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Historical Review

The power of methods

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ABSTRACT

Major advances in science are usually launched by new methods or techniques. Because this essay is not intended as a history of science, I shall not invoke the invention of the microscope or telescope as the gateways to inner and outer space, but will restrict myself to developments I have witnessed, or almost witnessed, during my scientific lifetime.

Contents

1. Weights and volumes

As a chemistry student at the University of Zurich in the late 1950s we weighed materials on a double-pan balance: this involved placing the object on one pan and adding weights to the other until the swinging of the beam became symmetrical. Single-pan balances, pioneered by Mettler, were available only to professors, if at all. Fluids were dispensed by glass pipettes the volumes of which ranged from 0.1 to as much as 20 ml. They were filled by sucking up the liquid by mouth, and then rapidly closing off the top with our index finger before the contents could escape. When dealing with toxic or noxious stuff, the sissies among us would use rubber bulbs or tubing to minimize a more intimate contact between subject and object. A memorable sight was that of a medical student closing off the tip of a vertically held pipette with a finger and trying to fill it with liquid from the top.

Many years were to go by until in 1961, as a postdoc in Ochoa's Department of Biochemistry, I became acquainted with Pedersen pipettes. These were delicate glass instruments, manufactured in Denmark, that permitted accurate delivery of volumes down to a few microliters; but you still had to fill them by sucking on a rubber tube with a plastic mouth piece. Protection was afforded, if at all, by a cotton plug in the tubing. Needless to say, these pipettes were non-disposable and were cleaned by overnight immersion in bichromate-sulfuric acid, a powerful oxidant that not only dissolved residues in pipettes but also our clothing and occasionally patches of skin. Extensive rinsing was required to remove all traces of the corrosive fluid, residues of which would

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potently inhibit enzymatic reactions. While large pipettes were cleansed by lab personnel, we handled our Pedersen pipettes ourselves, for fear of having them damaged or disappear. The quantum leap to Eppendorf pipettes with disposable tips was made only in the late 1970s; it turned out to be one of the major minor advances in laboratory technique.

2. Radioactive isotopes

War, while a scourge of mankind, has one major redeeming feature besides stimulating the economy ("war is good business – invest your son"): that of spawning technology and speeding up its application. While heavy, stable isotopes such as deuterium, ^{15}N and ^{18}O had been used in the early 1930s to trace metabolic pathways, it was the availability of radioactive isotopes after World War II that ushered in a revolution in biochemistry.

Early attempts in the 1950s to measure protein synthesis *in vitro* consisted of adding amino acids to cell-free extracts and attempting to measure a net increase of trichloroacetic acid-precipitable material. Needless to say, no useful information was gleaned by this approach because the increment of newly synthesized over pre-existing protein was infinitesimal. Only when radioactively labeled amino acids became available could cell-free protein synthesis be reliably documented. The availability of ^{32}P-labeled phosphate enabled Arthur Kornberg to synthesize radioactive nucleoside triphosphates, which he correctly guessed would be the substrate for enzymatic DNA synthesis; that led to the identification and purification of DNA polymerase I [1,2]. In seminal – now forgotten – experiments, Kornberg showed that his enzyme could synthesize a DNA strand complementary to a template, adding biochemical support to Watson and Crick's model of anti-parallel double-stranded DNA structure, proposed a few years earlier, in 1953. In the late 50s only those able to synthesize radioactive substrates on their own could participate in the brave new science soon to be named "molecular biology." Admission to this exclusive guild was liberalized when radioactive compounds became commercially available, albeit at exorbitant prices.

In the early 1960s little if any supervision restricted the liberal use of inorganic ^{32}P-phosphate to label bacterial and viral nucleic acids *in vivo*. With my mind's eye I still see one of my colleagues in the NYU Department of Biochemistry perched on the back of a chair with a flask containing radioactive growth medium in his hand, tipping over and flooding the floor with some 50 mCi of radioactive phosphate. After soaking up what we could, the hot spot was covered with sheets of lead foil and remained a *memento mori* for many half lives.

3. Biotechnology

An obscure area of phage biology, restriction and modification, spawned technology that ultimately made genetic engineering possible. The seminal observation was that phages grown on one strain of *Escherichia coli* were "restricted", *i.e.* virtually unable to propagate on a different strain of the same bacteria, but after a rare, successful infection could produce modified progeny phages no longer restricted. That finding led to the discovery of a plethora of bacterial "type 2 restriction enzymes" which had the ability to cleave DNA at a variety of precisely defined sequences. An important earlier event was the discovery of plasmids, circular, "self-replicating" DNAs which frequently carried antibiotic resistance genes, indigenous to bacteria, along with simple methods for their isolation and re-introduction into *E. coli*. Finally, the discovery of DNA ligase, an enzyme that covalently joins ends of DNA, in conjunction with the availability of restriction enzymes and plasmids, opened the gate to gene cloning and genetic engineering which revolutionized every field of biology and launched a thousand biotech companies.

The essential role played by the dozens of sequence-specific restriction enzymes discovered early on, a number that was eventually to grow into the thousands, required labs engaged in molecular biology to produce dozens of these enzymes at a high degree of purity, a laborious and time-consuming task. The enzyme trading system that developed among molecular biology labs lasted until the mid 1970s, when high-quality restriction enzymes became commercially available. I view the emergence of genetic engineering and the biotech industry from Joshua Lederberg's studies on sex life of bacteria [3] as a prime example of how "useless" research, which the late Senator Proxmire might have recognized by his "Golden Fleece" award, can revolutionize science and industry.

It bears mentioning that recombinant DNA technology, one of the most potent tools of molecular biology, could not have emerged but for simple and potent methods for separating nucleic acids or protein molecules by electrophoresis through agarose or SDS polyacrylamide gels, respectively. These techniques, developed in the 1960s and 70s, replaced analytical ultracentrifugation and its requirement of hugely expensive and bulky equipment for most practical purposes and provide another striking example of the impact of simple methods on the emergence of a seminal field. However, in a countertrend, the search for ever more rapid and accurate determination of protein molecular mass spawned methods ranging from light scattering to mass spectrometry, requiring sophisticated and expensive equipment.

4. Sequencing

With the recognition of DNA (and in some cases RNA) as the repository of genetic information, the burning question in the late 1950s became the relationship between nucleic acid and protein sequence, i.e. the nature of the "genetic code". The comparison of a protein sequence with the nucleotide sequence of the cognate gene would have yielded the desired answer, but while many protein sequences had been determined – albeit, it is said, none error-free except for that of insulin by Fred Sanger [4,5] – sequence determination of nucleic acids was still in its zygotic stage, limited to analysis of short oligonucleotides. The seminal technologies developed by Bob Holley in the early 1960s led to the first isolation of a pure tRNA species – by the back-breaking method of countercurrent distribution – and to the sequence determination of the oligonucleotides derived from it by cleavage with site-specific ribonucleases. However, the ordering of these oligonucleotides remained an insuperable problem until one night Holley's graduate student J. Penswick – performing what, in my lab, I used to call "secret experiments" [6] – discovered conditions of partial digestion under which the tRNA was cleaved into two about equal-sized halves, each of which could again be partially cleaved, thereby allowing the ordering of the fragments and resulting in the first determination of a nucleotide sequence of a biologically relevant RNA molecule. Walter Fiers subsequently used partial cleavage to elucidate the sequence of much larger viral RNAs, and even of SV40 viral DNA by sequencing its RNA transcripts [7]. Direct sequencing of DNA by chemical or enzymatic methods revolutionized the field and culminated in the incredibly potent "next-generation" methods in which hundreds of thousands of single DNA molecules are sequenced in parallel. Thus, the time-consuming chemical or enzymatic reactions, the arduous pouring of thin polyacrylamide gels and the tiresome reading of meter long autoradiograms has been replaced by the shipment of DNA samples to in-house or commercial sequencing facilities.

5. Kits

A major boon to research labs, and in particular to graduate students, was the creation of commercial laboratories that supplied not only pure enzymes and reagents, but a plethora of kits providing complete sets of pure and validated components, including water, required for various procedures in recombinant DNA technology. From the didactic point of view this was a negative development, because students could readily obtain diplomas in molecular biology or even biochemistry without ever having purified an enzyme: "Molecular biology," Erwin Chargaff quipped, "is the practice of biochemistry without a license." On the other hand, kits greatly accelerated the progress of actual research projects. Unlike the days of my apprenticeship, it was no longer necessary to spend weeks preparing enzymes and reagents required for a one-day experiment. Less than fondly I remember the day-long effort involved in isolating ATP from muscle of Mg++-treated rabbits, and gratefully I acknowledge the scientific community's debt to the unsung heroes of kit development.

6. Single-cell techniques

Experiments using isolated single cells go back a long way, perhaps most famously to John Gurdon's use of *Xenopus oocytes* for the translation of injected mRNA in the early seventies [8], and later for nuclear transplantation. Injecting oocytes with mRNA from virus-infected leukocytes to assay for interferon production was a critical step in the cloning and bacterial expression of the human interferon alpha genes by our Zurich laboratory in 1979 [9].

Methods to isolate cells by virtue of their surface properties initially required manual "panning" on plates, or collection on beads, coated with cell surface-directed antibodies, but the invention of the Fluorescence Activated Cell Sorter in the late 1960s eventually allowed the rapid selection of cells by up to twelve criteria and led, among other achievements, to the isolation of murine and human hematopoietic stem cells. The generation of monoclonal antibodies, one of the most potent tools in the biologist's technical repertoire, is also based on isolation of single cell clones. In a more general view, electrophysiological experiments monitor single cells within the context of tissues by using microelectrodes, and more recently voltage-sensitive dyes, but here I am getting out of my depth.

7. Embryonic stem cells

The stem-cell torrent arose from a rivulet of experiments, beginning with pluripotent, teratoma-derived murine "embryonal carcinoma cells". The propagation and manipulation of mouse embryonic stem cells garnered from the inner cell mass soon led to a flood of applications, ranging from genetically engineered mice to sheep, goats and cattle lacking or over-expressing genes of interest. Most recently, the generation and maintenance of human cells with stem-cell like properties, and its promise for medical applications, is capturing headlines.

In the late eighties, following the groundbreaking work of Capecchi, Evans and Smithies [10–12], we knocked out the prion protein-encoding gene *Prnp*, to determine whether indeed the PrP protein was essential for the propagation of prions. To our surprise, the inactivation of this gene, which is expressed in all vertebrates examined, and maximally in brain (according to Woody Allen our second most important organ), entailed no noticeable deficiencies in targeted mice, but conferred complete resistance to prion disease. This first instance of genetically engineered immunity to a disease was followed by the generation of scrapie-resistant goats and (at least one) cow resistant to mad cow disease, the erstwhile scourge of British husbandry. The long and strenuous task of creating knockout mice can now be readily circumvented by purchasing them from specialized companies. Potent as knockouts are for determining the function of a gene, conclusions regarding a resulting phenotype must be tempered by the consideration that the deletion of a DNA segment may result not only in the disruption of the targeted gene, but possibly also of elements encoding small RNAs, long non-coding RNAs or elements affecting the structure of chromatin, all of which may have direct or indirect consequences for a phenotype.

8. Information technology

One of the major banes of scientific activity in mid 20th century was the literature search; it meant leafing through sources such as Index Medicus or Chemical Abstracts for relevant publications, searching for the cognate volumes in the library, and actually reading them – were they only to be found. This tiresome activity was reduced with the advent of "Current Contents" but the problem of keeping track of and accessing publications of interest remained. In the late 1960s I attempted to devise a filing system using punch cards on which my secretary typed title and authors of papers I selected. These cards then piled up on my desk until I got around – if ever – to notch them, so that for a search by one or more criteria I could insert steel needles through the appropriate holes of the stack and shake out the relevant cards. Needless to say, this procedure was frustratingly inefficient and I relied more on gleaning references from relevant previous publications by myself and others. Doubtless this approach to quoting, if widely practiced, must have promoted the premature disappearance of valuable papers from the literature.

The mechanics of writing a paper were pretty daunting for the many of us unable to dictate a printable version of our thoughts. Typed drafts were reworked with white-out ink, scissors, tape and glue, indeed in the 1960s even copying a page was a messy two-step procedure: Xerox was a dream of the future. Some relief was provided by the advent of crude word processors in the early nineteen eighties, but the age of modern science writing really began when the first Mac computer settled on our desks.

The dramatic breakthrough in access to knowledge around the globe came with the development of hypertext and the internet which provided instant access to the gigantic resources of Medline, PubMed and other databases; these have provided access to otherwise practically inaccessible sources. More recently, the advent of Google and other search engines provide us with more information than we would ever wish to quote.

9. What's next?

While it is obviously impossible to predict seminal breakthroughs in methods and technology, we may speculate on where they could be applied. Perhaps we will be able to "understand" a cell, i.e. predict cellular processes and their regulation as a function of the cell's environment, a monumental task even in the simplest of mycoplasma and seemingly utopian for mammalian cells. And if we think that's tough, imagine trying to trace neuronal processes underlying discovery and invention. Happily, the history of biology teaches us that methods and techniques that today exist only in our imagination will materialize in some unexpected way.

Acknowledgments

Doubtless I have omitted important methods and developments because I have restricted myself to items of which I have some personal knowledge. I apologize for mentioning some names but

not others that are equally meritorious. I thank Tamas Bartfai, Piet Borst and Gerald Joyce for jogging my memory and Gerald Weissmann (no known family connection) for his efforts to improve my style.

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