

Ten commandments of enzymology, amended

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Reflections on advances and retreats in biochemistry have, over the years, generated several maxims. The late Efraim Racker's 'don't waste clean thinking on dirty enzymes' has been one of the most durable. On the occasion of a lecture in Jerusalem a decade ago, I framed a list of the ten 'best' maxims as 'commandments', which were later published as a 'Guest Commentary' in the *Journal of Bacteriology* [1].

These ten commandments have been useful in discussions of papers and reviews, as well as ongoing work. One simply says 'Commandment three' or simply 'five' and there is generally no need to explain an emphatic judgment. When *TIBS* invited me to submit my current version of the commandments, I realized that after only three years since their publication, the list needed to be judged again to qualify as my 'top ten'. The list is personal, and has been narrowed by my previous focus on DNA replication and my current one on inorganic polyphosphate. I hope this game of choosing a 'top-ten' will encourage others to try it and then compare their lists with mine and others'.

Thou shalt...

I. Rely on enzymology to resolve and reconstitute biologic events

With the ever-increasing reliance on genomics and proteomics, enzymes are no longer isolated or even assayed for activity but, rather, inferred from sequence (a violation of Commandment III). Repeated observations over the course of the past century have supported a dogma that all reactions in a cell are catalyzed and directed by enzymes. The activity of an enzyme can be assayed with accuracy even in a crude extract and its mass determined upon its isolation. This fundamental quantitation of measurement is often lacking in assays of biologic activities.

Beyond the catalytic face, enzymes have two additional faces: regulatory and social. The regulatory site binds a ligand that modulates the rate and specificity of the enzymes. The social face associates the enzyme with other components, such as a membrane or a scaffold, or complexes with other enzymes, such as nucleic acids or polysaccharides. These macromolecular assemblies are crucial for cellular operations, but are still largely unexplored. The cellular location of these assemblies has also not been thoroughly investigated.

When a specific enzyme is obtained from a commercial source, its purity and specific activity are unknown or unverified. With regard to the genes of the many sequenced genomes and the epigenetic systems that encode enzymes, few have been studied for their catalytic functions, let alone their regulatory and social sites.

Resolution of the components in a cell-free extract that can carry out a stage in a biologic event is achieved by purifying them to near homogeneity by fractionation procedures. Reconstitution by reassembling these isolated components can indicate whether they are necessary and sufficient to account for their biologic role. Reverse genetics and genomics (Commandment VI) by mutation, deletion and overexpression of each encoding gene can provide major insights into their physiologic functions.

Few multi-step pathways have been fully reconstituted and the urge to tackle them has diminished. The refined genetic analyses of events in cell biology and developmental biology cry out for efforts to observe them in a cell-free system, which could then be resolved and reconstituted.

II. Trust the universality of biochemistry and the power of microbiology

The increasing ease and surge of microbial genome sequencing has further confirmed the extraordinary revelation that basic metabolic functions are conserved between bacteria and humans. Many proteins and mechanisms of *Escherichia coli* DNA replication have proven homologous in sequence and structure to those found in eukaryotic systems. Remarkably, DnaA protein, which initiates replication of the *E. coli* chromosome at its unique origin, is structurally similar to the Orc1, Orc4 and Orc5 proteins that operate at the replication origins on eukaryotic chromosomes. Indeed, fascinating variations are abundant among various species, but the basic themes and even some structural components are highly conserved.

I chose to study DNA replication using bacteria rather than plants and animals because of their relative simplicity and my faith in the universality of biochemistry. I was confident that bacteria would hold the key to understanding replication and evolution. Two further revelations of the power of microbiology have come to me more recently from studies of inorganic polyphosphate (poly P). First, is the vital role of the stationary phase of replication shown by the requirement for poly P in bacteria to respond to stresses and stringencies and to withstand

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starvation for long periods. The genetic adaptations acquired to cope with hard times accounts for the survival of a species. Remarkably, studies directed at this stage of the life cycle have been largely neglected in favor of an emphasis on growth and development.

Second, is that ever since microbes were first isolated, we have failed to realize that they are multicellular in nature. More than 99% of all microbes live in aggregates on surfaces called biofilms. The microbes in these communities differ markedly in gene expression and morphology from the rare planktonic individuals or those that we cultivate in flasks or on a petri dish. In a biofilm, a bacterial colony is more resistant to drying, to antibiotics and to immune cells, it competes with neighboring colonies and, as in pathogens do, produces the many factors for virulence in a host cell. We humans are merely transient guests in a rapidly evolving microbial world.

How unfortunate that the studies of microbes, the geese that, for 50 years, laid so many of the golden eggs of basic biochemistry, molecular biology and genetics, are being deliberately starved. One of the most prominent of these geese is *E. coli*. 'Coli' is a four-letter word now banished from the language of granting agencies and academic search committees. Only the studies of commercially important pathogenic microbes are sustained, especially those that can be weaponized for bioterrorism.

III. Not believe something just because you can explain it

This commandment has wide social applications, but is most significant in hypothesis-driven science because serendipity and chance are often the source of novel observations. It has been said that 'chance favors the prepared mind', but there are many examples in which the mind might be too well prepared and, thus, inclined to believe something because it is anticipated. A mistake I made in 1954 was being distracted by a facile explanation of data that led us to abandon what proved to be template-directed RNA synthesis and its product, mRNA, many years before they were discovered by others.

When *E. coli* DNA polymerase was first isolated, its purity was doubted because the preparation still contained anomalous DNase activity that could remove nucleotides at the end of the growing chain. This activity in the homogeneous enzyme proved to be a proof-reading domain that increased fidelity of replication by excising a mismatched nucleotide. Again and again, a mechanism has emerged more complex and fascinating than the one that seemed to explain everything.

IV. Not waste clean thinking on dirty enzymes

Discovering an assay for a biological event in a cell-free extract opens the way to its molecular resolution and reconstitution (Commandment I). Trying to devise a mechanism with a crude extract, even with ingenious experiments, is generally a waste of effort. An extract is too dirty; purification is the only way to go.

DNA polymerase was first observed as the conversion of a minute amount of [¹⁴C] thymidine (0.01% of the input) to an acid-insoluble DNase-sensitive form. This assay provided a wedge in the discovery of DNA polymerase; the hammer that expanded the wedge was

enzyme fractionation. The starting assay mixture contained only a crude *E. coli* extract, ATP, calf thymus DNA and the labeled thymidine.

The isolation and characterization of DNA polymerase depended on the discovery of the four deoxynucleoside di- and tri-phosphates and six new enzymes (thymidine kinase, the four deoxynucleoside monophosphate kinases and exonuclease III). In addition, the discovery and isolation of DNA polymerase depended on four functions supplied by the DNA that was added to the incubation mixture. The DNA acted as: (i) a source of nuclease action of the four deoxynucleotides; (ii) as the template to direct the assembly of the deoxynucleotides into the correct sequence; (iii) the primer ends for extension of chains; (iv) and as a pool to protect and trap the synthetic product in the face of overwhelming nuclease activities in the crude extract.

With the isolation of pure enzymes comes novel opportunities: to explore structure–function relationships; the molecular reconstitution of a biological event; the possibility of identifying the components of related events (e.g. replication, repair and recombination); a means for site-directed mutagenesis; and the use of this unique reagent (see Commandment X) in a wide variety of basic studies and technologies.

Of these opportunities, none match the ultimate: replacing 16th century gross anatomy and physiology with a 21st century view at the molecular level. Current advances in crystallography, electron microscopy, NMR, mass spectrometry and genetic engineering have made it possible to see an enzyme machine in action. The highly effective transcriptosome – the massive, 57-subunit, highly regulated RNA polymerase II of yeast – can be seen, at near atomic resolution, building a mRNA in milliseconds.

V. Not waste clean enzymes on dirty substrates

This commandment was based on my long struggle to find the missing mechanism for starting a DNA chain, something the pure DNA polymerase could not do. The problem was that the DNA substrate needed to direct the start was hopelessly dirty; the DNA source, whether microbial or eukaryotic, was frayed, fragmented, gapped and heterogeneous. Only when we employed the intact, single-stranded, circular homogeneous DNA from certain phages were we able to discover that RNA priming is the mechanism.

A significant problem is the heterogeneity and non-specificity of the substrates for enzymes. For example, many of the protein kinases are assayed by their capacity to phosphorylate casein, but in almost every case, the physiologic substrate is unknown. Thus, the true functions of many vital enzymes remain in doubt.

VI. Use genetics and genomics

The enormous power of genetics, genomics and associated biotechnologies to obtain and annotate information about enzymes and to trace their evolution would probably put this commandment first on most lists. This choice would be fortified by the promise of transcriptomics, proteomics and other 'omics'. However, I place genetics and genomics lower on my list because of what these approaches do not

do (Commandments I and III). Also, the very ease with which sophisticated data are collated and interpreted has discouraged the slow and burdensome approach of resolving and reconstituting a complex enzyme system. DNA arrays, which measure the transcription of most genes in a genome, disclose neither the abundance nor any of the faces of the enzymes they encode. As the prescient F.G. Hopkins said in 1931; '(The biochemist's word) may not be the last in the description of life, but without his help, the last word will never be said'.

VII. Be aware that cells are molecularly crowded

Half of the cell dry weight is made up of tightly packed proteins. Cells are gels! Upon disruption of cells, there is a huge dilution, 20-fold or more, of the cellular components. This must be taken into account in developing an assay of a multistep pathway. With regard to macromolecules, the dilution factor can be overcome by the addition of a high concentration of a polymer, such as polyethylene glycol. These polymers encompass a large volume that excludes enzymes and other macromolecules, in effect crowding them into a very small space. Concentration can also be obtained by salt precipitation of the proteins in the extract and dissolving the precipitate in a reduced volume. Dilution can be so serious that, an activity might not survive even with only a twofold dilution of the extract. Such was the case in assays of replication at the origin of the *E. coli* genome. If resolution and reconstitution of cellular events in a cell-free system are attempted, emphasis should be placed on correcting for a severe dilution of the components.

VIII. Depend on viruses to open windows

Previously, the study of phages provided key insights into many of the basic elements of replication of the host chromosome: helicases, topoisomerases, DNA-binding proteins, RNA priming and DNA modifications. Now technologic advances that enable the introduction of DNA by plasmids or even uncoated DNA has reduced the dependence on viral vectors. Yet, the molecular reconstitution of a viral life cycle (Commandment I) would contribute to the medically urgent problem of finding the drugs and vaccines needed to cope with virulent viruses.

IX. Remain mindful of the power of radioactive tracers

This technology has been, and remains, of great value in charting pathways and mechanisms in all sectors of biochemistry. The commandment to use radioactive tracers had not been included previously, but now makes the list because the use of radioactive tracers has fallen out of fashion. One reason is the grossly exaggerated fear of the health hazard, not only by regulatory agencies that have exploited this fear, but even by scientists as well.

The ^{14}C and ^3H isotopes pose no conceivable hazard (an 'EXIT' sign in a hallway contains ten Curies of ^3H), and the other isotopes are generally used at sub-microCurie levels. Another reason for the decline in the use of radioactive tracers is that sensitive fluorescent tags and luminescence became available. But there are no substitutes for tracing bioactive elements in an unambiguous way that can be used both *in vitro* and *in vivo*. In cellular events, radioactivity cannot be created or destroyed and can be readily accounted for.

X. Employ enzymes as unique reagents

The use of enzymes in industrial processes and as components of kits for, for example, clinical diagnosis, forensics, apoptosis and PCR, is well established. But there are no enzymes available to identify and measure the levels of novel compounds in unexplored pathways of biosynthesis and metabolism.

In our pursuit of the origin and functions of the mysterious inorganic poly P, enzymes as reagents played a crucial role. Poly P is a polymer of hundreds of phosphate residues linked by anhydride bonds. Despite its presence in all cells (bacterial, fungal, plant and animal), it was regarded, for lack of any functions, as a 'molecular fossil'. Most investigators would approach the poly P problem by making mutants with altered levels of poly P. But there were no phenotypes to guide the selection of mutants and the available assays were cumbersome, insensitive and ambiguous. The discovery and purification of enzymes that make and use poly P opened the way to identifying their encoding genes. Deleting them provided insights into the many poly P functions, and their overexpression supplied abundant reagents, which made assays of poly P facile, sensitive and definitive.

Envoi

In the movie *History of the World, Part 1*, depicting The Ten Commandments, the comedian Mel Brooks is Moses descending from Mt. Sinai carrying three tablets. He drops one and it shatters, he sighs: 'Oh well, ten commandments are enough!' Not so! The most important of the commandments was on the lost tablet: 'thou shalt support basic research'.

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Further Reading

- 1 Kornberg, A. (2000) Ten commandments: lessons from the enzymology of DNA replication. *J. Bacteriol.* 182, 3613–3618