137 mM/L and the cell sodium is 20 mM/L. Medium pH is 7.2. Cell pH has dropped from 7.05 to 6.5 because of the entry of propionic acid and in spite of the buffering efforts of cell buffers. What happens if the sodium/proton exchanger is turned on? Sodium will enter the cells and protons will leave. Therefore, the cells will gain additional sodium ion. The loss of protons from the cells will shift cell pH back toward its normal value. As cell pH rises, propionic acid will dissociate to form additional protons and propionate anion. The end result will be additional production of propionate anion as well. The added solute in the cell will upset the osmotic

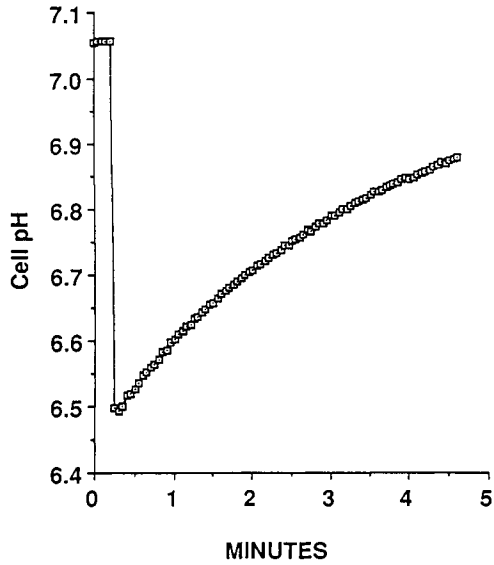

Figure 5a. Changes in cell pH when lymphocytes were suspended in isosmotic sodium propionate at pH 7.2. Only the sodium/proton exchangers were **functional.**

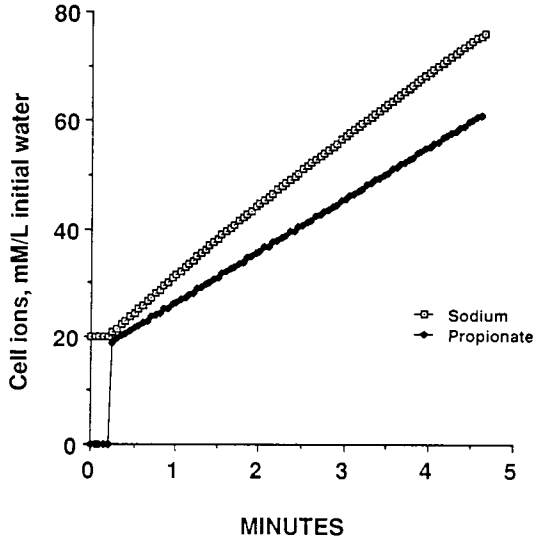

Figure 5b. Changes in cell sodium and propionate when lymphocytes were suspended in isosmotic sodium propionate at pH 7.2. Only the sodium/proton exchangers were **functional.**

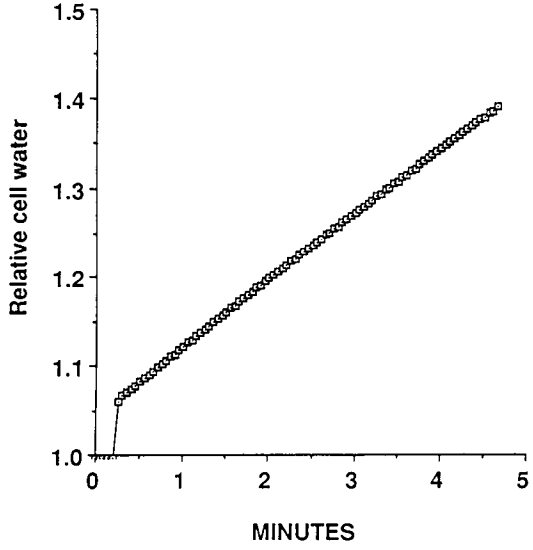

Figure 5c. Changes in cell water when lymphocytes were suspended in isosmotic sodium propionate at pH 7.2. Only the sodium/proton exchangers were **functional.**

balance, water will enter, and the cells will swell. How long will this continue? Remember that two factors control the entry of solutes: driving force (concentration gradient) and permeability of the sodium/proton exchanger. If the sodium/proton exchanger is not turned off, then the entry of sodium and the exit of protons will continue until the sodium gradient is equal to and opposite to the proton gradient.

In this example, equilibrium will not be reached because the entry of water dilutes the cell sodium and keeps its concentration much less than the medium concentration. Similarly, the cell pH does not reach a value which exceeds the medium pH. The inevitable consequence is cell lysis. In effect, we are getting the same outcome that we saw with erythrocytes placed in ammonium chloride. When both solutes of the medium become permeant, the cells respond as if they were placed in distilled water, i.e., with cell swelling and lysis. Figures 5a–c summarize the predicted changes in cell pH, solute content, and cell water according to our model.

THE REGULATION OF CELL VOLUME: REGULATORY VOLUME DECREASE (RVD)

Let us now put what we have learned about ion channels into a physiological context. Let us discuss how the cells use ion channels to regulate their cell volume. To do so, let us challenge the cells by putting them into a hypoosmotic medium. The cell of choice for our example will be the lymphocyte. When lymphocytes are placed into 0.6 isosmotic sodium chloride, i.e., only 0.6 the cell's osmolality, they swell immediately as water enters the cells and dilutes the cell solutes until their concentration is also 0.6 isosmotic. This response of the cells occurs in seconds because the membrane of the lymphocyte is very permeable to water. However, during the next few minutes, the cells lose water and cell volume returns toward normal.

Two ion channels are responsible for this regulatory volume decrease (RVD): a potassium channel and a chloride channel. Let us see how this regulatory volume decrease operates in lymphocytes.

The concentration of cell potassium is 140 mM/L while the medium may contain 4 mM/L. This concentration gradient is maintained by the operation of the sodium/potassium pump which extrudes three sodium ions from the cell in exchange for two potassium ions. The imbalance in the ion exchange leads to a potential difference across the membrane of the lymphocyte, which is taken to mean this sodium/potassium pump is electrogenic. In the lymphocyte, the tendency of the high potassium to move out of the cells by the driving force of its concentration gradient is balanced by the restraining force of the negative potential inside the cell produced by the sodium/potassium pump.

The chloride concentration gradient favors the entry of chloride into the cells. The negative potential inside the cell favors the expulsion of chloride from the cell. The question now is, "Which ion is dominant when the cell is under isosmotic

conditions?" The choice is determined by that other important factor which we have discussed. Under isosmotic conditions, the membrane is far more permeable to potassium than to chloride. As a result, the electrogenic potential produced by the sodium/potassium pump is determined by the concentration of potassium inside and outside the cell, as predicted by the Goldman equation. The cell membrane potential is dominated by the potassium ion. Little chloride moves in or out of the cells because of its low permeability.

When the lymphocytes are placed in 0.6 isosmotic medium, the cells swell and this deformation in cell structure triggers a large increase in the permeability of the membrane to chloride. It becomes the most permeant ion. As a result, the Goldman equation predicts that the membrane potential difference should depolarize, because the ratio of cell chloride concentration to medium concentration will generate a much lower potential difference. When the potential difference becomes less negative inside the cell, the restraining force on potassium exit from the cells is reduced and potassium is able to leave the cell. However, it cannot go far without an accompanying anion, and that anion is the chloride anion. The net result is the exit of potassium chloride.

How fast will the potassium chloride leave the cells? Again we have to come back to our two major factors: the driving force and the permeability. The driving force will be determined by the net free energy, i.e., the algebraic sum of the chloride

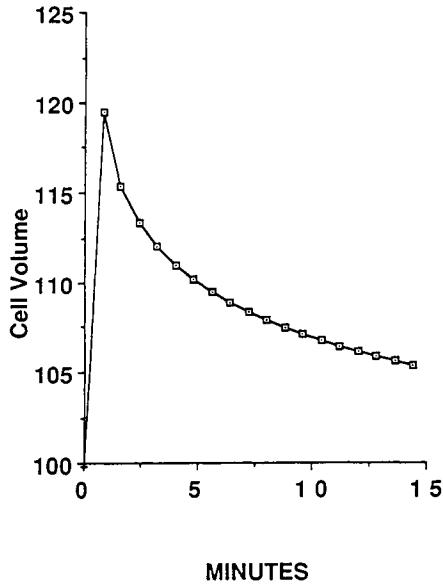

Figure 6a. Changes in lymphocyte volume during regulatory volume decrease (RVD). Lymphocytes were suspended in 0.6 isosmotic sodium chloride. Potassium and chloride channels were **functional.**

and potassium electrochemical gradients. The ion with the slowest membrane permeability will set the rate at which potassium chloride is lost. In this case, it will be the potassium ion because the stretching of the cell had increased the membrane permeability to chloride far more than the permeability to potassium.

Figures 6a–c summarize the predictions from experimental data. In Figure 6a, we see the lymphocyte volume increase sharply within the first few seconds after suspension in 0.6 isosmotic medium. Swelling has been produced by the rapid entry of water in response to the hypoosmolality. During the next 15 minutes, cell volume decreases. Figure 6b indicates why. During this time, potassium and chloride are leaving the cells in equimolal amounts. Note that the rate of loss diminishes and approaches an asymptote at a lower cell content. Why does the rate taper off? Is it because the driving force for exit has been dissipated? The answer is no. The cell potassium concentration is still far greater than its value of 4 mM/L in the medium. The answer comes in Figure 6c. This figure summarizes the changes in membrane permeability of the slowest member of the pair. Before suspension of the lymphocytes in the hypoosmotic medium, permeability was set at 2.5×10^{-8} cm/min to signify the permeability of the membrane to chloride ion. Under these circumstances, no net exit of potassium chloride occurs because the potential of the membrane is dominated by the potassium distribution ratio. However, when the cells swell, the permeability to potassium chloride increases. Is this the permeability of the membrane to the potassium ion or to the chloride ion? It is the permeability

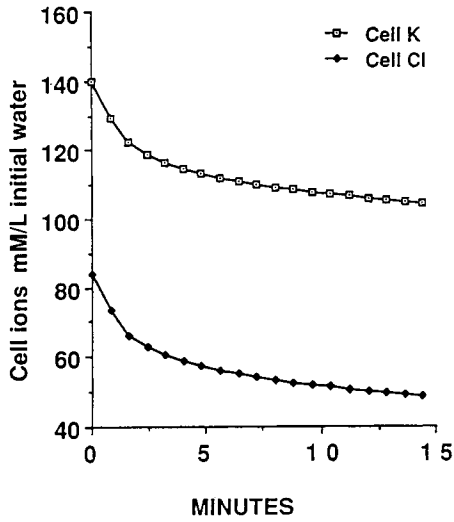

Figure 6b. Changes in lymphocyte potassium and chloride during regulatory volume decrease (RVD). Lymphocytes suspended in 0.6 isosmotic sodium chloride. Potassium and chloride channels were **functional.**

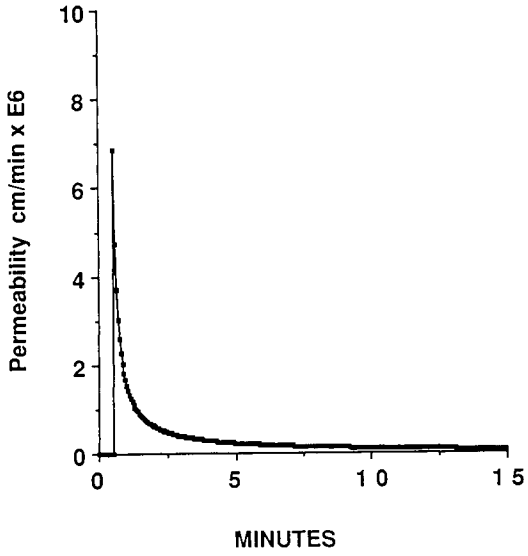

Figure 6c. Predicted changes in the permeability of the potassium and/or chloride channel during regulatory volume decrease.

of the slowest ion, the potassium ion. Experimentally, it turns out that this permeability of potassium may be as much as ten times its permeability under unstimulated conditions, but still much slower than the permeability of the chloride channel.

If this permeability was maintained during the following 15 minutes, cell volume would decrease well below the starting level and far more rapidly than seen experimentally. Our hypothesis demands that the permeability of potassium chloride falls. The mechanism for this decrease in the permeability of potassium and also of chloride to their unstimulated values may be the removal of the stress placed on the cell since the cell volume is returning toward normal values.

After a cycle of cell swelling followed by regulatory volume decrease, we are left with lymphocytes having reduced intracellular concentrations of potassium and chloride.

THE REGULATION OF CELL VOLUME: REGULATORY VOLUME INCREASE (RVI)

In this section, we will analyze an example of the physiological utility of two channels that have been discussed already: the sodium/proton exchanger and the chloride/bicarbonate exchanger. Several mammalian cells use these channels to protect themselves against cell shrinkage due to increases in medium osmolality. To be consistent, we will continue to use the lymphocyte as our cell, recognizing that other cells may adopt different variations of these two channel mechanisms.

We have noted that when lymphocytes recovered from a hypoosmotic stress, they lost potassium chloride. What happens if we now resuspend these lymphocytes in their normal medium? Since the lymphocytes have lost solute, they are now hypoosmotic to the normal medium. As a result, the cells shrink. The deformation in cell structure turns on the sodium/proton exchanger. The sodium gradient favors the entry of sodium into the cells since the medium sodium concentration is 137 mM/L and the cell sodium concentration is 20 mM/L or less. Cell pH did not change significantly during recovery from cell swelling; its value is still 7.05. Medium pH is 7.2. Therefore, we still have a significant proton gradient for the exit of protons from the cell. As a result, sodium enters the cells and protons leave. Figures 7a and 7b model what happens under these conditions. Figure 7a is a plot of cell volume versus time. Regulatory volume decrease is recapitulated during the first 7.5 min. At that point, the cells were resuspended in a normal medium at 298 mOsm/L. Cells shrank rapidly, as water was lost to the medium, which is now hyperosmotic to the cells. Shrinkage is followed by a slow reswelling. Let us look at the open squares first. In this model, only the sodium/proton exchanger was activated. Cell swelling is minimal and soon levels off at a volume well below the normal cell volume. Apparently, regulatory volume increase has not been successful in this case. Little sodium has entered the cells and little water has followed. Figure 7b explains why.

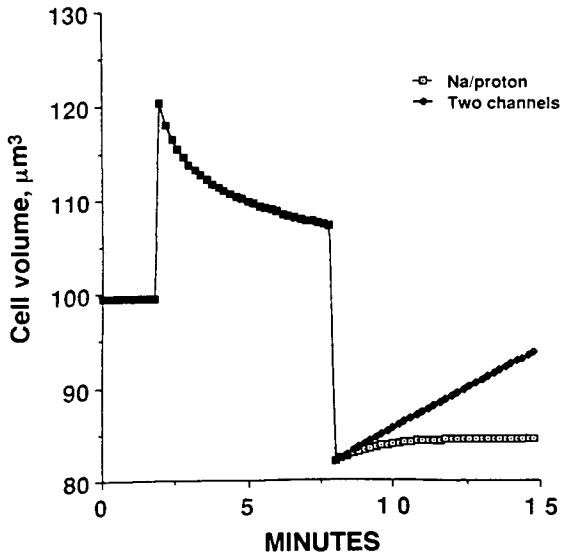

Figure 7a. Changes in cell volume during regulatory volume decrease (RVD) followed by regulatory volume increase (RVI) in lymphocytes. Only sodium/proton exchangers were **functional** (square). Both sodium/proton exchangers and chloride/bicarbonate exchangers were **functional** (diamond).

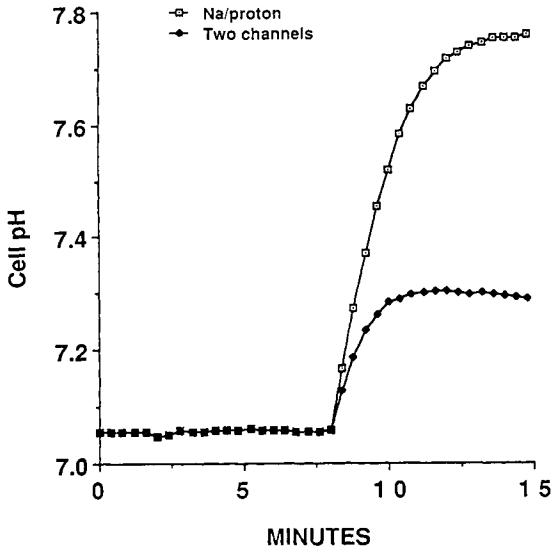

Figure 7b. Changes in cell pH during regulatory volume decrease (RVD) followed by regulatory volume increase (RVI) in lymphocytes, only sodium/proton exchangers were **functional** (square). Both sodium/proton exchangers and chloride/bicarbonate exchangers were **functional** (diamond).

In Figure 7b, cell pH is plotted versus time. First note that little change in cell pH occurred during regulatory volume decrease when potassium chloride was being lost. However, as soon as the sodium/proton exchanger was turned on in response to cell shrinkage, the entry of cell sodium produced a corresponding loss of protons and cell pH rose rapidly to pH 7.8 in spite of the buffering power of the lymphocyte. Very little sodium had to enter to produce this major rise in cell pH. At a cell pH of 7.8 and a medium pH of 7.2, the proton gradient favors the entry of protons. The inward sodium gradient is not high enough to push the protons out against this pH gradient. In fact the two free energies of the gradients are equal. The exchange system is now in equilibrium and no further net exchange occurs. It is for this reason that no additional sodium enters the cells; the protons cannot move out against their gradient.

The situation becomes quite different if we also turn on the chloride/bicarbonate channel (filled diamond). Cell volume increases almost linearly with time. Cell pH rises to 7.3 and levels off. At this cell pH, the sodium gradient has enough free energy to overcome the gradient against which the protons have to move. As a result, sodium enters the cells. The reduction in cell pH has been produced by the entry of chloride into the cells in exchange for bicarbonate. As the bicarbonate leaves, cell buffers replace them with hydroxyl ions to keep the pH steady. The net

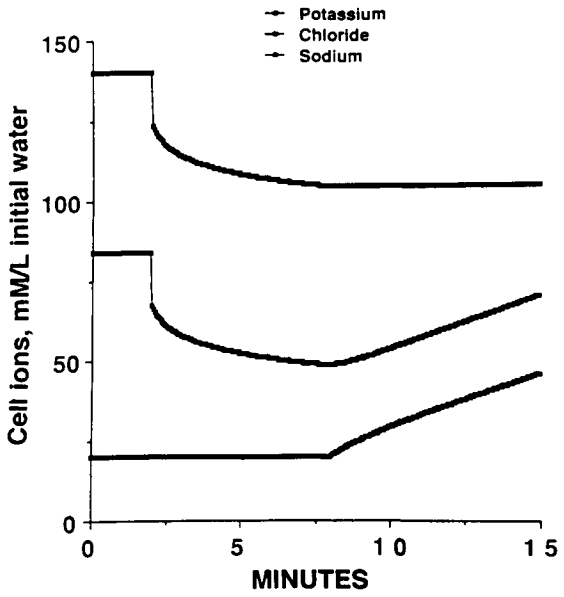

Figure 7c. Changes in lymphocyte ions during regulatory volume decrease followed by regulatory volume increase.

gain of solute is sodium chloride. We now have the same conditions that we found with ammonium chloride. Both sodium and chloride of the medium are able to enter the cells. The inevitable outcome is cell swelling. If no other regulatory mechanism intervenes, the cells will lyse. The concomitant movements of sodium and chloride are shown in Figure 7c. During the first 7.5 minutes we note the exit of potassium chloride during regulatory volume decrease. After resuspension in normal sodium chloride medium, with both channels open, we note the concomitant entry of sodium chloride in a parallel and linear fashion.

The lymphocyte is faced with a dilemma. In order to recover volume after shrinkage, it needs solute. To get enough sodium from the medium, it risks alkalinization and the dissipation of the proton gradient. If it turns on the chloride/bicarbonate exchanger also, it can modulate the rise in cell pH, but it opens the possibility of cytolysis. Cell survival, therefore, requires regulation of these two channels. As the cells swell and approach their normal volume, the sodium/proton exchanger will have to be turned off. Figure 7d plots what happens to cell pH. During the first 7.5 minutes both the sodium/proton exchanger and the chloride/bicarbonate exchanger are activated. Cell pH rises from 7.05 and levels off at 7.3. When the sodium/proton exchanger is turned off, and the chloride/bicarbonate exchanger remains on, cell pH falls from 7.3 and levels off at pH 6.93, slightly

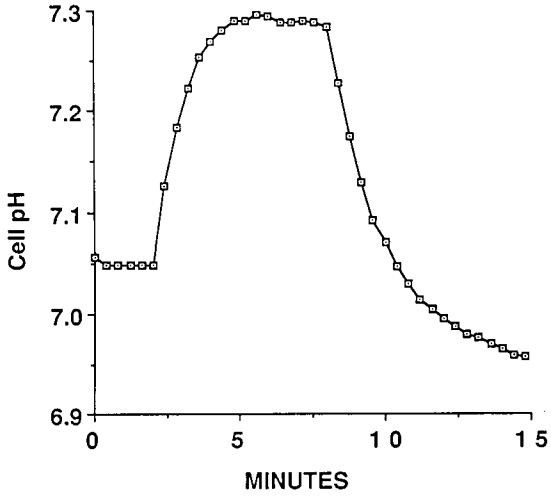

Figure 7d. Changes in cell pH after lymphocytes suspended in isosmotic medium were resuspended in 1.5 isosmotic medium. Both the sodium/proton exchangers and the chloride/bicarbonate exchangers were **functional.** At 7.5 min, the sodium/proton exchanger was turned off, but the chloride/bicarbonate exchanger was **functional.**

lower than the normal cell pH of 7.05. Although chloride has entered the cells in exchange for bicarbonate to restore cell pH, not enough chloride has entered to produce significant cell swelling. Therefore, the cell earns a bonus in allowing this channel to remain open. Ideally, when the normal cell pH has been reached, it too should turn off.

What happens if lymphocytes are in their normal medium and then are resuspended in a hyperosmotic medium? Experimental evidence indicates that the pH of the cells rises. Values were recorded in the range of 7.2 to 7.5. These values are much lower than a value of 8.1, which is what is predicted if the sodium/proton exchangers were turned on alone. The implication, therefore, is that the chloride/bicarbonate exchanger was also turned on. Figure 7e graphs the rate of swelling predicted under these conditions and compares it with the rate if the cells had first undergone regulatory volume decrease before exposure to the hyperosmotic medium. The pattern of response is similar under the two protocols, but the rate is slower under regulatory volume increase alone. The explanation for this difference in rates is found in the difference in the osmolalities of the suspending media. After regulatory volume decrease, the cells were suspended in a normal medium of 298 mOsm/L. In contrast when only regulatory volume increase was initiated, cells in 298 mOsm/L were resuspended in 1.5 times that osmolality or 450 mOsm/L. Therefore, to move the same amount of water into the cells would require 1.5 times as much solute to enter under conditions of regulatory volume increase alone. If

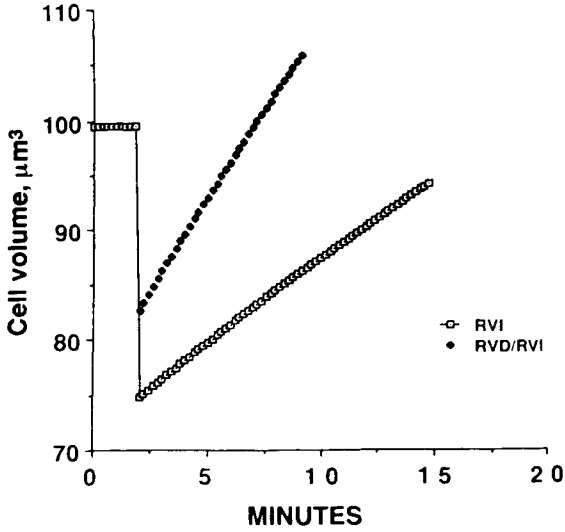

Figure 7e. Comparison of changes in cell volume between lymphocytes suspended in 0.6 isosmotic medium, followed by resuspension in normal medium (RVD/RVI, diamond) and lymphocytes suspended in normal, isosmotic medium followed by resuspension in 1.5 isosmotic medium (RVI, square).

the permeability of the sodium/proton exchanger is the same in both experimental procedures, one would expect the rate of cell swelling to be slower.

SUMMARY

This chapter on intracellular water has emphasized that the net transport of water into or out of cells determines cell volume. The regulation of cell volume is the regulation of cell water. Two factors are necessary for the transport of water across cell membranes: the free energy gradient for water and the permeability of the membrane to the solvent. The free energy of water is affected by its interaction with solutes so that the regulation of cell water in turn becomes the regulation of the solutes which interact with water. The transport of solutes also requires an energy gradient and specific membrane permeabilities. Weak acids and bases affect cell volume because they are very permeable and form osmotically active salts. These osmotic changes in cell volume may be used to determine the buffering power of cells and how intracellular pH is regulated. Specific ion channels for potassium, chloride, sodium, protons, and bicarbonate contribute to volume regulation. These channels also require a free energy gradient and membrane permeability to be effective. Changes in cell pH will affect both factors and lead to an interaction between the regulation of cell volume and cell pH.

RECOMMENDED READINGS

Grinstein, S. & Foskett, J. K. (1990). Ionic mechanisms of cell volume regulation in leukocytes. Ann. Rev. Physiol. 52, 399–414.

Hempling, H. G. (1989). Analysis of ion channels by modeling the osmotic effects of weak acids and bases. J. Membrane Biol. 110, 122–137.

Kedem, O. & Katchalsky, A. (1958). Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. Biochim. Biophys. Acta 27, 229–246.

Chapter 10

Protein Synthesis and Regulation in Eukaryotes

SURESH I. S. RATTAN

INTRODUCTION

The genetic information encoded in DNA becomes functionally meaningful only when it is accurately transcribed and translated into RNA and protein. Two types of RNA, transfer (t) RNA and ribosomal (r) RNA, are themselves functional

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molecules. However, the genetic information transcribed into the third RNA, messenger (m) RNA, has to be translated from a language of nucleic acids into a language of amino acids in order to produce proteins which are functional gene products. It is generally estimated that in a human cell, there are about 100,000 genes, of which between 3,000 and 10,000 are expressed and translated into a spectrum of proteins specific for every differentiated cell type.

Proteins are the most versatile macromolecules necessary for the organization of internal cellular structures, for the formation of the energy-creating and metabolic-utilizing systems in the cell, for the transport of ions and larger molecules across cell membranes and for maintaining intracellular and intercellular communication pathways. Furthermore, proteins interact with all other macromolecules, including DNA, RNA, carbohydrates, and lipids, and are required for maintenance and repair at all levels of biological organization.

Protein synthesis is thus crucial for the survival of a living system, and any disturbance at this level can cause large imbalances and deficiencies. For example, inhibition of protein synthesis is followed rapidly by cell death. If the rate of protein synthesis slows down, it affects not only the basic metabolic enzymatic processes but also the energy-supporting system, the membrane-mediated signaling pathways, and the processes for the removal of damaged and abnormal molecules. Similarly, if the process of protein synthesis becomes less accurate, that is, if unfaithful translation of the genetically specified sequence of amino acids occurs, it can result in the loss of activity and specificity of proteins with far reaching and damaging consequences for the cell and the organism. Therefore, the process of protein synthesis and its regulation during different stages of growth, division, differentiation, development, and aging is one of the most crucial aspects of a living system.

MECHANISMS OF PROTEIN SYNTHESIS

Since protein synthesis in eukaryotes occurs both in the cytoplasm and in certain cellular organelles such as mitochondria (and chloroplasts in the case of plants), the mechanisms of cytoplasmic protein synthesis are described first, followed by a discussion of the similarities and differences in protein synthesis in the organelles, particularly the mitochondria.

The primary structure of a protein is its sequence of amino acids, translated from the sequence of three-letter nucleotide codons in mRNA that itself is copied (transcribed) from DNA. The transcription of RNA, its posttranscriptional editing, processing, and transport from the nucleus to the site of translation is a huge subject, beyond the scope of this article. Interested readers are advised to consult other review articles, for example Bag (1991), Kozak (1991), and Ross (1989).

Protein synthesis is one of the most complex processes in the cell. In order to translate one mRNA molecule, almost 200 small and large components are required to function effectively and accurately while using large quantities of cellular energy

Table 1. Major Components of the Protein Synthetic Machinery Required for Translation

Component	*Subcomponents*	*Function*
1. Translational particle		
Ribosome	40S and 60S subunits 4 rRNAs and about 80 ribosomal proteins	Recognizing and translating the genetic codons in mRNA
2. Charging system		
Amino acids	At least 20	Building blocks for proteins
tRNAs	About 60	Matching codons with respective amino acids
tRNA synthetases	At least 20	Adding amino acids to tRNA
3. Translational factors		
Initiation factors	more than 24 proteins	Initiating protein synthesis
Elongation factors	4 proteins	Addition of amino acids to growing peptide chain
Release factor	1 protein	Terminating protein synthesis

in the form of GTP. There are three major components of the translational apparatus: (1) the translational particle, the ribosome; (2) the amino acid-transfer system or charging system; and (3) the translational factors. Table 1 gives an overview of the components and the subcomponents involved in eukaryotic protein synthesis, along with their major functional characteristics.

Protein synthesis can be envisaged as proceeding in three steps—initiation, elongation, and termination, followed by posttranslational modifications, including folding, which give the protein a functional tertiary structure. Of these steps, the initiation and termination steps occur only once for each polypeptide chain, whereas the elongation step is repeated as many times as the number of amino acids in a complete chain. Although there are no minimum and maximum limits on the number of amino acids required to make a functional protein, the lengths of the shortest and longest mRNA molecules in eukaryotes are not yet known.

Initiation

The translation of an mRNA begins with the formation of a so-called initiation complex between the ribosome and the initiator codon, AUG. Figure 1 is a simplified schematic representation of the major steps in the generation of an initiation complex. It is an intricate process, which consumes energy and involves at least seven-initiation factors (eIFs; e stands for eukaryotic) consisting of 24-different subunits, two subunits of ribosomes, and an initiating tRNA called Met-$tRNA_i$.

The process of translational initiation begins with the dissociation of inactive 80S (S stands for Svedberg) ribosomes, called monosomes, to generate free 60S

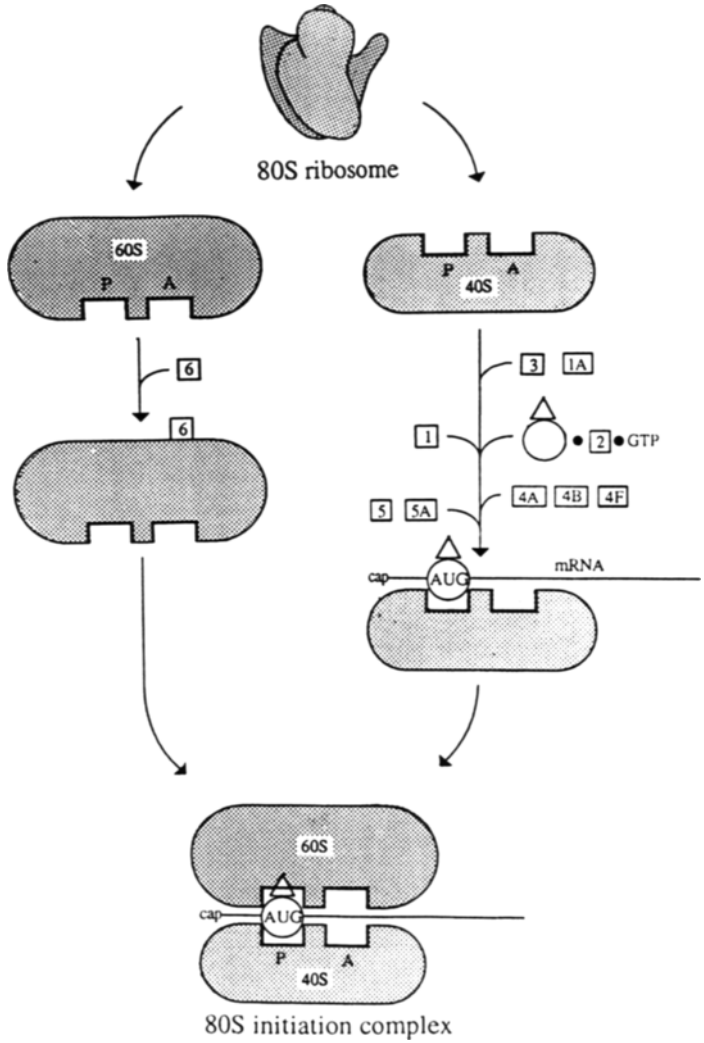

Figure 1. Schematic diagram showing the major steps of the initiation of protein synthesis in eukaryotic cells. The formation of an 80S initiation complex involves the joining of the 40S subunit bound to mRNA and met-tRNA$_i$, and the 60S subunit. Numbers in □ represent various initiation factors, ○ represents tRNA, and △ represents the amino acid. (From Johansen and Rattan, 1993).

and 40S subunits. Although far less is known about eukaryotic ribosomes than prokaryotic ribosomes, significant progress is being made in understanding the structure of eukaryotic ribosomes, particularly from yeast and from rat (Wool, 1991). Ribosomes are cellular organelles that comprise at least four types of rRNA and about 80 proteins, and are pivotal in the translation of the language of nucleic acids into a language of amino acids.

The nucleotide sequences of the four species (5S, 5.8S, 18S, and 28S) of rRNA have been determined, and these show remarkable homology among the three kingdoms (archaebacteria, eubacteria, and eukaryotes). In the case of ribosomal proteins, the amino acid sequences of about 35 proteins out of a total of about 80 have been determined. However, the exact roles of various rRNAs and proteins and their interactions in determining the activity, efficiency, and accuracy of the ribosomes is not very well understood at present. The coordinated synthesis of rRNAs and proteins, and the assembly of ribosomes is a process whose complexity is only beginning to be unravelled (Wool, 1991).

The dissociation of the inactive ribosome into its subunits is dependent on the activity of two initiation factors, eIF-3 and eIF-6, which keep subunits apart by binding to the 40S and the 60S subunits, respectively (Hershey, 1991; Rhoads, 1991; Merrick, 1992). eIF-3 is the largest of the initiation factors (molecular mass 600–650 kDa) comprising 8–10 different polypeptides. In comparison, eIF-6 is a single polypeptide of about 25 kDa.

The next step is the formation of the 43S ternary complex of met-tRNA$_i$, GTP, and eIF-2. This binds to the 40S subunit in the absence of mRNA. eIF-2 is one of the most widely studied initiation factors and is known to contain three polypeptides, α, β, and γ, with molecular masses of 35, 38, and 52 kDa, respectively. The primary function of eIF-2 is to bind met-tRNA$_i$ in a process that is dependent on GTP. Thereafter, through a process that is still largely a mystery, the binding of the 43S complex with mRNA occurs at its methyl-7-guanine (m^7G) cap at the 5′ end (Nygård and Nilsson, 1990). Three initiation factors, eIF-4A, 4B, and 4F, are required for optimal binding of mRNA while using one molecule of ATP. eIF-4A is a highly conserved single-polypeptide chain of about 45 kDa and is characterized as an ATP-dependent RNA helicase involved in the unwinding of RNA. A particular feature of all putative RNA helicases is the presence of the D-E-A-D (Asp-Glu-Ala-Asp) amino acid sequence motif, thought to be associated with the processing of RNA. In comparison, eIF-4B is a dimer of identical subunits of about 80 kDa and is necessary for the cross-linking of eIF-4A to mRNA. eIF-4F also contains two subunits of 26 kDa and 220 kDa, both of which interact with the m^7G cap of the mRNA.

The binding of the 43S complex to the capped 5′-end of the mRNA is followed by its migration and scanning until the first AUG codon is encountered (Kozak, 1989). Although there are some exceptions, more than 90% of cellular mRNAs appear to have "GCC GCC A/GCC **AUG** GG" as the consensus sequence for the

initiation of protein synthesis. However, it is not known what recognizes the consensus sequence and brings about a transient stopping of the complex at the AUG start codon to effect the correct positioning of mRNA for translation.

Once correct positioning occurs, and the match is made between the anticodon of the met-tRNA$_i$ and the start codon, the GTP molecule bound to eIF-2 is hydrolyzed in a reaction promoted by eIF-5. The physical nature of this reaction remains controversial. There are thought to be two forms of eIF-5 with molecular masses of 125 kDa and 60 kDa without, however, any differences in their biological properties (Hershey, 1991; Merrick, 1992). The hydrolysis of GTP causes the release of the initiation factors from the surface of the 40S ribosomal subunit, and allows attachment of the 60S subunit by triggering the release of eIF-6 from it. The formation of the 80S initiation complex culminates in the formation of the first peptide bond at the ribosomal P site. The initiation factor eIF-4D is required for the formation of the first peptide bond. eIF-4D is a small protein (about 16 kDa), and has a unique posttranslational modification of its lysine-50 residue by the action of a polyamine, spermidine, to form a hypusine residue essential for its activity (Hershey, 1991; Merrick, 1992). Furthermore, in order to allow efficient and catalytic use of eIF-2 after GTP hydrolysis and its release from the complex, another factor, eIF-2B, facilitates the exchange of eIF-2 bound GDP for GTP.

The whole process of the formation of the 80S initiation complex takes about 2–3 s in cell-free assays and is thought to occur much faster *in vivo*. Each mRNA can participate in multiple rounds of initiation, thus giving rise to a string of ribosomes called polysomes, engaged at different stages of translation. Polysomes can be observed both attached to the endoplasmic reticulum and in the cytoplasm. It is estimated that an efficiently translated mRNA at 37° C initiates protein synthesis once every 5–6 s (Hershey, 1991). How many times an mRNA can be translated depends on several aspects of its structure, including the context surrounding the AUG codon, and its lifespan expressed in terms of the rate of degradation (Ross, 1989; Kozak, 1991). What is important in a biological context is that the initiation step is considered the main target for the regulation of protein synthesis during cell cycle, growth, development, hormonal response, and under stress conditions including heat shock, irradiation, and starvation. How this regulation is brought about will be discussed in a separate section.

Elongation

The formation of the 80S initiation complex is followed by the repetitive cyclic event of peptide-chain elongation. How many times the elongation cycle will be repeated is determined by the total number of amino acids to be incorporated into the polypeptide chain, as specified by the number of codons in the mRNA. The transfer of amino acids from the cytoplasm to the ribosome is carried out by tRNAs followed by their joining in a chain, a series of reactions catalyzed by elongation

Table 2. Categories and Names of the Most Common Amino Acids, Their Abbreviations, and Respective Translatable Codons in mRNA

Name	*Abbreviations*		*Translatable Codons*
	Three letter	*One letter*	
Aliphatic amino acids			
1. Glycine	Gly	G	GGU/GGC/GGA/GGG
2. Alanine	Ala	A	GCU/GCC/GCA/GCG
3. Valine	Val	V	GUU/GUC/GUA/GUG
4. Leucine	Leu	L	CUU/CUC/CUA/CUG
5. Isoleucine	Ile	I	AUU/AUC/AUA
Hydroxy amino acids			
6. Serine	Ser	S	UCU/UCC/UCA/UCG/AGU/AGC
7. Threonine	Thr	T	ACU/ACC/ACA/ACG
Dicarboxylic amino acids			
8. Aspartic acid	Asp	D	GAU/GAC
9. Asparagine	Asn	N	AAU/AAC
10. Glutamic acid	Glu	E	GAA/GAG
11. Glutamine	Gln	Q	CAA/CAG
Basic amino acids			
12. Lysine	Lys	K	AAA/AAG
13. Histidine	His	H	CAU/CAC
14. Arginine	Arg	R	CGU/CGC/CGA/CGG/AGA/AGG
Aromatic amino acids			
15. Phenylalanine	Phe	F	UUU/UUC
16. Tyrosine	Tyr	Y	UAU/UAC
17. Tryptophan	Trp	W	UGG
Sulfur-containing amino acids			
18. Methionine	Met	M	AUG
19. Cysteine	Cys	C	UGU/UGC
Imino acids			
20. Proline	Pro	P	CCU/CCC/CCA/CCG

factors (EFs; also abbreviated as eEFs). In this section, we shall first describe various components, particularly amino acids, tRNAs, aminoacyl–tRNA synthetases, and elongation factors involved in the elongation phase of protein synthesis and shall then describe the elongation cycle.

Amino Acids

Most commonly, there are 20-amino acids used in protein synthesis. For reasons not well understood, all the amino acids that occur in proteins are in the L form of the two enantiomers, D and L. The sequence of amino acids in a polypeptide chain gives rise to its primary structure that is, in turn, the basis of the secondary and tertiary forms of a protein. Table 2 gives a list of amino acids, their three-letter and single-letter abbreviations, and the codons on the mRNA that can be translated for each of them.

Although not every protein contains all the amino acids, lack of a single amino acid in the cytoplasmic pool can result in the inhibition of total protein synthesis.

In addition to the twenty "standard" amino acids, many nonstandard amino acids are also found in almost all proteins. Generally, these amino acids arise as a consequence of various chemical modifications after they have been incorporated into protein. Posttranslational modification of amino acids is one basis of the regulation of protein activity, specificity, and stability.

tRNAs and Aminoacyl–tRNA Synthetases

RNA molecules that physically bring the amino acids onto the template codons of mRNAs bound to ribosomes are tRNAs. There is at least one tRNA for each of the 61 codons translated into amino acids, but there are no tRNAs for the stop codons UAA, UAG, and UGA. Therefore, for several of the amino acids, for example G, A, V, L, S, and R there are 4 to 6 isoacceptor tRNA species, and their relative abundance is correlated with the codon usage in the mRNAs being translated (Lapointe and Giegé, 1991). More than a thousand sequences of tRNAs and their genes have been determined. All tRNAs are between 70 and 95 nucleotides long and can be folded into a cloverleaf secondary structure. A unique characteristic of tRNA is the presence of several modified nucleotides near and around the anticodon loop. More than 50 modified nucleotides have been discovered in eukaryotic tRNAs and these include dihydrouridine (D), inosine (I), N^6-isopentenyladenosine (i^6A), queusine (Q), and wyosine (Y). Although the exact mechanisms are not known, these modified nucleotides in tRNA are considered to be involved in codon recognition.

The function of a tRNA in transferring the amino acid to the ribosome–mRNA complex is dependent upon a specific enzyme that catalyzes the ligation of the appropriate amino acid to its acceptor arm at the 3′ end. This process of amino-acylation of an amino acid to a tRNA is also known as "charging", and the enzymes involved in this process are called aminoacyl–tRNA synthetases (aaRSs) or, more accurately, aminoacyl–tRNA ligases. A group of isoaccepting tRNAs are charged only by the single aaRS specific for their amino acid. Thus, there are only about 20 aaRSs for all tRNA species.

Functionally, it is the anticodon on tRNA that determines the specificity for an amino acid as recognized by aaRSs. Charging of tRNA is an energy-dependent process that consumes two molecules of ATP for every event of amino-acylation. The correct charging of a tRNA molecule requires specificity of tRNA recognition by aaRSs along with proofreading and editing of incorrect amino acids. Through a process of what has been called "double-sieve editing", the error frequency of tRNA charging is usually maintained at less than 1 in 10^3 amino acid additions (Fersht, 1986). Furthermore, levels of tRNAs and aaRSs are considered to be rate limiting for protein synthesis.

Elongation Factors

The addition of an amino acid to the growing polypeptide chain is facilitated by at least two elongation factors, EF-1 and EF-2. A third factor, EF-3, is reported only in some fungi and yeast. EF-1 is composed of three distinct parts: a G-binding protein, EF-1α, and a nucleotide-exchange protein complex, EF-1βγ. EF-1 usually occurs in multiple molecular forms, composed of varying amounts of EF-1α and EF-1βγ. Sequences of EF-1α genes, cDNAs and protein from more than 15 different species have been determined and found to be highly conserved during evolution. For example, in comparison with human EF-1α, the most distant eukaryotic sequence is that of tomato (78% homology), the closest nonmammal is *Xenopus laevis* (95% homology), whereas almost 100% homology exists among mammals (Riis et al., 1990a).

Some other interesting features of EF-1α include its high abundance (between 3% and 10% of the soluble protein) and multiple copies of the gene that in some cases undergo cell type and/or developmental stage-specific expression as reported in yeast, fungi, brine shrimp, fruit fly, toad, and human cells (Merrick, 1992). Furthermore, EF-1α appears to have several other functions in addition to its requirement in protein synthesis. For example, EF-1α has been reported to bind to cytoskeletal elements, particularly actin. It is associated with the endoplasmic reticulum, and is part of the valyl–tRNA synthetase complex; it is also associated with the mitotic apparatus, and is involved in protein degradation and ribosome association (Merrick, 1992). Similarly, the role of an extra copy of the EF-1α gene in life prolongation, (e.g., transgenic *Drosophila* at high temperature, Shepherd et al., 1989), and in an increase in the accuracy of protein synthesis in yeast cells (Song et al., 1989) indicate the pluripotent nature of this factor.

The other elongation factors, EF-1βγ and EF-2, are involved, respectively, in the posthydrolytic exchange of GDP with GTP and in the translocation of peptidyl–tRNA on the ribosome. Of these, EF-2 (95 kDa) has a unique characteristic in the form of a histidine residue at position 715 modified into diphthamide, as a result of which it can be ADP-ribosylated either endogenously or by bacterial toxins such as diphtheria toxin (Riis et al., 1990b). EF-3, which is present in some yeast and fungal species, is a 125 kDa polypetide chain. A unique property of EF-3 is its ability to function with any of the three purine nucleosides (A, G, and I), perhaps to facilitate the interaction of EF-1α, GTP, and aminoacyl–tRNA. The requirement for EF-3, even *in vitro*, is an exclusive property of yeast ribosomes. When yeast factors are tested with mammalian ribosomes, EF-3 has no effect (Riis et al., 1990).

The elongation cycle proceeds in three steps (Figure 2). Once the initiator tRNA is bound in the ribosomal P site thereby forming the 80S initiation complex, the codon-directed repetitive cycle of peptide-chain elongation sets in. In the first step, the binding of an aminoacyl–tRNA carrying the appropriate amino acid is directed by EF-1α bound to GTP. After the correct match has been made and a process of

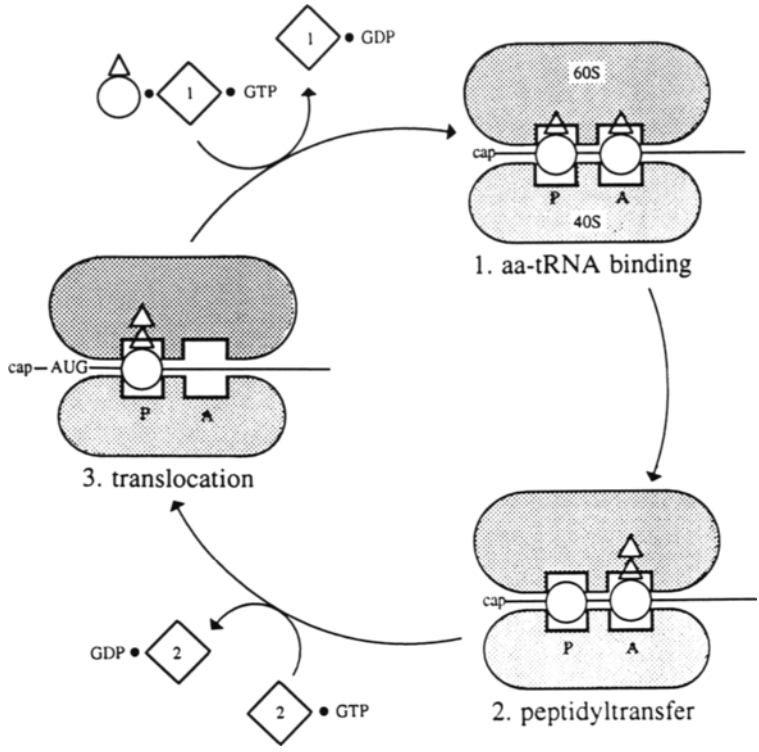

Figure 2. Schematic representation of the cyclic and repetitive event of polypeptide chain elongation. Numbers in ◇ represent various elongation factors, ○ represents aminoacyl–tRNA, and △ represents the amino acid. (From Johansen and Rattan, 1993).

proofreading completed, the hydrolysis of GTP is initiated by some as yet unidentified signal, followed by the placement of the aminoacyl–tRNA in the A site and the release of the EF-1α·GDP complex. The GDP on EF-1α is exchanged for GTP through a process that involves EF-1βγ, thereby regenerating an active EF-1α.

The second step in the elongation of protein synthesis is the peptidyl-transferase reaction in which a peptide bond is formed between the amino acid in the P site and the amino acid coupled to aminoacyl–tRNA in the A site. This reaction is facilitated by the intrinsic activity of the ribosome. As a result of this reaction, the growing polypeptide chain becomes elongated without moving forward. The movement or translocation of the dipeptide–tRNA from the A site to the P site is achieved by the action of EF-2, while another molecule of GTP is hydrolyzed. This process results in the relative movement of the mRNA by three nucleotides, so that a new codon becomes readable in the A site. The deacylated tRNA is pushed out of the ribosome after a transient halt at the so-called exit (E) site. At this point, all the components

involved in the elongation cycle become ready to undergo the next cycle. In terms of energy consumption, the addition of each new amino acid to a growing polypeptide chain costs 4 high-energy phosphates from 2 molecules of ATP during amino-acylation and 2 molecules of GTP during elongation. Various estimates of the elongation rates in eukaryotic cells give a value in the range of 3–6 amino acids incorporated per ribosome per second, which is several times slower than the prokaryotic elongation rate of 15–18 amino acids incorporated per second (Hershey, 1991).

Termination

The cycle of peptide-chain elongation continues until one of the three stop codons (UAA, UAG, UGA) is reached. There is no aminoacyl–tRNA complementary to these codons, and instead a termination factor or a release factor (RF) with bound GTP binds to the ribosome and induces hydrolysis of both the aminoacyl-linkage and GTP, thereby releasing the completed polypeptide chain from the ribosome. The 475 amino acid-long sequence of rabbit liver RF has been deduced from its cDNA sequence, and it shows 90% homology with mammalian tryptophanyl–tRNA synthetase (Lee et al., 1990). It has also been reported that for efficient and accurate termination, an additional fourth nucleotide (most commonly an A or a G) after the stop codon is required (Tate and Brown, 1992). The exact role of the fourth nucleotide in the termination of protein synthesis is not fully understood at present.

Organeller Protein Synthesis

Mitochondria and chloroplasts are the two main eukaryotic cellular organelles that contain their own genome and undertake the semi-independent processes of transcription and translation. Most of the constituent proteins of these organelles are imported from the surrounding cytoplasm, but each organelle also synthesizes its own proteins. Here we shall limit our discussion to mitochondrial protein synthesis, because the chloroplast is not present in animal cells.

Since nucleic acids generally cannot go in and out of mitochondria, all mitochondria appear to code for their own rRNAs and tRNAs. For the same reason, only the mRNAs that have been transcribed from the mitochondrial genome are translated in the mitochondria. A unique feature of mitochondrial mRNAs is the lack of a m^7G cap at the 5′ end (reviewed by Bag, 1991). There are only 22–25 tRNA species in the mitochondria, indicating that a single tRNA can recognize more than one codon. There are some structural and sequence differences in the mitochondrial tRNAs. Furthermore, deviations from the standard genetic code, for example, utilization of AUA as the initiation codon instead of AUG, and reading UGA as a tryptophan instead of a stop codon, are a unique feature of mitochondria (Lapointe

and Giegé, 1991). The proteins imported from the cytoplasm into mitochondria include ribosomal proteins, initiation, elongation, and termination factors, and aminoacyl–tRNA synthetases.

Although there is a 5-fold difference between the sizes of the mitochondrial genomes of yeast (84 kb) and mammals (16 kb), the number of proteins synthesized within mitochondria is similar. Proteins produced by mammalian mitochondria are those involved in electron-transport and oxidative-phosphorylation systems. These include cytochrome *b*, three subunits of cytochrome oxidase, one subunit of ATPase, and six subunits of NADH dehydrogenase. Apart from these differences, protein synthesis in mitochondria follows the same steps and mechanisms as those in the cytoplasm.

REGULATION OF PROTEIN SYNTHESIS

The regulation of protein synthesis means two things: first, the regulation of translation of individual mRNA species, and second, the regulation of the rates of total protein synthesis in a cell. For specific proteins, the rate-limiting factor is usually the mRNA, whose levels and several secondary and tertiary features such as the presence and accessibility of the cap, the placement and context of the AUG initiator codon, and the presence and length of the poly(A) tail determine the rate of translation. Some of the most important examples of translational regulation at the level of individual mRNAs include mRNAs for ribosomal proteins, heat shock proteins, several hormonally regulated proteins, transcription activator protein GCN4 (in yeast), and proteins involved in iron metabolism (e.g., ferritin). The regulation of the rates of mRNA production, processing, transport, stability, and turnover is a complex and wide subject beyond the scope of this chapter, and other review articles (Ross, 1989; Bag, 1991; Kozak, 1991; Rhoads, 1991), should be consulted.

The regulation of total- or bulk-protein synthesis is independent of mRNA levels and the rate-limiting factors, in principle, can be any of the components of the protein synthetic machinery. Therefore, all three steps of initiation, elongation, and termination are targets in the regulation of protein synthesis.

Regulation at the Level of Initiation

Global regulation of protein synthesis occurs most commonly at the level of initiation as exemplified by studies carried out on protein-synthetic rates during the cell cycle, differentiation, embryonic development, and by altered physiological conditions such as serum starvation, amino acid starvation, glucose starvation, hypertonic conditions, ionic changes, heat shock, and other stresses.

The availability of the 43S ternary complex of met-tRNA$_i$, GTP, and eIF-2 is the rate-limiting step for translation initiation. In this context, both the levels of ribosomes and the amounts and activities of soluble factors including eIFs are

critical. However, it is generally believed that the regulation of specific activities rather than levels of translational components allows the cell to alter rates of bulk protein synthesis rapidly (Hershey, 1991). Posttranslational modification, particularly phosphorylation of eIFs, is considered the main mode of regulation of their activities.

The phosphorylation of the α subunit of eIF-2 at ser 48 and ser 51 appears to regulate its activity in terms of increasing its binding with GDP, hindering the GDP/GTP exchange reaction, and thus abolishing its recycling activity (Mathews et al., 1990; Hershey, 1991). At least two protein kinases and phosphatases are involved in the phosphorylation and dephosphorylation of eIF-2. Examples of the regulation of the ternary complex by the phosphorylation of eIF-2 include physiological conditions such as heat shock, nutrient deprivation, heme deficiency, and viral infection.

Other soluble factors whose activities are regulated by phosphorylation and which are involved in determining the rates of protein initiation include eIF-3, eIF-4A, eIF-4B, eIF-4F, and eIF-5. However, unlike eIF-2 in which phosphorylation is related to inhibition of protein synthesis, phosphorylation of all other factors is correlated with stimulation of protein synthesis at the level of initiation. Similarly, phosphorylation of S6 ribosomal protein is also considered to increase the rates of initiation by increasing the activity of 40S ribosomal subunits. In some cases (increased amounts and activities of eIFs, for example), overexpression of eIF-4E has been shown to cause malignant transformation of mammalian fibroblasts (Lazaris-Karatzas et al., 1990). In addition, the initiation factor eIF-4D, which is required for the formation of the first peptide bond, has a unique posttranslational modification, hypusine, whose absence can block the initiation of protein synthesis (Park et al., 1991).

Regulation at the Level of Elongation

The regulation of protein synthesis can also occur totally and differentially at the level of polypeptide-chain elongation. Examples of differential regulation include the rapid translation of heat shock-induced mRNAs in *Drosophila* and chick reticulocytes, translation of viral S1 mRNA in reovirus-infected cells, synthesis of vitellogenin in cockerel liver after estradiol injection, and the synthesis of tyrosine aminotransferase in cultured hepatoma cells treated with cAMP (Spirin and Ryazanov, 1991). The regulation of bulk-protein synthesis at the level of elongation has been reported for normal and transformed cells during cell cycle transition, amino acid starvation, serum stimulation, and phorbol ester treatment (Spirin and Ryazanov, 1991; Johansen and Rattan, 1993). Similarly, alterations in the rates of elongation have also been reported in full-term human placenta from diabetic mothers, in rat livers during fasting and refeeding, and during aging in various cells, tissues, and organs of *Drosophila*, rats, and mice (Rattan, 1992).

In principle, elongation rates can be regulated through changes in the concentration of aminoacyl-tRNAs, modifications of ribosomes, and changes in the amounts and activities of elongation factors. However, most of the available evidence points towards elongation factors EF-1 and EF-2 as the main regulators of protein-elongation rates. For example, changes in the total-protein-synthesis rate have been correlated with the amounts and activities of EF-1 in mammalian cells in culture, both during the cell cycle and during aging, in rat and mouse organs during regeneration and aging, in rat spleen during the immune response, in sea urchin eggs after fertilization, and in a fungus *Mucor* during spore germination (Spirin and Ryazanov, 1991). Similarly, the amount of active EF-2 has been reported to vary with the protein synthetic status of mammalian cells in different phases of the cell cycle and during aging (Riis et al., 1990b).

As in the case of initiation factors, posttranslational modifications of EF-1 and EF-2 are considered important for determining their activities. Methylation of EF-1α at five-lysine positions appears to regulate its activity in a way that is correlated with changes in protein-synthetic rates (Riis et al., 1990a). Furthermore, phosphorylation and the addition of glycerol-phosphoryl-ethanolamine to EF-1α have been suggested to alter its activity. Similarly, phosphorylation of the recycling protein, EF-1$\beta\gamma\delta$, is reported to regulate its activity either positively or negatively, depending on the kinase responsible for phosphorylation (Merrick, 1992).

The site of regulation of the activity of EF-2 is considered to be his715, which is modified to diphthamide (Riis et al., 1990a). ADP-ribosylation of this diphthamide residue then results in abolition of the translocation activity of EF-2. Phosphorylation of EF-2 by a calcium/calmodulin-dependent protein kinase III (CaM PK III), also known as the EF-2 kinase (Redpath and Proud, 1993), is considered another mode of regulation of EF-2 activity. Changes in the amounts of phosphorylated EF-2 during the mammalian cell cycle have been correlated with the changes in protein-synthetic rates (Celis et al., 1990; Spirin and Ryazanov, 1991). Furthermore, the level of phosphorylated EF-2 can be regulated by specific protein phosphatases, for example the type 2A, whose activities are increased by treatment with phorbol esters (Merrick, 1992).

Regulation at the Level of Termination

It was once commonly believed that the termination step of polypeptide-chain synthesis was not a target for regulation of protein synthesis. However, in recent years significant evidence has accumulated showing that in several instances a termination codon can be translated as a sense codon (nonsense suppression), or it can be skipped by frame-shift resulting in the synthesis of elongated proteins. For example, the opal termination codon, UGA, can be translated as trp in mitochondria, and UAA and UAG termination codons can be translated as gln in ciliates (Valle and Morch, 1988). Similarly, owing to ribosomal frame-shifting (when a ribosome

shifts from one reading frame to another at a position in the mRNA before reaching a termination codon), elongated proteins can be produced, such as during the expression of retroviruses and coronavirus in eukaryotes (Valle and Morch, 1988; Tate and Brown, 1992). Furthermore, in some proteins, for example mammalian glutathione peroxidase, the incorporation of selenium-cysteine (Se-cys) is facilitated by an opal suppressor tRNA that can translate UGA as Se-cys (Valle and Morch, 1988). Thus, although the termination step of protein synthesis is not a common site of regulation, in special cases it can regulate the rate and extent of expression of specific proteins.

SOME GENERAL COMMENTS

Although faithful translation of the genetic information encoded in mRNA into a polypeptide chain is a prerequisite for accurate protein synthesis, it is not enough to guarantee efficient functioning of the protein. According to one estimate, more than 140 types of posttranslational modifications of proteins have been described that determine the activity, stability, specificity, and transportability of a protein (Alix and Hayes, 1983; Rattan et al., 1992). These modifications include covalent modification reactions involving amino acid side-chain residues (for example, phosphorylation, oxidation, methylation, acetylation, glycation, and ADP-ribosylation), deamidation, racemization, and noncovalent spontaneous changes in protein conformation and folding.

In addition to posttranslational modifications that can regulate translational rates by regulating the activities and efficiencies of various components of protein synthetic machinery, regulation of protein synthesis can also be achieved by intracellular ionic levels, energy charge, pH, nutrient availability, growth factors, heat shock, heavy metals, and other physiological conditions. Thus, the subject of protein synthesis and its regulation still awaits the filling of several gaps and the gathering of more knowledge in addition to what is already known about this most fundamental process in the cell.

SUMMARY

Protein synthesis is one of the most complex processes in the cell. Its regulation during different stages of growth, division, differentiation, development, aging, and death is a crucial aspect of a living system. In order to translate one mRNA molecule transcribed from a gene, almost 200 small and large components are required to function effectively and accurately, while using large quantities of cellular energy. Ribosomes, initiation factors, elongation factors, amino acids, tRNAs, and aminoacyl–tRNA synthetases are the major components of the protein synthetic apparatus. Protein synthesis proceeds in three steps—initiation, elongation, and termination, followed by posttranslational modifications. The rate-limiting factors for the regulation of total protein synthesis can be any of the components of the

protein synthetic machinery. The availability of mRNA and the amounts and activities of ribosomes, initiation factors, and elongation factors are the major regulators of protein synthesis. Posttranslational modifications such as phosphorylation of various protein synthetic components are involved in determining their activity and stability.

REFERENCES

Alix, J. H. & Hayes, D. (1983). Why are macromolecules modified post-synthetically? Biol. Cell. 47, 139–160.

Bag, J. (1991). mRNA and mRNP. In: Translation in Eukaryotes. (Trachsel, H., ed.), pp. 71–95, CRC Press, Boca Raton.

Celis, J. E., Madsen, P., & Ryazanov, A. G. (1990). Increased phosphorylation of elongation factor 2 during mitosis in transformed human amnion cells correlates with a decreased rate of protein synthesis. Proc. Natl. Acad. Sci. USA 87, 4231–4235.

Fersht, A. R. (1986). The charging of tRNA. In: Accuracy in Molecular Processes. Its Control and Relevance to Living Systems. (Kirkwood, T. B. L., Rosenberger, R. F., & Galas, D. J., eds.), pp. 67–82, Chapman and Hall, London.

Hershey, J. W. B. (1991). Translational control in mammalian cells. Ann. Rev. Biochem. 60, 717–755.

Johansen, L. B. & Rattan, S. I. S. (1993). Protein synthesis and aging. Rev. Clin. Gerontol. 3, 3–12.

Kozak, M. (1989). The scanning model for translation: an update. J. Cell Biol. 108, 229–241.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Biol. 115, 887–903.

Lapointe, J. & Giegé, R. (1991). Transfer RNAs and aminoacyl–tRNA synthetases. In: Translation in Eukaryotes. (Trachsel, H., ed.), pp. 35–69, CRC Press, Boca Raton.

Lazaris-Karatzas, A., Montine, K. S., & Sonenberg, N. (1990). Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature 345, 544–547.

Lee, C. C., Craigen, W. J., Munzy, D. M., Harlow, E., & Caskey, C. T. (1990). Cloning and expression of a mammalian peptide chain release factor with sequence similarity to tryptophanyl–tRNA synthetase. Proc. Natl. Acad. Sci. USA 87, 3508–3512.

Mathews, M. B., Gunnery, S., Manche, L., Mellits, K. H., & Pe'ery, T. (1990). Control of protein synthesis by RNA regulators. In: Post-transcriptional Control of Gene Expression. (McCarthy, J. E. G., & Tuite, M. F., eds.), pp. 377–388, Springer-Verlag, Berlin.

Merrick, W. C. (1992). Mechanism and regulation of eukaryotic protein synthesis. Microbiol. Rev. 56, 291–315.

Nygård, O. & Nilsson, L. (1990). Translational dynamics. Interactions between the translational factors, tRNA and ribosomes during eukaryotic protein synthesis. Eur. J. Biochem. 191, 1–17.

Park, M. H., Wolff, E. C., Smit-McBride, Z., Hershey, J. W. B., & Folk, J. B. (1991). Comparison of the activities of variant forms of eIF-4D. The requirement for hypusine or deoxyhypusine. J. Biol. Chem. 266, 7988–7994.

Rattan, S. I. S. (1992). Regulation of protein synthesis during ageing. Eur. J. Gerontol. 1, 128–136.

Rattan, S. I. S., Derventzi, A., & Clark, B. F. C. (1992). Protein synthesis, post-translational modifications and aging. Ann. NY Acad. Sci. 663, 48–62.

Redpath, N. T. & Proud, C. G. (1993). Purification and phosphorylation of elongation factor-2 kinase from rabbit reticulocytes. Eur. J. Biochem. 212, 511–520.

Rhoads, R. E. (1991). Initiation: mRNA and 60S subunit binding. In: Translation in Eukaryotes. (Trachsel, H., ed.), pp. 109–148, CRC Press, Boca Raton.

Riis, B., Rattan, S. I. S., Clark, B. F. C., & Merrick, W. C. (1990a). Eukaryotic protein elongation factors. Trends Biochem. Sci. 15, 420–424.

Riis, B., Rattan, S. I. S., Derventzi, A., & Clark, B. F. C. (1990b). Reduced levels of ADP-ribosylatable elongation factor-2 in aged and SV40-transformed human cells. FEBS Lett. 266, 45–47.

Ross, J. (1989). The turnover of messenger RNA. Sci. American 260, 28–35.

Shepherd, J. C. W., Walldorf, U., Hug, P., & Gehring, W. J. (1989). Fruitflies with additional expression of the elongation factor EF-1α live longer. Proc. Natl. Acad. Sci. USA 86, 7520–7521.

Song, J. M., Picologlou, S., Grant, C. M., Firoozan, M., Tuite, M. F., & Liebman, S. (1989). Elongation factor EF-1α gene dosage alters translational fidelity in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 9, 4571–4575.

Spirin, A. S. & Ryazanov, A. G. (1991). Regulation of elongation rate. In: Translation in Eukaryotes. (Trachsel, H., ed.), pp. 325–350, CRC Press, Boca Raton.

Tate, W. P. & Brown, C. M. (1992). Translational termination: "stop" for protein synthesis of "pause" for regulation of gene expression. Biochem. 31, 2443–2450.

Valle, R. P. C. & Morch, M. D. (1988). Stop making sense or regulation at the level of termination in eukaryotic protein synthesis. FEBS Lett. 235, 1–15.

Wool, I. G. (1991). Eukaryotic ribosomes: structure, function, biogenesis, and evolution. In: Translation in Eukaryotes. (Trachsel, H., ed.), pp. 3–33, CRC Press, Boca Raton.

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Chapter 11

The Role of Glycosylation in Cell Regulation

ELIZABETH F. HOUNSELL

INTRODUCTION

There are a large series of enzymes called glycosyltransferases encoded in the genome of all organisms except viruses which use their host machinery. The enzymes catalyze the linkage of monosaccharides to each other to form a) oligosaccharides of glycoproteins and glycolipids, b) repeating disaccharides of proteoglycans, and c) polysaccharides. Due to the nature of certain monosaccharides (e.g., glucose having the formula $C_6H_{12}O_6$ arranged as shown in Figure 1), these have been studied under the general title of Carbohydrate Chemistry, but other monosac-

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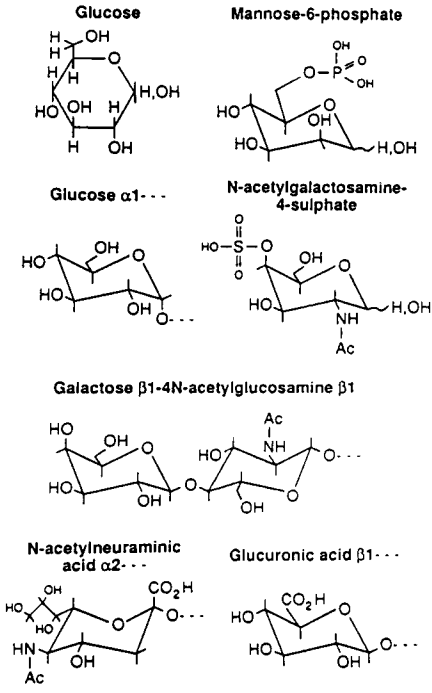


Figure 1. Some examples of the structures of monosaccharides occurring in mammalian glycoconjugates (glycoproteins, glycolipids, and proteoglycans).

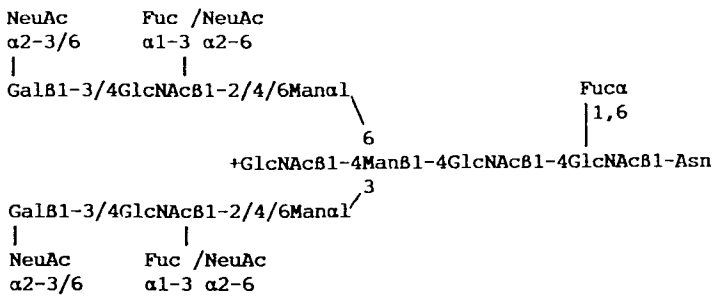
charides can be of higher molecular weight and contain nitrogen (N), sulphur (S) and phosphorus (P). Monosaccharide diversity is therefore large, depending (see Figure 1) on the orientation of hydroxyl-groups around each monosaccharide glycosidic-ring, the anomeric configuration at C1 (either α or β) and the array of additional functional groups such as NH_2, $NHCOCH_3$ (NAc), OAc, SO_3, PO_4 and CO_2H (Table 1). The monosaccharides are linked together between hydroxyl-groups around the glycosidic-ring by a condensation reaction catalyzed by the glycosyltransferases, which are specific for donor and acceptor, the underlying sequence, the α or β configuration, and the linkage position. More than one hydroxyl-group within a monosaccharide can be linked, leading to branching. Oligosaccharide diversity can, therefore, be very large indeed and the inherent chemical information contained within the various oligosaccharide structures provides many specific messages that are involved in the molecular recognition events of cellular regulation.

The potential areas of influence of oligosaccharides are many. The various types of mammalian protein glycosylation discussed in detail in this chapter can be

Table 1. A Summary of Monosaccharides and their Substituents Commonly Making Up Mammalian Glycoconjugates (Glycoproteins, Glycolipids, and Proteoglycans)

Family	*Monosaccharide*	*Abb*^{n}	*SO*$_4$	*PO*$_4$	*OAc*
Hexose	D-Galactose α/β	(Gal)	+		+
	D-Mannose α/β	(Man)		+	
	D-Glucose α/β	(Glc)		+	
Deoxy-hexose	L-Fucose α/β	(Fuc)			
Pentose	L-Xylose β	(Xyl)		+	
Acetamido	N-acetyl-D-galactosamine α/β	(GalNAc)	+		
-sugars	N-acetyl-D-glucosamine α/β	(GlcNAc)	+		
Uronic acids	D-Galactouronic acid β	(GalA)	+		
	D-Glucuronic acid β	(GlcA)	+		
	L-Iduronic acid α	(Ido A)	+		
Sialic acids	N-acetylneuraminic acid α	(NANA)	+	+	+
	N-glycolylneuraminic acid α	(NGNA)	+	+	+

summarized as follows. Starting from the inside of the cell and working outward: a single monosaccharide, GlcNAcβ (Table 1), is attached to nuclear pore (Hart et al., 1989) and cytoskeletal (King and Hounsell, 1989) proteins via a cytoplasmically oriented intracellular membrane transferase (Haltiwanger et al., 1992); an oligosaccharide having the sequence $(GlcNAc\beta)_2(Man\ \beta)_1(Man\alpha)_8(Glc\alpha)_3$ is built up on a lipid core (dolichol) and transferred to the lumenal side of intracellular membranes where it is attached to Asn moieties of nascent polypeptides as they are being synthesized on the ribosome, i.e., N-linked protein glycosylation to the nitrogen of Asn in the consensus sequence Asn-X-Ser/Thr where X is any amino acid except proline (Gavel and von Heijne, 1990); as the new (glyco)protein travels through the endoplasmic reticulum (ER) to the Golgi apparatus, the N-linked oligosaccharide chains are processed first by glycosidases and then by glycosyltranferases to a diversity of structures, with additional processing occurring within the Golgi; at some stage from the ER onwards, O-linked oligosaccharide chains can be added through the hydroxyl-groups of Ser/Thr residues, usually in areas of the peptide having several such residues and a preponderance of proline (Wilson et al., 1991); other proteins receive proteoglycan chains of repeating disaccharides such as keratan sulphate (Gal-GlcNAc), heparin (GlcNAc-GlcA), and chondroitin- and dermatan-sulphates (GalNAc-GlcA) where the uronic acid (GlcA) can be changed by epimerization to iduronic acid (IdUA) and the proteoglycan chains are further altered by O- and N-sulphation (Poole, 1986; Hardingham and Fosang, 1992); high molecular weight multiply O-linked glycoproteins (mucins) and proteoglycans tend to be packaged in secretory vesicles for secretion into the extracellular space (Poole, 1986; Fransson, 1987), other glycoproteins and glycolipids (Hakomori, 1986) are integrated into the cell membrane and, still further glycoproteins of approximately 20 – 200 kDa are secreted into the serum; for some proteins, the



Polylactosamine backbone

Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3/6Galß1-

Figure 2. Generalized N-linked oligosaccharide chain to which are attached peripheral-chain terminating sequences, for example those expressing the blood group and related antigens on single or repeating lactosamine-type sequences. (*continued*)

transmembrane signal sequence is removed and they are transferred to membrane bound, lipid-linked anchors via an inositol phosphate-GlcNH_2-glycan-Man-6-phosphate-ethanolamine (glycophosphatidyl-inositol) bridge attached to the amino acid carboxy-terminus of the protein (Thomas et al., 1990; Ferguson, 1991); outside the cell, proteoglycans and glycoproteins can be sequestered to the extracellular matrix, or passed into the blood stream; mucins largely take up their positions lining the respiratory and gastro-intestinal tracts.

In each of these different scenarios, the oligosaccharides can have various effects on the underlying protein and oligosaccharide sequences themselves can be important as antigens and molecular recognition signals. The first area where the importance of human oligosaccharide sequences became apparent was in blood transfusion. The erythrocyte carries on its cell surface, glycoproteins and glycolipids bearing the oligosaccharide antigens synthesized using the glycosyltranferases encoded by the blood group genes. The blood group H gene encodes an α-fucosyltransferase which catalyzes the addition of Fucα to the C2 hydroxyl of Gal in the following blood group backbone sequences:

Type 1: Galβ1-3GlcNAcβ---

Type 2: Galβ1-4GlcNAcβ---

Type 3: Galβ1-3GalNAcα---

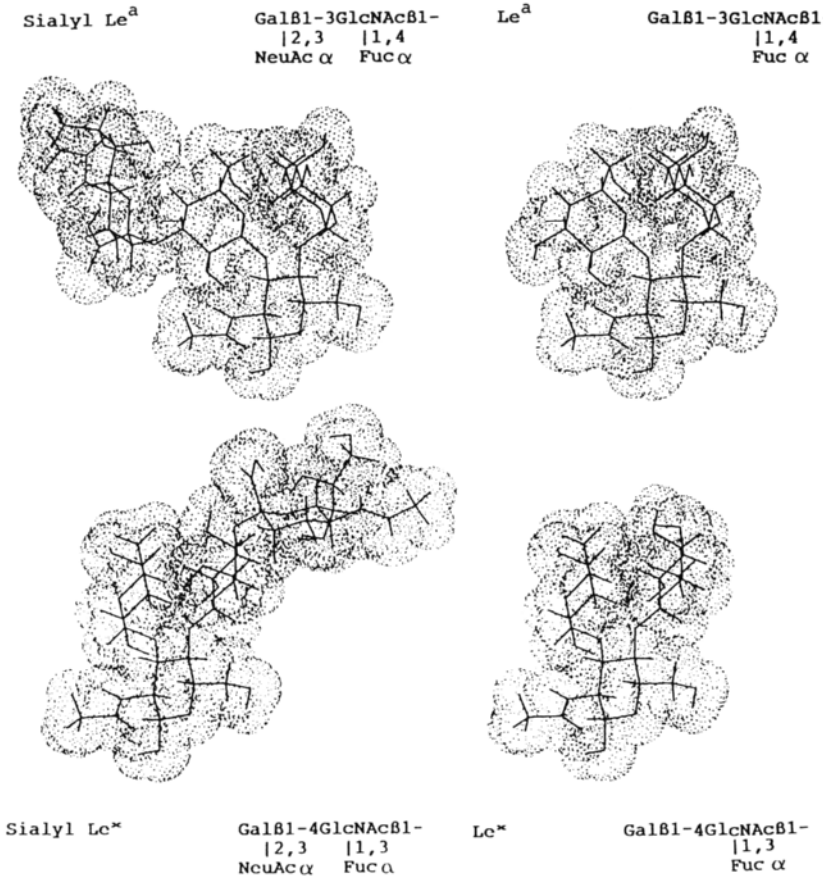


Figure 2. (Continued)

Type 1 and type 2 sequences are part of longer oligosaccharide chains linked through either complex N-linked chains of glycoproteins (Figure 2) or to lactosamine (Figure 2) sequences of O-linked chains and glycolipids. Type 3 is the O-linked glycoprotein core I (Table 2) where the GalNAcα is linked to Ser/Thr. The blood group A and B genes encode GalNAcα- and Galα-transferases, respectively, which add GalNAcα or Galα to the preformed blood group H structure at C3 of Gal giving:

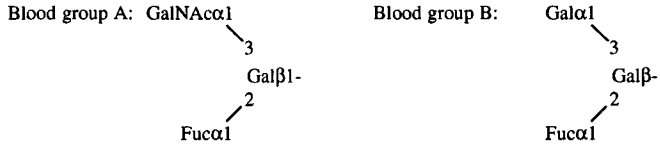


Table 2. Nomenclature for the O-linked Chain Core Region Structures Linked to Ser/thr Hydroxyl Groups

Structure	*Type*
GalNAcα1-	0
Galβ1-3GalNAcα1-	I
Galβ1-3[GlcNAcβ1-6]GalNAcα1-	II
GlcNAcβ1-3GalNAcα1-	III
GlcNAcβ1-3[GlcNAcβ1-6]GalNAcα1-	IV
GalNAcα1-3GalNAcα1-	V
GlcNAcα1-6GalNAcα1-	VI
GalNAcα1-6GalNAcα1-	VII

The problem with blood transfusion is that if, for example, the recipient is blood group A and has this sequence on their erythrocytes, they also have in circulation, antibodies to blood group B which would interact with the blood group B antigen of a blood group B donor. Where do these circulating antibodies arise? The consensus view is that blood group A people become tolerant to the blood group A antigen during fetal thymic development, i.e., they cannot make antibodies to these antigens, but the blood group B antigen on bacterial polysaccharides to which one is exposed postpartum (e.g., in the normal gut flora) will initiate an immune response and produce circulating antibodies. Obviously, this situation is reversed for blood group B donors and it follows that in transfusion, universal acceptors are blood group AB (no antibodies) and universal donors are blood group O (only having the H sequence, but antibodies to both A and B).

The blood group ABO(H) antigens are not restricted to erythrocytes, but also occur on body tissues and, for the approximate 75% of the population who are 'secretors' (Watkins, 1987), on secreted mucins. Except for the general roles that oligosaccharides have with respect to glycoprotein conformation, protease stability, etc. (discussed in more detail in the sections of this chapter on intracellular, cell membrane, and secreted glycoproteins) the functions of the blood group antigens remains obscure. Little influence of different blood groups on disease has been found (Race and Sanger, 1968). However it has been shown that blood group antigens on tumor tissues vary from their normal expression (Feizi, 1985; Hounsell et al., 1985). With the advent of the hybridoma technique for raising monoclonal antibodies, it was found that a series of oligosaccharide antigens related to the blood group sequences also changed during tumorigenesis and normal differentiation. They were hence called onco-developmental antigens (Feizi, 1985; Hakomori, 1986; Hounsell, 1987). The first of these to be characterized was a stage-specific embryonic antigen of the mouse (SSEA-1) which was shown (Hounsell et al., 1981) to be the sequence Le^x, Galβ1-4[Fucα1-3]GlcNAc (Figure 2).

Table 3. Examples of Specific Oligosaccharide Sequences Recognized by Endogenous Mammalian Lectins

Structure of Ligand	*Function of Lectin*	*Reference*
Galβ1-3/4GlcNAcβ1-2/4/6Manα	Hepatocyte gal-binding lectin for recognition of asialoglycoproteins	Morell et al., 1968
6-Phospho-Manα1-	Lysosomal membrane lectin recognizing lysosomal enzymes bearing Man-6-phosphate on N-Linked chains	Haslik and Neufeld, 1980 Kornfeld, 1990
Galβ1-4GlcNAcβ1-	Galactose-binding lectins found, for example, in bovine heart muscle	Feizi and Childs, 1987 Abbott et al., 1988 Barondes, 1984
Man, GlcNAc, Fuc	Mannose-binding proteins	Stahl et al., 1980 Weiss et al., 1992
NeuAcα2-3Galβ1-3-[Fucα1-4]GlcNAcβ1-	Sialyl Le^x recognized by ELAM-1 of activated endothelial cells	Lowe et al., 1990
SO_4-4GalNAcβ1-4GlcNAcβ1-2Manα	Hepatic reticuloendothelial cell receptor for glycopeptide hormones	Fiete et al., 1991

The relevance of cell-specific oligosaccharide sequences remained obscure until it was shown that such sequences could be recognized by cell surface carbohydrate-binding proteins. More recently, these studies were complemented by molecular biology. For example, a cell line lacking a fucosyltransferase, but which had the sequence NeuAcα2-3Galβ1-4GlcNAc was transfected with a fucosyltransferase gene (Lowe et al., 1990) to make the sequence NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc (called SLe^x), and it was then found that this cell line could be recognized by activated endothelial cells (Lowe et al., 1990; Larkin et al., 1992). The reciprocal molecule on endothelial cells was called ELAM-1 or E-selectin and the ligand, SLe^x, was found on specific cells of the lymphocyte lineage. E-selectin, two other selectins, P-selectin (Aruffo et al., 1991) and L-selectin (Lasky et al., 1992), and a mannose-binding protein (Weiss et al., 1992) all belong to a family of molecules called C-type lectins (C for calcium dependent), and several oligosaccharide sequences have been characterized with various affinities for each (Larkin et al., 1992; Weiss et al., 1992; Yuen et al., 1992). Several examples of specific recognition of oligosaccharide sequences by mammalian carbohydrate-binding proteins of nonimmune origin (lectins) are now known (Table 3), dating from the original observation of Ashwell and Morell in 1968 on the galactose-binding lectin of liver, which is one of a series of S-type lectins (S for thiodependent).

Now that examples of specific recognition of oligosaccharides have been introduced, let us look at the overall function of these molecules starting again from the intracellular and moving outward.

INTRACELLULAR GLYCOSYLATION

Several important proteins which function cytoplasmically have been shown to be modified by the addition of a single GlcNAcβ residue. Although this was originally discovered by the use of a galactosyltransferase to add radioactively labeled Gal for detection purposes (Hart et al., 1989), there is no evidence that this happens *in vivo*, nor have GlcNAcβ-binding properties been ascribed to any cytoplasmic proteins. The function of this modification therefore remains obscure, but its presence on proteins which travel to the nucleus (Hart et al., 1989), or otherwise organize proteins in the cytoskeleton (King and Hounsell, 1989; Chou et al., 1992) suggests it does have a recognition role, and increases in O-GlcNAc of nuclear and cytoplasmic glycoproteins appear to correlate with cellular activation (Kearse and Hart, 1991). The types of protein sequences glycosylated are similar to those phosphorylated at Ser/Thr in cell regulation and evidence is accumulating that they have a reciprocal relationship. It may be that a particular GlcNAcβ–protein motif is recognized or that the presence of GlcNAc orientates the surrounding peptide sequence to the correct conformation for functions in cell regulation. The ability of glycosylation to affect protein conformation is well known from the field of oligosaccharide O-linked chains of cell surface and secreted glycoproteins (discussed in more detail later).

INTRACELLULAR PROTEIN TRAFFICKING

The last section dealt with glycosylation occurring on the cytoplasmic side of intracellular membranes. For those proteins designated to proceed through the inside (lumenal side) of the intracellular membranes to the outside of the plasma membrane or into vesicles for secretion, two more types of glycosylation are found. This section discusses one of these, called N-glycosylation, and the role it may have in directing intracellular protein trafficking.

There is an enzyme called an oligosaccharyltransferase which has recently been identified as being associated with a protein complex composed of ribophorin I and II ribosomal proteins (Kelleher et al., 1992). This enzyme uses a preformed oligosaccharide chain attached to lipid-dolichol as a donor to glycosylate the Asn-X-Thr/Ser acceptor sequence of nascent proteins as they are being synthesized (Figure 3). Although the mechanism of this is not understood, it is obvious that at some point, the growing peptide chain crosses into the lumen of the ER (Kornfeld, 1990) and this process is likely coincident with addition of the oligosaccharide as the lipid is most probably constrained to being membrane bound. It may be that the growing chain can be quite long and loosely associated with the lumenal side of the membrane before glycosylation, because the oligosaccharide is often found on Asn side-chains in exposed protein folds called β-turns (Brockbank and Vogel, 1990), and is always found on areas of the protein where it can be orientated on the outside, suggesting that some protein folding has occurred before oligosaccharide

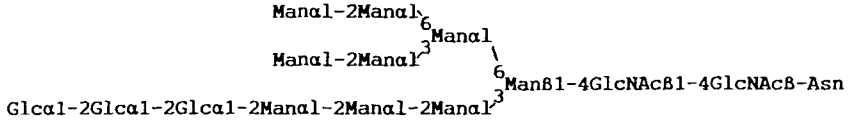


Figure 3. The oligosaccharide sequence associated with nascent polypeptides.

addition. I refer you to other chapters in this book for a more detailed account of protein synthesis, but keep in mind that until recently, the fact that proteins are glycosylated at an early stage has been largely ignored. The mechanisms of *in vivo* protein folding are only just being unraveled and, therefore, it is too early to have any fixed idea on the role of glycosylation in this process. We do know that chaperonins are involved, and it may be that some of these such as calnexin function

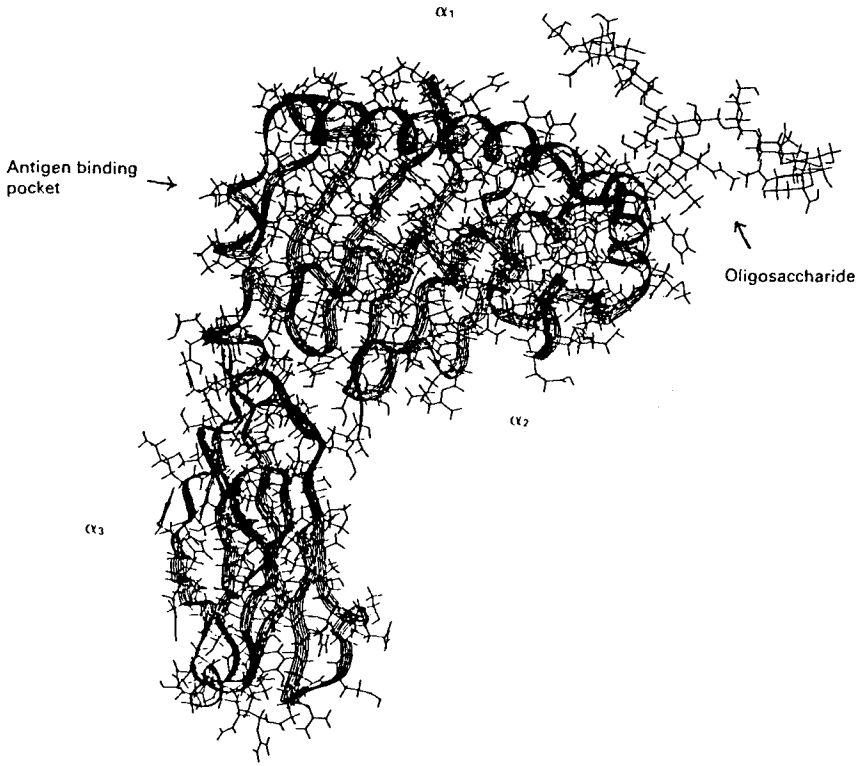


Figure 4. A model glycoprotein showing the X-ray crystallographic data for the human major histocompatibility (MHC) antigen, HLA-A2, (Bjorkman et al., 1987) to which has been added one possible oligosaccharide chain (a di-sialylated biantennary complex N-linked undecasaccharide, Figure 2) at the consensus N-glycosylation site, Asn 86. This represents a snapshot of one of many possible conformations. (Printed with permission of the BMA from the review Hounsell and Davies, 1993).

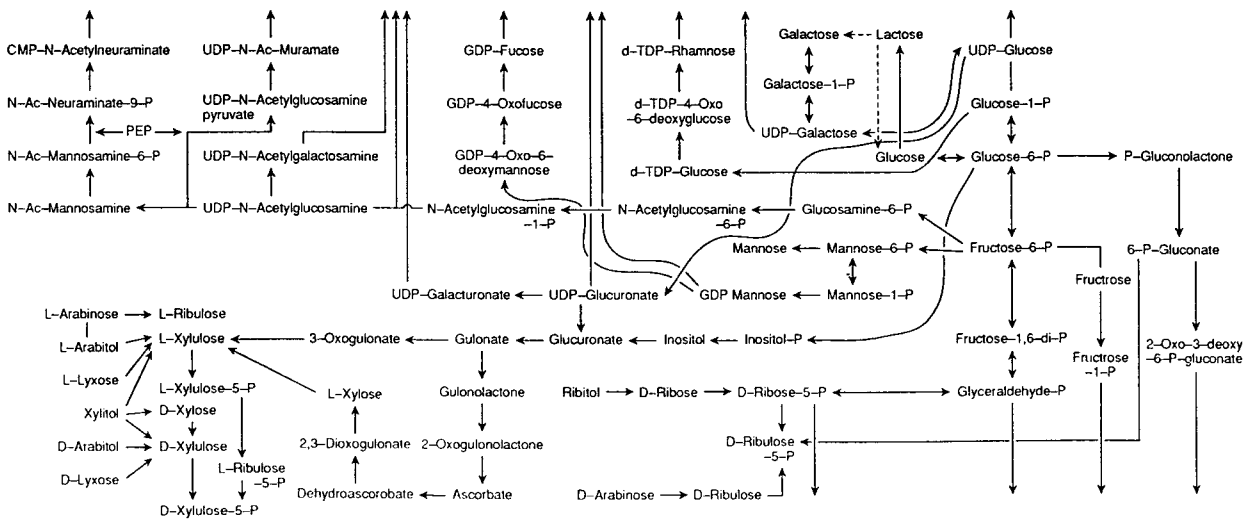


Figure 5. The biosynthetic pathways of monosaccharide interconversions (printed with permission of Koch Light).

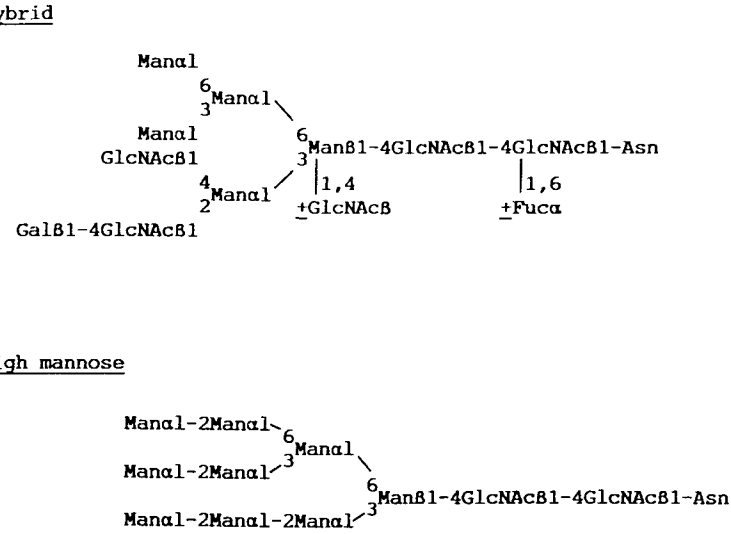


Figure 6. High-mannose and hybrid-type N-linked oligosaccharide chains.

as carbohydrate-binding proteins which are 'cognizant' of the protein glycosylation status. Because of the large hydrodynamic mass imparted by the oligosaccharide (Figure 4), this may be very important in protein folding. We also have to remember that usually more than one N-linked oligosaccharide chain is added to each protein at different consensus sequences along its length and these often cover a large percentage of the protein surface area. An additional factor only just becoming appreciated, is that there seems to be a large amount of degeneracy in protein synthesis, i.e., only a small percentage of the early formed proteins make it through to the later stages of the ER. This appears to be the result of an active process of degradation and may depend on correct glycosylation (Kuznetsov et al., 1993). It may even be that the availability of the glycosylation enzymes and substrates is an important regulation mechanism, i.e., homeostasis of cell regulation may be maintained by a cell being in a healthy state of carbohydrate homeostasis. The monosaccharides themselves are under strict biosynthetic control (Figure 5).

In addition, the position a protein occupies within the intracellular membrane pathway is reflected in the status of its glycosylation. Hence, the nascent glucose-containing chains (Figure 3) and mature-sialylated chains (Figure 2) are indicative of the distance the protein has travelled on its path from the ribosome, through the ER, into the Golgi apparatus, and on to the plasma membrane. Processing of the nascent chain occurs in an ordered sequence, by removal of the glucose residues to form high-mannose chains (Figure 6), further cleavage of mannose residues, and then addition of specific monosaccharides to give hybrid (Figure 6) and complex

(Figure 2) chains. Cell trafficking can, therefore, be followed by the susceptibility of high-mannose chains only to cleavage by the enzyme, endo-β-glucosaminidase H (endo H) or the ability of high-mannose chains and biantennary-complex chains (having only Gal-GlcNAc branches linked to the C2 positions of Manα; Figure 2) to be bound by the plant lectin, concanavalin A. Complex chains having additional Gal-GlcNAc branches at C4 or C6 of Manα (Figure 2) are cleaved by peptide N-glycanase F (PNGase F) and bound variably by several plant lectins (Hounsell, 1993). Thus, specific enzymes (glycosidases and glycosyltransferases) and plant lectins are very useful classes of reagents for sensitive studies of glycosylation status. These, together with physico-chemical analysis methods are discussed at length in *Glycoprotein Analysis in Biomedicine* (Hounsell, 1993). Also included is a discussion of glycosylphosphatidyl-inositol anchors which are not dealt with further in this review.

ANTIGEN PRESENTATION AND VIRAL GLYCOSYLATION

There are three particular areas of intracellular trafficking which differ from the normal biosynthetic pathways leading to cell membrane expression. The first is that enzymes destined for lysosomal storage are glycoproteins which have a particular glycosylation pattern, the presence of Man-6-phosphate, which is recognized by receptors on the lysosome in the context of additional peptide sequences on the enzyme, and this acts as the signal for transport of the enzymes into the lysosomes (Kornfeld, 1990). Second, and related to the first, is that glycoproteins destined for secretion, including mucins, are translocated to secretory granules by an as yet unknown mechanism. These molecules will be discussed in the next section. Third is the recognition by the machinery of immune cells of nonself and self proteins and glycoproteins. This falls into two categories depending on the class of the major histocompatibility (MHC) molecule which presents processed antigen to T lymphocytes. MHC class I molecules are expressed on most somatic cells where their function is to take up peptides from endogenously synthesized (glyco)proteins. MHC class II molecules are displayed on a more specialized subset of dedicated antigen-presenting cells (APC's), where their function is to bind proteolytic fragments derived from endocytosed extracellular (glyco)proteins.

Thus MHC class I monitors the intracellular *milieu* for foreign antigens and MHC class II, the extracellular. They do this by binding self antigens to which they are normally exposed until infection or autoimmunity provides new sequences, to which they have previously not been exposed, to be recognized. The presence of foreign peptide in the antigen presentation groove of MHC triggers an activation of cells of the immune system via interaction with the T-cell receptor (TCR). Obviously, this is a very complicated area of cell regulation which I will not discuss in detail here, but one important aspect is germane to the present discussion, i.e., intracellular trafficking of MHC and the peptides they recognize is a prime area of

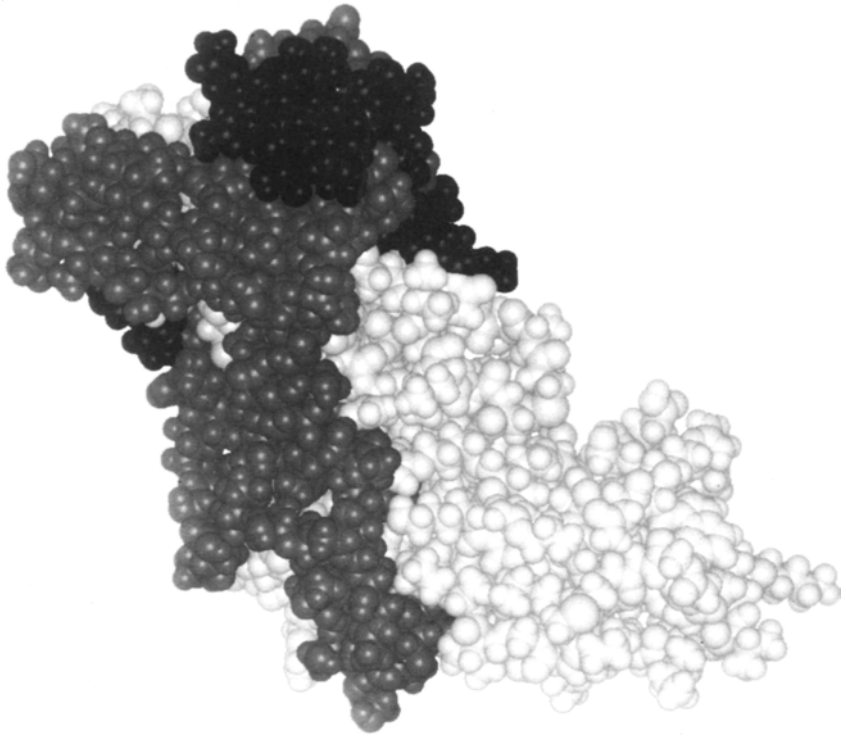


Figure 7. A space-filling computer graphics model of a multiply N-glycosylated polypeptide showing from light to dark grey, respectively, exposed peptide motifs, 4-complex di-sialylated biantennary undecasaccharides, and 3-high-mannose chains. The model is of a possible conformation for the 233-amino acid carboxy-terminus of HIV-gp120, with added glycosylation at predicted sites (Leonard et al., 1990; Hounsell et al., 1991). The modeling was carried out using energy minimization and molecular dynamics simulation to obtain an energetically favorable solution conformation.

control, in that they have to come into contact with each other. The involvement of glycosylation is twofold:

1. the MHC molecules are themselves glycosylated (Figure 4) which may be important in intracellular trafficking and stability (Neefjes et al., 1990; Nag et al., 1992; Moore and Spiro, 1993).

2. many of the peptides presented by MHC come from glycoproteins, the oligosaccharides of which will dictate which parts of glycoproteins are processed for peptide antigen presentation and may also be used to capture the glycoprotein for the processing machinery.

Further, there is some evidence that posttranslationally modified peptides may be recognized (Shawar et at., 1990; Pfeffer et al., 1992) and the invariant chain which associates with MHC class II before peptide recognition can be variously glycosylated (Sant et al., 1985; Steward et al., 1990).

The major area of immune surveillance mediated by class I MHC is of viruses synthesized within cells. Virus surface proteins are some of the most highly glycosylated proteins found. This is probably because viruses can escape immune surveillance by 'mutating in' additional consensus glycosylation sites, the oligosaccharide chains of which can 'cover up' peptide recognition areas, but are themselves poorly antigenic because the host cell transferase machinery is used by the virus to synthesize mammalian self structures. Figure 7 shows a model for the carboxy-terminal 25% of the glycoprotein, gp120, of the AIDS virus, HIV_1 (Hounsell et al., 1991), showing seven-predicted glycosylation patterns (Leonard et al., 1990) of the area of the molecule between the V3 loop (Leonard et al., 1990) and the gp120-gp41 cleavage site. The model is a computer-generated snapshot of one possible conformation and the exact structure is not known, but the figure serves to show the relationship of carbohydrate to exposed peptide. Proteins with this amount of glycosylation are not amenable to X-ray crystallography studies and, therefore, a true picture of virus coats may never be known.

SECRETED GLYCOPROTEINS

Apart from the virus glycoproteins discussed in the last section and bacterial cell wall polysaccharides, which are not the main area for discussion here, the highest density of oligosaccharide sequences is usually found on mammalian secreted glycoproteins called mucins. These are mainly of O-linked type having the core regions shown in Table 2 with additional glycosylation of the lactosamine type with blood group and related chain terminating sequences (Figure 2). Mucins are very high molecular weight glycoproteins ($>10^6$Da) which have clusters of repeating peptide sequences having a high proportion of Ser and Thr amino acids, a large number of which are acceptors for one or more of the N-acetylgalactosamine-α-transferases necessary to initiate oligosaccharide chain synthesis. In some circumstances, the GalNAc can remain as the only monosaccharide present at a particular site, but usually additional monosaccharides are added, depending on the availability of the substrate and its specific transferase. There is still some controversy over where in the cell the first GalNAc is added, but it seems to be added posttranslationally and the additional glycosylation machinery is probably restricted to the Golgi apparatus. This is interesting because the first transferase would appear to

need a stretch of unfolded peptide sequence over which multiple O-glycosylation can occur with extreme overcrowding leading to an extended glycopeptide region.

At some point in their biosynthesis, the mucins are transferred to vesicles for secretion into the extracellular space. Although mucins are found in serum, and mucin-type sequences can be present attached to the cell membrane, their primary role is in the lining of the respiratory and gastro-intestinal tracts. Here they function as physico-chemical barriers against acid, visco-elastic stress, etc. and contain receptor sequences for bacteria. The oligosaccharide sequences they carry are also present on the cell surfaces lining the respiratory and gastro-intestinal tracts, and they are also represented on tissue and circulating cells. These structures are blood group and onco-developmental antigens which are important markers of differentiation, health, and disease (Feizi, 1985; Hounsell, 1987). Mucins have, therefore, been an important large-scale source of oligosaccharides for characterization of the specificities of the antibodies and lectins which bind to specific cell surfaces.

A number of studies have set out to characterize the oligosaccharide sequences of mucins as reviewed in Hounsell (1994). A huge variety of sequences have been found, a small percentage of which are also represented on serum glycoproteins which can have both O- and N-linked chains. By far, the most prevalent oligosaccharides linked via GalNAc to Ser/Thr of serum and cell-surface glycoproteins are based on the type I core (Table 2), with one or two sialic acids attached, i.e., ±NeuAcα2-3Galβ1-3[±NeuAcα2-6]GalNAcα1-Ser/Thr (in humans, the sialic present in all normal tissues is restricted to NeuAc rather than NeuGc; see Table 1).

GLYCOSYLATION OF CELL SURFACE AND SERUM GLYCOPROTEINS

Glycosylation is a significant part of the majority of proteins which follow the intracellular routes toward the plasma membrane for expression on the extracellular face and those which are released into the serum. In addition to having specific recognition roles, the oligosaccharides effect protein expression, stability, conformation, and antigenicity. The O-linked type chains probably do this by interacting tightly with sequences of the peptide backbone. For example, two cell surface molecules, CD8 and the receptor for low density lipoproteins (LDLR), have sequences bearing a number of small clustered O-linked chains thought to form extended glycopeptide regions linking more globular, functional protein epitopes (Leahy et al., 1992). The O-linked chains on CD8 made crystallization of the molecule impossible (Leahy et al., 1992), and those on LDLR have been implicated in surface expression (Kuwano et al., 1991). On erythrocytes, the molecule glycophorin A has similar sequences which express the M and N blood group structures shown to rely on both oligosaccharide and peptide for antibody recognition and for cell surface expression (Remaley et al., 1991). These multiply-glycosylated sequences are also particularly resistant to protease digestion. N-linked chains, on the other hand, may not affect the conformation of the underlying protein to such an

extent, having a relatively flexible amino acid (Asn)-oligosaccharide linkage and some very flexible regions within the oligosaccharide itself (Hounsell, 1994). However, the resulting relatively large oligosaccharides can conceal underlying peptide sequences from proteases and antigen recognition (Figure 7).

In addition to roles in controlling the surface expression of proteins and their conformational and structural stability, intact oligosaccharide structures are important in determining the circulatory half-life of glycoproteins, primarily controlled by the mammalian liver-galactose binding lectin which binds cells and glycoproteins lacking chain-terminating sialic acid residues (Morell et al., 1968). More recent studies have shown that the circulatory half-lives of glycoproteins have a complicated relationship to their glycosylation patterns (Varki, 1993) and that previous studies *in vitro* may not reflect the *in vivo* situation.

Many studies have correlated glycosylation pattern with specific changes in differentiation, health, and disease, but in few cases so far has this been shown to be involved in cell regulation mechanisms. Changes in glycosylation have been implicated in tumor cell evasion of the immune system and in metastatic spread. A general role for the myriad of relatively highly glycosylated proteins circulating in the serum in the regulation of receptor interactions can be proposed (Graziadei et al., 1993; White et al., 1993). For example, several acute-phase glycoproteins are now being studied to consider the relevance of glycosylation changes to function. For orosomucoid or α_1-acid glycoprotein (AGP), acute inflammation induces a strong increase in SLe^x-substituted AGP molecules that persists at a high level throughout the inflammatory period (De Graaf et al., 1993). This may function to "dampen down" the neutrophil–endothelial cell interaction mediated by SLe^x binding to ELAM-1. Another well characterized glycosylation change in autoimmunity is the lack of sialylation and galactosylation of N-linked chains of immunoglobulins in rheumatoid arthritis and several other possibly related syndromes (Axford, 1991). As discussed previously (Hounsell and Davis, 1993), the area of glycosylation in immune regulation is a complicated one, underscored by the experimental use of plant lectins as potent T cell mitogens. These can bind the oligosaccharide sequences of cell-surface receptors for cytokines and tissue growth factors which, together with some of the factors themselves, are glycoproteins. A direct role for receptor oligosaccharides in the binding of cytokines or growth factors has generally been dismissed, although they may possibly have a role in the receptor clustering necessary for cellular activation (Feizi and Childs, 1987). Most importantly, the control of growth factor concentrations at the cell surface and their trafficking between cells is controlled by the interactions of basic amino acid protein motifs with anionic oligosaccharides of the extracellular *milieu*. Cells are, in general, constrained within a bed of basement membrane molecules and a complex ordered solution of glycosylated molecules which are being increasingly implicated in cellular regulation. These are discussed next in the section on

proteoglycans, which are major components of the extracellular matrix, and oligosaccharide sequences of which are also found on membrane bound glycoproteins.

PROTEOGLYCANS

Structurally, proteoglycans fall into three main categories depending on the initial disaccharide building blocks as discussed in the Introduction. Heparin and heparan sulphate, dermatan, and chondroitan sulphate sequences are linked via a Gal-Xyl core to the hydroxyl-group of Ser/Thr amino acids, whereas keratan sulphate sequences are linked through the previously discussed glycoprotein N- and O-linked cores. In several situations, the protein to which the oligosaccharide is attached is now being characterized, and specific names are being given to proteoglycans based on the underlying protein. In addition, the diversity of structures formed by possible GlcA epimerization to IdoA and different N- and O-sulphation patterns is being dissected so that specific sequences can be identified with particular functions. The techniques used in their structural and conformational characterization are the same as those used for classical glycoprotein glycosylation (Hounsell, 1993, 1994).

Until recently, the proteoglycan oligosaccharide components (called glycosaminoglycans) were primarily thought of as extracellular matrix components where they interact with other basement membrane and cartilage molecules, in particular hyaluronan (a polysaccharide of repeating unsulphated GlcA and GlcNAc disaccharides), laminin, fibronectin, and collagen. It is now realized that the core proteins target the glycosaminoglycans to their strategic positons on the cell surface and in the extracellular matrix (Hardingham and Fosang, 1992; Mertens et al., 1992). Several core proteins which constitute different gene products have been described, such as perlecan (primarily basement membrane), glypican (cell membrane-linked through a glycophosphatidyl-inositol anchor), syndecan (epithelial cell surfaces), and aggrecan (noncovalently bound to hyaluronan in cartilage).

In addition to their roles in producing a functional extracellular matrix, proteoglycans specifically bind the relatively low molecular weight, protein cell growth factors and regulate their concentrations and interactions with cell surface receptors. Cytokines and growth factors such as interleukin 7 (IL-7), transforming growth factor β (TGF-β), granulocyte-macrophage colony stimulating factor (GM-CSF) factor, hepatocyte growth factor (HGF), and basic- and acidic-fibroblast growth factors (bFGF and aFGF) possess glycosaminoglycan binding sites (Saksela et al., 1988). From studies of heparin binding to antithrombin III (Grootenhuis and Van Boeckel, 1991), the specific interactions of polyanionic ligands to basic amino acid protein motifs has been characterized. For aFGF, the specific heparin sequence has also begun to be established (Barzu et al., 1989), which amino acid binds to basic areas of the aFGF.

Several membrane bound molecules are now known to bind glycosaminoglycan chains, such as the transferrin receptor and the enzyme, lipoprotein lipase, and also molecules which mediate cellular adhesion, such as the neural cell adhesion molecule, NCAM, which also has polysialic acid chains. This, together with binding to fibronectin and laminin and their interactions with integrins, suggests the importance of proteoglycans in signals subsequent to selectin binding in lymphocyte trafficking (see above). Heparin also binds to thymic stroma-derived T-cell growth factor (TSTGF; Kimura et al., 1991) and macrophage inflammatory protein-1β, (MIP-1β; Tanaka et al., 1993a,b) to regulate T-cell growth and adhesion. For MIP-1β, the cell surface receptor has been shown to be heparin sequences on the glycoprotein CD44 (Underhill, 1992). Different forms of this molecule, which are involved in cell interactions of macrophages (Tanaka, et al., 1993a,b), keratinocytes (Brown et al., 1991), lymphocytes (Goldstein et al., 1989), and tumor cells (Hardwick et al., 1992; Slupsky et al., 1993) vary in the extent of glycosylation with N-linked, O-linked, and glycosaminoglycan sequences. When the latter sequences are present, CD44 is the major cell surface hyaluronan binding protein (hyaladherin) important in binding this ubiquitous extracellular molecule. The intricate pericellular network of hyaluronan; glycosaminoglycans; the glycoproteins fibronectin, laminin, and cell adhesion molecules; integrins; cytokines (growth factors); and selectins is now beginning to be appreciated (Matsuura et al., 1987; Iida et al., 1992; Mackay and Imhof, 1993; Klein et al., 1993; Tanaka et al., 1993a,b). At the core of this network are oligosaccharide–oligosaccharide and oligosaccharide–protein interactions. We have mounting evidence that specific oligosaccharide sequences are functionally regulated to orchestrate the many players involved.

Proteoglycans are also implicated in regulation inside the cell. In mast cells, they control the localization and secretion of proteases released during allergic reactions (Stevens, 1986). In *Drosophila*, a proteoglycan sequence has been found to be one of the gene products (the per clock locus) involved in the development of circadian rhythms (Reddy et al., 1986). Heparan sulphate has been directly implicated in nuclear regulation in hepatocytes where prevention of entry of heparin sulphate into the nucleus results in loss of contact inhibition (Ishihara et al., 1987). Our discussion has hence taken us back to the nucleus from where we started. It is a complicated path, full of many interconnecting threads. For those of us who are cognizant of the intricacies of oligosaccharide structure and function, the threads weave a fascinating picture of the fine tuning of cellular regulation by protein glycosylation.

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REFERENCES

Abbott, W. M., Hounsell, E. F., & Feizi, T. (1988). Further studies of oligosaccharide recognition by the soluble 13kDa lectin of bovine heart muscle. Biochem. J. 252, 283–387.

Aruffo, A., Kolanus, W., Gerd, W., Fredman, P., & Seed, B. (1991). CD62/P-selectin recognition of myeloid and tumor cell sulfatides. Cell 67, 35–44.

Axford, J. (1991). Oligosaccharides: an optional extra of relevance to disease mechanism in rheumatology? J. Rheumatol. 18, 1124–1127.

Barondes, S. H. (1984). Soluble lectins: a new class of extracellular proteins. Science 233, 1259–1264.

Barzu, T., Lormeau, J.-C., Petitou, M., Michelson, S., & Choay, J. (1989). Heparin-derived oligosaccharides: affinity for acidic fibroblast growth factor and effect on its growth-promoting activity for human endothelial cells. J. Cell. Physiol. 140, 538–548.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., & Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen HLA-A2. Nature 329, 506–512.

Brockbank, R. L. & Vogel, H. J. (1990). Structure of the oligosaccharide of hen phosvitin as determined by two dimensional ^{1}H NMR of the intact glycoprotein. Biochem. 29, 5574–5583.

Brown, T. A., Bouchard, T., St. John, T., Wayner, E., & Carter, W. G. (1991). Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. J. Cell Biol. 113, 207–221.

Chou, C. F., Smith, A. J., & Omary, M. B. (1992). Characterization and dynamics of O-linked glycosylation of human cytokeratin 8 and 18. J. Biol. Chem. 267, 3901–3906.

DeGraff, T. W., Van der Stelt, M. E., Anbergen, M. G., & van Dijk, W. (1993). Inflammation-induced expression of sialyl lewis X-containing glycan structures on α_1-acid glycoprotein (orosomucoid) in human sera. J. Exp. Med. 177, 657–666.

Feizi, T. (1985). Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. Nature, 314, 53–57.

Feizi, T. & Childs, R. A. (1987). Carbohydrates as antigenic determinants of glycoproteins. Biochem. J. 245, 1–11.

Ferguson, M. A. J. (1991). Lipid anchors on membrane proteins. Curr. Opinions in Struct. Biol. 1, 522–529.

Fiete, D., Srivastava, V., Hindsgaul, O., & Baenziger, J. U. (1991). A hepatic reticuloendothelial cell receptor specific for SO_4-4GalNAcβ1, 4GlcNAcβ1,2Manα that mediates rapid clearance of lutropin. Cell 67, 1103–1110.

Fransson, L.-A. (1987). Structure and function of cell-associated proteoglycans. Trends Biochem. Sci. 12, 406–411.

Gavel, Y. & von Heijne, G. (1990). Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sited: implications for protein engineering. Prot. Eng. 3, 433–442.

Goldstein, L. A., Zhou, D. F. H., Picker, L. J., Minty, C. N., Bargatze, R. F., Ding, J. F., & Butcher, E. C. (1989). A human lymphocyte homing receptor the hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell 56, 1063–1072.

Graziadei, I., Kaserbacher, R., Braunsteiner, H., & Vogel, W. (1993). The hepatic acute-phase proteins α_1-antitrypsin and α_2-macroglobulin inhibit binding of transferrin to its receptor. J. Biochem. 290, 109–113.

Grootenhuis, P. D. J. & van Boeckel, C. A. A. (1991). Constructing a molecular model of the interaction between antithrombin III and a potent heparin analogue. J. Amer. Chem. Soc. 113, 2743–3747.

Hakomori, S. (1986). Glycosphingolipids. Sci. Amer. 254, 32–41.

Haltiwanger, R. S., Blomberg, M. A., & Hart, G. W. (1992). Glycosylation of nuclear and cytoplasmic proteins. J. Biol. Chem. 267, 9005–9013.

Hardingham, T. E. & Fosang, A. J. (1992). Proteoglycans: many forms and many functions. FASEB J. 6, 861–870.

Hardwick, C., Hoare, K., Owens, R., Hohn, H. P., Hook, M., Moore, D., Cripps, V., Austen, L., Nance, D. M., & Turley, E. A. (1992). Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J. Cell Biol. 117, 1343–1350.

Hart, G. W., Haltiwanger, R. S., Holt, G. D., & Kelly, W. G. (1989). Glycosylation in the nucleus and cytoplasm. Ann. Rev. Biochem. 58, 841–874.

Haslik, A. & Neufeld, E. F. (1980). Biosynthesis of lysosomal enzymes in fibroblasts. Phosphorylation of mannose residues. J. Biol. Chem. 285, 4946–4950.

Hounsell, E. F. (1987). Structural and conformational characterization of carbohydrate differentiation antigens. Chem. Soc. Rev. 16, 161–185.

Hounsell, E. F. (Ed.) (1993). Glycoprotein Analysis in Biomedicine. Humana Press, Totowa, New Jersey.

Hounsell, E. F. (1994). Physicochemical analysis of oligosaccharide determinants. Adv. Carb. Chem. Biochem. 50, 311–350.

Hounsell, E. F., Gooi, H., & Feizi, T. (1981). The monoclonal antibody anti-SSEA-1 discriminates between fucosylated type 1 and type 2 blood group chains. FEBS Letters 131, 279–282.

Hounsell, E. F., Gooi, H. C., & Feizi, T. (1985). Tumour-associated carbohydrate antigens of glycoproteins. Methodological Surveys in Biochemistry and Analysis Vol. 15 (Reid, E., Cook, G. M. W., and Morré, D. J., Eds.), pp. 317–322, Plenum, New York.

Hounsell, E. F., Renouf, D. V., Liney, D., Dalgleish, A. G., & Habeshaw, J. A. (1991). A proposed molecular model for the carboxy terminus of HIV-1 gp 120 showing structural features consistent with the presence of a T-cell alloepitope. Molec. Aspects Med. 12, 283–296.

Hounsell, E. F. & Davies, M. J. (1993). Protein glycosylation in immune regulation. Annals Rheum. Dis. 52, 522–529.

Iida, J., Skubitz., A. P. N., Furcht, L. T., Wayner, E. A., & McCarthy, J. B. (1992). Coordinate role for cell surface chondroitin sulfate proteoglycan and $\alpha 4\beta 1$ integrin in mediating melanoma cell adhesion to fibronectin. J. Cell Biol. 118, 431–444.

Ishihara, M., Fedarko, N. S., & Conrad, H. E. (1987). Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. J. Biol. Chem. 262, 4708–4716.

Kearse, K. P. & Hart, G. W. (1991). Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc. Natl. Acad. Sci. USA. 88, 1701–1705.

Kelleher, D. J., Kreibich, G., & Gilmore, R. (1992). Oligosaccharyltransferase activity is associated with a protein complex composed of ribophorins I and II and a 48 kd protein. Cell, 69, 55–65.

Kimura, K., Matsubara, H., Sogoh, S., Kita, Y., Sakata, T., Nishitani, Y., Watanabe, S., Hamaoka, T., & Fujiwara, H. (1991). Role of glycosaminoglycans in the regulation of T-cell proliferation induced by thymic stroma-derived T-cell growth factor. J. Immunol. 146, 2618–2624.

King, I. A. & Hounsell, E. F. (1989). Cytokeratin 13 contains O-glycosidically linked N-acetylglucosamine residues. J. Biol. Chem. 264, 14022–14028.

Klein, N. J., Levin, M., Strobel, S., & Finn, A. (1993). Degradation of glycosaminoglycans and fibronectin on endotoxin-stimulated endothelium by adherent neutrophils: relationship to CD11/CD18 and L-selectin expression.

Kornfeld, S. (1990). Lysosomal enzyme targeting. Biochem. Soc. Trans. 18, 367–374.

Kuwano, M., Seguchi, T., & Ono, M. (1991). Glycosylation mutations of serine/threonine-linked oligosaccharides in low-density lipoprotein receptor: indispensable roles of O-glycosylation. J. Cell Sci. 98, 131–134.

Kuznetsov, G., Brostrom, M. A., & Brostrom, C. O. (1993). Role of endoplasmic reticular calcium in oligosaccharide processing of α_1-antitrypsin. J. Biol. Chem. 268, 2001–2008.

Larkin, M., Ahern, T. J., Stoll, M. S., Shaffer, M., Sako, D., O'Brien, J., Yuen, C-T., Lawson, A. M., Childs, R. A., Barone, K. M., Langer-Safer, P. R., Hasegawa, A., Kiso, M., Larsen, G. R., & Feizi, T. (1992). Spectrum of sialylated and nonsialylated fuco-oligosaccharides bound by the endothelial-leukocyte adhesion molecule E-selectin. J. Biol. Chem. 267, 13661–13668.

Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R., & Rosen, S. D. (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 69, 927–938.

Leahy, D. J., Axel, R., & Hendrickson, W. A. (1992). Crystal structure of a soluble form of the human T-cell coreceptor CD8 at 2.6 Å resolution. Cell 68, 1145–1162.

Lowe, J. B., Stoolman, L. M., Nair, R. P., Larson, R. D., Berheid, T. L., & Marks, R. M. (1990). ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase. Cell, 63, 475–484.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N., & Gregory, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp 120) expressed in Chinese hamster ovary cells. J. Biol. Chem. 265, 10373–10382.

Mackay, C. R. & Imhof, B. A. (1993). Cell adhesion in the immune system. Immunol. Today 14, 99–101.

Matsuura, H., Greene, T., & Hakomori, S. (1989). An α-N-acetylgalactosaminylation at the Thr residue of a defined peptide sequence creates the oncofetal peptide epitope in human fibronectin. J. Biol. Chem. 264, 10472–10476.

Mertens, G., Cassiman, J-J., van den Berghe, H., Vermylen, J., & David, G. (1992). Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. J. Biol. Chem. 267, 20435–20443.

Moore, S. E. H. & Spiro, R. G. (1993). Inhibition of glucose trimming by castanospermine results in rapid degradation of unassembled major histocompatibility complex class I molecules. J. Biol. Chem. 268, 3809–3812.

Morell, A. G., Irvine, R. A., Sternlieb, I., Scheinberg, I. H., & Ashwell, G. (1968). Physical and chemical studies of ceruloplasmin. J. Biol. Chem. 243, 155–159.

Nag, B., Passmore, D., Kendrick, T., Bhayani, H., & Sharma, S. D. (1992). N-linked oligosaccharides of murine major histocompatibility complex class II molecule. J. Biol. Chem. 267, 22624–22629.

Neefjes, J. J., De Bruijn, M. L. H., Boog, C. J. P., Nieland, J. D., Boes, J., Melief, C. J. M., & Ploegh, H. L. (1990). N-linked glycan modification on antigen-presenting cells restores an allospecific cytotoxic T-cell response. J. Exp. Med. 171, 583–588.

Pfeffer, K., Schoel, B., Plesnila, N., Lipford, G. B., Kromer, L. S., Deusch, K., & Wagner, H. (1992). A lectin-binding, protease-resistant mycobacterial ligand specifically activates $V\gamma9^+$ human γδT cells. J. Immunol. 148, 575–583.

Poole, A. R. (1986). Proteoglycans in health and disease: structures and functions. Biochem. J. 236, 1–14.

Race, R. R. & Sanger, R. (1968). Blood Groups in Man. Blackwell, Oxford.

Reddy, P., Jacquier, A. C., Abovich, N., Petersen, G., & Rosbash, M. (1986). The period clock locus of *D. melanogaster* codes for a proteoglycan. Cell 46, 53–61.

Remaley, A. T., Ugorski, M., Wu, N., Litzky, L., Burger, S. R., Moore, J. S., Fukuda, M., & Spitalink, S. L. (1991). Expression of human glycophorin A in wild type and glycosylation-deficient Chinese hamster ovary cells. J. Biol. Chem. 266, 24176–24183.

Saksela, O., Moscatelli, D., Sommer A., & Rifkin, D. B. (1988). Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J. Cell Biol. 107, 743–751.

Sant, A. J., Cullen, S. E., Giacolitto, K. S., & Schwartz, B. D. (1985). Invariant chain is the core protein of the Ia-associated chondroitin sulfate proteoglycan. J. Exp. Med. 162, 1916–1934.

Shawar, S. M., Cook, R. G., Rodgers, J. R., & Rich, R. R. (1990). Specialized functions of MHC class I molecules. J. Exp. Med. 171, 897–912.

Slupsky, J. R., Duggan-Keen, M., Booth, L. A., Karpas, A., Rhodes, E. G. H., Cawley, J. C., & Zuzel, M. (1993). The peanut-agglutinin (PNA)-binding surface components of malignant plasma cells incompletely sialylated CD44 *vs* sialyl 2-6Tn. Brit. J. Haematol. 83, 567–573.

Stahl, P., Schlesinger, P. H., Sigardson, E., Rodman, J. S., & Lee, Y. C. (1980). Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages; characterisation and evidence for receptor recycling. Cell, 19, 207–215.

Stevens, R. L. (1986). Secretory granule proteoglycans of mast cells and natural killer cells. Ciba Foundation Symposium Function of Proteoglycans, 124, 272–285.

Steward, W. P., Christmas, S. E., Lyon, M., & Gallagher, J. T. (1990). The synthesis of proteoglycans by human T lymphocytes. Biochim. Biophys. Acta, 1052, 416–425.

Tanaka, Y., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U., & Stephen, S. (1993a). T. Cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. Nature 361, 79–82.

Tanaka, Y., Adams, D. H., & Shaw, S. (1993b). Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. Immunol. Today 14, 111–113.

Thomas, J. R., Dwek, R. A., & Rademacher, T. W. (1990). Structure, biosynthesis and function of glycosylphosphatidylinositols. Biochem. 29, 5413–5422.

Underhill, C. (1992). CD44: the hyaluronan receptor. J. Cell Sci. 103, 293–398.

Varki, A. (1993). Biological roles of oligosaccharides: all the theories are correct. Glycobiol. 3, 97–130.

Watkins, W. M. (1987). Biochemical genetics of blood group antigens: retrospect and prospect. Biochem. Soc. Trans. 15, 620–624.

Weiss, W. I., Drickamer, K., & Hendrickson, W. A. (1992). Structure of a C-type mannose-binding protein complexed with an oligosaccharide. Nature, 360, 127–134.

White, H., Totty, N., & Panayotou, G. (1993). Haemonectin, a granulocytic-cell-binding protein, is related to the plasma glycoprotein fetuin. Eur. J. Biochem. 213, 523–528.

Wilson, I. B. H., Gavel, Y., & von Heijne, G. (1991). Amino acid distributions around O-linked glycosylation sites. Biochem. J. 275, 529–534.

Yuen, C-T., Lawson, A. M., Chai, W., Larkin, M., Stoll, M. S., Stuart, A. C., Sullivan, F. X., Ahern, T. J., & Feizi, T. (1992). Novel sulfated ligands for the cell adhesion molecule E-selectin revealed by the neoglycolipid technology among O-linked oligosaccharides on an ovarian cystadenoma glycoprotein. Biochem. 31, 9126–9131.

Chapter 12

Why Are Proteins Methylated?

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INTRODUCTION

The twenty amino acids encoded into proteins during ribosomal synthesis are insufficient to endow these macromolecules with all of their functional and structural properties. Accordingly, a series of covalent modification reactions occurs after protein synthesis and results in new types of amino acid residues. A large group of these reactions involve the addition of methyl (CH_3) groups to proteins. Enzymes that effect these modifications are termed protein methyltransferases, each catalyzing the transfer of a methyl group from the biological donor, S-adenosylmethionine, to nucleophilic oxygen, nitrogen, or sulfur atoms on the polypeptide chain. These reactions result in the formation of methyl esters, methyl amides, methyl amines, and other derivatives on the side-chains of 9 of the 20 common amino acids, as well as on several additional amino acids when located at the amino- and carboxyl-termini of the polypeptide chain (Table 1). Interestingly, the only nucleophilic sites on these twenty amino acid residues that have not yet been demonstrated as sites for methylation are the hydroxyl groups on serine, threonine, and tyrosine residues.

What cellular functions necessitate the presence of methylated amino acids in proteins? From the number of different types of methylated products formed in nature (Table 1), one might also expect a number of different physiological roles played by the methylation reactions. Recent research has now allowed us to gain some insight into the importance of at least some of these reactions.

Protein methylation reactions can be separated into two major classes. The first class involves methyl ester formation on carboxylic acid groups. These methylation reactions are generally reversible and at least one of their functions is similar to that of protein phosphorylation reactions in switching a modified species from one type of activity to another. However, other types of methyl esterification reactions appear to play more novel roles in the metabolism of aging proteins and in the targeting of proteins to membranes.

A second group of protein methylation reactions, involving methyl transfer to protein sulfur and nitrogen nucleophiles, can irreversibly modify a protein. Significantly, not all of these latter types of methylation reactions appear "essential" for the function of the target protein in the cell. For example, one enzyme target may be methylated in one group of organisms and not in another group, even though the overall function of the protein apparently remains the same. Furthermore, experiments designed to test the role of these modifications, either by blocking the methylation reaction or by genetically removing the site of methylation, have generally not shown large changes in the function of the target protein. In these cases, either the small change in the measured activity is physiologically significant or protein methylation may affect unrecognized functions of these species. Alternatively, the methylation reaction may only be crucial during specific metabolic states—for example, when cells are under certain environmental stresses.

Table 1. Sites of Methylation on Procaryotic and Eucaryotic Proteins

Methylation Site	*Group Modified*	*Residue Formed*	*Distribution*
N-terminal modifications			
methionine	α-amino	N-methylmethionyl	bacterial ribosomal and chemotaxis proteins
alanine	α-amino	N-methylalanyl	bacterial ribosomes
		N,N-dimethylalanyl	bacterial ribosomes
		N,N,N-trimethylalanyl	bacterial ribosomes, myosin light chains, protozoan histone H2b
phenylalanine	α-amino	N-methylphenylalanyl	bacterial pili
proline	α-amino	N,N-dimethylprolyl	starfish histone H2b, protozoan cytochrome c-557
Side-chain modifications			
glutamate	γ-carboxyl	glutamyl γ-methyl ester	bacterial chemotactic receptors
L-isoaspartate	α-carboxyl	L-isoaspartyl α-methyl ester	widespread in procaryotes and eucaryotes
D-aspartate	β-carboxyl	D-aspartyl β-methyl ester	widespread in eucaryotes
lysine	ε-amino	monomethyllysyl	widespread in procaryotes and eucaryotes
		dimethyllysyl	widespread in procaryotes and eucaryotes
		trimethyllysyl	widespread in procaryotes and eucaryotes
histidine	τ-amino	τ-methylhistidyl	actin, myosin, histones
	α-amino of modified histidine	diphthamide	eucaryotic elongation factor EF-2
arginine	guanidino-nitrogen	monomethylarginyl	myelin basic protein
		N^G,N^G-dimethylarginyl	histone and nonhistone nuclear proteins
		N^G,N'^G-dimethylarginyl	myelin basic protein
glutamine	amide nitrogen	N-methylglutaminyl	bacterial ribosomal protein L3
asparagine	amide nitrogen	N-methylasparaginyl	bacterial and algal photosynthetic accessory proteins
cysteine	sulfhydryl	S-methylcysteinyl	bacterial CO dehydrogenase
methionine	sulfur	S-methylmethionyl	algal cytochrome c
C-terminal modifications			
isoprenylcysteine	α-carboxyl	isoprenylcysteinyl α-methyl ester	widespread in eucaryotes
leucine	α-carboxyl	leucyl α-methyl ester	widespread in eucaryotes

Much of the work in this area prior to 1980 has been reviewed by Woon Ki Paik and Sangduk Kim in a monograph *Protein Methylation* (Paik and Kim, 1980). This work remains an excellent source for both a historical view of this field and for a discussion of the results obtained in the initial investigations.

MODIFICATION OF CARBOXYLIC ACID GROUPS

Because proteins are synthesized with carboxyl groups at the C-terminus, at glutamate residues, and at aspartate residues, one might expect that there would be three sites of protein methyl ester formation. Although examples of the first two reactions have been characterized, no example of the last type of reaction has been observed to date. The absence of a physiological mechanism utilizing esterification of L-aspartate residues may be due to the instability of aspartate methyl esters which undergo a rapid and spontaneous intramolecular reaction with the polypeptide-chain backbone that results in their racemization and isomerization (Stephenson and Clarke, 1989). Such spontaneous reactions would make a mechanism involving reversible modification reactions impossible, because the original L-aspartate residue would not be quantitatively regenerated upon demethylation.

L-Glutamate Methylation of Bacterial Chemoreceptors

Enzymes which form and hydrolyze γ-methyl esters at glutamate residues have been found in chemotactic bacteria (for a review, see Stock and Simms, 1988). Mutant strains lacking either the chemotaxic-specific methyltransferase or the chemotaxic-specific methylesterase cannot swim toward attractants or away from repellents, and these results have suggested that the methylation system is essential to chemotaxis. However, it has also been shown that strains lacking *both* modifying enzymes have a rudimentary ability to chemotax (Stock, Borczuk, Chiou, and Burchenal, 1985). These results suggest that the methylation system plays a role in balancing the output of the chemoreceptors. Receptor molecules that are either too highly methylated (in the absence of the esterase, for example) or not methylated enough (absence of the methyltransferase) might generate signals that overload the rest of the chemotaxis information processing apparatus (Dunten and Koshland, 1991).

It was initially thought that these bacterial enzymes were the first examples of a large family of glutamate methyltransferases that could reversibly modify other bacterial and animal cell proteins and regulate their activity (Springer, Goy, and Adler, 1979). However, no further examples of this modification have been found to date in nature.

Why does a methylation reaction regulate the chemotaxis system when phosphorylation reactions are so widely used throughout nature (including elsewhere in the bacterial chemotaxis system! [Stock, Surette, McCleary, and Stock, 1992]) for this type of role? The question is even more pointed when it is realized that the metabolic cost of a methylation reaction can be 12 to 13 times greater than a phosphorylation reaction (Stock and Simms, 1988). In the chemotaxis system, methylation of glutamate residues appears to function by mimicking the signaling properties of a glutamine residue originally present in the chemoreceptor. Thus, the methylation/demethylation system may function to convert receptors with glu-

tamine/γ-glutamate methyl ester residues with one signaling function to receptors with glutamic acid residues and an altered signaling function.

C-terminal Methylation as Part of a Signal for Membrane Attachment and/or Signaling Interactions

Two systems have recently been described that result in methyl ester formation at the C-terminus of a protein. Both appear to be widely distributed in eucaryotic cells, but have not yet been observed in procaryotic cells. In one system, the C-terminal leucine residue of one or more 36 kDa cytosolic polypeptides is the target of methylation (Xie and Clarke, 1993). These methyl ester linkages are much more labile in cell extracts than would be predicted on the basis of their chemistry and this observation suggests the presence of a methylesterase activity (Xie and Clarke, 1994a). The protein substrate for this methyltransferase has recently been identified as the catalytic subunit of protein phosphatase 2A and its reversible methylation may modulate its activity (Xie and Clarke, 1994b).

A second system, identified several years ago, results in the methylation of isoprenylated C-terminal cysteine residues in eucaryotic cells (Clarke, Vogel, Deschenes, and Stock, 1988). Here, the cysteine residue that may be methylated is not a substrate until it itself is modified by the addition of a C_{15} farnesyl group or a C_{20} geranylgeranyl group to the side-chain sulfhydryl group (Stephenson and Clarke, 1990). The largest class of proteins modified by this type of reaction is synthesized as precursor proteins with -Cys-Xaa-Xaa-Xaa C-terminal sequences, where Xaa represents a variety of amino acids (Clarke, 1992). Polypeptides containing a terminal residue such as leucine are first modified by a cytosolic enzyme that transfers the geranylgeranyl group from geranylgeranyl pyrophosphate to the sulfhydryl group of the cysteine residue. On the other hand, precursors with C-terminal serine, alanine, or methionine residues are modified by a distinct cytosolic enzyme that transfers the farnesyl group from farnesyl pyrophosphate to the cysteine residue. The three terminal amino acids in both of these derivatives are then removed by one or more membrane-bound and/or cytosolic proteases, exposing the S-isoprenylated cysteine residue at the C-terminus (Hrycyna and Clarke, 1993). The final reaction is methylation of the exposed α-carboxyl group by a membrane-bound methyltransferase.

Polypeptides that have these C-terminal modifications include the ras proto-oncogene proteins and many of their small G-protein analogs, the γ-subunits of the large G-proteins, and the retinal cGMP phosphodiesterase (Clarke, 1992). These proteins are all involved in the transduction of information from the exterior environment of a cell to its interior. The modification of these proteins can allow for specific interactions with the membrane bilayer or with specific receptor proteins on the plasma membrane or internal membrane systems. The specific role of the C-terminal methylation reaction has been tested recently in the yeast system,

where it has been possible to identify the gene encoding the methyltransferase and to prepare strains entirely lacking this activity (Hrycyna, Sapperstein, Clarke, and Michaelis, 1991). Here, the biological activity of the **a**-mating factor is reduced more than 90% in the absence of its C-terminal methyl esterification (Marcus, Caldwell, Miller, Xue, Naider, and Becker, 1991). Interestingly, the RAS signaling protein that is modified by this enzyme still functions in these strains where it is not methylated, although its maturation to a membrane-bound species is markedly abnormal (Hrycyna, Sapperstein, Clarke and Michaelis, 1991). Recently, inhibitor studies have suggested more specific roles of the methylation reaction in platelet signaling and in the response of neutrophils to chemotactic stimulation (Huzoor-Akbar, Wang, Kornhauser, Volker, and Stock, 1993; Philips, Pillinger, Staud, Volker, Rosenfeld, Weissmann, and Stock, 1993).

D-Aspartate/L-Isoaspartate Methylation and the Recognition and Possible Repair of Damaged Proteins

One of the most widely distributed methyltransferases, found in both procaryotic and eucaryotic cells, does not recognize any of the 20 ribosomally synthesized amino acid residues or their enzymatically formed derivatives. Almost all proteins are substrates for this methyltransferase, but the number of methyl groups added is generally very small, on the order of one methyl group per 100 to 1,000,000 polypeptide chains. Both the broad methyl-acceptor specificity and the sub-stoichiometric nature of the methylation reaction can be explained by the observations that this class of enzymes specifically recognizes two degradation products of asparagine and aspartate residues in proteins (Clarke, 1985; Aswad and Johnson, 1987).

Proteins are not thermodynamically stable and undergo spontaneous chemical degradation. A major site of such damage is at aspartate and asparagine residues, where deamidation, racemization, and isomerization reactions can occur. (Geiger and Clarke, 1987; Stephenson and Clarke, 1989). The cumulative effect of the substitution of D-aspartate or L-isoaspartate residues for normal L-aspartate and L-asparagine residues in a protein would be expected to disrupt its structure and function. The ability of this methyltransferase to recognize damaged proteins containing altered amino acids suggests two hypotheses for its function (McFadden and Clarke, 1982). The first hypothesis is that the methylation reaction marks the protein for proteolytic reactions that result in the conversion of the altered protein back to free amino acids. A second hypothesis proposes that the methylation reaction sets the stage for subsequent reactions in which the damaged residue is restored to its original configuration. Protein repair reactions would make even more sense for cells that have greatly diminished capacities for protein synthesis or have lost the ability to synthesize proteins altogether. In these cells, including mammalian red blood cells and the fiber cells of the eye lens, the possible consequences of spontaneous chemical damage to proteins are especially severe.

Evidence has been provided that these methyltransferases can, in fact, catalyze repair reactions. The regulatory protein calmodulin can be aged *in vitro* to produce species that are deficient in the activation of Ca^{2+}/calmodulin-dependent protein kinase. The methylation of these species by the D-aspartate/L-isoaspartate methyltransferase has been shown to restore the activity to levels that approach that of native calmodulin (Johnson, Langmack, and Aswad, 1987). Based on peptide models, a likely mechanism is that spontaneous demethylation of the methylated protein proceeds via the same succinimide that may have been originally responsible for the formation of the isoaspartate residue. The regeneration of the succinimide after ester formation would now allow for repair because its hydrolysis generates normal aspartate as well as isoaspartate residues. Since the isoaspartate residue would be recycled by the methyltransferase back to the ester (and thus the succinimide), the net result of these reactions would be the conversion of isoaspartate residues to aspartate residues. In several peptide systems, this is exactly what has been found to occur (Johnson, Murray, Clarke, Glass, and Aswad, 1987; McFadden and Clarke, 1987).

Recently, a genetic approach has been used to better understand the function of this methyltransferase. The high degree of amino acid sequence similarity between the bacterial and mammalian enzymes (Fu, Ding, and Clarke, 1991) suggests that investigating methylation in *Escherichia coli* may be a good model for studies to understand the physiology of this reaction in higher cells. It has recently been possible to generate deletions of the gene encoding this enzyme in *E. coli* and to examine the phenotype of the resulting methyltransferase-lacking cells (Li and Clarke, 1992). Remarkably, no changes in logarithmic growth were found between methyltransferase-containing and methyltransferase-lacking strains. However, cells lacking methyltransferase were found to be abnormally sensitive to heat-shock and survived poorly in stationary phase. These results suggest that the accumulation of proteins with altered aspartate residues in methyltransferase-lacking cells can be detrimental and that the presence of the methyltransferase normally limits this potentially toxic accumulation (Li and Clarke, 1992).

MODIFICATION OF AMINO GROUPS

In contrast to the reactions described above, the methylation of amino groups generally results in a permanent modification of the protein. These reactions can be viewed as steps to enlarge the repertoire of amino acid residues available to cellular proteins. In some of the reactions described below, a particular modification is limited to one type of protein molecule. For example, only elongation factor-2 appears to contain a trimethylated derivative of a histidine residue. On the other hand, a very large number of different proteins have been found to be methylated at lysine and arginine residues. However, there may be a distinct methyltransferase designed for each of the lysine and arginine methylation reactions described below, even when the same methylated residue is formed.

N-terminal Methylation

The addition of one or more methyl groups to the amino-terminus has been found in proteins that have different overall functions and structures, but that all appear to be involved in macromolecular complexes such as ribosomes, myofibrils, and nucleosomes (Stock, Clarke, Clarke, and Stock, 1987). These methylation reactions can be divided into two major classes depending on the extent of the modification. The first class represents the mono-methylation of methionine, alanine, and phenylalanine residues in procaryotic cells. The second class includes reactions that form quaternary-nitrogen derivatives in both eucaryotic and procaryotic cells. For example, amino-terminal alanine and proline residues can be fully methylated in several eucaryotic proteins, including histone H2b, myosin light chains, and cytochrome c-557, and in at least one procaryotic protein, the ribosomal large subunit protein L11 (Table 1). In each of these cases, it appears that the N-terminus may be particularly prone to proteolytic attack and the presence of a methyl group may prevent such degradation (Pettigrew and Smith, 1977). The quaternarization of the amino group in these latter N-terminal residues results in the presence of a fixed positive charge and the loss of the chemical reactivity of the amino group, two properties that may also be important for these species.

L-Lysine Methylation

A very large number of proteins contain internal mono-, di-, and tri-methyllysine residues. These proteins include bacterial flagellins, ribosomal proteins, and protein synthesis factors, as well as eucaryotic histones, myosin, actin, ribosomal proteins, rhodopsin, tooth matrix proteins, cytochrome c, calmodulin, citrate synthase, and ribulose bisphosphate carboxylase/oxygenase (for a review see Paik and Kim, 1980). While most of these proteins are methylated at only one or two of the available lysine residues, a fungal elongation factor can be methylated at up to 16 sites (Hiatt, Garcia, Merrick, and Sypherd, 1982). At this time, the physiological roles for most of these methylation reactions have not been established with certainty. As discussed above, it is tempting to speculate that the quaternary nitrogen of trimethyllysine residues can function by providing a fixed positive charge. There is at least one example of such a residue in the hydrophobic active site of myosin where the charge may be essential to the catalytic reaction (Okamoto and Yount, 1985).

Calmodulin

Calmodulin is a highly conserved intracellular calcium-binding protein that regulates several different enzymes in eucaryotic cells. With the exception of calmodulins from several plants and lower eucaryotes, these proteins all contain a

trimethyllysine residue at position 115 (reviewed in Wylie and Vanaman, 1988). An enzyme specific for this reaction has been purified from rat testes (Rowe, Wright, and Siegel, 1986). Comparison of the activities of unmethylated and enzymatically methylated calmodulin has revealed that methylation does not alter the activation of cyclic nucleotide phosphodiesterase by calmodulin, but does result in a lower degree of activation of NAD kinase (Roberts, Rowe, Siegel, Lukas, and Watterson, 1986).

Cytochrome c

This protein has been found to be methylated immediately after its synthesis in fungi and higher plants (for a review see Paik, Polastro, and Kim, 1980). The products of the reaction are trimethyllysine residues at positions 72 and 86. The enzyme specific for methylation of lysine-72 has been purified from wheat germ (DiMaria, Kim, and Paik, 1982). Differences between the behavior of methylated and unmethylated cytochrome c suggest that methylation may lead to enhanced mitochondrial import and binding to the mitochondrial inner membrane (Park, Frost, Tuck, Ho, Kim, and Paik, 1987).

Histones

One of the major sites of protein methylation in cells occurs on components of nucleosomes, the histones. Evidence has been presented for the methylation of histones H1, H2a, H3, and H4 in different organisms. These histones are modified at lysine residues on their exposed N-terminal domain by a specific nuclear methyltransferase, resulting in a mixture of mono-, di-, and tri-methyllysine residues. For example, two histone H1-specific methyltransferases were partially purified from the alga *Euglena gracilis* (Tuck, Farooqui, and Paik, 1985), and H4-specific enzymes were partially purified from calf lymphocytes and thymus (Sarnow, Rasched, and Knippers, 1981). Histone methylation occurs in the late S-phase of the cell cycle when heterochromatin is replicated (Sarnow, Rasched, and Knippers, 1981) and appears to follow lysine acetylation and chromosomal assembly. It has been proposed that the methylation reaction is necessary to keep chromatin in a particular conformation prior to mitosis (Sarnow, Rasched, and Knippers, 1981).

Heat-shock Proteins

At least three heat-shock proteins in chicken fibroblast cells are methylated shortly after their synthesis to give trimethyllysine residues (Wang, Lin, and Lazarides, 1992).

Procaryotic Cells

Elongation factor EF-Tu of *Escherichia coli* is mono- and di-methylated at lysine-56. Methylation of this lysine residue can reduce the GTPase activity of the protein and possibly result in more accurate translation, particularly under conditions of limited growth, such as stationary phase (Van Noort, Krall, Sinjorgo, Persoon, Johanns, and Bosch, 1986). Ribosomal proteins, including the large subunit proteins L7/L12 and L11 in *E. coli*, are also methylated at lysine residues (Alix, 1988). In the latter case, a trimethyllysine is present at position 3, nearly adjacent to the trimethylalanine-N-terminus, and thus two fixed positive charges are located within a small area. Interestingly, a similar situation is found in protozoan cytochrome c-557 and in mammalian myosin light chains (see Stock, Clarke, Clarke, and Stock, 1987 for a review).

L-Arginine Methylation

Both mono- and di-methylated arginine residues have been described in proteins such as myelin basic protein, myosin, heat shock proteins, nuclear proteins, ribosomal proteins, and tooth matrix proteins (reviewed in Paik and Kim, 1980). N^G,N'^G-dimethylarginine residues are found mainly in myelin basic proteins, while N^G,N^G-dimethylarginine residues are found largely in nonhistone nuclear proteins.

Myelin Basic Protein

One of the major protein components of the myelin membrane that serves to insulate axons is a family of related proteins known as myelin basic protein. These species contain an arginine residue at position 107 that is partially methylated to give N^G-monomethylarginine and N^G,N'^G-dimethylarginine residues. A specific enzyme that catalyzes this modification has been purified from bovine brain (Ghosh, Paik, and Kim, 1988). The functional role of this modification is unclear. In one study, it was shown that a type of mutant mice ("Jimpy") with defective incorporation of myelin basic protein into the myelin sheath have reduced levels of the arginine methyltransferase as well (Kim, Tuck, Kim, Campagnoni, and Paik, 1984). *In vitro* studies have also supported a role for the methylation reaction in increasing the interaction of myelin basic protein with the membrane (Young, Vacante, and Waickus, 1987).

Nuclear Proteins

Two of the most highly methylated proteins described to date are found in the nucleolus of mammalian cells and include a 34 kDa scleroderma antigen (Lischwe, Ochs, Reddy, Cook, Yeoman, Tan, Reichlin, and Busch, 1985) and the 110 kDa

Protein C23 (Lischwe, Cook, Ahn, Yeoman, and Busch, 1985). Each protein contains about 13 clustered N^G,N^G-dimethylarginine residues, the bulk of which are flanked by two glycine residues on each side. Other nuclear proteins containing methylated arginine residues include the high-mobility groups 1 and 2, as well as a group of heterogeneous nuclear RNA binding proteins similar to the nucleolar proteins that contain multiple N^G,N^G-dimethylarginine residues and have a high glycine content (Lischwe, Ochs, Reddy, Cook, Yeoman, Tan, Reichlin, and Busch, 1985; Christensen and Fuxa, 1988). It is interesting to note that the arginine residue methylated in myelin basic protein is also flanked by a glycyl residue on each side. Histones are also substrates for a specific arginine methyltransferase (Ghosh, Paik, and Kim, 1988).

L-Histidine Methylation

Methylation of histidine residues has been described in actin, myosin, histones, and rhodopsin (Paik and Kim, 1980). Of these substrates, actin is the most widely studied. Residue 73 of actin is fully methylated in all eucaryotic species with the exception of the amoeba *Naegleria gluberi* (Sussman, Sellers, Flicker, Lai, Cannon, Szent-Gyorgyi, and Fulton, 1984). The ability of the *Naegleria* actin to form filaments and interact with myosin suggests that the methylation is not crucial to basic actin functions, unless this unmodified actin is structurally different from the others. The role of actin methylation has recently been tested by creating actin variants (by site-specific mutagenesis) that contain an arginine or tyrosine residue in place of histidine-73 (Solomon and Rubenstein, 1987). The properties of these variant actins, including their ability to become incorporated into the cytoskeleton of a nonmuscle cell, were identical to the methylated normal actin. However, these authors point out that actin interacts with a very large number of binding proteins within the cell and that the presence of the conserved methylhistidine residue may be crucial for one or more of these interactions.

Diphthamide Methylation and Protein Synthesis

Elongation factor EF-2 is a central component of the eucaryotic and archaebacterial protein synthetic apparatus and contains a uniquely modified histidine residue. This residue, diphthamide, is synthesized in a series of steps that includes the trimethylation of the α-amino group created by the addition of elements of a methionine residue to the peptide-bound histidine. The methyltransferase activity involved has been characterized from a number of cell types (Moehring and Moehring, 1988) and its gene has been recently characterized in yeast (Mattheakis, Shen, and Collier, 1992). This residue is the target of the ADP-ribosylation action of diphtheria toxin, and it has been proposed that endogenous ADP-ribosylation enzymes with specificities similar to that of the toxin may regulate protein synthesis

under some conditions (Lee and Iglewski, 1984). Interestingly, diphthamide in EF2 is not essential to the survival of yeast cells, although cells lacking this residue have slightly slower growth rates (Phan, Perentesis, and Bodley, 1993).

MODIFICATION OF AMIDE GROUPS

L-Asparagine Methylation on Light-gathering Proteins

The side-chain amide nitrogen of a specific asparagine residue is methylated in a variety of photosynthetic accessory proteins present in cyanobacteria and red algae (Swanson and Glazer, 1990). These proteins, including the C- and R-phycocyanins and the B-, C-, and R-phycoerythrins, contain a methylated asparagine residue at position 72 on their β-subunit. On the C-phycocyanin, this methylated residue is located near one of the chromatophoric groups, and evidence has been presented that methylation serves to enhance the efficiency of energy transfer in the light-harvesting complexes.

L-Glutamine Methylation on Bacterial Ribosomes

Evidence has been presented for the presence of N^5-methylglutamine in ribosomal protein L3 of *Escherichia coli*. Mutants of this organism, *prm-2*, are defective in the methyltransferase activity that catalyzes this reaction. These mutants have impaired ribosomal assembly at low temperatures, suggesting a role for this modification in allowing bacteria to grow at suboptimal temperatures (Lhoest and Colson, 1981).

MODIFICATION OF SULFHYDRYL AND THIOETHER GROUPS

Although the methylated free amino acids S-methylcysteine and S-methylmethionine are found widely distributed in nature, especially in plants, there are only a few instances reported so far of the methylation of these residues in proteins. In the alga *Euglena gracilis*, an enzyme that catalyzes the formation of an S-methylmethionine residue in cytochrome c has been characterized (Farooqui, Tuck, and Paik, 1985). This enzyme is apparently specific for the Met-65 residue of cytochrome c and does not recognize other methyl-accepting substrates. The physiological role of this methylation reaction is unknown. An S-methylcysteine residue is formed as an enzymatic intermediate in a bacterial carbon monoxide dehydrogenase (Pezacka and Wood, 1988). Very low levels of S-methylated cysteine residues have also been reported in hemoglobin and it has been postulated that these result from a nonenzymatic reaction of an active cysteine sulfhydryl group with xenobiotic alkylating agents (Bailey, Farmer, and Lamb, 1980).

CONCLUSIONS

A large variety of protein methylation reactions occur in nature. It appears that the function of many of these reactions is to create new types of residues that complement the twenty amino acid residues available from ribosomal synthesis. To date, it has been difficult to address the question of what specific advantages the methylated residues impart to the protein, especially in cases where methylation does not occur and where there is little or no gross change in the physiology of these cells or organisms. It is tempting to speculate that many of these modifications may be only crucial to the cell under certain circumstances—nutrient deprivation or oxidative stress for example.

Particularly interesting are the methylation reactions that are designed to be reversible. This feature allows the modulation of protein activities in response to cell needs under particular circumstances. Some of these reactions, such as those that occur on the C-terminus or on glutamate residues, may thus function to regulate the membrane localization or signaling properties of plasma membrane proteins or other proteins. A widely distributed class of reactions modifies only altered aspartate residues. These residues result from spontaneous deamidation, racemization, and isomerization reactions that are the inevitable consequences of aging proteins. Present evidence indicates that the enzymatic methylation system may limit the damage caused by aging and allow cells and organisms to enjoy a longer existence.

It is clear that many questions are left unanswered in the field of protein methylation. What are the precise physiological roles of each of the methylation reactions described? In how many cases do de-methylating enzymes reverse the reaction? Do alterations of these pathways result in pathological conditions? How many new methylated proteins of known types are yet to be found? Finally, how many entirely new types of pathways remain to be discovered?

SUMMARY

Many intracellular proteins can be modified after their biosynthesis by the enzymatic addition of a methyl group from S-adenosylmethionine. These posttranslational reactions can permanently or temporarily modify the structure and function of the target proteins. Importantly, these modifications can expand the repertoire of the cellular chemistry performed by proteins. Unmodified proteins must function with only the 20 amino acid residues incorporated in ribosomal protein synthesis, while methylation reactions can create a variety of new types of residues for specialized cellular roles. At this point, we understand best the processes that reversibly form methyl esters at carboxylic acid residues. One such reaction in bacteria methylates glutamate residues on several membrane-bound chemoreceptors whose signaling properties are modulated by the degree of modification at multiple methylation sites. Another methylation system in higher cells leads to C-terminal methyl ester formation on a variety of proteins such as the small and

large G-proteins and may regulate their ability to interact with their upstream and downstream partners in signal transduction reactions. A third reaction involves the response of the cell to spontaneous damage at abnormal protein residues. Here, aged proteins containing isomerized and racemized aspartyl residues can be recognized for removal from the cell by repair or degradation reactions. Less is known about the physiological role of a wide variety of reactions that stably modify proteins by methylation at the amino terminus and at the side-chains of lysine and arginine residues. These latter modifications often are found on structural proteins, and one role of these methylation reactions may be to stabilize polypeptides in complex cellular structures.

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REFERENCES

Alix, J.-H. (1988). Post-translational methylations of ribosomal proteins. Adv. Exper. Med. Biol. 231, 371–385.

Aswad, D. W. & Johnson, B. A. (1987). The unusual substrate specificity of eukaryotic protein carboxyl methyltransferases. Trends Biochem. Sci. 12, 155–159.

Bailey, E., Farmer, P. B., & Lamb, J. H. (1980). The enantiomer as internal standard for the quantitation of the alkylated amino acid S-methyl-L-cysteine in haemoglobin by gas chromatography–chemical ionisation mass spectrometry with single ion detection. J. Chromat. 200, 145–152.

Christensen, M. E. & Fuxa, K. P. (1988). The nucleolar protein, B-36, contains a glycine and dimethylarginine-rich sequence conserved in several other nuclear RNA-binding proteins. Biochem. Biophys. Res. Commun. 155, 1278–1283.

Clarke, S. (1985). Protein carboxyl methyltransferases: two distinct classes of enzymes. Ann. Rev. Biochem. 54, 479–506.

Clarke, S. (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Ann. Rev. Biochem. 61, 355–386.

Clarke, S., Vogel, J. P., Deschenes, R. J., & Stock, J. (1988). Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. Proc. Natl. Acad. Sci. USA 85, 4643–4647.

Dimaria, P., Kim, S., & Paik, W. K. (1982). Cytochrome c specific methylase from wheat germ. Biochemistry 21, 1036–1044.

Dunten, P. & Koshland, D. E., Jr. (1991). Tuning the responsiveness of a sensory receptor via covalent modification. J. Biol. Chem. 266, 1491–1496.

Farooqui, J. Z., Tuck, M., & Paik, W. K. (1985). Purification and characterization of enzymes from *Euglena gracilis* that methylate methionine and arginine residues. J. Biol. Chem. 260, 537–545.

Fu, J., Ding, L., & Clarke, S. (1991). Purification, gene cloning, and sequence analysis of an L-isoaspartyl protein carboxyl methyltransferase from *Escherichia coli*. J. Biol. Chem. 266, 14562–14572.

Geiger, T. & Clarke, S. (1987). Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides : succinimide-linked reactions that contribute to protein degradation. J. Biol. Chem. 262, 785–794.

Ghosh, S. K., Paik, W. K., & Kim, S. (1988). Purification and molecular identification of two protein methylases I from calf brain: myelin basic protein- and histone-specific enzyme. J. Biol. Chem. 263, 19024–19033.

Hiatt, W. R., Garcia, R., Merrick, W. C., & Sypherd, P. S. (1982). Methylation of elongation factor 1-alpha from the fungus *Mucor*. Proc. Natl. Acad. Sci. USA 79, 3433–3437.

Hrycyna, C. A., Sapperstein, S. K., Clarke, S., & Michaelis, S. (1991). The *Saccharomyces cerevisiae STE14* gene encodes a methyltransferase that mediates C-terminal methylation of **a**-factor and RAS proteins. EMBO J. 10, 1699–1709.

Hrycyna, C. A. & Clarke, S. (1993). Modification of eucaryotic signaling proteins by C-terminal methylation reactions *Pharmacol. Therapeut* 59, 281–300.

Huzoor-Akbar, Wang, W., Kornhauser, R., Volker, C., & Stock, J. B. (1993). Protein prenylcysteine analog inhibits agonist-receptor-mediated signal transduction in human platelets. Proc. Natl. Acad. Sci. USA 90, 868–872.

Johnson, B. A., Langmack, E. L., & Aswad, D. W. (1987). Partial repair of deamidation-damaged calmodulin by protein carboxyl methyltransferase, J. Biol. Chem. 262, 12283–12287.

Johnson, B. A., Murray, E. D., Jr., Clarke, S., Glass, D. B., & Aswad, D. W. (1987). Protein carboxyl methyltransferase facilitiates conversion of atypical L-isoaspartyl peptides to normal L-aspartyl peptides. J. Biol. Chem. 262, 5622–5629.

Kim, S., Tuck, M., Kim, M., Campagnoni, A. T., & Paik, W. K. (1984). Studies on myelin basic protein-specific protein methylase I in various dysmyelinating mutant mice. Biochem. Biophys. Res. Commun. 123, 468–474.

Lee, H. & Iglewski, W. J. (1984). Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and *Pseudomonas* toxin A. Proc. Natl. Acad. Sci. USA 81, 2703–2707.

Lhoest, J. & Colson, C. (1981). Cold-sensitive ribosome assembly in an *E. coli* mutant lacking a single methyl group in ribosomal protein L3. Eur. J. Biochem. 121, 33–37.

Li, C. & Clarke, S. (1992). A protein methyltransferase specific for altered aspartyl residues is important in *Escherichia coli* stationary-phase survival and heat-shock resistance. Proc. Natl. Acad. Sci. USA 89, 9885–9889.

Lischwe, M. A., Cook, R. G., Ahn, Y. S., Yeoman, L. C., & Busch, H. (1985). Clustering of glycine and N^G,N^G-dimethylarginine in nucleolar protein C23. Biochemistry 24, 6025–6028.

Lischwe, M. A., Ochs, R. L., Reddy, R., Cook, R. G., Yeoman, L. C., Tan, E. M., Reichlin, M., & Busch, H. (1985). Purification and partial characterization of a nucleolar schleroderma antigen (M_r = 34,000; pI, 8.5) rich in N^G,N^G-dimethylarginine. J. Biol. Chem. 260, 14304–14310.

Marcus, S., Caldwell, G. A., Miller, D., Xue, C. B., Naider, F., & Becker, J. M. (1991). Significance of C-terminal cysteine modifications to the biological activity of the *Saccharomyces cerevisiae* **a**-factor mating pheromone. Mol. Cell. Biol. 11, 3603–3612.

Mattheakis, L. C., Shen, W. H., & Collier, R. J. (1992). DPH5, a methyltransferase gene required for diphthamide biosynthesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 12, 4026–4027.

McFadden, P. N. & Clarke, S. (1982). Methylation at D-aspartyl residues in erythrocytes: Possible step in the repair of aged membrane proteins. Proc. Natl. Acad. Sci. USA 79, 2460–2464.

McFadden, P. N. & Clarke, S. (1987). Conversion of isoaspartyl peptides to normal peptides: implications for the cellular repair of damaged proteins. Proc. Natl. Acad. Sci. USA 84, 2595–2599.

Moehring, J. M. & Moehring, T. J. (1988). The post-translational trimethylation of diphthamide studied *in vitro*. J. Biol. Chem. 263, 3840–3844.

Okamoto, Y. & Yount, R. G. (1985). Identification of an active site peptide of skeletal myosin after photoaffinity labeling with N-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate. Proc. Natl. Acad. Sci. USA 82, 1575–1579.

Paik, W. K. & Kim, S. (1980). Protein Methylation. John Wiley & Sons, New York.

Paik, W. K., Plastro, E., & Kim, S. (1980). Cytochrome c methylation: enzymology and biologic significance. Curr. Top. Cell. Regul. 16, 87–115.

Park, K. W., Frost, B., Tuck, M., Ho, L. L., Kim, S., & Paik, W. K. (1987). Enzymatic methylation of *in vitro* synthesized apocytochrome c enhances its transport into mitochondria. J. Biol. Chem. 262, 14702–14708.

Pettigrew, G. W. & Smith, G. M. (1977). Novel N-terminal protein blocking group identified as dimethylproline. Nature 265, 661–662.

Pezacka, E. & Wood, H. G. (1988). Acetyl-CoA pathway of autotropic growth: identification of the methyl-binding site of the CO dehydrogenase. J. Biol. Chem. 263, 16000–16006.

Phan, L. D., Perentesis, J. P., & Bodley, J. W. (1993). *Saccharomyces cerevisiae* elongation factor 2. Mutagenesis of the histidine precursor of diphthamide yields a functional protein that is resistant to diphtheria toxin. J. Biol. Chem. 268, 8665–8668.

Philips, M. R., Pillinger, M. H., Staud, R., Volker, C., Rosenfeld, M. G., Weissmann, G., & Stock, J. B. (1993). Carboxyl methylation of ras-related proteins during signal transduction in neutrophils. Science 259, 977–980.

Roberts, D. M., Rowe, P. M., Siegel, F. L., Lukas, T. J., & Watterson, D. M. (1986). Trimethyllysine and protein function: effect of methylation and mutagenesis of lysine 115 of calmodulin on NAD kinase activation. J. Biol. Chem. 261, 1491–1494.

Rowe, P. M., Wright, L. S., & Siegel, F. L. (1986). Calmodulin N-methyltransferase: partial purification and characterization. J. Biol. Chem. 261, 7060–7069.

Sarnow, P., Rasched, I., & Knippers, R. (1981). A histone H4-specific methyltransferase: properties, specificity and effects on nucleosomal histones. Biochim. Biophys. Acta, 655, 349–358.

Solomon, L. R. & Rubenstein, P. A. (1987). Studies on the role of actin's N^{tao}-methylhistidine using oligodeoxynucleotide-directed site-specific mutagenesis. J. Biol. Chem. 262, 11382–11388.

Springer, M. S., Goy, M. F., & Adler, J. (1979). Protein methylation in behavioural control mechanisms and in signal transduction. Nature 280, 279–284.

Stephenson, R. C. & Clarke, S. (1989). Succinimide formation from aspartyl and asparginyl peptides as a model for the spontaneous degradation of proteins. J. Biol. Chem. 264, 6164–6170.

Stephenson, R. C. & Clarke, S. (1990). Identification of a C-terminal protein carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesyl cysteine-containing peptide substrate. J. Biol. Chem. 265, 16248–16254.

Stock, J., Borczuk, A., Chiou, F., & Burchenal, J. E. B. (1985). Compensatory mutations in receptor function: a re-evaluation of the role of methylation in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA 82, 8364–8368.

Stock, A., Clarke, S., Clarke, C., & Stock, J. (1987). N-terminal methylation of proteins: structure, function and specificity. FEBS Lett. 220, 8–14.

Stock, J. & Simms, S. (1988). Methylation, demethylation, and deamidation at glutamate residues in membrane chemoreceptor proteins. Adv. Exp. Med. Biol. 231, 201–212.

Stock, J. B., Surette, M. G., Mcclearly, W. R., & Stock, A. M. (1992). Signal transduction in bacterial chemotaxis. J. Biol. Chem. 267, 19753–19756.

Sussman, D., Sellers, J. R., Flicker, P., Lai, E. Y., Cannon, E., Szent-Gyorgyi, A. G., & Fulton, C. (1984). Actin of *Naegleria gruberi*: absence of N^{tao}-methylhistidine. J. Biol. Chem. 259, 7349–7354.

Swanson, R. V. & Glazer, A. N. (1990). Phycobiliprotein methylation. Effect of the γ-N-methylasparagine residue on energy transfer in phycocyanin and the phycobilisome. J. Mol. Biol. 214, 787–796.

Tuck, M. T., Farooqui, J. Z., & Paik, W. K. (1985). Two histone H1-specific protein-lysine N-methyltransferases from *Euglena gracilis*: Purification and characterization. J. Biol. Chem. 260, 7114–7121.

Van Noort, J. M., Krall, B., Sinjorgo, K. M. C., Persoon, N. L. M., Johanns, E. S. D., & Bosch, L. (1986). Methylation *in vivo* of elongation factor EF-Tu at lysine-56 decreases the rate of tRNA-dependent GTP hydrolysis. Eur. J. Biochem. 160, 557–561.

Wang, C., Lin, J.-M., & Lazarides, E. (1992). Methylations of 70,000-Da heat shock proteins in 3T3 cells: Alterations by arsenite treatments, by different stages of growth and by virus transformation. Arch. Biochem. Biophys. 297, 169–175.

Wylie, D. C. & Vanaman, T. C. (1988). Structure and evolution of the calmodulin family of calcium regulatory proteins. In: Calmodulin (Cohen, P., & Klee, C. B., eds.). pp. 1–15, Elsevier, Amsterdam.

Xie, H. & Clarke, S. (1993). Methyl esterification of C-terminal leucine residues in cytosolic 36 kDa polypeptides of bovine brain: A novel eucaryotic protein carboxyl methylation reaction. J. Biol. Chem. 268, 13364–13371.

Xie, H. & Clarke, S. (1994a). An enzymatic activity in bovine brain that catalyzes the reversal of the C-terminal methyl esterification of protein phosphatase 2A. Biochem. Biophys. Res. Commun. 203, 1710–1715.

Xie, H. & Clarke, S. (1994b). Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain. J. Biol. Chem. 269, 1981–1984.

Young, P. R., Vacante, D. A., & Waickus, C. M. (1987). Mechanism of the interaction between myelin basic protein and the myelin membrane; the role of arginine methylation. Biochem. Biophys. Res. Commun. 145, 1112–1118.

RECOMMENDED READINGS

Borchardt, R. T., Creveling, C. R., & Ueland, P. M. (eds.), (1986). Biological Methylation and Drug Design, Humana Press, Clifton, New Jersey.

Paik, W. K. & Kim, S. (1980). Protein Methylation, John Wiley & Sons, New York.

Paik, W. K. & Kim, S. (eds.), (1990). Protein Methylation, CRC Press, Boca Raton, FL.

Zappia, V., Galletti, P., Porta, R., & Wold, F. (eds.), (1988). Advances in Post-translational Modifications of Proteins and Aging, Plenum Press, New York.

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Chapter 13

ADP-ribosylation Reactions

COLIN K. PEARSON

INTRODUCTION: DEFINITIONS AND NOMENCLATURE

What is an ADP-ribosylation Reaction?

An ADP-ribosylation reaction is the cleavage of the oxidized form of nicotinamide adenine dinucleotide (NAD^+), the co-enzyme of numerous redox reactions,

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into nicotinamide and adenosine diphosphate-ribose (ADP-ribose), with the concomitant covalent attachment of the ADP-ribose moiety to an acceptor protein:

$$\text{Acceptor Protein} + \beta\text{-NAD}^+ \longrightarrow \text{Nicotinamide} + \text{Protein-(ADP-ribose)}_n$$

This is, thus, another example of a posttranslational modification of proteins, like phosphorylation, methylation, acetylation, etc.

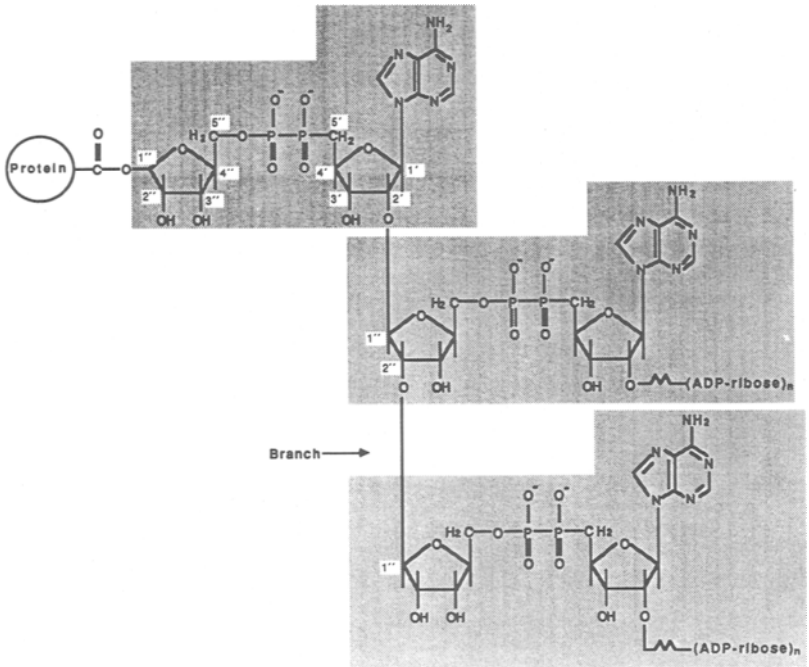

Figure 1. Structure of poly(ADP-ribose) showing a single branch link. Points to note are: (1) the shaded areas show respective ADP-ribose moieties in the polymer, (2) the polymer is shown attached to the acceptor protein via an ester linkage, (3) in each ADP-ribose moiety, the carbon atoms on the ribose nearest the adenine residues are numbered 1′ to 5′, and those on the distal ribose from 1″ to 5″. The two ribose residues are oriented with their respective 5′ to 5″ C atoms through the two phosphates, (4) polymer chains are joined by α-glycosidic links from the 1″ position of a ribose furthest from the adenine to a 2′ position of a ribose nearest to the adenine in the neighboring ADP-ribose moiety (i.e., the polymer has a ribose (1″ → 2′) ribose–phosphate–phosphate backbone); branch points are also the result of glycosidic links between two ribose residues but both of these are distal to the adenine in their respective ADP-ribose moieties. The link is a 1‴ to 2″. Note: in the literature, this first ribose is sometimes referred to as 1‴ to distinguish it from other riboses distal to adenines that form the polymer backbone.

When a single ADP-ribose moiety (n = 1) becomes attached to an acceptor site, the reaction is referred to as mono(ADP-ribosyl)ation, and when a homopolymer chain of repeating units is attached, the process is referred to as poly(ADP-ribosyl)ation (n > 1). Polymer chains may consist of over 200 ADP-ribose residues and may also contain branches, occurring some three times per 100 residues (see Figure 1).

Where do ADP-ribosylation Reactions Occur?

ADP-ribosylation reactions are quite ubiquitous and are found in animals, plants, microorganisms, and some viruses. In eukaryotic cells, monomeric ADP-ribosylation occurs essentially in the cytoplasm and the plasma membrane, whereas poly(ADP-ribosyl)ation is found predominantly in the nucleus. Some terminally differentiated cells lack the polymerizing enzyme activity, e.g., mature granulocytes. The polymerase also seems to be absent in prokaryotic organisms.

ENZYMOLOGY OF ADP-RIBOSYLATION REACTIONS

Synthesizing Enzymes

Enzymes which catalyze monomeric ADP-ribosylation are called **mono(ADP-ribosyl) transferases** (EC 2.4.2.31). The eukaryotic nuclear enzyme catalyzing the synthesis of polymers is referred to as **poly(ADP-ribose) polymerase** (EC 2.4.2.30). Other names for this enzyme in the literature include poly(ADP-ribose) synthetase and also ADP-ribosyl transferase; this latter, unfortunately, does not distinguish it from the mono(ADP-ribosyl) transferase. The polymerase is remarkable in that it can catalyze a number of reactions, including: (1) the initiation event, in which the first ADP-ribose moiety becomes covalently attached to protein; (2) the elongation of this to form a polymeric chain; (3) branching, and (4) cleavage of NAD^+ without attaching the released ADP-ribose to protein, the so-called 'abortive glycohydrolase activity' of the polymerase.

It is important to note that other enzymes also exist which catalyze the cleavage of NAD^+ without attaching the ADP-ribose moiety to proteins; these are called NAD^+ glycohydrolases and are not ADP-ribosylating enzymes.

ADP-ribose Attachment to Protein

A variety of amino acid-acceptor sites have been discovered and these are summarized as follows:

Protein – lysine – ADPR (ADPR = ADP-ribose)

Protein – glutamate – ADPR

These are ester bonds linking the ADP-ribose to the amino acid and are formed by the nuclear polymerizing enzyme.

Protein – arginine – ADPR

Protein – cysteine – ADPR

Protein – diphthamide – ADPR

Protein – asparagine – ADPR

These bonds are characteristic of the various mono(ADP-ribosyl) transferase catalyzed reactions that are known (see section on mono(ADP-ribosyl)ation reactions below).

Nonenzymatic Attachment to Protein

Free ADP-ribose, produced from cleavage of NAD^+ by the action of NAD^+ glycohydrolase or the abortive glycohydrolase activity of the polymerase, may react nonenzymatically with proteins to form a Schiff's base with the amino acid acceptor. Such nonenzymatic attachments of ADP-ribose have been found in mammalian cell mitochondria.

Degrading Enzymes

Two different enzymes degrade poly(ADP-ribose) and a third releases the remaining monomeric ADP-ribose from its attachment to the protein acceptor (Figure 2).

Poly(ADP-ribose) glycohydrolase. The glycohydrolase is the major degrading enzyme *in vivo*, and attacks the ribose–ribose bond between successive ADP-ribose moieties in the polymer, producing free ADP-ribose in the process. The purified enzyme degrades poly(ADP-ribose) of length greater than 20 residues in a biphasic manner. More than half the polymer is degraded processively with the remainder being degraded nonprocessively. The K_m for the large polymers is some 100-fold lower than that for the smaller molecules, suggesting that the glycohydrolase may differentially regulate the levels of large and small poly(ADP-ribose) in the cell.

Phosphodiesterase. This enzyme has a K_m for poly(ADP-ribose) of about 28 μM, some 15-fold higher than that of the glycohydrolase, and is not considered to play a significant role in degrading the polymer *in vivo* except perhaps when polymer levels are high.

ADP-protein lyase. The single ADP-ribose residue still attached to protein after glycohydrolase action on the polymer can be cleaved by ADP-ribosyl protein lyase. The enzyme is specific for ester linkages to the protein. [Clinical note: Williams and collaborators at the University of Texas Health Center have described the case

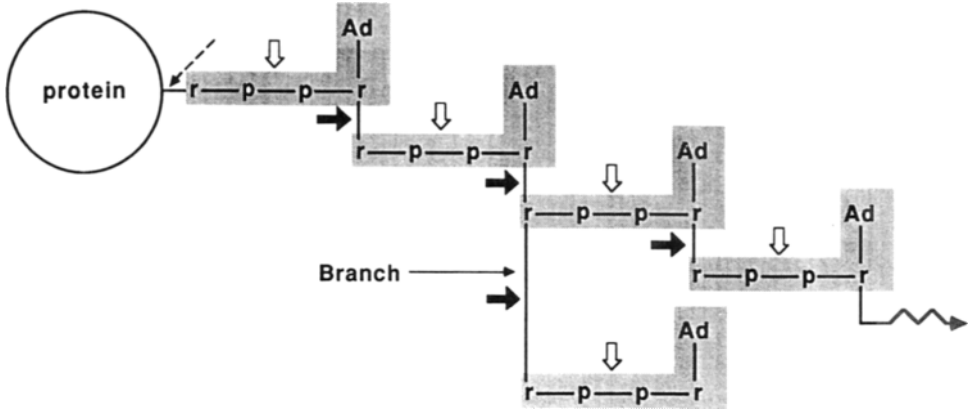

Figure 2. Enzymatic degradation of poly(ADP-ribose). The arrows indicate the covalent bonds that are cleaved by poly(ADP-ribose) glycohydrolase (➡), ADP-ribosyl protein lyase (⇢), and phosphodiesterase (⇩). The reader can work out what the respective reaction products would be in each case (refer also to Figure 1).

of a boy with a lysosomal storage disease who had a period of progressive neurological disorder, finally dying at the age of 6 from renal failure. The cause appears to be due to a deficiency of the lyase (Williams, 1985)].

An enzyme catalyzing the cleavage of monomeric ADP-ribose attached to protein via an arginine residue was first isolated from the turkey erythrocytes in 1985 (Moss et al., 1985) and is referred to as **ADP-ribosylarginine hydrolase**. A number of these enzymes have since been characterized in a variety of animal species and tissues.

REVERSIBILITY: A FUNDAMENTAL REQUIREMENT OF A METABOLIC REGULATORY PROCESS

The presence of both synthesizing and degrading enzymes in cells, together with the established rapid turnover of many of the ADP-ribose residues, supports the concept that ADP-ribosylation is, indeed, a reversible process which plays a regulatory role in cell metabolism.

Constitutive poly(ADP-ribose) is degraded in a biphasic manner in intact cells with reported half-lives of about 5 minutes and one to several hours. When polymer levels are enhanced by experimentally activating the polymerase, the polymer is turned over rapidly with a half-life measured in minutes or even less.

Perhaps the best characterized example of the turnover of monomeric ADP-ribose residues is the regulation of nitrogenase in the photosynthetic bacterium *Rhodospirillum rubrum* by reversible ADP-ribosylation.

FUNCTIONS OF PROTEIN ADP-RIBOSYLATION

Nuclear Poly(ADP-ribosyl)ation

Poly(ADP-ribose) polymerase is activated by DNA strand breaks and this property is central to its regulatory role in cell metabolism. The enzyme is tightly bound to chromatin where it ADP-ribosylates chromosomal proteins including histones, nonhistones, and itself (automodification), and its activity influences and is influenced by chromatin structural changes. These properties point to a funda-

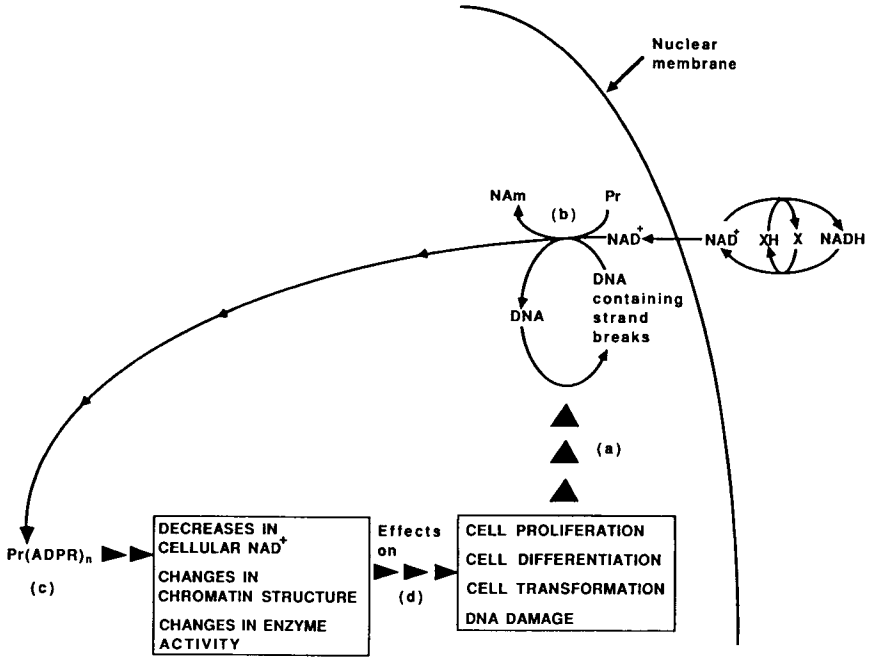

Figure 3. Proposed pleiotropic functions carried out by nuclear ADP-ribosylation reactions. Events such as cellular proliferation, differentiation, transformation, and DNA damage caused by external agents (e.g., ionizing radiation, drugs) involve changes in the integrity of DNA and/or chromatin architecture (**a**) which activate the poly(ADP-ribose) polymerase to catalyze the ADP-ribosylation of nuclear proteins predominantly at the expense of cytoplasmic NAD^+, (**b**). The consequences of protein ADP-ribosylation are a decrease in cellular NAD^+ content, alterations in chromatin structure, and possibly also the activity of various enzymes involved in chromatin function (**c**). This tripartite system operates, either wholly or partly, to ameliorate the activation of the polymerase by modulating the repair of DNA strand breaks, thereby affecting those processes which initially triggered the activation of the enzyme (**d**). Pr, protein; NAm, nicotinamide; $(ADPR)_n$, poly(ADP-ribose). (From Gaal and Pearson, 1986).

mental role, or roles, in major nuclear processes. These include, for example, DNA repair, DNA replication, cell differentiation and gene expression, and perhaps transposition and gene rearranging events in which some breakage and rejoining of DNA strands may occur (see the Reading List for further information on these topics). The proposed pleiotropic role of nuclear poly(ADP-ribosyl)ation is summarized in Figure 3.

What is it then about poly(ADP-ribose) polymerase and protein ADP-ribosylation that would explain such a global involvement in nuclear metabolism? Recent advances in two selected areas of the field help to answer this question:

Poly(ADP-ribose) Polymerase Structure and Molecular Cloning of the Gene

Poly(ADP-ribose) polymerase is a multifunctional, highly conserved enzyme in which three functional domains have been identified following limited proteolysis (Figure 4). These are a DNA binding domain, an automodification domain, and a catalytic domain, which includes the binding site for the substrate, NAD^+.

The human polymerase gene consists of 23 exons located within 43 kb of DNA (Figure 4). The nucleotide sequence, contained in a single open reading frame of 3,042 nucleotides, encodes a 113,153 kDa polypeptide containing 1014 amino acids. Computer data base comparisons have revealed several protein-sequence motifs within the different domains with putative functions. The N-terminal DNA-binding domain contains two zinc fingers. This metal ion is required for polymerase activity and for DNA binding. There is also a nuclear location signal that targets large proteins to the cell nucleus. The automodification domain contains 15 glutamic acid residues, which are potential auto(ADP-ribosyl)ation sites. The C-terminal region contains a G-rich segment (GKG in the figure; i.e., glycine-lysine-glycine) containing a lysine residue essential for catalytic activity, and also a motif that may bind the ADP part of the substrate NAD^+ molecule.

As we shall see in the next section, the automodification of the polymerase, which is consequent upon activation by DNA damage, has a major role to play in ensuing nuclear events.

ADP-ribosylation and Chromatin Structure

Recent work, particularly in Felix Althaus's laboratory in Switzerland, has enabled a general hypothesis to be made about nuclear poly(ADP-ribosyl)ation (Realini and Althaus, 1992). The proposal, referred to as 'histone shuttling', is summarized in Figure 5. It begins by assuming that poly(ADP-ribose) polymerase is targeted to sites of DNA strand breaks where it then becomes activated. A number of branched polymer ADP-ribose chains are synthesized, and the polymerase itself is the major acceptor becoming covalently automodified. These polymers then

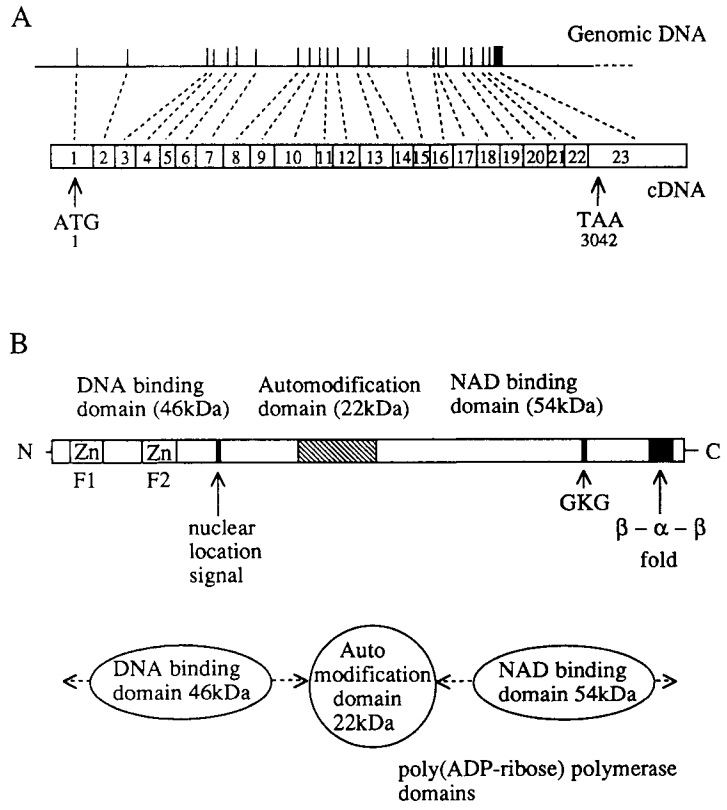

Figure 4. (**A**) The human nuclear poly(ADP-ribose) polymerase gene. The figure shows the relative distribution of the 23 exons in the genomic DNA (the gene covers 43 kb) and the corresponding transcript cDNA. The length of the open reading frame in the cDNA is indicated by the positions of the initiation and termination codons. (From Auer et al., 1989). (**B**) Poly(ADP-ribose) polymerase functional domains. The enzyme contains three functional domains as indicated (Kameshita et al., 1984). In addition, the diagram (upper part) shows the positions of the two zinc finger regions required for DNA binding, a nuclear location signal, the motif GKG in the NAD^+ binding domain involved in the catalytic activity, and a putative β– α– β fold, thought to interact with the ADP moiety of NAD^+. (From de Murcia et al., 1991).

associate noncovalently with the histones forming a histone–polymer–polymerase complex which dissociates from the DNA. With increasing ADP-ribosylation and concomitant complex formation with the histones, the DNA can be increasingly denuded of histones. The presence of ADP-ribose polymers stimulates the activity of poly(ADP-ribose) glycohydrolase, which then begins to degrade the ADP-ribose chains. As a consequence of this action, the histones then become free to reassociate with the DNA.

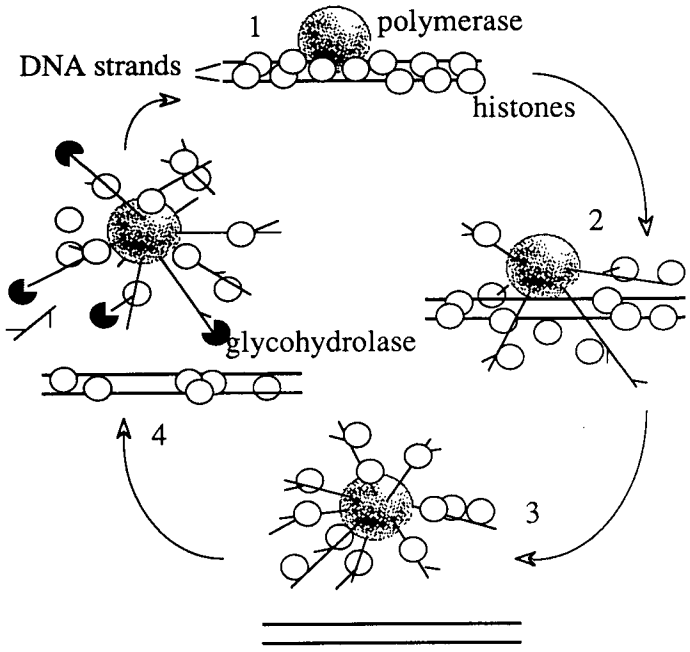

Figure 5. Poly(ADP-ribosyl)ation-dependent histone shuttle on DNA. Step (**1**) shows a poly(ADP-ribose) polymerase molecule bound to chromatin. Step (**2**): auto(ADP-ribosyl)ation of the polymerase attracts histones from the DNA so they become noncovalently bound to the polymeric ADP-ribose chains attached to the polymerase. Step (**3**) indicates an extreme case where the local DNA has been completely denuded of histones. Step (**4**): upon degradation of the poly(ADP-ribose) by poly(ADP-ribose) glycohydrolase the histones reassociate with the DNA. (From Realini and Althaus, 1992).

This model is supported by other recent studies showing that poly(ADP-ribose) chains added in an *in vitro* system increase the accessibility of chromatin histones to histone-specific antibodies (Thiebault et al., 1992).

It now becomes easier to envisage how nuclear ADP-ribosylation can influence so many major nuclear processes. Any initial relaxation of chromatin structure caused by DNA strand breaks would be further emphasized by the ensuing ADP-ribosylation of the activated polymerase. This would allow access to other enzymes and regulatory molecules involved, for example, in DNA repair, cell differentiation, gene expression, etc.

It must be emphasized that this model does not explain the functions of heteromodification, whereby nuclear proteins other than the poly(ADP-ribose) polymerase also become covalently modified, including both histones and nonhistone proteins.

Mono(ADP-ribosyl)ation Reactions: Bacterial ADP-ribosylating Toxins

The best understood reactions are those catalyzed by ADP-ribosyl transferase components of certain bacterial toxins. These include, for example, toxins produced by *Vibrio cholerae* (the causative agent of cholera), pertussis toxin (from *Bordetella pertussis*, which causes whooping cough), diphtheria toxin, exotoxin A from *Pseudomonas aeruginosa* (an opportunistic pathogen), and toxins from *Clostridium botulinum*.

Most of these toxins consist of two functional moieties, A and B. The A moiety carries the ADP-ribosyl transferase activity and the B moiety mediates cellular uptake of the toxin. All bacterial toxins so far studied, modify nucleotide-binding proteins in their target cells, and are proving to be valuable experimental tools with which to study the functions of their target proteins. Also, their use has paved the way for the discovery of ADP-ribosyl transferases in uninfected mammalian cells which may carry out similar reactions.

Some examples illustrating current knowledge on the action of toxin ADP-ribosyl transferases are given below:

Toxins and Signal Transduction: ADP-ribosylation of G-proteins

G-proteins mediate the activation or inhibition of an effector molecule situated on the inside surface of cell membranes, as a result of an external agonist (growth factor, hormone, neurotransmitter, etc.) binding to or activating (e.g., light) a specific receptor in the cell membrane (Figure 6). The agonist first binds to receptor sites on the cell surface. This activates the receptor molecule to interact with a heterotrimeric G-protein situated within the membrane and facilitates the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP), which is already bound to the α-subunit of the trimer. Activation of the GTP-bound form of the G-protein appears to coincide with its dissociation from the receptor, as well as its own dissociation into a free α-subunit and the $\beta\gamma$-dimer. In many cases, the free GTP-bound α-subunit is then responsible for effector regulation (e.g., the stimulation of adenylyl cyclase), although in other cases it is possible that the $\beta\gamma$-dimer may play a role. Deactivation of the G-protein subsequently occurs via the GTPase activity of the α-subunit, which results in cleavage of the bound GTP to GDP and the subsequent association of the GDP-bound α-subunit with the $\beta\gamma$-dimer.

Current ideas regarding toxin-mediated ADP-ribosylation of G-proteins are also summarized in Figure 6 and the model enables us to understand how agonist action can be stimulated or inhibited by the action of various toxins. We can consider toxin-mediated modulation of adenylyl cyclase as an example.

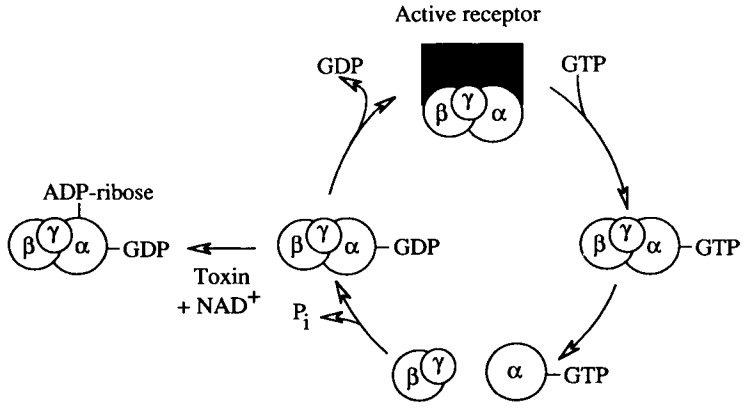

Figure 6. General models for agonist activation of G-proteins and the action of bacterial toxins on G-proteins. The receptor is activated when a specific ligand binds to it. The activated receptor promotes the exchange of GTP for GDP, which is already bound to the quiescent trimeric G-protein on the α-subunit. Binding of GTP facilitates the dissociation of the βγ-dimer from the GTP-α subunit. This liganded α-subunit is thought to be responsible for effector regulation. Deactivation is initiated by cleavage of bound GTP to GDP by the α-subunit, which leads to reassociation of the quiescent, trimeric G-protein.

ADP-ribosylation occurs on the GDP-liganded subunit, probably when it is in the trimeric state. With some toxin-mediated ADP-ribosylation reactions, this may lead to effector activation by promoting exchange of GTP for the bound GDP (e.g., cholera toxin activation of adenylyl cyclase). In other cases, ADP-ribosylation prevents the reassociation of the trimeric G-protein with the receptor, thus negating the effect of agonist binding (e.g., the action of pertussis toxin).

Agonist Activation of Adenylyl Cyclase: Modulation by Cholera Toxin

The G-protein α-subunit bound to GDP is the substrate for cholera toxin ADP-ribosylation; probably while it is in the trimeric form (this subunit is called $G_s\alpha$, to indicate its stimulatory mode of action). Cholera toxin activation of adenylyl cyclase is mediated by a protein called ADP-ribosylation factor (ARF). ARF is also a GTP-binding protein. The proposed involvement of ARF is illustrated in Figure 7. In this model, ARF binds GTP, forming an active ARF·GTP complex, which subsequently interacts with the thiol-activated cholera toxin, A1 protein, and enhances its enzymatic activities. In the presence of NAD^+, $G_s\alpha$·GDP is ADP-ribosylated on an arginine residue by ARF-stimulated toxin. The ADP-ribosylated $G_s\alpha$ binds GTP in exchange for GDP, and subsequently activates adenylyl cyclase. ADP-ribosylation of α_s by cholera toxin inhibits its GTPase activity, which leads to a prolonged activation of the protein.

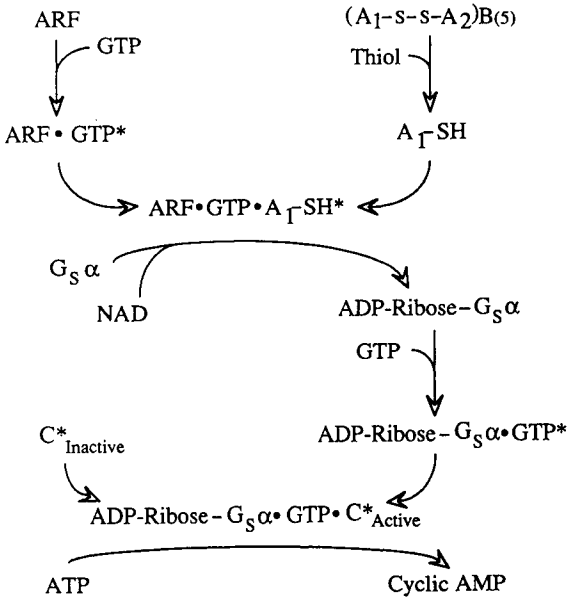

Figure 7. Proposed G-protein cascade for activation of adenylyl cyclase by cholera toxin. Holotoxin is shown as $(A_1\text{-s-s-}A_2)B_{(5)}$. ARF·GTP interacts with thiol-activated toxin (A_1-SH) and stimulates the ADPribosyltransferase activity of A_1-SH. ARF-stimulated A_1-SH ADP-ribosylates $G_s\alpha$, which binds GTP, becomes constitutively active, and subsequently activates the catalytic subunit of adenylyl cyclase, (C). (From Tsai et al., 1988).

Agonist Inhibition of Adenylyl Cyclase: Modulation by Pertussis Toxin

When G-proteins are ADP-ribosylated by pertussis toxin, the host cell no longer responds to a variety of hormones and neurotransmitters. Referring again to Figure 6, it is now generally thought that the major effect of ADP-ribosylation by pertussis toxin is to prevent the interaction of the agonist-activated receptor with the GDP-liganded G-protein. Pertussis toxin ADP-ribosylates cysteine residues in G-proteins. At least twenty different G-proteins have been discovered so far. The substrate for pertussis toxin in the adenylyl cyclase system is referred to as $G_i\alpha$, where the i indicates an inhibitory role. The effect of the toxin in this case leads essentially to an activation of the adenylyl cyclase by thwarting the action of the inhibitory agonist.

Toxin Inhibition of Protein Synthesis

Some toxins ADP-ribosylate eukaryotic elongation factor 2 (EF-2), an essential component of the protein biosynthesis machinery of the cell:

$$\text{EF-2} + \text{NAD}^+ \longrightarrow \text{EF-2(ADP-ribose)} + \text{nicotinamide}$$
toxin ADP-ribosyl transferase activity

EF-2 is a GTP-binding protein. When it is ADP-ribosylated, it cannot mediate polypeptide-chain elongation, consequently the affected cells lose the ability to synthesize protein and die.

Toxins capable of catalyzing such a reaction include diphtheria toxin, secreted by lysogenic strains of *Corynebacterium diphtheriae* carrying the phage-encoded toxin gene (DT), and the exotoxin A from *Pseudomonas aeruginosa* (ETA). Both catalyze the ADP-ribosylation of a posttranslationally-modified histidine residue (called diphthamide) in EF-2.

Clostridial Toxins ADP-ribosylating Actin

ADP-ribosylation of actin by *Clostridium botulinum* C2 toxin causes dramatic changes to its properties and can result in destruction of the cellular microfilament network (Figure 8). After receptor binding of the binary toxin to the cell surface, the ADP-ribosylating component, C21, is translocated into the cell where it ADP-ribosylates an arginine residue in monomeric G-actin. The modified G-actin is turned into a capping protein, which caps the fast-growing end of actin filaments. At the other end of the filaments, monomeric actin is still released. This can then be a substrate for ADP-ribosylation. Since the previously growing ends of the actin filaments are capped, these newly released moieties are trapped as ADP-ribosylated monomers. These processes, whereby filament polymerization is prevented by capping, and modified G-actin accumulates by trapping, disturb the dynamic equilibrium between G- and F-actin, resulting in destruction of the microfilament network (Aktories and Wegner, 1989).

Most of the toxicological effects of the botulinum C2 toxin on animals can be attributed to an increase in vascular permeability. In rats, for instance, it causes hypotension, hemorrhaging in the lungs, and fluid accumulation around the lungs.

ADP-Ribosylation of "Small" GTP-binding Proteins

Clostridium botulinum also produces another ADP-ribosylating toxin called C3. Substrates for this toxin are the Rho and Rac proteins, which become ADP-ribosylated at an asparagine residue. These belong to the family of "small" G-proteins (molecular weight about 20–26 kDa), to which the well known *ras* proteins belong, mutations in which are associated with tumorigenesis. It is thought that Rho proteins are involved in the regulation of cytoskeletal proteins, particularly microfilament proteins and that their modification with ADP-ribose renders them biologically inactive.

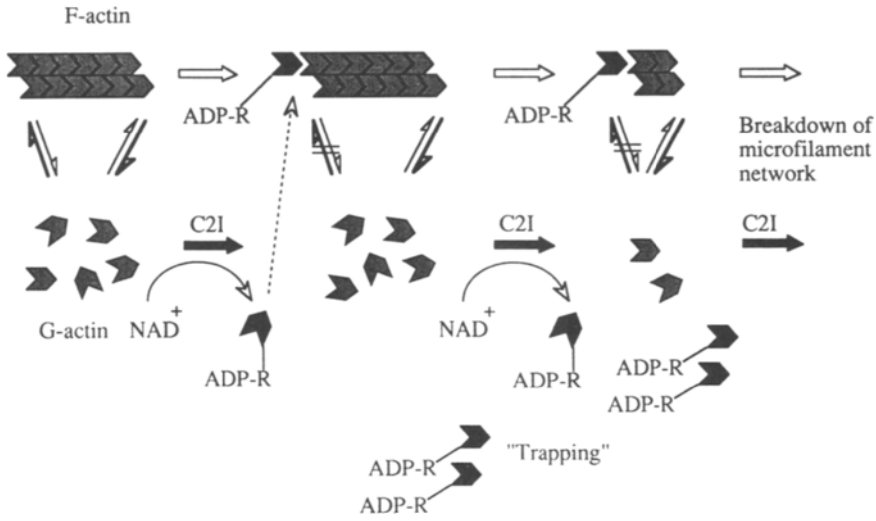

Figure 8. Model for the action of actin ADP-ribosylating toxins exemplified by the action of *Botulinum* C2 toxin. This toxin consists of two subunits, C2I and C2II. When the C2I component of the toxin (possessing ADP-ribosylating activity) enters the target cell, its activity disturbs the equilibrium between actin polymerization and depolymerization. ADP-ribosylation of actin inhibits polymerization and turns G-actin into a capping protein which binds to the fast-growing (barbed) ends of actin filaments. Capping of the barbed ends increases the critical concentration for actin polymerization. Since the slow-growing (pointed) ends of actin filaments are free, depolymerization of actin occurs at these ends. Released actin, however, is a substrate of the toxin and will be withdrawn from the pool of available actin by ADP-ribosylation. This trapping of G-actin and capping of F-actin will finally induce the breakdown of the microfilament network. (From Aktories and Wegner, 1989).

Cellular Mono(ADP-ribosyl) Transferases

Identification of toxin substrates inevitably led to the question of whether cells possess comparable endogenous reactions. A brief summary is given as follows:

G-proteins. Studies have demonstrated endogenous ADP-ribosylation of G-proteins in various mammalian tissues (Duman et al., 1991), and recent experiments have shown that endogenous ADP-ribosylation is under the control of specific hormones and second messenger systems (Brune et al., 1990). These findings suggest that regulation of ADP-ribosylation may represent a mechanism by which receptor-coupled signal transduction systems modulate cell function.

Elongation Factor 2. Eukaryotic ADP-ribosyl transferases, which modify EF-2, have also now been isolated from a variety of animal species and tissues. The *in*

vivo activity of the transferase has been demonstrated by immunoprecipitation of ADP-ribosylated EF-2. Interestingly, the cellular enzyme does not require the diphthamide modification of the histidine ring for transfer of ADP-ribose to the ring (Fendrick et al., 1992).

Actin. This is one of the most important components in the cytoskeletal architecture, and in the movement of cytoplasm in eukaryotic cells. In addition to muscle contraction, actin is involved in cellular processes such as phagocytosis, secretion, cell migration, and the maintenance of cell shape. A recent report shows that a mammalian cell arginine-specific ADP-ribosyl transferase can ADP-ribosylate actin molecules, suggesting that this could be a regulatory mechanism in the above cellular functions (Terashima et al., 1992).

Mitochondrial ADP-ribosylation. Other protein substrates for mono(ADP-ribosyl) transferases continue to be reported, but the best characterized reaction is that of mammalian cell mitochondria. Most mono-ADP-ribosyl–protein conjugates in eukaryotic cells are associated with mitochondria. A specific function, namely, stimulation of calcium release from mitochondria, has been ascribed to ADP-ribosylation activity in this organelle. This could, therefore, be an important cell regulatory mechanism, since numerous calcium-dependent enzymes play an important role in cell functioning.

Release of Ca^{2+} from rat liver mitochondria is induced by oxidation of mitochondrial pyridine nucleotides, but also requires that these be cleaved into ADP-ribose and nicotinamide. Mitochondrial protein ADP-ribosylation has been proposed as the link between this cleavage and Ca^{2+} release (Figure 9). Recent work

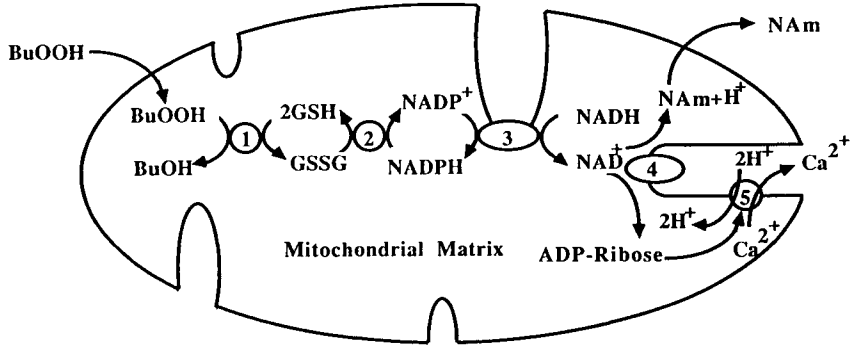

Figure 9. Proposed link between pyridine nucleotide-related Ca^{2+} release from rat liver mitochondria and ADP-ribosylation in the inner mitochondrial membrane. Oxidation of mitochondrial pyridine nucleotides can be brought about by various compounds; tertiary butylhydroperoxide (BuOOH) is shown: (**1**) glutathione peroxidase; (**2**) glutathione reductase; (**3**) energy-linked pyridine nucleotide transhydrogenase; (**4**) NAD^+ glycohydrolase; (**5**) Ca^{2+}/H^+ antiporter, modified by ADP-ribose. NAm, nicotinamide. (From Richter et al., 1985).

which supports this hypothesis shows that cyclosporin, an immunosuppressive drug, inhibits mitochondrial Ca^{2+} release induced by pro-oxidants (Schlegel et al., 1991), the analgesic drug paracetamol, or a cytochrome P450-mediated metabolite of the drug (Weis et al., 1992). Oxidation of NADH still occurs in the presence of the cyclosporin, but cleavage of the pyridine nucleotide to nicotinamide and ADP-ribose no longer takes place. These studies also showed that an inhibitor of mono(ADP-ribosyl) transferase, MIBG (meta-iodobenzylguanidine), prevented both NAD^+ cleavage and Ca^{2+} release.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Although a precise definition of the role of nuclear poly(ADP-ribosyl)ation is not available, the histone-shuttle mechanism proposed by Althaus and colleagues offers a possible unifying explanation of numerous experimental findings. While this model will come under further experimental scrutiny, the effects of ADP-ribosylating individual chromosomal proteins other than the polymerase itself (automodification) still needs to be elucidated.

Cloning the nuclear polymerase gene has allowed identification of a number of sequence motifs which mediate nuclear targeting of the protein and DNA strand break recognition, to which the catalytic activity of the enzyme is linked. de Murcia and colleagues have pointed out how detailed structural information from the crystallized protein will eventually facilitate an understanding of the mechanism of poly(ADP-ribose) polymerase activation by DNA strand breaks. The availability of gene sequences which can be used as probes is facilitating studies on the expression of the gene in a variety of tissues under different physiological circumstances. It is also facilitating polymerase gene cloning from a growing variety of living organisms, thus enabling informed comment about the evolution of the gene. However, despite this rapid growth of information about the gene and the enzyme, it still remains to define more precisely the *in vivo* roles of nuclear protein ADP-ribosylation.

The use of bacterial toxins as molecular probes will continue to provide valuable information on the functions of their various substrates. In addition, studies on endogenous cellular mono(ADP-ribosyl) transferases look set to expand. New substrates will be identified and the biochemical consequences of the different modifications will reveal the roles played by mono(ADP-ribosylation) reactions in different cell compartments. For example, the case of cytoskeletal actin has been discussed (see Figure 8). Work in Mandel's laboratory (Mandel, 1992) has revealed that other cytoskeletal proteins are also substrates for endogenous ADP-ribosyl transferase, including components of the microfilaments (tubulin, intermediate filaments, and the neurofilament proteins L, M, and H).

It is clear that the attachment of ADP-ribose to protein is a widespread mechanism of posttranslational modification within the cell. Further study will reveal the magnitude of its importance to the regulation of cellular activities.

REFERENCES

Aktories, K. & Wegner, A. (1989). ADP-ribosylation of actin by clostridial toxins. J. Cell Biol. 109, 1385–1387.

Auer, B., Ulrich, N., Herzog, H., Schneider, R., & Schweiger, M. (1989). Human nuclear NAD^+ ADP-ribosyltransferase(polymerizing): organization of the gene. DNA 8, 575–580.

Brune, B., Molina y Vedia, L., & Lapetina, E. G. (1990). Agonist-induced ADP-ribosylation of a cytosolic protein in human platelets. Proc. Natl. Acad. Sci. USA 87, 3304–3308.

de Murcia, G., de Murcia, J. M., & Schreiber, V. (1991). Poly(ADP-ribose) polymerase: molecular biological aspects. BioEssays 13, 455–462.

Duman, R. S., Terwilliger, R. Z., & Nestler, E. J. (1991). Endogenous ADP-ribosylation in brain: initial characterization of substrate. J. Neurochem. 57, 2124–2132.

Fendrick, J. L., Iglewski, W. J., Moehring, J. M., & Moehring, T. J. (1992). Characterization of the endogenous ADP-ribosylation of wild type and mutant elongation factor 2 in eukaryotic cells. Eur. J. Biochem. 205, 25–31.

Gaal, J. C. & Pearson, C. K. (1986). Covalent modification of proteins by ADP-ribosylation. Trends Biochem. Sci. 11, 171–175.

Kameshita, I., Matsuda, Z., Tanigushi, T., & Shizuta, Y. (1984). Poly(ADP-ribose)synthetase. Separation and identification of three proteolytic fragments as the substrate-binding domain, the DNA binding domain, and the automodification domain. J. Biol. Chem. 259, 4770–4776.

Mandel, P. (1992). Some aspects of nuclear and cytoplasmic ADP-ribosylation. Biological and pharmacological perspectives. In: ADP-Ribosylation Reactions (Poirier, G. G., & Moreau, P. eds.), pp. 163–172, Springer Verlag, Berlin.

Moss, J., Jacobson, M. K., & Stanley, S. J. (1985). Reversibility of arginine-specific mono(ADP-ribosyl)ation: identification in erythrocytes of an ADP-ribose-L-arginine cleavage enzyme. Proc. Natl. Acad. Sci. USA 82, 5603–5607.

Realini, C. A. & Althaus, F. R. (1992). Histone shuttling by poly(ADP-ribosylation). J. Biol. Chem. 267, 18858–18865.

Richter, C., Frei, B., & Schlegel, J. (1985). Calcium transport and mono(ADP-ribosylation) in mitochondria. In: ADP-Ribosylation of Proteins (Althaus, F. R., Hilz, H., & Shall, S., Eds.), pp. 530–535. Springer-Verlag, Berlin.

Schlegel, J., Meier, P., Kass, G. E. N., & Richter, C. (1991). Inhibition by cyclosporine A of the prooxidant-induced but not of the sodium-induced calcium release from rat kidney mitochondria. Biochem. Pharmacol. 42, 2193–2198.

Terashima, M., Mishima, K., Yamada, K., Tsuchiya, M., Wakutani, T., & Shimoyama, M. (1992). ADP-ribosylation of actins by arginine-specific ADP-ribosyltransferase purified from chicken heterophils. Eur. J. Biochem. 204, 306–311.

Thibeault, L., Hengartner, M., Lagueux, J., Poirier, G. G., & Muller, S. (1992). Rearrangements of the nucleosome structure in chromatin by poly(ADP-ribose) Biochim. Biophys. Acta 1121, 317–324..

Tsai, S.-C., Noda, M., Adamik, R., Chang, P. P., Chen, H-C., Moss, J., & Vaughan, M. (1988). Stimulation of choleragen enzymatic activities by GTP and two soluble proteins purified from bovine brain. J. Biol. Chem. 263, 1768–1772.

Weis, M., Kass, G. E. N., Orrenius, S., & Moldeus, P. (1992). N-acetyl-p-benzoquinoneimine induces calcium release from mitochondria by stimulating pyridine nucleotide hydrolysis. J. Biol. Chem. 267, 804–809.

Williams, J. C. (1985). Glutamyl ribose-5-phosphate storage disease: Clinical description and characterization of stored material. In: ADP-Ribosylation of Proteins (Althaus, F. R., Hilz, H., & Shall, S., Eds.), pp. 167–177. Springer-Verlag, Berlin.

RECOMMENDED READINGS

Aktories, K. (ed.), (1992). ADP-ribosylating Toxins In: Current Topics in Microbiology and Immunology, Vol. 175, Springer–Verlag, Berlin.

Althaus, F. R. & Richter, C. (eds.), (1987). ADP-ribosylation of proteins: enzymology and biological significance. In: Molecular Biology Biochemistry and Biophysics, Vol. 37, pp. 1–237, Springer–Verlag, Berlin.

Ludden, P. W. & Roberts, G. P. (1989). Regulation of nitrogenase activity by reversible ADP-ribosylation. Curr. Topics in Cell. Reg. 30, 23–56.

Pearson, C. K. (1991). ADP-ribosylation reactions. In: Fundamentals of Medical Cell Biology, Vol. 3B, (Bittar, E. E., ed.), pp. 437–452, JAI Press Inc., Greenwich.

Mandel, P. (1991). ADP-ribosylation : approach to molecular basis of aging. Adv. in Exp. Med. Biol., 296, 329–343.

Shall, S. (1989). ADP-ribosylation of proteins: a ubiquitous cellular control mechanism. Biochem. Soc. Trans. 17, 317–322.

Chapter 14

Modification of Proteins by Prenyl Groups

MICHAEL H. GELB

INTRODUCTION

Most proteins in eukaryotic cells undergo one or more types of posttranslational modifications, including lipidation, phosphorylation, methylation, and proteolysis, as well as many other types. There are four types of lipids attached to proteins: 14-carbon myristoyl groups, 16-carbon palmitoyl groups, the structurally complex glycosyl phoshatidylinositol groups, and prenyl groups (Olson, 1988; Glomset et al., 1990). The precise roles of these protein-lipid groups in cell function are not fully understood, but they appear to be involved in anchoring proteins to cell membranes. Thus, water soluble proteins can be attached to cellular membranes by

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the addition of one or more lipid chains. Interest in proteins that contain prenyl groups has escalated in recent years since oncogenic *ras* proteins have been shown to be prenylated, and it is now known that this modification is obligatory for cellular transformation by these proteins (Hancock et al., 1989; Jackson et al., 1990; Gibbs, 1991). In addition, several other important proteins have been shown to be prenylated, as will be discussed below.

This chapter discusses protein prenylation. The structural diversity of this type of lipidation is discussed, along with the enzymes that attach prenyl groups to proteins. Finally, possible roles of protein prenyl groups in cellular processes are discussed. More detailed reviews on the subject of protein prenylation have been published recently (Glomset et al., 1990; Casey, 1992; Clark, 1992).

STRUCTURES OF PRENYLATED PROTEINS

The first prenylated protein-like substances discovered were the mating factors, Rhodotorucine A and Tremerogen A-10, isolated from the fungi *Rhodosporidium toruloides* and *Tremella mesenterica*, respectively (Kamiya et al., 1979; Sakagami et al., 1979). These substances are peptides that contain a prenyl group attached to the C-terminal cysteine residue (Figure 1). Rhodotorucine A has a 15-carbon prenyl group, called a farnesyl group, attached to the cysteine-sulfhydryl group via a thioether linkage. Tremerogen A-10 has a farnesyl group bearing a hydroxyl group on one of its terminal methyl groups.

Farnesyl groups are produced in the same biosynthetic pathway as cholesterol; both are derived from a building block called mevalonic acid (Figure 2). In this pathway, the 15-carbon compound, farnesyl pyrophosphate, is produced, and this is dimerized to give the 30-carbon product squalene, which is converted into cholesterol. As will be described below, farnesyl pyrophosphate is the source of the farnesyl group in farnesylated peptides and proteins.

In the early 1980s, Glomset and co-workers discovered that adding radiolabeled mevalonic acid to mammalian cells in culture resulted in the radiolabeling of a specific set of proteins (Schmidt et al., 1984). This suggested that these proteins contained an attached compound derived from mevalonic acid. Subsequent studies showed that this phenomenon occurs in many types of animal cells (Sinensky and Logel, 1985; Maltese and Sheridan, 1987; Sepp-Lorenzino et al., 1989). The first protein of this type to be identified was lamin B, which is bound to the nuclear envelope of cells where it probably functions as a structural component (Beck et al., 1988; Wolda and Glomset, 1988). Structural studies revealed that this mevalonic acid-derived compound is a farnesyl group attached to the C-terminal cysteine of lamin B via a thioether linkage (Farnsworth et al., 1989), exactly as was found in the fungal mating factors nearly a decade earlier.

Some of the proteins now known to be farnesylated are listed in Table 1 along with their functions. All of these proteins are synthesized in cells in an unmodified form, and this is followed by a series of posttranslational modifications. The first

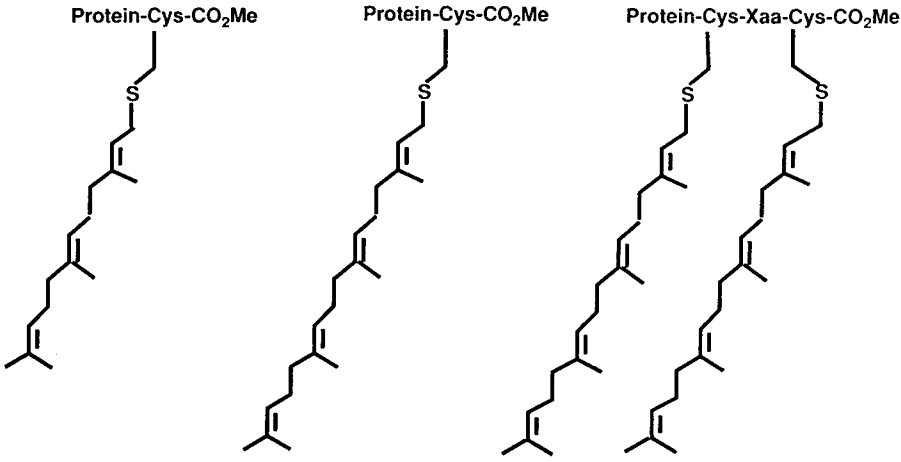

Figure 1. The structure of the C-termini of farnesylated proteins (left), geranylgeranylated proteins (middle), and doubly geranylgeranylated proteins (right). Xaa designates a variety of different amino acids.

step is the farnesylation of the cysteine residue that is part of the C-terminal sequence motif, CaaX (a is often, but not necessarily, an aliphatic amino acid and X designates a variety of different amino acids) (Figure 3). The CaaX sequence, by itself, seems to be the signal for the subsequent protein modification events. The first step is the attachment of the farnesyl group to the cysteine of the CaaX sequence. This reaction occurs in the cytosol of cells and is catalyzed by an enzyme called protein farnesyltransferase (Reiss et al., 1990). This enzyme uses the cholesterol pathway intermediate, farnesyl pyrophosphate, as the source of the farnesyl group which is transferred to the sulfhydryl group of the cysteine residue (Figure 3). After farnesylation, the last three amino acids, aaX, are removed as an intact tripeptide by a membrane-bound prenyl protein-specific endoprotease (Figure 3) (Ashby et al., 1992; Ma and Rando, 1992; Jang et al., 1993). The last step in the maturation pathway is the methylation of the α-carboxyl group of the now C-terminal farnesylated-cysteine residue (Figure 3). This methylation is catalyzed by a membrane-bound methyltransferase that uses S-adenosylmethionine as the source of the methyl group (Stephenson and Clarke, 1990).

The farnesyl group is not the only prenyl group attached to proteins in eukaryotic cells. The more prevalent protein-bound prenyl group is the 20-carbon geranylgeranyl group which is one isoprene unit longer than the farnesyl group (Figure 1). This type of modification was first discovered in mammalian cells and is now thought to occur in all eukaryotic cells, including fungi (Farnsworth, et al., 1990; Rilling et al., 1990). Like farnesyl groups, geranylgeranyl groups are attached via a thioether linkage to the cysteine sulfhydryl of a CaaX C-terminal motif, and this is followed by proteolysis and methylation. Some of the proteins known to be

Isoprenoid Metabolism in Animals

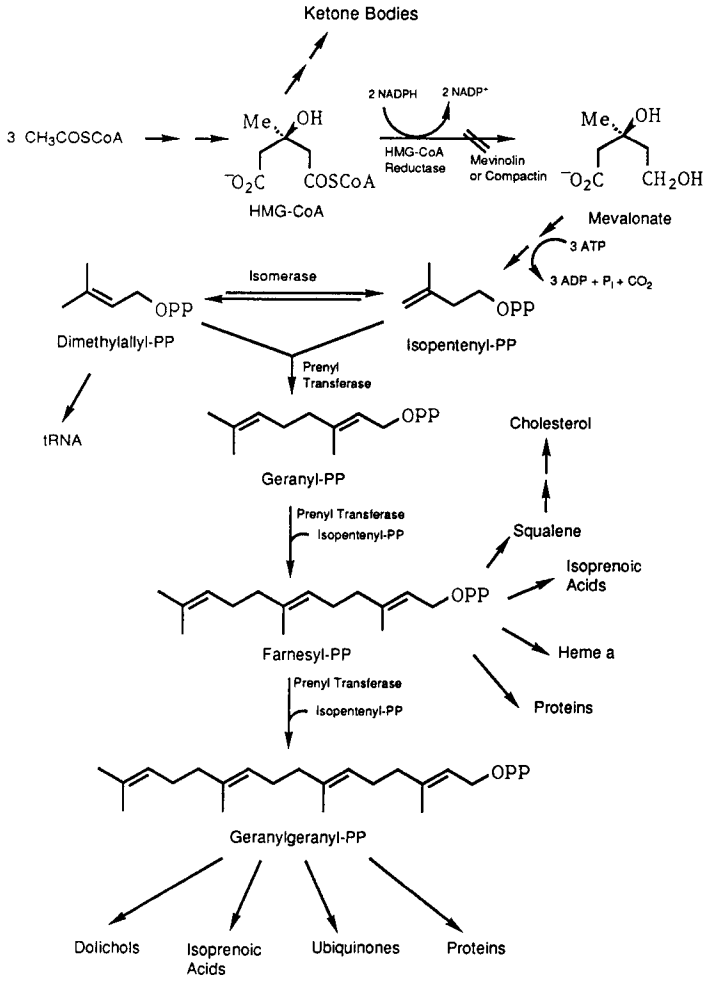

Figure 2. The mevalonic acid biosynthetic pathway. The transformation of hydroxymethyl-coenzyme A (HMG-CoA) to mevalonic acid is the first committed step of the pathway. The enzyme, HMG-CoA reductase, catalyzes this step and is inhibited by the compounds, mevinolin and compactin. Note that farnesyl-pyrophosphate (Farnesyl-PP), the substrate of the protein, farnesyltransferase, can be used to make cholesterol or elongated to make geranylgeranyl-pyrophosphate (Geranylgeranyl-PP). The later compound is the substrate for the protein, geranylgeranyltransferase, or is further elongated to make the long-chain isoprenoids, dolichols, ubiquinones, and isoprenoic acids.

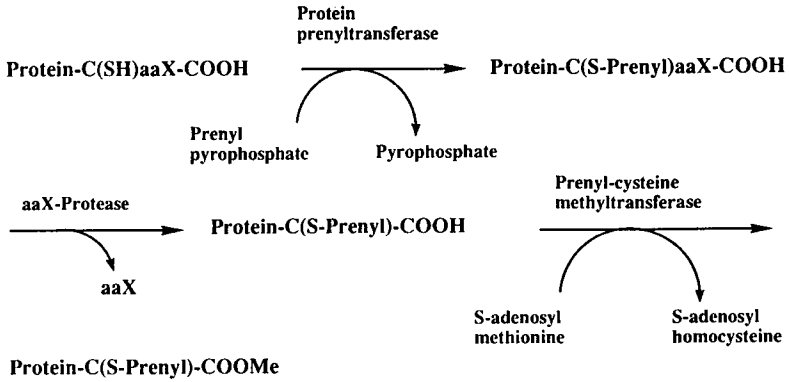

Figure 3. The 3-step pathway for the posttranslational processing of the C-termini of prenylated proteins that contain the CaaX-motif.

geranylgeranylated are listed in Table 1. Geranylgeranylation of proteins occurs in the cytoplasm of cells and is catalyzed by the enzyme, protein geranylgeranyltransferase-I, which uses geranylgeranyl pyrophosphate as the prenyl-group donor (Moomaw and Casey, 1992; Yokoyama and Gelb, 1993). Geranylgeranyl pyrophosphate is a normal metabolite of the mevalonic acid pathway. The synthesis of this species from farnesyl pyrophosphate is the first committed step for the synthesis of long-chain isoprenoids such as dolichols and ubiquinones (see Figure 2).

The X residue of the CaaX sequence motif is the major determinant for specifying the type of prenyl group that is attached to proteins. For example, when X is S, Q, M, A, and probably a few other residues, the protein is farnesylated. All of the known geranylgeranylated proteins contain L as the X residue. Interestingly, if the X residue of a normally farnesylated protein is changed to L, the protein is now geranylgeranylated. Likewise, if the X-residue of a normally geranylgeranylated protein is changed to S, for example, the protein now becomes farnesylated.

Table 1. Some Farnesylated Proteins

Protein Name	*Function*	*Reference*
ras	cell growth, insulin response	see text
transducin	visual transduction	see text
rhodopsin kinase	visual transduction	see text
fungal mating factors	mating pheromone	see text
lamin B	forms part of the nuclear envelope protein matrix	see text
cyclic GMP phosphodiester α-subunit	visual transduction	see text
phosphorylase kinase α- and β-subunits (membrane-bound form)	regulation of muscle calcium-transport ATPase	see text

Such specificity results have been observed in living cells, as well as *in vitro*, with purified protein prenyltransferases.

There is a third structural class of prenylated proteins exemplified by a protein called rab 3A. This protein is a member of the *ras*-like superfamily of small GTP-binding proteins. Rab 3A is localized on the synaptic vesicle membranes in neurons where it plays a role in the fusion of the vesicles with the plasma membrane in the process of neurotransmitter release. This protein has a C-terminal sequence of CAC and is geranylgeranylated on the sulfhydryl groups of both cysteine residues, as well as methylated on the C-terminal residue (Figure 1). A distinct protein, geranylgeranyltransferase, has been identified that operates on rab proteins (Seabra et al., 1993). This rab geranylgeranyltransferase recognizes structural features of the protein substrates other than the C-terminus since peptides having a sequence of the C-terminal position of rab proteins are not substrates. This is in marked contrast to the protein farnesyltransferase, and the protein geranylgeranyltransferase, described above which do prenylate short peptides. Interestingly, patients suffering from the retinal degeneration disease, choroideremia, are deficient in one of the protein subunits of rab geranylgeranyltransferase activity and lack the activity of this enzyme (Seabra et al., 1993).

BIOLOGICAL FUNCTIONS OF PROTEIN PRENYL GROUPS

An understanding of the biological functions of protein prenyl groups is only starting to emerge. It has already been mentioned that prenylation causes proteins to bind to cellular membranes, but it is not yet known whether the membrane attachment is via the interaction of the hydrophobic prenyl group with phospholipids in the membrane or whether the prenyl group binds to other proteins already present in the membrane. Recent research on the role of protein prenylation for a few proteins is discussed below and more examples can be found in the references listed in Table 2.

Visual Transduction

The visual transduction system of retinal membranes contains a plethora of prenylated proteins. One of the prenylated components is a protein called transducin. This protein is a member of a family of G proteins that contain three distinct subunits (α, β, and γ) and function in mediating signal transduction from cell membrane receptors to downstream effector proteins. In the case of visual transduction, photoactivation of rhodopsin leads to a conformational change that is sensed by transducin. In the absence of photoactivation of rhodopsin, transducin exists in a "resting" state in which GDP is bound to its α-subunit. Photoactivated rhodopsin catalyzes the exchange of GDP bound to transducin with GTP. This GTP-bound "activated" transducin then stimulates the enzymatic activity of another membrane-bound protein termed cyclic GMP phosphodiesterase. The latter

enzyme, when activated, hydrolyzes soluble cyclic GMP, and the decrease in the intracellular concentration of this compound leads to the closure of cyclic GMP-regulated cation channels, resulting in a decrease in Na^+ conductance and hyperpolarization of the rod cell. The γ-subunit of transducin contains a C-terminal S-farnesyl-cysteine methyl ester (Fukada et al., 1990; Lai et al., 1990). These C-terminal modifications to the γ-subunit are important in promoting its interaction with GDP-bound form of the transducin α-subunit, and this effect is seen with the purified protein components in the absence of membranes (Fukada et al., 1990). This suggests that the C-terminal modifications play a role in promoting protein–protein interactions. The farnesyl group is also important for the membrane binding of transducin. In the absence of photoactivation of rhodopsin, transducin is partitioned between the membrane and aqueous phases of retinal cells. After photoactivation, most of the transducin translocates to the membrane and this effect requires that the protein be farnesylated (Ohguro et al., 1991). C-terminal methylation also enhances membrane binding of transducin. Thus, the C-terminal modifications on the γ-subunit of transducin are not sufficient to promote strong membrane binding. Additional interactions of transducin with membrane-bound photoactivated, but not resting, rhodopsin are required for membrane translocation as well.

Another farnesylated protein involved in visual transduction is rhodopsin kinase. This enzyme selectively phosphorylates photoactivated rhodopsin and this terminates its interaction with transducin. Thus, rhodopsin kinase is responsible for terminating the visual process in those retinal cells that have been activated by light, so that one does not continue to see what one has seen in the past. Like transducin, farnesylated rhodopsin kinase binds weakly to membranes, but translocation to the membranes is enhanced when rhodopsin is photoactivated, and this requires that rhodopsin kinase be farnesylated (Inglese et al., 1992).

Table 2. Some Geranylgeranylated Proteins

Protein Name	*Function*	*Reference*
heterotrimeric G protein γ-subunit	receptor-mediated signal transduction	(Yamane et al., 1990)
cyclic GMP phosphodiester β-subunit	visual transduction	see text
rab	intracellular vesicle trafficking and secretion	(Farnsworth et al., 1991)
rap	precise function is unknown, but it can reverse ras-dependent cell transformation	(Buss et al., 1991)
rac	regulation of NADPH oxidase, the enzyme that produces cytotoxic superoxide by certain leukocytes	(Kinsella et al., 1991)
ral	function unknown	(Kinsella et al., 1991)
G25K	function in mammalian cells is unknown, cell budding in yeast	(Yamane et al., 1991)

The cyclic GMP phosphodiesterase described above is also prenylated. This enzyme is composed of four subunits (α, β, and two-γ subunits). Recent studies have shown that the α-subunit contains a C-terminal farnesyl-cysteine methyl ester, whereas the β-subunit is geranylgeranylated (Anant et al., 1992). The C-terminal region of the α-subunit has also been shown to be important for attachment of the phosphodiesterase to the retinal membranes.

Ras Proteins

Ras proteins have been intensively studied because certain mutated forms of these proteins cause oncogenic transformation of various types of eukaryotic cells. These proteins reside primarily on the intracellular face of the plasma membrane. *Ras* proteins are now known to play key roles in signal transduction processes that lead, for example, to cell proliferation and cellular responses to insulin.

It has been known for more than a decade that a subset of *ras* proteins (H-*ras* and N-*ras*) contain a covalently attached palmitoyl group. Both of these proteins contain a C-terminal CaaX sequence and mutation of the cysteine to serine abolishes both the palmitoylation and membrane binding properties. This provided circumstantial evidence that the palmitoyl group resides on the cysteine residue of the CaaX sequence, but recent studies indicate that this is not correct. It is now known that both H-*ras* and N-*ras* are first farnesylated on this cysteine, and this is followed by palmitoylation of cysteines that lie a few residues on the N-terminal side of the farnesylated-cysteine (Casey et al., 1989; Hancock et al., 1989; Schafer et al., 1990) (Figure 4). Sometime during this dual lipidation, the *ras* proteins are also proteolyzed to remove the C-terminal aaX sequence, and the α-carboxyl group of the farnesyl-cysteine residue is methylated. Farnesylation is thus a prerequisite to palmitoylation, and the latter modification increases the affinity of *ras* proteins for membranes (Hancock et al., 1990). Interestingly, K-*ras* contains a C-terminal

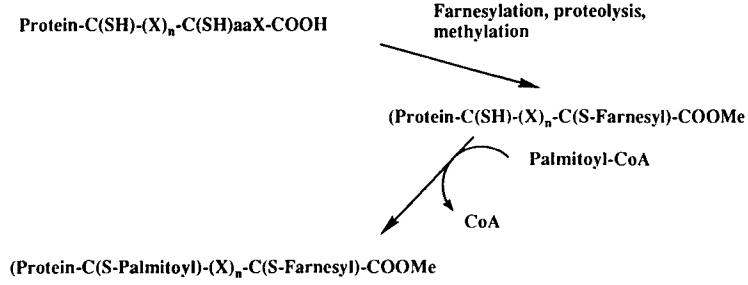

Figure 4. Posttranslational C-terminal modifications of *ras* proteins that are prenylated and palmitoylated (H-*ras* and N-*ras*). The residues $(X)_n$ designate the fact that the two-cysteine residues that are lipidated are separate by a variable distance.

CaaX sequence and is farnesylated, proteolyzed, and methylated, but this protein lacks upstream cysteines and is thus not palmitoylated. Instead, K-*ras* has a run of several lysine residues that have been shown to increase the affinity of the protein for the membrane, possibly by electrostatic interactions with the negatively charged phospholipid bilayer (Hancock et al., 1990).

It is now clear that farnesylation of *ras* proteins is absolutely essential for transformation of cells expressing oncogenic forms of these proteins (Cox and Der, 1992), and there are ongoing efforts worldwide to identify inhibitors of the protein, farnesyltransferase, that attaches the farnesyl group to *ras* proteins and other farnesylated proteins. Farnesylation of *ras* proteins together with either palmitoylation or a polylysine sequence, directs these proteins to the plasma membrane, but the basis for the membrane-selective targeting is not known. There have been numerous recent breakthroughs in the identification of proteins that are part of the *ras*-dependent signal transduction pathway in eukaryotic cells, and the possible role of the farnesyl group in promoting the interaction of *ras* proteins with other proteins in the pathway can now be addressed. *Ras* proteins, like transducin, contain bound GDP, and activation of the *ras* proteins is accomplished by replacement of the GDP by GTP. It is now clear that the activated GTP-bound form of *ras* leads eventually to the activation of a protein kinase called mitogen-activated protein kinase (MAP-kinase), and this latter enzyme plays a critical role in the regulation of gene transcription in the nucleus. Interestingly, *ras*-dependent activation of MAP-kinase has been demonstrated in membrane-free cell homogenates, and the farnesyl group is essential for this activation. This result shows that the farnesyl group of *ras* can be important for *ras*-dependent processes that do not occur in membranes; the farnesyl group may dictate protein–protein interactions.

SUMMARY

Both farnesyl (15-carbon) and geranylgeranyl (20-carbon) prenyl groups are covalently attached to cysteine residues of a subset of proteins in eukaryotic cells. Prenyl groups are derived from mevalonic acid, which is also the starting material for the biosynthesis of cholesterol. These posttranslational modifications occur at the C-terminus of the protein, and the modified-cysteine residue is often methylated on its α-carboxyl group. Many prenylated proteins such as *ras*, transducin, and heterotrimeric G proteins are involved in signal transduction across cell membranes. Prenyl groups most likely help to anchor proteins in membranes, but in many cases, protein–protein interactions are also required to support tight membrane binding. In the case of *ras* proteins, the farnesyl group is required for the functions of this protein that occur in the absence of membranes. This suggests that prenyl groups may act as molecular handles for the recognition by other proteins.

REFERENCES

Anant, J. A., Ong, O. C., Xie, H., Clarke, S., O'Brien, P. J., & Fung, B. K.-K. (1992). *In vivo* differential prenylation of retinal cyclic GMP phosphodiesterase catalytic subunits. J. Biol. Chem. 267, 687–690.

Ashby, M. N., King, D. S., & Rine, J. (1992). Endoproteolytic processing of a farnesylated peptide *in vitro*. Proc. Natl. Acad. Sci. USA 89, 4613–4617.

Beck, L. A., Hosick, T. J., & Sinensky, M. (1988). Incorporation of a product of mevalonic acid metabolism into proteins of Chinese hamster ovary cell nuclei. J. Cell. Biol. 107, 1307–1316.

Buss, J. E., Quilliam, L. A., Kato, K., Casey, P. J., Solski, P. A., Wong, G., Clark, R., McCormick, F., Bokoch, G. M., & Der, C. J. (1991). The COOH-terminal domain of the Rap1A (Krev-1) protein is isoprenylated and supports transformation by an H-*ras*:Rabp1A chimeric protein. Mol. Cell. Biol. 11, 1523–1530.

Casey, P. J. (1992). Biochemistry of protein prenylation. J. Lipid Res. 33, 1731–1740.

Casey, P. J., Solski, P. A., Der, C. J., & Buss, J. E. (1989). p21*ras* is modified by a farnesyl isoprenoid. Proc. Natl. Acad. Sci. USA 86, 8323–8327.

Clarke, S. (1992). Protein isoprenylation and methylation at carboxy-terminal cysteine residues. Ann. Rev. Biochem. 61, 355–386.

Cox, A. D. & Der, C. J. (1992). The *ras*/cholesterol connection: implications for *ras* oncogenicity. Crit. Rev. Oncogenesis 3, 365–400.

Farnsworth, C. C., Wolda, S. L., Gelb, M. H., & Glomset, J. A. (1989). Human lamin B contains a farnesylated cysteine residue. J. Biol. Chem. 264, 20422–20429.

Farnsworth, C. C., Gelb, M. H., & Glomset, J. A. (1990). Identification of geranylgeranyl-modified proteins in HeLa cells. Science 247, 320–322.

Farnsworth, C. C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M. H., & Glomset, J. A. (1991). C-terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. Proc. Natl. Acad. Sci. USA 88, 6196–6200.

Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T., & Shimonishi, Y. (1990). Farnesylated gamma-subunit of photoreceptor G protein indispensable for GTP-binding. Nature 346, 658–660.

Gibbs, J. B. (1991). Ras c-terminal processing enzymes—new drug targets? Cell 65, 1–4.

Glomset, J. A., Gelb, M. H., & Farnsworth, C. C. (1990). Prenyl proteins in eukaryotic cells: a new type of membrane anchor. Trends Biochem. Sci. 15, 139–142.

Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989). All *ras* proteins are polyisoprenylated but only some are palmitoylated. Cell 57, 1167–1177.

Hancock, J. F., Paterson, H., & Marshall, C. J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21^{ras} to the plasma membrane. Cell 63, 133–139.

Heilmeyer, L. M. J., Serwe, M., Weber, C., Metzger, J., Hoffmann-Posorske, E., & Meyer, H. E. (1993). Farnesyl cysteine is a constituent of the alpha and beta subunits of rabbit skeletal muscle phosphorylase kinase. Proc. Natl. Acad. Sci. USA 89, 9554–9558.

Inglese, J., Koch, W. J., Caron, M. G., & Lefkowitz, R. J. (1992). Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. Nature 359, 147–150.

Jackson, J. H., Cochrane, C. G., Bourne, J. R., Solski, P. A., Buss, J. E., & Der, C. J. (1990). Farnesol modification of kirsten-*ras* exon 4B protein is essential for transformation. Proc. Natl. Acad. Sci. USA 87, 3042–3046.

Jang, G.-F., Yokoyama, K., & Gelb, M. H. (1993). A prenyl protein-specific endoprotease in rat liver microsomes that produces a carboxy-terminal tripeptide. Biochemistry 32, 9500–9507.

Kamiya, Y., Sakurai, A., Tamura, S., & Takahashi, N. (1979). Structure of rhodotorucine A, a peptidyl factor, inducing mating tube formation in *Rhodosporidium toruloides*. Agric. Biol. Chem. 43, 1049–1053.

Khosravi-Far, R., Lutz, R. J., Cox, A. D., Conroy, L., Bourne, J. R., Sinensky, M., Balch, W. E., Buss, J. E., & Der, C. J. (1991). Isoprenoid modification of rab proteins terminating in CC/CXC motifs. Proc. Natl. Acad. Sci. USA 88, 6264–6268.

Kinsella, B. T., Erdman, R. A., & Maltese, W. A. (1991). Carboxyl-terminal isoprenylation of *ras*-related GTP-binding proteins encoded by rac1, rac2, and ralA. J. Biol. Chem. 266, 9786–9794.

Lai, R. K., Perez-Sala, D., Canada, F. J., & Rando, R. R. (1990). The γ subunit of transducin is farnesylated. Proc. Natl. Acad. Sci. USA 87, 7673–7677.

Ma, Y.-T. & Rando, R. R. (1992). A microsomal endoprotease that specifically cleaves isoprenylated peptides. Proc. Natl. Acad. Sci. USA 89, 6275–6279.

Maltese, W. A. & Sheridan, K. M. (1987). Isoprenylated proteins in cultured cells: subcellular distribution and changes related to altered morphology and growth arrest induced by mevalonate deprivation. J. Cell Physiol. 133, 471–481.

Moomaw, J. F. & Casey, P. J. (1992). Mammalian protein geranylgeranyltransferase. Subunit composition and metal requirements. J. Biol. Chem. 267, 17438–17443.

Ohguro, H., Fukada, Y., Takao, T., Shimonishi, Y., Yoshizawa, T., & Akino, T. (1991). Carboxyl methylation and farnesylation of transducin γ-subunit synergistically enhances its coupling with metarhodopsin II. EMBO J. 10, 3669–3674.

Olson, E. N. (1988). Modification of proteins with covalent lipids. Prog. Lipid. Res. 27, 177–197.

Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., & Brown, M. S. (1990). Inhibition of purified $p21^{ras}$ farnesyl:protein transferase by cys-AAX tetrapeptides. Cell 62, 81–88.

Rilling, H. C., Bruenger, F., Epstein, W. W., & Crain, P. F. (1990). Prenylated proteins: the structure of the isoprenoid group. Science 247, 318–320.

Sakagami, Y., Isogai, A., Suzuki, A., Tamura, S., Kitada, C., & Fujino, M. (1979). Structure of tremerogen A-10, a peptidal hormone inducing conjugation tube formation in *Tremella mesenterica*. Agric. Biol. Chem. 43, 2643–2645.

Schafer, W. R., Trueblood, C. E., Yang, C.-C., Mayer, M. P., Rosenberg, S., Poulter, C. D., Kim, S.-H., & Rine, J. (1990). Enzymatic coupling of cholesterol intermediates to a mating pheromone precursor and to the *ras* protein. Science 249, 1133–1139.

Schmidt, R. A., Schneider, C. J., & Glomset, J. A. (1984). Evidence for post-translational incorporation of a product of mevanolic acid into Swiss 3T3 cell proteins. J. Biol. Chem. 259, 10175–10180.

Seabra, M. C., Brown, M. S., & Goldstein, J. L. (1993). Retinal degeneration in choroideremia: deficiency of rab geranylgeranyl transferase. Science 259, 377–381.

Sepp-Lorenzino, L., Azrolan, N., & Coleman, P. S. (1989). Cellular distribution of cholesterogenesis-linked, phosphoisoprenylated proteins in proliferating cells. FEBS Lett. 245, 110–116.

Sinensky, M. & Logel, J. (1985). Defective macromolecule biosynthesis and cell-cycle progression in a mammalian cell starved for mevalonate. Proc. Natl. Acad. Sci. USA 82, 3257–3261.

Stephenson, R. C. & Clarke, S. (1990). Identification of a C-terminal protein carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesyl cysteine containing peptide substrate. J. Biol. Chem. 265, 16248–16254.

Wolda, S. L. & Glomset, J. A. (1988). Evidence for modification of lamin B by a product of mevalonic acid. J. Biol. Chem. 263, 5997–6000.

Yamane, H. K., Farnsworth, C. C., Xie, H., Howald, W., Fung, B. K.-K., Clarke, S., Gelb, M. H., & Glomset, J. A. (1990). Brain G protein γ subunits contain an all-*trans*-geranylgeranyl cysteine methyl ester at their carboxyl termini. Proc. Natl. Acad. Sci. USA 87, 5868–5872.

Yamane, H. K., Farnsworth, C. C., Xie, H., Evans, T., Gelb, M. H., Glomset, J. A., Clarke, S., & Fung, B. K.-K. (1991). Membrane-binding domain of the small G protein G25K contains an S-(all-*trans*-geranylgeranyl)cysteine methyl ester at its carboxyl terminus. Proc. Natl. Acad. Sci. USA 88, 286–290.

Yokoyama, K. & Gelb, M. H. (1993). Purification of a mammalian protein geranylgeranyltransferase. J. Biol. Chem. 268, 4055–4060.

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Chapter 15

Lipobiology

DAVID A. FORD and RICHARD W. GROSS

INTRODUCTION

The evolutionary development of an integrated network of specialized compartments in mammalian cells has facilitated the development of biologic control systems which evaluate, store, and process chemical information. Mammalian membranes are macromolecular lipid complexes which physically separate specialized intracellular compartments, provide an interfacial matrix for the interaction of

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hydrophobic constituents, and store the chemical precursors of lipid-derived second messengers which communicate information between cellular compartments.

Lipid-derived second messengers of signal transduction are typically short-lived lipid metabolites synthesized from membrane-derived lipid precursors in response to cellular stimulation (e.g., ligand–receptor coupling, electrical stimulation, and elevation in intracellular calcium). The production of lipid-derived second messengers is initiated by activation of intracellular phospholipases which liberate metabolites capable of propagating a cell-specific cascade of biochemical events that collectively result in cellular activation. Each cell's response to a given stimulus is individually tailored to its biological function by specific genetic programming to ensure that the appropriate functional complement of phospholipases, oxygenases, and receptors are available to allow each cell to fulfill its physiological function.

Although biologic membranes serve as physical barriers, it is important to recognize that their molecular constituents are in a constant state of motional flux. Many different types of molecular motion are present in biological membranes including rotation, translation and libration, each of which contributes in important ways to the physical properties of cellular membranes. Since alterations in membrane physical properties have profound effects on the kinetics of many transmembrane enzymes and modulate the rates and types of interactions between proteins, it comes as no surprise that the molecular dynamics of a cell membrane is an important modulator of signal transduction (e.g., Lenaz, 1987). Thus, biological

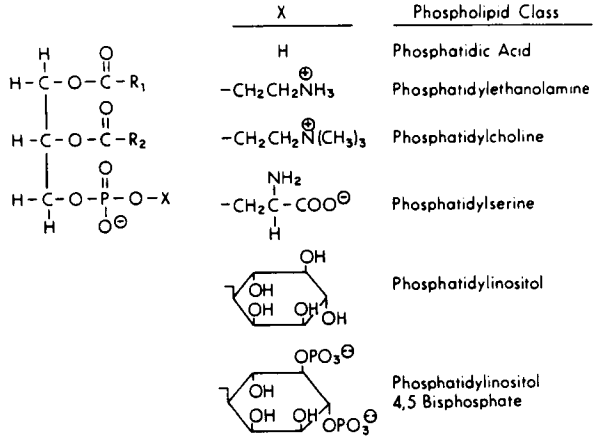

Figure 1. Phospholipid classes. Phospholipid classes are categorized by the polar head group (X) covalently attached to the *sn*-3 carbon of the glycerol backbone by a phosphodiester bond. The *sn*-1 aliphatic chain (R_1) is usually a saturated long-chain moiety (e.g., palmitic or stearic acids), while the *sn*-2 aliphatic chain (R_2) usually contains long-chain unsaturated aliphatic moieties (e.g., oleic, linoleic, or arachidonic acids).

membranes have multiple functional roles. The macromolecular lipid complex provides an efficient physical boundary between cellular compartments, while specific chemical entities which comprise this boundary serve as substrates for the enzymes which transduce chemical information. Furthermore, biological membranes simultaneously provide an appropriate matrix for the interaction of multiple cellular constituents to facilitate the precise interaction of water-soluble and membrane-bound chemical entities which mediate signal transduction.

To understand how biological membranes fulfill these multiple functional roles, it is first necessary to consider the diverse nature of their chemical structures. In large part, biological membranes are comprised of polar lipids (e.g., phospholipids), nonpolar lipids (e.g., cholesterol), and protein. The chemical structure of most mammalian membrane phospholipids is based on a glycerol backbone to which two aliphatic chains and one polar head group are covalently attached by two ester and one phosphodiester linkages, respectively. Phospholipids are divided into individual phospholipid classes by the nature of their polar head group constituents (Figure 1). Each phospholipid class, in turn, is divided into three distinct subclasses based upon the covalent nature of attachment of the aliphatic chain to the *sn*-1 glycerol hydroxyl group (Figure 2). Although the majority of mammalian phospholipids in most cell types consist of diacyl-phospholipids, some subcellular membranes are predominantly comprised of ether-linked choline- and/or ethanolamine-glycerophospholipids (e.g., myocardial sarcolemma; Gross, 1984). The diversity of mammalian phospholipids is further amplified by the presence of multiple aliphatic constituents (different chain-lengths and degrees of unsaturation) which are covalently linked to the *sn*-1 and *sn*-2 positions. Thus, mammalian phospholipids are comprised of hundreds of distinct molecules which differ in their polar head group composition (class), covalent attachment to the *sn*-1 position (subclass), and aliphatic constituents at the *sn*-1 and *sn*-2 positions (individual molecular species).

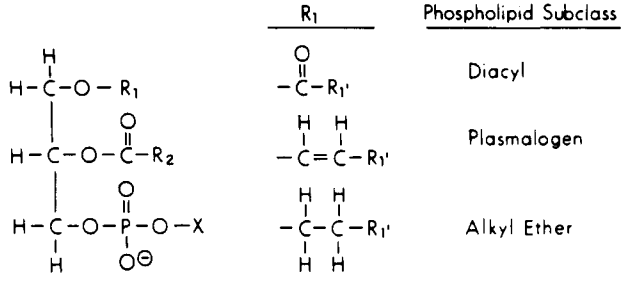

Figure 2. Phospholipid subclasses. Phospholipid subclasses are categorized by the type of covalent attachment of aliphatic constituents to the *sn*-l carbon of the glycerol backbone. The aliphatic chain of diacyl, plasmalogen, and alkyl-ether subclasses of phospholipids contain an ester, vinyl-ether, or alkyl-ether bond at the *sn*-l position, respectively.

Each individual molecular species of phospholipid possesses distinct physical properties and affinities for the enzymes involved in signal transduction pathways. Accordingly, one current goal of biomembrane and signal transduction research is to identify how such diversity contributes to the specificity and complexity of the signal transduction process.

LIPID-DERIVED MEDIATORS OF SIGNAL TRANSDUCTION

Eicosanoids

Eicosanoids are a family of oxygenated arachidonic acid derivatives which interact with specific cellular receptors to amplify and propagate the flow of biological information. One unifying biochemical mechanism of signal transduction in the majority of mammalian cells is the release of arachidonic acid from endogenous phospholipid storage pools after ligand–receptor coupling (cf, Samuelsson et al., 1978). The concentration of free arachidonic acid in resting cells is diminutive since the overwhelming majority of cellular arachidonic acid is covalently linked to the *sn*-2 position of phospholipids (including choline-, ethanolamine-, and inositol-glycerophospholipids). Esterified arachidonic acid is released from endogenous phospholipid storage pools during cellular activation by intracellular phospholipases. Released arachidonic acid is rapidly oxygenated by one of several oxidative enzymes (e.g., cyclooxygenase or lipoxygenase) to initiate an enzymatic cascade that results in the generation of a multiplicity of different oxygenated metabolites of arachidonic acid (i.e., eicosanoids). Since the eicosanoid-producing oxidative enzymes can not oxygenate esterified arachidonic acid present in phospholipids, arachidonic acid is biologically inactive until it is released from endogenous phospholipids by intracellular phospholipases (Lands and Samuelsson, 1968). Furthermore, since the release of arachidonic acid from endogenous membrane stores by phospholipases is the rate-limiting step in eicosanoid production, it is clear that the extent of phospholipase activation represents a primary biochemical determinant of the magnitude and type of each cell's response to stimulation. Since the K_m of arachidonic acid for either cyclooxygenase or lipoxygenase is well above the concentration of free arachidonic acid in resting cells, the potential for the rapid catalytic amplification of a single chemical stimulus by the activation of phospholipase is inherent in the design of this signal transduction system.

After arachidonic acid is released from endogenous phospholipid stores, a highly specific cascade of enzyme-catalyzed reactions is initiated, leading to the production of a family of structurally related metabolites which have separate and distinct biological functions. These oxygenated eicosanoids are highly lipid soluble; thus, they readily traverse cellular membranes thereby facilitating their interaction with receptors on adjacent cells (paracrine) or with receptors on their cell of origin

(autocrine). To add to the complexity of this system, arachidonic acid may be released from phospholipid stores in one cell type and subsequently enter an adjacent cell where it can be oxygenated. The precise complement of eicosanoid-specific oxidative and degradative enzymes which each cell possesses determines the type of eicosanoid a cell produces, its relative rate of accumulation, and its biological half-life. Thus, in a real sense, mammalian cells are poised to respond to a multiplicity of stimuli with a genetically predetermined program tailored to each cell's physiological function.

This section will examine mechanisms through which eicosanoids regulate biological systems by focusing on: 1) the release of free arachidonic acid by phospholipases, 2) the generation of bioactive metabolites initiated through the actions of cyclooxgenase and lipoxygenase, and 3) the biochemical mechanisms responsible for the inactivation of these potent metabolites.

Generation of Arachidonic Acid by Phospholipases

Since eicosanoid production is limited by the availability of free arachidonic acid, it follows that liberation of arachidonic acid from membrane phospholipids is the rate-limiting step for the formation of biologically active eicosanoid metabolites. Arachidonic acid may be released directly by the action of phospholipase A_2 or by sequential enzymic reactions initiated by phospholipase-A_1, -C or -D. Over the last two decades, the existence of many separate and distinct types of intracellular phospholipase activities have been characterized (cf, Dennis, 1994). Due to its obvious physiological importance, the precise identification of the phospholipases involved in the release of arachidonic acid during signal transduction has been the subject of intense investigation. Recent experiments have demonstrated that arachidonic acid released during cellular stimulation typically results from activation of at least two types of phospholipase activities, phospholipase A_2 and phospholipase C (Figure 3). For example, platelet activation by thrombin results in the highly selective release of arachidonic acid from endogenous phospholipids, accompanied by the concomitant accumulation of lysophospholipids, diglycerides, and phosphatidic acid (Broekman et al., 1980). Accumulation of these intermediates within seconds after agonist stimulation, demonstrates that both phospholipase A_2 and phospholipase C are activated. However, the relative contributions of each of these phospholipases to the net mass of arachidonic acid released during platelet stimulus-response coupling has been the subject of intense controversy. The hypothesis that phospholipase C-mediated hydrolysis of phosphatidylinositol was the major enzymic pathway responsible for arachidonic acid release was initially received enthusiastically because of the known rapid turnover of the phosphatidylinositol pool in stimulated platelets and the marked enrichment of arachidonic acid in platelet phosphatidylinositol molecular species. However, during the last several years this enthusiasm has been tempered by several independent pieces of experi-

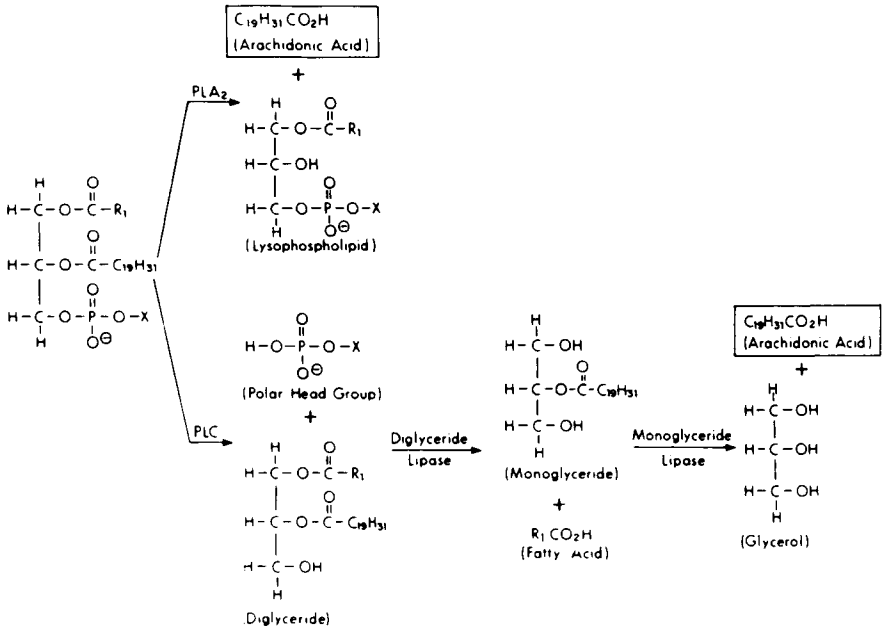

Figure 3. Arachidonic acid release is mediated by phospholipase A_2 or phospholipase C. Arachidonic acid esterified to the *sn*-2 position of phospholipids can be released directly by the action of phospholipase A_2 or by the sequential actions of phospholipase C, diglyceride lipase, and monoglyceride lipase.

mental evidence, including the demonstration that the mass of arachidonic acid released during thrombin stimulation of platelets exceeded the mass of the entire phosphatidylinositol pool, and that the decreases in choline- and ethanolamine-glycerophospholipid mass during platelet stimulation could account for over 70% of released arachidonic acid (Purdon and Smith, 1985). Accordingly, attention has subsequently refocused on the role of phospholipase A_2 as the major enzymic mediator of arachidonic acid mass released during platelet activation. The recent demonstration of a novel phospholipase A_2 activity in platelets, which selectively hydrolyzes choline- and ethanolamine-glycerophospholipid molecular species enriched in arachidonic acid, and is stimulated by physiologic increments in calcium ion concentration, has implicated this polypeptide as an important contributor to arachidonic acid released during platelet stimulation (Loeb and Gross, 1986). Thus, although there has been substantial debate regarding the phospholipid class, as well as the enzymic mediators responsible for the release of arachidonic acid, it seems likely that the combined actions of both phospholipase A_2 (which utilizes choline- and ethanolamine-glycerophospholipids as substrates) and phospholipase C (which utilizes inositol-glycerophospholipids as substrates) are important contributors to arachidonic acid release during platelet stimulation. In general, the amount of

arachidonic acid released from each phospholipid class in individual cell types during activation is a function of the magnitude and types of activating processes that a signal elicits, the integrity of the coupling of the receptor to the phospholipase, and the relative abundance of each type of phospholipase activity present.

Cyclooxygenase Reaction Products

PGG_2 and PGH_2. Cyclooxygenase catalyzes the incorporation of two molecules of oxygen into one molecule of arachidonic acid. Several excellent reviews are available that describe the reaction mechanism employed by cyclooxygenase, their subsequent metabolism, and their biological activities (cf, Samuelsson et al., 1978; Samuelsson, 1983; Needleman et al., 1986). Prostaglandin production from arachidonic acid is catalyzed by the enzyme prostaglandin endoperoxide synthase (PES), which catalyzes the insertion of two oxygen molecules into arachidonic acid (cyclooxygenase activity) to produce the prostaglandin, PGG_2. Subsequently, this same polypeptide can catalyze the reduction of PGG_2 to its 15-hydroxy analog, PGH_2, by an endogenous peroxidase activity (Figure 4). PES requires a hydroperoxide activator and yet is auto-inactivated by a radical-initiated suicide mechanism. This auto-inactivation has been suggested to limit PGG_2 synthesis, and thus, to modulate the biological response to cell activation.

PGH_2 is a branch point intermediate in eicosanoid synthesis since the relative rates of thromboxane synthase, prostacyclin synthase, or prostaglandin isomerase activities are the primary biochemical determinants of the fractional proportion of PGH_2 destined for conversion into thromboxanes, prostacyclin, or other prostaglandins (*vide infra*). Accordingly, PES represents a strategic target for pharmacological manipulation of biological responses to signal transduction, since inhibition of a single enzyme can attenuate flux through several divergent metabolic cascades resulting in the modulation of the production of multiple eicosanoids. PES is inhibited by several mechanistically distinct inhibitors including nonsteroidal anti-inflammatory agents, eicosa-5,8,11,14-tetrynoic acid (ETYA), and aspirin. The cyclooxygenase activity of PES is inhibited by nonsteroidal anti-inflammatory agents, as well as the suicide substrate, ETYA. Aspirin irreversibly inhibits PES by acetylation of a critical serine residue on cyclooxygenase.

Thromboxanes. Since platelets are highly enriched with thromboxane synthase, the principal metabolites of PGH_2 in platelets are metabolites of the thromboxane series. Thromboxane A_2 (TxA_2) production is catalyzed by the enzyme, thromboxane synthase (Figure 4). TxA_2 is a short-lived bioactive metabolite with a biological half-life of less than 30 seconds due to the spontaneous (nonenzymatic) hydrolysis of TxA_2, yielding the inactive, but stable metabolite, thromboxane B_2 (TxB_2). Thromboxane production can be attenuated by several thromboxane synthase inhibitors including imidazole and pyridine derivatives. The predominant biological effects of TxA_2 are the induction of platelet aggregation and smooth

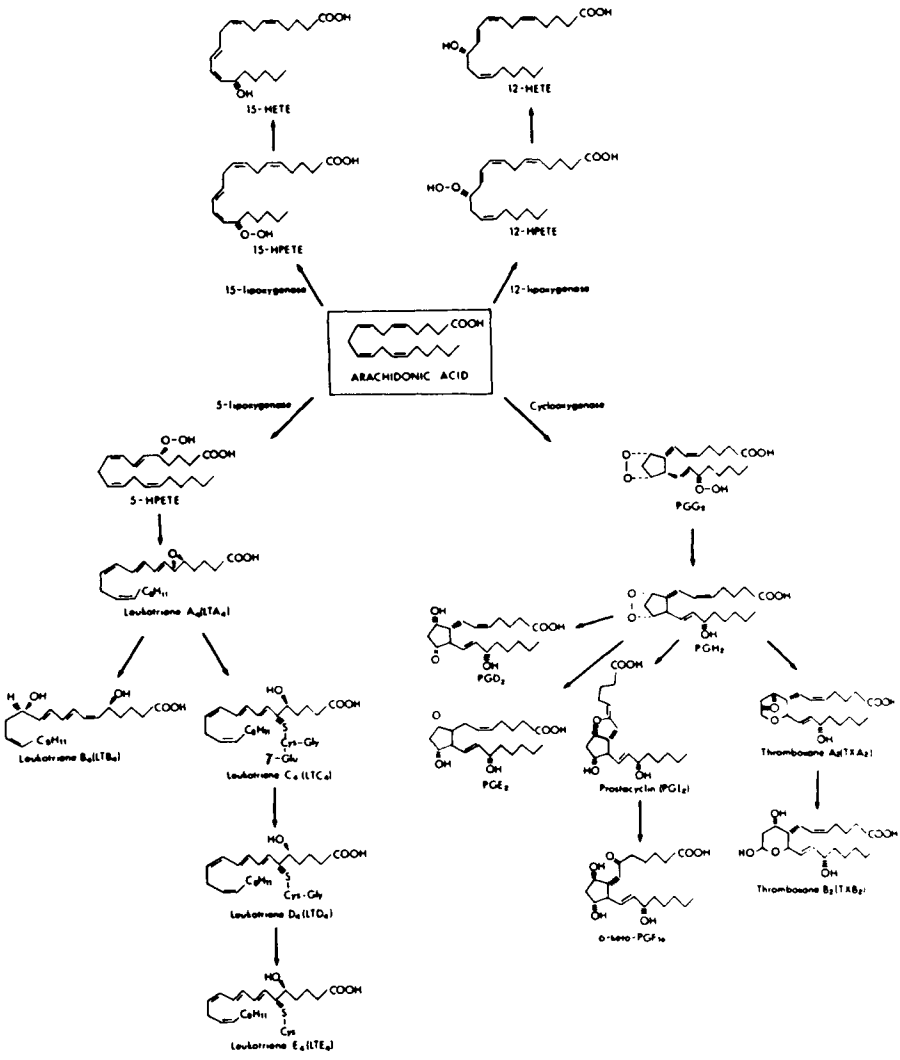

Figure 4. Biosynthetic pathways of eicosanoid production. Unesterified arachidonic acid is oxygenated by cyclooxygenase or by 5-, 12-, or 15-lipoxygenases. Cyclooxygenase catalyzes the incorporation of two molecules of molecular oxygen into arachidonic acid and is the first committed step for the production of prostaglandins and thromboxanes. Alternatively, incorporation of one molecule of oxygen into the 5-position of arachidonic acid (as catalyzed by 5-lipoxygenase) is the first committed step in leukotriene biosynthesis.

muscle contraction. It is likely that TxA_2 and PGH_2 share a common receptor which activates platelets, and that both PGH_2 and TxA_2 analogs act as antagonists at a common TxA_2/PGH_2 receptor.

Prostacyclin. The generation of prostacyclin (PGI_2) from PGH_2 is catalyzed by the enzyme, prostacyclin synthase (Figure 4). PGI_2 is the predominant eicosanoid produced in vascular endothelial cells and it is also synthesized in both vascular and nonvascular smooth muscle. Prostacyclin synthase is inhibited by lipid hydroperoxides as a result of the direct interaction of the hydroperoxide with the heme moiety of prostacyclin synthase. The biological half-life of PGI_2 is very short since it spontaneously hydrolyzes in water to produce the inactive prostaglandin, 6-keto $PGF_{1\alpha}$ (Figure 4). Prostacyclin is a potent vasodilator and is responsible in large part for arachidonate-induced coronary vasodilation. Furthermore, PGI_2 is also a potent inhibitor of platelet aggregation. Since PGI_2 stimulates cAMP accumulation in both smooth muscle and platelets, it seems likely that PGI_2-mediated vasodilation, as well as platelet anti-aggregatory activity result from elevations in intracellular cAMP concentration. It should be noted that PGI_2 and TxA_2 have opposing effects on vascular tone and thrombosis. This has led many investigators to suggest that these two arachidonic acid-derived metabolites serve to feed-back regulate each cell's response to stimulation. For example, platelet activation results in the accumulation of thrombin and ADP, each of which stimulate PGI_2 synthesis by endothelial and vascular smooth muscle cells. Accordingly, it has been suggested that PGI_2 regulates platelet adhesion in regions of vascular injury to prevent an overactive response to tissue injury which could have adverse sequelae (e.g., complete vessel obstruction by a platelet thrombus). Thus, the production of PGI_2 by the endothelial and vascular smooth muscle cells in conjunction with TxA_2 synthesis by platelets regulate, at least in part, vascular wall tone and repair.

PGD_2. PGD_2 isomerase activity catalyzes the conversion of PGH_2 to PGD_2 (Figure 4). It is likely that PGD_2 has an important role in central nervous system physiological function since: 1) PGH_2 to PGD_2 isomerase activity is enriched in the central nervous system, 2) PGD_2 augments cAMP levels and induces depolarization in neuroblastoma cells, 3) PGD_2 attenuates norepinephrine release from noradrenergic nerve terminals, and 4) injection of PGD_2 into the third ventricle of rat brain induces slow-wave sleep.

In addition, PGD_2 has important physiological functions in non-neural tissues. For example, in platelets, PGD_2 has a distinct receptor which inhibits aggregation and elevates cAMP through a specific receptor. PGD_2 is also the predominant eicosanoid produced by mast cells. Additional biological effects of PGD_2 include vasodilation, pulmonary constriction, and bronchoconstriction.

PGE_2. PGH_2 to PGE_2 isomerase activity catalyzes the production of PGE_2 (Figure 4). PGE_2 is the predominant eicosanoid produced in the kidney where it

attenuates antidiuretic hormone-induced water reabsorption and augments renin release. PGE_2 affects the cardiovascular system, both indirectly through renal mechanisms which increase blood volume, and directly by exerting positive-inotropic effects on the heart, as well as by inducing peripheral vasodilation. PGE_2 also contributes to the initiation of labor since PGE_2 levels increase during the third trimester of pregnancy and PGE_2 can independently induce uterine contraction.

Lipoxygenase Reaction Products

Whereas cyclooxygenase catalyzes the incorporation of two oxygen molecules into one molecule of arachidonic acid resulting in the production of prostaglandins and thromboxanes, lipoxygenase catalyzes the incorporation of one oxygen molecule into one molecule of arachidonic acid resulting in the production of 5-HPETE, 12-HPETE, or 15-HPETE. Incorporation of molecular oxygen into the 5-position of arachidonic acid to produce 5-HPETE is the first committed step in the production of the leukotrienes (Figure 4).

Leukotrienes. Leukotrienes are potent, biologically active compounds synthesized in neutrophils, eosinophils, monocytes, mast cells, keratinocytes, lung, spleen, brain, and heart. The biological effects of leukotrienes were identified in 1938 and collectively categorized as the slow-reacting substance (SRS) released from lung after stimulation with cobra venom, and as the slow-reacting substance of anaphylaxis (SRS-A) (Feldberg and Kellaway, 1938; Kellaway and Trethewie, 1940). The chemical structures of the molecular entities which comprise SRS-A were identified as arachidonic acid metabolites with covalently linked peptides, and have been categorized into a class of lipid-derived second messengers known as leukotrienes (Hammarstrom et al., 1979; Murphy et al., 1979). Leukotriene production is initiated by 5-lipoxygenase mediated oxygenation of arachidonic acid which produces 5-HPETE. 5-HPETE is subsequently converted nonenzymatically to the leukotriene, LTA_4 (Figure 4). LTA_4 can be enzymatically hydrolyzed to produce LTB_4 or alternatively, LTA_4 can be converted to LTC_4 by a glutathione transferase-catalyzed addition of glutathione. LTD_4 is produced by the removal of glutamic acid from LTC_4 by the enzyme γ-glutamyl transpeptidase. Next, LTE_4 is produced by the enzymatic removal of a glycine from LTD_4 as catalyzed by a dipeptidase. Since LTC_4, LTD_4, and LTE_4 each have amino acid constituents, they are frequently referred to as peptidoleukotrienes.

The peptidoleukotrienes play a major role in the pathophysiological sequelae of anaphylaxis by exerting profound effects on smooth muscle tone. Peptidoleukotrienes contract respiratory, vascular, and intestinal smooth muscle. Furthermore, peptidoleukotrienes have potent effects on the cardiovascular system including coronary vasoconstriction, reduced heart rate, arteriolar constriction, venule dila-

tion, and plasma exudation (cf, Samuelsson, 1983). In general, LTC_4 and LTD_4 are more potent than LTE_4. The nonpeptidoleukotriene, LTB_4, is a potent chemotactic agent for both neutrophils and eosinophils, and increases vascular permeability. Thus, peptidoleukotrienes are important mediators of acute hypersensitivity reactions.

12-HPETE and 12-HETE. 12-HPETE production is catalyzed by the enzyme 12-lipoxygenase which incorporates molecular O_2 into the 12-position of arachidonic acid. Lipoxygenase has a kinetic lag period and is inhibited by ETYA through a suicide-substrate mechanism. 12-lipoxygenase also has endogenous peroxidase activity that results in the production of 12-HETE. The peroxidase activity of 12-lipoxygenase is inhibited by nonsteroidal anti-inflammatory agents. 12-HPETE inhibits collagen-induced platelet aggregation and stimulates neutrophil leukotriene production. 12-HETE also attenuates platelet-induced migration of smooth muscle, potentially attenuating the development of atherosclerotic lesions. Furthermore, 12-HETE has been suggested to be intimately involved in glucose-induced insulin secretion by pancreatic islet cells.

15-HPETE and 15-HETE. Arachidonic acid is converted to 15-HPETE by the enzyme 15-lipoxygenase. 15-HPETE rapidly decomposes (nonenzymatically) to produce 15-HETE. 15-HETE has been suggested to modulate both 5-lipoxygenase and 12-lipoxygenase activities since 15-HETE inhibits both 5-lipoxygenase and 12-lipoxygenase activities in neutrophils. However, in a mast/basophil cell line, 15-HETE stimulates 5-lipoxygenase and 12-lipoxygenase activity. The mechanisms underlying these tissue-specific effects remain to be elucidated.

Platelet Activating Factor

Platelet activating factor (PAF) was initially described as a factor released from activated leukocytes that induced platelet degranulation. Elegant studies demonstrated that PAF was a choline-glycerophospholipid which contained an alkyl-ether linkage at the *sn*-1 position, and an acetate moiety at the *sn*-2 position (Demopoulos et al., 1979). In parallel studies, a potent hypotensive factor isolated from renal medulla was demonstrated to possess an identical structure (Blank et al., 1979). Although PAF can contain from 14 to 18 carbons in the *sn*-1 alkyl-aliphatic moiety without alterations in its biologic potency, the structural requirements for biological activity in the remainder of the molecule are rigid. Biological potency is virtually lost when the alkyl-ether, acetate, or phosphocholine moieties are altered to structurally related chemical entities. This section, consisting of two parts, will cover the mechanisms responsible for PAF synthesis and catabolism in the first part, and will review some of PAF's physiological and pathophysiological effects in the second part.

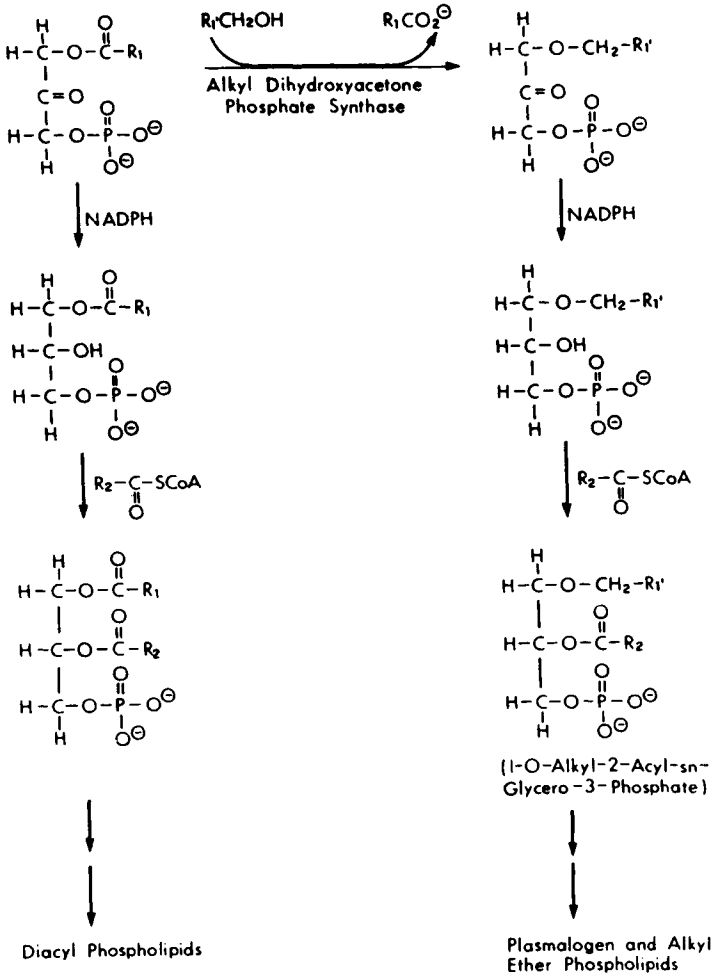

Figure 5. Biosynthetic pathways for diacyl, plasmalogen and alkyl-ether molecular subclasses of phospholipids. Monoacyl dihydroxyacetone phosphate is the key branch-point intermediate whose utilization determines the phospholipid subclass distribution of newly synthesized phospholipids. Reduction of monoacyl dihydroxyacetone phosphate leads to the biosynthesis of diacyl phospholipids. Fatty alcohol exchange, catalyzed by alkyl dihydroxyacetone phosphate synthase, is the first committed step in the biosynthesis of alkyl-ether and plasmalogen subclasses of phospholipids.

Platelet Activating Factor Biosynthesis

A wide variety of cells produce PAF when stimulated including, but not limited to, platelets, basophils, neutrophils, macrophages, and endothelial cells (cf, Snyder, 1982; Hanahan, 1986). An essential biochemical requirement for the production of PAF is that PAF-producing cells contain the enzymic machinery necessary to synthesize the l-O-alkyl bond. Biosynthesis of the l-O-alkyl bond is catalyzed by the enzyme, alkyl dihydroxyacetone phosphate synthase, which utilizes acyl-dihydroxyacetone phosphate and fatty alcohol as substrates to produce alkyl dihydroxyacetone phosphate and fatty acid (Figure 5). The cellular utilization of acyldihydroxyacetone phosphate is the primary determinant of the specific subclass distribution of each cell's phospholipid constituents (e.g., diacyl, alkyl-ether, or plasmalogen subclasses of phospholipids) (Figure 5). Through multiple biosynthetic steps, alkyl dihydroxyacetone phosphate is converted into l-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkyl-acyl-GPC). The *sn*-2 fatty acid of alkyl-acyl-GPC is highly enriched in arachidonic acid due to the selective incorporation of arachidonic acid into alkyl-acyl-GPC during *de novo* synthesis or phospholipid remodeling (phospholipid deacylation–reacylation cycles).

Three mechanisms for PAF biosynthesis occur in mammalian cells (Figure 6). PAF can be synthesized from endogenous alkyl-acyl-GPC by the initial hydrolysis of the *sn*-2 fatty acid (typically arachidonic acid) by phospholipase A_2, followed by acetylation of lyso-PAF to produce PAF (Figure 6, Mechanism I) (Wykle et al., 1980). Recently, the strategy engendered by Mechanism I has been expanded to describe a modified mechanism for the generation of lyso-PAF. Lyso-PAF biosynthesis in neutrophils and HL60 cells is also initiated by PLA_2-catalyzed hydrolysis of plasmenylethanolamine and completed by the direct transfer of arachidonic acid from 1-O-alkyl-2-arachidonyl-GPC to lysoplasmenylethanolamine through a transacylase reaction (Nieto et al., 1991; Uemura et al., 1991). Alternatively, PAF can be synthesized through a *de novo* pathway in which 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphate is sequentially dephosphorylated and acetylated (to produce 1-O-alkyl-2-acetyl-*sn*-glycerol) prior to its condensation with CDP-choline as catalyzed by choline phosphotransferase (Figure 6) (Lee et al., 1986). All of these biosynthetic pathways contribute to PAF production during cellular stimulation and the relative flux through each pathway is determined by the specific enzymic machinery present in each cell type. It is important to note that the first pathway (*sn*-2 hydrolysis of 1-O-alkyl-2-acyl-GPC and subsequent *sn*-2 acetylation) typically results in the concomitant production of both PAF and arachidonic acid, while the second mechanism results only in PAF synthesis. Thus, two mediators of signal transduction are concomitantly released by a single enzymic reaction. The relative amounts of arachidonic acid and PAF that a cell produces in response to stimulation is determined by the subclass specificities of the phospholipase which is activated,

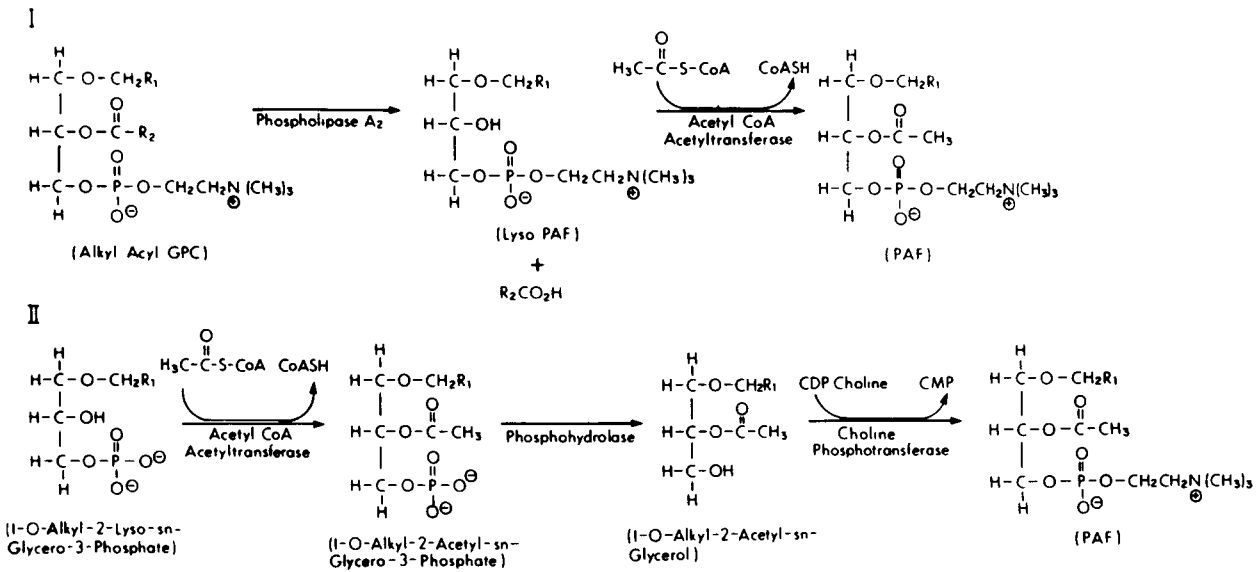

Figure 6. Biosynthetic pathways for PAF. PAF can be synthesized by two different metabolic pathways. The first pathway (I) is initiated by phospholipase A_2 hydrolysis of alkyl-acyl-GPC, followed by acetylation of lyso-PAF. The second pathway (II) acetylates 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphate, followed by dephosphorylation to yield 1-O-alkyl-2-acetyl-*sn*-glycerol. This moiety subsequently condenses with CDP-choline to produce PAF.

the relative amounts of lyso-PAF acetylation versus acylation, and the amount of PAF production through *de novo* synthesis.

PAF can act on the cell where it originates or can partition into plasma and affect other target cells by interaction with a specific receptor. As with many other secreted humoral agents, PAF has a short biological half-life since it is inactivated by an acetylhydrolase present in plasma. PAF-acetylhydrolase is highly selective for the hydrolysis of lipids with a short-chain at the *sn*-2 position. Lyso-PAF can also be transacylated to regenerate 1-O-alkyl-2-acyl-GPC by an enzyme which has a high selectivity for arachidonic acid, thereby maintaining the high enrichment of arachidonic acid in 1-O-alkyl-2-acyl-GPC.

Biological Roles of Platelet Activating Factor

Many of the biological effects of PAF have been reviewed by Snyder (1982) and Hanahan (1986). Platelet activation by thrombin or collagen results in PAF synthesis. Subsequent interaction of PAF with its plasma membrane receptor induces serotonin release from intracellular granules and platelet aggregation. PAF also modulates neutrophil degranulation, phagocytosis, exocytosis, chemotaxis, and superoxide production. Macrophage phagocytosis of zymosan particles, antibody-coated erythrocytes, and immune complexes are accompanied by PAF production which modulates a variety of subsequent macrophage-mediated events.

PAF is also a lipid mediator of anaphylactic responses. PAF produced by anti-IgE challenge of IgE-sensitized basophils results in degranulation and histamine release. PAF can induce rapid and shallow breathing, transient apnea, and edema in the respiratory system. In the cardiovascular system, PAF directly induces bradycardia, hypotension, elevated right ventricular pressure, vascular spasms, and increased vascular permeability.

PAF also has potent effects on many other biological systems. For example, PAF induces hepatic phosphoinositide turnover and glycogenolysis which is accompanied by glucose release into the plasma. PAF has also been implicated as a mediator of ischemic bowel necrosis since it can independently induce lesions morphologically similar to those present during human necrotizing enterocolitis. Furthermore, the role of PAF or a PAF-like lipid as an endogenous antihypertensive substance is currently under intense experimental scrutiny.

Diacylglycerol and Inositol Polyphosphates

Since the initial demonstration that phosphoinositide turnover is dramatically accelerated in pancreatic slices stimulated with acetylcholine (Hokin and Hokin, 1953), the biological significance of this observation has captured the imagination of investigators in diverse fields. However, it was over twenty years before the relationship between accelerated phosphoinositide turnover, intracellular calcium

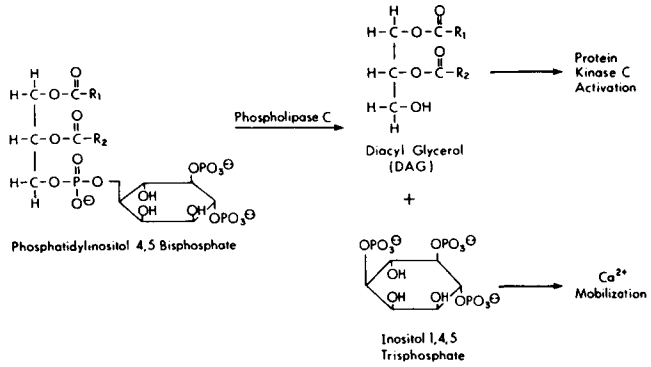

Figure 7. Phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate. Phosphoinositide-specific phospholipase C is activated during cellular stimulation and mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate. The two products of this reaction, DAG and IP_3, are both intracellular second messengers. Thus, a single hydrolytic reaction initiates a bifurcating pathway of signal transduction mediated by protein kinase C activation and calcium mobilization, respectively.

mobilization, and activation of protein kinase C became clear. The demonstration that phospholipase C-catalyzed hydrolysis of inositol-glycerophospholipids initiated a bifurcating pathway, resulting in the concomitant release of intracellular calcium and the activation of protein kinase C, provided a unifying biochemical mechanism which explained seemingly unrelated observations (Berridge and Irvine, 1984) (Figure 7). The key element of this hypothesis was the ability of inositol 1,4,5-trisphosphate (IP_3) to act as an intracellular second messenger that releases calcium ions from intracellular storage pools. Recent results have demonstrated that inositol trisphosphate production is regulated by a guanine nucleotide-regulated phospholipase C, which selectively hydrolyzes phosphatidylinositol 4,5-bisphosphate during cellular stimulation. Stored intracellular calcium is subsequently released after interaction of the liberated IP_3 with its intracellular membrane receptor(s). The coproduct of phospholipase C-mediated cleavage of inositol-glycerophospholipids, diacylglycerol (DAG), is also a lipid-derived second messenger which, in conjunction with calcium, synergistically activates protein kinase C (cf, Kikkawa and Nishizuka, 1986; Bell, 1987).

Recently, the potential biological diversity inherent in this signal transduction pathway has become increasingly apparent as it is now clear that multiple phospholipases C and D are activated during signal transduction in mammalian cells. The differential substrate specificities of each of these phospholipases C result in the release of specific molecular species of diglycerides, which contribute to the diversity of biological responses elicited after cell activation. This section will focus on the role of diglycerides and inositol phosphates as mediators of signal transduc-

tion in individual cell types, and demonstrate how the activation of phospholipase C and phospholipase A_2 are tightly interwoven enzymic mediators of biologic signal transduction.

Diacylglycerol

1,2-Diacylglycerol (DAG) is released from endogenous inositol- and choline-glycerophospholipids during cellular activation by phospholipase C-catalyzed hydrolysis, resulting in the activation of protein kinase C. Detailed studies on the temporal course of molecular species of DAG which accumulate during cellular stimulation have demonstrated that phospholipase C-catalyzed inositol-glycero-phospholipid hydrolysis substantially precedes phospholipase C (and/or D)-catalyzed hydrolysis of choline-glycerophospholipids. The accumulation of DAG mass during cellular activation is determined by the relative rate of its release from endogenous phospholipid storage pools in comparison to the rate of its metabolic removal by diglyceride kinase, diglyceride lipase, and choline (or ethanolamine) phosphotransferase. Indeed, in many cell types (e.g., platelets), the majority of the diglyceride released is rapidly phosphorylated by diglyceride kinase, resulting in the accumulation of phosphatidic acid, which is incapable of activating protein kinase C. Thus, the magnitude and temporal course of protein kinase C activation are determined not only by the extent of phospholipase C hydrolysis, but also by the combined activities of the above-mentioned enzymes in conjunction with the local calcium concentration.

Although it is well accepted that the synergistic interaction between DAG and calcium is responsible for protein kinase C activation, the biochemical mechanisms that mediate this activation *in vivo* are complex. Several studies have demonstrated that cellular activation is accompanied by the translocation of protein kinase C from the cytosolic compartment to cellular membranes (e.g., Ganong et al., 1986). Increases in cytosolic calcium promote protein kinase C translocation to cellular membranes, and removal of calcium accelerates the dissociation of protein kinase C from cellular membranes in a process that appears to be modulated by ATP (Bell, 1986).

Recent results have demonstrated that protein kinase C activation does not require a phospholipid bilayer (e.g., protein kinase C can be activated by micelles comprised of phosphatidylserine, diglyceride, and detergent), and have suggested that the activated lipid complex likely contains four molecules of phosphatidylserine complexed with one molecule of diglyceride and one molecule of calcium. Bell and co-workers proposed that four molecules of phosphatidylserine aggregate on the surface of a cellular membrane, where their negatively charged carboxyl groups bind to calcium, leading to a complex of phosphatidylserine and calcium which can sequester protein kinase C (Ganong et al., 1986). It has been proposed that in this state, the enzyme is primed but inactive (Bell, 1986). During cellular activation,

diglycerides produced by phospholipase C activation interact with this complex, enhancing its affinity for calcium and rendering the enzyme–lipid complex catalytically competent. Although this model is compatible with the available evidence, the salient mechanistic features of protein kinase C activation *in vivo* are necessarily more complex, since multiple isoforms of protein kinase C activity have been described which have different calcium requirements, and these isoforms are selectively modulated by different lipids that accumulate during cellular activation including arachidonic acid, lysophospholipids, and sphingosine. Thus, although a rudimentary understanding of the elements which contribute to protein kinase C activation during cellular stimulation has been obtained, the precise biochemical mechanism responsible for the regulation of protein kinase C activity *in vivo* remains a major focus of current research.

Identification of the physiological significance of protein kinase C activation in intact cells has been complicated by difficulties inherent in incorporating naturally occurring long-chain diglycerides into cell membranes. Accordingly, the role of diacylglycerols as activators of protein kinase C in intact cells was, by necessity, explored utilizing cell-permeable diglycerides (e.g., 1-oleoyl-2-acetyl-*sn*-glycerol), as well as tumor promoting phorbol esters. Phorbol esters act as diglyceride analogs in that they fulfill the diglyceride requirements for protein kinase C activation. Unfortunately, the responses elicited by phorbol esters, while similar, do not completely parallel the effects of agonist-induced increases in diglyceride mass (cf, Kikkawa and Nishizuka, 1986). One reason underlying such disparate responses is the transient nature of the increase in endogenous diglyceride mass after cellular activation in comparison to phorbol esters, which have long biological half-lives. Thus, while phorbol ester treatment usually produces an initial activation of protein kinase C, the long-term effects of phorbol esters are markedly different than those elicited by cellular activation *in vivo*.

It is now clear that protein kinase C-catalyzed phosphorylation of specific cellular proteins plays an essential role in the orchestration of the biochemical events responsible for cellular activation. Some of the cellular proteins phosphorylated by protein kinase C are listed in Table 1. The effects of protein kinase C-mediated phosphorylation include the modulation of ion channels and ion pumps, alterations in cellular endocytosis and exocytosis (e.g., the release of neurotransmitters and humoral agents), and modulation of gene expression and cellular proliferation.

Protein kinase C activation has been implicated in augmenting the transmembrane flux of calcium ions, the activities of the Na^+K^+-ATPase, as well as the activity of the Na^+/H^+-antiporter. Substantial evidence suggests that protein kinase C augments the uptake of calcium released during cellular stimulation, thereby limiting the temporal duration of its activation (e.g., phorbol esters activate the sarcoplasmic reticulum Ca^{2+}-ATPase).

Table 1. Proteins Phosphorylated by Protein Kinase C

Contractile and cytoskeletal proteins
Caldesmon
Cardiac C-protein
Myosin light chain
Troponin I
Troponin T
Vinculin
Membrane and receptor proteins
Ca^{2+}-ATPase
GTP-binding protein
Glucose transport protein
Na^{+}/H^{+} exchange protein
β-adrenergic receptor
EGF receptor
Insulin receptor
Interleukin 2 receptor
Nicotinic cholinergic receptor
Enzymes
Glycogen phosphorylase kinase
Glycogen synthase
Guanylate cyclase
Myosin light-chain kinase
NADPH oxidase
Phosphofructokinase

Protein kinase C also has an important role in hormone and neurotransmitter release. For example, activated protein kinase C augments catecholamine release from the adrenal medulla, aldosterone secretion from the adrenal cortex, and acetylcholine release from the caudate nucleus. Furthermore, protein kinase C-elicited hormone and neurotransmitter release augment Ca^{2+}-induced release mechanisms.

Gene expression and cellular proliferation are induced by the concomitant activation of protein kinase C and mobilization of Ca^{2+}. The activation of both of these pathways by phosphatidylinositol 4,5-bisphosphate hydrolysis, is necessary for DNA synthesis in macrophage-depleted human peripheral lymphocytes. However, the mechanisms regulating rapid cell proliferation are complex as demonstrated by their dependence on the combined presence of both activated protein kinase C and growth factors.

Although it is beyond the scope of this chapter to review all the effects of protein kinase C-mediated phosphorylation of cellular constituents, it is important to realize that the synergistic actions of diglyceride and calcium ion on protein kinase C activity have profound biological sequelae.

Inositol Polyphosphates

In mammalian cells, there are three major types of phosphoinositides (PI) which contain either one phosphate (phosphatidylinositol, ~90% of PI mass), two phosphates (phosphatidylinositol 4-phosphate, ~8% of PI mass), or three phosphates (phosphatidylinositol 4,5-bisphosphate, ~2% of PI mass). Accordingly, hydrolysis of this series of inositol-glycerophospholipids by phospholipase C results in the production of inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate (IP_3), respectively (cf, Berridge, 1987). Furthermore, it should be recognized that cyclic inositol phosphates are also produced during phospholipase C cleavage (by internal nucleophilic attack), resulting in additional products including inositol cyclic 1:2-phosphate, inositol cyclic 1:2,4-bisphosphate and inositol cyclic 1:2,4,5-trisphosphate (cf, Majerus et al., 1988). Inositol phosphates are metabolized by a complex array of phosphatases which catalyze the highly ordered removal of these phosphates. The majority of released inositol 1,4,5-trisphosphate is initially metabolized to inositol 1,4-bisphosphate, and subsequently, to inositol l-phosphate by the enzymes, 5-phosphomonoesterase and 4-phosphomonoesterase, respectively.

Although many different inositol polyphosphates are produced during cellular stimulation by phospholipase C-mediated cleavage of inositol-glycerophospholipids, IP_3 has received major attention since it rapidly accumulates in stimulated tissue and even diminutive concentrations of this metabolite result in the mobilization of calcium ion (Streb et al., 1983). IP_3 selectively accumulates in the initial stage of cell activation because the preferentially activated phospholipases C hydrolyze phosphatidylinositol 4,5-bisphosphate. The intracellular Ca^{2+} signal elicited by IP_3 is transient, due to the rapid dephosphorylation of IP_3 by the specific intracellular inositol monoesterases. Furthermore, since one IP_3 molecule results in the release of at least 20 calcium ions from intracellular stores, the potential for the intracellular amplification of a single hydrolytic cleavage is inherent in the design of this system (Joseph et al., 1984). Thus, the IP_3 second messenger system represents a mechanism through which mammalian cells can transduce extracellular signals by mobilizing the release of stored intracellular Ca^{2+} which, in turn, activates a number of different enzymes and biological cascades. It should be appreciated that mobilization of intracellular calcium results in the concomitant activation of calcium-dependent phospholipases. Similarly, activation of intracellular phospholipases A_2 can activate inositol-glycerophospholipid hydrolysis by the effects of both eicosanoids (e.g., thromboxane) and platelet activating factor, which are both potent agonists that activate phosphoinositide-specific phospholipase C. Thus, activation of phospholipase A_2 and phospholipase C are closely coupled since metabolites of each pathway can cross-modulate the activity of the other pathway.

Inositol polyphosphates have been implicated as mediators of muscarinic-, angiotensin II-, and vasopressin-mediated stimulation of smooth muscle; muscarinic and noradrenergic activation of myocardium; muscarinic stimulation of neural tissue; ADP, thrombin, and PAF activation of platelets; phototransduction in *Limulus* photoreceptors; and cellular activation in a vast range of other tissues (cf, Berridge and Irvine, 1984). Introduction of IP_3 into permeabilized cells results in increases in calcium ion and initiates numerous Ca^{2+} dependent events. For example, IP_3 induces the contraction of permeabilized vascular smooth muscle, stomach smooth muscle, and skeletal muscle and, therefore, has been proposed as a second messenger mediating pharmaco-mechanical transduction. Furthermore, in permeabilized platelets, IP_3 stimulates shape change, aggregation, serotonin secretion, and protein phosphorylation. In intact cells, IP_3 injection into *Limulus* photoreceptors induces phototransduction, and IP_3 injection into *Xenopus* eggs elicits membrane depolarization. Although the effects elicited by inositol polyphosphates differ with respect to cell type, all of the effects found to date are attributable to increases in intracellular calcium. Furthermore, it should be appreciated that the IP_3-mediated Ca^{2+} signal represents only one arm of the dual pathway initiated by phosphatidylinositol 4,5-bisphosphate cleavage (i.e., the Ca^{2+} response is synergistic with DAG in the activation of protein kinase C).

The biological function of the large diversity of other less-abundant inositol polyphosphates is the subject of intense research, but no definitive conclusions can be made at present. For instance, several groups have demonstrated that inositol trisphosphates in some cell types, contain large fractions of inositol 1,3,4-trisphosphate which is synthesized from IP_3 by sequential 3′-kinase and 5′-phosphomonoesterase activities. However, since inositol 1,3,4-trisphosphate does not efficiently mobilize intracellular Ca^{2+}, and no known functions of inositol 1,3,4-trisphosphate have been described, the significance underlying this pathway of inositol trisphosphate metabolism remains obscure (cf, Berridge, 1987).

ELECTROPHYSIOLOGICAL DYSFUNCTION DURING MYOCARDIAL INFARCTION: A PATHOPHYSIOLOGICAL PROCESS MEDIATED BY ALTERATIONS IN CELLULAR LIPID METABOLISM

Ischemic heart disease results in over 500,000 deaths in the United States each year. The overwhelming majority of deaths attributable to atherosclerotic heart disease results from electrophysiological alterations in zones of ischemic myocardium which predispose to ventricular fibrillation and tachycardia. Accordingly, a substantial amount of attention has focused on identifying the biochemical mechanisms which precipitate the lethal electrophysiological sequelae of coronary artery disease.

The electrophysiological characteristics of the myocardium are, in large part, mediated by the chemical constituents present in the sarcolemmal membrane.

Electrical conduction in normal myocardium is dependent upon the precise temporal orchestration of the gating kinetics of ion channels, which collectively result in the initiation and propagation of an action potential. During myocardial ischemia, the kinetics of ion channel gating are altered, resulting in changes in electrophysiological properties which predispose ischemic myocardium to lethal ventricular dysrhythmias, such as ventricular tachycardia and fibrillation.

It is well known that the kinetics of transmembrane proteins such as ion channels and ion pumps are profoundly modulated by alterations in the physical characteristics of their surrounding lipid constituents (cf, Lenaz, 1987). Myocardial ischemia is accompanied by the accumulation of amphiphilic metabolites (molecules containing both hydrophobic and hydrophilic constituents) which possess potent membrane perturbing properties. Accordingly, the hypothesis that accumulation of amphiphilic moieties during myocardial ischemia results in alterations of the sarcolemmal membrane, leading to electrophysiological dysfunction has been extensively studied (cf, Corr et al., 1984). Indeed, there is now substantial experimental evidence which strongly suggests that the accumulation of amphiphilic moieties during myocardial ischemia is the biochemical progenitor of electrophysiological dysfunction in ischemic zones. This section will focus on alterations in lipid metabolism and membrane composition which occur during myocardial ischemia, and will demonstrate how such alterations contribute to the lethal sequelae of atherosclerotic heart disease.

Inhibition of Myocardial Fatty Acid Oxidation During Ischemia

The myocardium preferentially oxidizes fatty acids to fulfill the energy requirements necessary for efficient pump function. However, during myocardial ischemia, decreased cellular oxygen content, in conjunction with reduction of the redox potential, result in the inhibition of fatty acid β-oxidation leading to the accumulation of fatty acyl metabolites, including acylCoA and acylcarnitine (cf, Neely and Morgan, 1974).

In normal myocardium, the first step in fatty acid utilization is thioesterification catalyzed by acylCoA synthetase. There are three potential metabolic fates of the synthesized acylCoA including: 1) transport into the mitochondrial matrix for subsequent β-oxidation, 2) utilization as an intermediate in polar and nonpolar lipid synthesis, and 3) hydrolysis by acylCoA hydrolase (i.e., a net futile cycle). In normal myocardium, the major fraction of synthesized acylCoA is transported into the mitochondrial matrix by sequential transesterification reactions catalyzed by carnitine acyltransferase. AcylCoA in the mitochondrial matrix space is sequentially oxidized in two-carbon units to produce acetylCoA, which is accompanied by the production of the reducing equivalents, NADH and $FADH_2$.

In ischemic myocardium, the flux through fatty acid β-oxidation is dramatically attenuated since it is tightly coupled to oxidative phosphorylation. The result of

inhibition of fatty acid β-oxidation is the accumulation of the proximal metabolites in this pathway, including acylCoA and acylcarnitine. Acylcarnitine readily diffuses across the mitochondrial membrane and selectively partitions into the sarcolemmal membrane. Since even small amounts of acyl carnitine have profound effects on sarcolemmal molecular dynamics, it seems likely that this series of events contributes to alterations in the kinetics of critical sarcolemmal proteins, such as ion channels. Indeed, attenuation of acylcarnitine production dramatically decreases the frequency of lethal ventricular dysrhythmias in an *in vivo* model of myocardial ischemia.

Activation of Phospholipases During Myocardial Ischemia

Myocardial ischemia is accompanied by the release of arachidonic acid and the accumulation of lysophosphatidylcholine and lysophosphatidylethanolamine (cf, Corr et al., 1984). Arachidonic acid is predominantly stored in choline- and ethanolamine-glycerophospholipids in myocardium. Thus, these findings demonstrate that phospholipase A_2 is activated during myocardial ischemia. Since lysophospholipids are potent amphiphilic compounds which have profound effects on the physical properties of myocardial sarcolemma, the accumulation of these moieties has also been implicated in arrhythmogenesis.

Accordingly, substantial work has focused on the biochemical mechanisms responsible for the accumulation of lysophospholipids during myocardial ischemia. Initial studies have examined the hypothesis that increases in myocytic calcium result in the activation of a calcium-dependent phospholipase A_2 which catalyzes the hydrolysis of endogenous phospholipids. However, recent work has demonstrated that the major measurable phospholipase A_2 in myocardium is calcium-independent and selectively hydrolyzes plasmalogen substrate. In fact, microsomal calcium-independent plasmalogen-selective phospholipase A_2 is activated during brief intervals of myocardial ischemia, demonstrating a four-fold activation of phospholipase A_2 after 2 min of myocardial ischemia and over a ten-fold activation after 5 min of myocardial ischemia (Ford et al., 1991 and Hazen et al., 1991). The activation of calcium-independent plasmalogen-selective phospholipase A_2 during myocardial ischemia is readily reversible during reperfusion of ischemic tissue and occurs prior to irreversible injury. The significance of plasmalogen catabolism during myocardial ischemia is further underscored by the recent demonstration that arachidonic acid content in lysoplasmenylethanolamine pools is depleted in reperfused myocardium (Davies et al., 1992). The mechanism responsible for the activation of this calcium-independent phospholipase A_2 during myocardial ischemia is presently unknown, but this phenomenon represents an attractive pharmacological target since inhibition of this phospholipase could reduce the accumulation of lysophospholipids in myocardium, and potentially attenuate the lethal electrophysiological sequelae of myocardial ischemia.

Of course, the rate of lysophospholipid accumulation during myocardial ischemia is dependent upon the net flux of activities of the enzymes which result in their production (phospholipases) in comparison to the activities which metabolically degrade these noxious metabolites. Lysophospholipids are metabolized in the myocardium by the enzymes lysophospholipase, lysophospholipase-transacylase, and lysophospholipid acyltransferase. The relative amounts of the measurable activities which generate lysophospholipids in myocardium are dwarfed in comparison to the overall amount of catabolic activity which can remove these noxious metabolites. Since lysophospholipids accumulate during myocardial ischemia, these results suggested that lysophospholipid metabolism was attenuated in ischemic myocardium. Detailed kinetic studies demonstrated that long-chain acyl carnitines are potent inhibitors of both lysophospholipase and lysophospholipase-transacylase (Gross et al., 1983; Gross and Sobel, 1983). Furthermore, pharmacological inhibition of carnitine acyltransferase results in decreased amounts of acyl carnitine accumulation and is accompanied by a concomitant reduction in lysophospholipid mass (Corr et al., 1989). Taken together, substantial evidence demonstrates that myocardial ischemia is accompanied by both phospholipase A_2 activation and inhibition of lysophospholipases, which together contribute to the accumulation of lysophospholipids in ischemic myocardium.

Biological Effects of Amphiphile Accumulation During Myocardial Ischemia

Since substantial perturbations in membrane dynamics and physical properties are often elicited by even diminutive alterations in the content of some amphiphilic metabolites (e.g., acyl carnitine and lysophospholipids), the effects of amphiphilic compounds on the kinetics of critical transmembrane proteins have been extensively explored (cf, Han and Gross, 1992). For example, long-chain acyl carnitine alters the activity of the sarcoplasmic reticular calcium pump and augments the release of sequestered calcium ions from the sarcoplasmic reticulum. Acyl carnitines are also potent inhibitors of the Na^+/Ca^{2+}-antiporter and the Na^+K^+-ATPase in sarcolemma. Similarly, lysophospholipids inhibit Na^+K^+-ATPase, adenylyl cyclase, and protein kinase C. Since amphiphilic constituents affect the activity of separate and distinct proteins with similar dose-response curves, it seems likely that their effects are mediated through a common mechanism which perturbs the membrane as a whole and not the result of specific amphiphile–protein interactions. Accordingly, it is no surprise that the electrophysiological characteristics of ventricular-muscle strips and Purkinje fibers are dramatically altered by exposure to low concentrations of either lysophosphatidylcholine or acyl carnitine. Electrophysiological alterations elicited by amphiphilic compounds include decreases in action potential total amplitude, resting membrane potential, and V_{max} of phase O depolarization. These alterations are strikingly similar to the electrophysiological

alterations seen in ischemic tissue. Furthermore, quantitative electron-microscopic autoradiography demonstrated that these alterations were present when only 1–3 mole % of sarcolemmal phospholipid was comprised of these amphiphiles. Thus, even modest perturbations of the lipid composition of the sarcolemmal membrane result in striking alterations of ion channel kinetics. Accordingly, since amphiphilic compounds accumulate during myocardial ischemia, alter the biophysical properties of the sarcolemmal membrane, and modulate the electrophysiological characteristics of myocytic ion channels, it seems likely that the accumulation of amphiphilic compounds is responsible for electrophysiological dysfunction in ischemic zones. Recently, "proof of concept" of the amphiphile hypothesis has emerged through specific pharmacological inhibition of altered lipid metabolism present in ischemic zones. Remarkably POCA, (phenylalkyloxirane carboxylic acid), a potent inhibitor of carnitine acyltransferase, decreases the levels of both acyl carnitine (by inhibition of carnitine acyltransferase) and lysophosphatidylcholine (presumably by attenuating the inhibition of lysophospholipid catabolic pathways). The POCA-mediated decreases in acyl carnitine and lysophosphatidylcholine were accompanied by the complete ablation of ventricular dysrhythmias during *in vivo* myocardial ischemia in a dog (Corr et al., 1989). Taken together, alterations in lipid catabolism during myocardial ischemia appear to be intimately involved in arrhythmogenesis. Thus, the implementation of specific pharmaceutical agents which prevent accumulation of amphiphilic constituents in ischemic zones hopefully will attenuate the morbidity and mortality of atherosclerotic heart disease in man.

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REFERENCES

Bell, R. M. (1986). Protein kinase C activation by diacylglycerol second messengers. Cell 45, 631–632.

Berridge, M. J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann. Rev. Biochem. 56, 159–193.

Berridge, M. J. & Irvine, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315–321.

Blank, M. L., Synder, F., Byers, L. W., Brooks, B., & Muirhead, E. E. (1979). Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. Biochem. Biophys. Res. Commun. 90, 1194–1200.

Broekman, M. J., Ward, J. W., & Marcus, A. J. (1980). Phospholipid metabolism in stimulated human platelets. Am. Soc. Clin. Invest. 66, 275–283.

Corr, P. B., Gross, R. W., & Sobel, B. E. (1984). Amphipathic metabolites and membrane dysfunction in ischemic myocardium. Circ. Res. 55, 135–154.

Corr, P. B., Creer, M. H., Yamada, K. A., Saffitz, J. E., & Sobel, B. E. (1989). Prophylaxis of early ventricular fibrillation by inhibition of acylcarnitine accumulation. J. Clin. Invest. 83, 927–936.

Davies, N. J., Schulz, R., Olley, P. M., Strynadka, K. D., Panas, D. L., & Lopaschuk, G. D. (1992). Lysoplasmenylethanolamine accumulation in ischemic/reperfused isolated fatty acid-perfused hearts. Circ. Res. 70, 1161–1168.

Demopoulos, C. A., Pinckard, R. N., & Hanahan, D. J. (1979). Platelet activating factor. Evidence for 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). J. Biol. Chem. 254, 9355–9358.

Dennis, E. A. (1994). Diversity of group types, regulation, and function of phospholipase A_2. J. Biol. Chem. 269, 13057–13060.

Feldberg, W. & Kellaway, C. H. (1938). Liberation of histamine and formation of lysolecithin-like substances by cobra venom. J. Physiol. 94, 187–226.

Ford, D. A., Hazen, S. L., Saffitz, J. E., & Gross, R. W. (1991). The rapid and reversible activation of a calcium-independent plasmalogen-selective phospholipase A_2 during myocardial ischemia. J. Clin. Inv. 88, 331–335.

Ganong, B. R., Loomis, C. R., Hannun, Y. A., & Bell, R. M. (1986). Specificity and mechanism of protein kinase C activation by *sn*-1,2-diacylglycerols. Proc. Natl. Acad. Sci. USA 83, 1184–1188.

Gross, R. W. (1984). High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: a fast atom bombardment mass spectroscopic and gas chromatography-mass spectroscopic characterization. Biochemistry 23, 158–165.

Gross, R. W., Drisdel, R. C., & Sobel, B. E. (1983). Rabbit myocardial lysophospholipase-transacylase. Purification, characterization and inhibition by endogenous cardiac amphiphiles. J. Biol. Chem. 258, 15165–15172.

Gross, R. W. & Sobel, B. E. (1983). Rabbit myocardial cytosolic lysophospholipase. Purification, characterization and competitive inhibition by L-palmitoyl carnitine. J. Biol. Chem. 258, 5221–5226.

Hammarstrom, S., Murphy, R. C., Samuelsson, B., Clark, D. A., Mioskowski, C., & Corey, E. J. (1979). Structure of leukotriene C. Identification of the amino acid part. Biochem. Biophys. Res. Commun. 91, 1266–1272.

Han, X. & Gross, R. W. (1992). Modulation of cardiac membrane fluidity by amphiphilic compounds. In: Advances in Membrane Fluidity (Aloia, R. C., ed.), Vol 5, pp. 225–243. Alan R. Liss, Inc., New York.

Hanahan, D. J. (1986). Platelet activating factor: a biologically active phosphoglyceride. Ann. Rev. Biochem. 55, 483–509.

Hazen, S. L., Ford, D. A., & Gross, R. W. (1991). Activation of a membrane-associated phospholipase A_2 during rabbit myocardial ischemia which is highly selective for plasmalogen substrate. J. Biol. Chem. 266, 5629–5633.

Hokin, M. R. & Hokin, L. E. (1953). Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreatic slices. J. Biol. Chem. 203, 967–977.

Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F., & Williamson, J. R. (1984). Myoinositol 1,4,5-trisphosphate. A second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. J. Biol. Chem. 259, 3077–3081.

Kellaway, C. H. & Trethewie, E. R. (1940). The liberation of a slow-reacting smooth muscle-stimulating substance in anaphylaxis. Quant. J. Exp. Physiol. Cogn. Med. Sci. 30, 121–145.

Kikkawa, U. & Nishizuka, Y. (1986). The role of protein kinase C in transmembrane signalling. Ann. Rev. Cell Biol. 2, 149–178.

Lands, W. E. M. & Samuelsson, B. (1968). Phospholipid precursors of prostaglandins. Biochim. Biophys. Acta 164, 426–429.

Lee, T., Malone, B., & Snyder, F. (1986). A new *de novo* pathway for the formation of 1-alkyl-2-acetyl-*sn*-glycerols, precursors of platelet activating factor. J. Biol. Chem. 261, 5373–5377.

Lenaz, G. (1987). Lipid fluidity and membrane protein dynamics. Bioscience Reports 7, 823–837.

Loeb, L. A. & Gross, R. W. (1986). Identification and purification of sheep platelet phospholipase A_2 isoforms. J. Biol. Chem. 261, 10467–10470.

Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S., & Lips, D. L. (1988). Inositol phosphates: synthesis and degradation. J. Biol. Chem. 263, 3051–3054.

Murphy, R. C., Hammarstrom, S., & Samuelsson, B. (1979). Leukotriene C: a slow reacting substance from murine mastocytoma cells. Proc. Natl. Acad. Sci. USA 76, 4275–4279.

Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R., & Lefkowith, J. B. (1986). Arachidonic acid metabolism. Ann. Rev. Biochem. 55, 69–102.

Neely, J. R. & Morgan, H. E. (1974). Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. Ann. Rev. Physiol. 36, 413–459.

Nieto, M. L., Venable, M. E., Bauldry, S. A., Greene, D. G., Kennedy, M., Bass, D. A., & Wykle, R. L. (1991). Evidence that hydrolysis of ethanolamine plasmalogens triggers synthesis of platelet-activating factor via a transacylation reaction. J. Biol. Chem. 266, 18699–18706.

Purdon, A. D. & Smith, J. B. (1985). Turnover of arachidonic acid in the major diacyl and ether phospholipids of human platelets. J. Biol. Chem. 260, 12700–12704.

Samuelsson, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 220, 568–575.

Samuelsson, B., Goldyne, M., Granstrom, E., Hamberg, M., Hammarstrom, S., & Malmsten, C. (1978). Prostaglandins and thromboxanes. Ann. Rev. Biochem. 47, 997–1029.

Snyder, F. (1982). Platelet activating factor (PAF), a novel type of phospholipid with diverse biological properties. Ann. Rep. Med. Chem. 17, 243–252.

Streb, H., Irvine, R. F., Berridge, M. J., & Schulz, I. (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306, 67–69.

Uemura, Y., Lee, T., & Snyder, F. (1991). A coenzyme A-independent transacylase is linked to the formation of platelet-activating factor (PAF) by generating the lyso-PAF intermediate in the remodeling pathway. J. Biol. Chem. 266, 8268–8272.

Wykle, R. L., Malone, B., & Snyder, F. (1980). Enzymatic synthesis of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, a hypotensive and platelet aggregating lipid. J. Biol. Chem. 255, 10256–10260.

RECOMMENDED READINGS

Kuo, J. F. (ed.) (1985). Phospholipids and Cellular Regulation, Vol. 2, CRC Press, Boca Raton.

Michell, R. H., Drummond, A. H., & Downes, C. P. (eds.) (1989). Inositol Lipids in Cell Signalling, Academic Press, New York.

Willis, A. L. (ed.) (1987). Handbook of Eicosanoids: Prostaglandins and Related Lipids, Vol. 1. CRC Press, Boca Raton.

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INDEX