Regulation, Restriction, and Reminiscences

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Choose a job you love, and you will never have to work a day in your life—Confucius

These reflections cover more than 60 years of my research, selected from among those that I consider to be of greatest scientific interest. It is a personal account, not a general review of all contributions, and so I have not included the numerous references into which my contributions are imbedded. I regret that I could not include other topics and colleagues. Some details, anecdotes, etc. are described in my previous reviews and overviews.

Subjects of my research may appear to be quite diverse. This is because from childhood I was fascinated by reading about explorers of new territories. They are my heroes, rather than the developers who came later. So in science my goal is always to explore new questions. There is, however, a central theme: the molecular basis of regulation. It can be observed at all levels of biological complexity. The goal and theme that bind together the disparate subjects of my investigation are to gain understanding of the general molecular mechanisms that underlie regulatory processes and their defects in diseases, principally in cancer.

Beginnings

Biological science has changed dramatically since I began research. One can liken the evolution of techniques to slide rules becoming computers. My undergraduate training was in chemistry at University of California, Berkeley (1938–1942), where I was fortunate in being taught by Nobel laureates William Giaque (freshman chemistry) and Melvin Calvin (undergraduate research). I did my Ph.D. under Linus Pauling at the California Institute of Technology (1942–1947), performing some of the first studies with purified antibodies. I was pretty cheeky; when Pauling noted that a graduate student should know how to spell “phenolphthalein,” I noted back that “so should a professor.” Graduate studies were interrupted by World War II, during which I performed research on chemical warfare agents and then on uranium, learning very directly about toxic substances and radioactivity, respectively. The death of my mother in 1942 directed me toward doing what I could against cancer, so I then took a Merck postdoctoral fellowship with Van Potter at the University of Wisconsin (1947–1949). His research was on deregulation in cancers of oxidative phosphorylation and the Krebs cycle, studied mainly with tissue homogenates. I learned a great deal under his wise guidance and was very productive. However, I decided that the time was not ripe for me to pursue cancer problems because of my inadequate knowledge of metabolic pathways, their regulation, and techniques for investigation. Therefore I shifted to more amenable bacterial systems.

I joined the faculty of the biochemistry department and the virus laboratory at University of California, Berkeley, of which Wendell Stanley was Director, in 1949. Subsequently I made several novel discoveries. We, simultaneously with others, discovered ribonucleoprotein particles in bacteria, later named ribosomes, and also found photosynthetic particles that we called chromatophores (1). I wanted to learn whether virus infection changes the metabolism of the host and discovered that nine enzyme activities changed after infection of Escherichia coli with bacteriophage (2). These included deoxyribonuclease, suggesting involvement of
phage DNA in infection. Later when we replaced thymidine with bromodeoxyuridine in phage DNA many mutants were produced, which is consistent with DNA being the genetic material of the virus (3). This research led to my being chosen a Young Biochemist in 1953 to represent the United States at the International Biochemistry Congress in Paris and to tour Europe, both remarkable experiences.

Regulation of Enzyme Activity

I first thought about metabolic regulation while I was a postdoctoral fellow. We demonstrated that oxalacetate inhibits succinic dehydrogenase, several steps back in the Krebs cycle (4). I wondered whether this “feedback” could keep reactions of the complex cycle in balance. I initially investigated coordination between molecular syntheses in *E. coli* and found linkages: that nucleic acid precursors are required for protein synthesis (5) and furthermore nucleic acid synthesis depends on the presence of amino acids (6). However, concurrent synthesis of DNA was not necessary.

Feedback and Allosteric Inhibition—Living organisms usually produce their constituent molecules in amounts sufficient to meet their needs, no more or less. Is there a general mechanism to explain this economical metabolic regulation? In 1950 biochemists did not ask this question; they were very busy creating a map of metabolism in which all roads were of the same intensity although traffic flow along some was far greater than on others. Richard Yates and I (and independently Ed Umbarger) reported a general control mechanism: we for the pyrimidine pathway and he for the isoleucine-valine biosynthetic pathway. Its principle is similar to regulating heat production of a furnace by a thermostat. An end product biosynthetic pathway can be an inhibitor of its initial enzymatic reaction. Thereby, in a living cell end product in excess economically shuts down its own synthesis. The feedback mechanism has now been verified for numerous pathways, and it remains a subject of active investigation (7).

Regulation is complex for some of these, involving branching to produce several end products as studied by Earl Stadtman or of an enzyme with several substrates like ribonucleotide reductase as elucidated by Peter Reichard.

The breakthrough came when I was using mutants defective in steps of the pathway that synthesizes the pyrimidines by seven successive enzyme-catalyzed reactions. I noticed that the metabolite synthesized prior to the absent reaction of a mutant accumulated as expected, but not when I made available the end product of the pathway. This observation was literally breathtaking, because I perceived that it suggests a novel mechanism for control of metabolism. Richard Yates and I reported at a 1954 AAAS meeting that “uracil blocks an enzyme step between aspartate and ureidosuccinate formation, and this block may be an important regulatory mechanism in the cell.” In three subsequent papers (8–10) we established this regulation. These discoveries led to perhaps the first review on regulation of metabolism (11).

Regulatory Sites—The molecular mechanism of feedback inhibition immediately created a problem. The general conception of inhibitors then was that they compete with substrate quite specifically for binding to an active site of the enzyme. How can a pyrimidine inhibit the enzyme aspartate transcarbamylase since it is structurally very dissimilar to the substrates, aspartate and carbamyl phosphate? I addressed this question after I returned from a sabbatical in Jacques Monod’s laboratory (see below). The uncertainties posed by results obtained with crude extracts made me decide to first obtain the pure enzyme, which Margaret Shepherdson and I isolated and crystallized (12). With this pure enzyme, John Gerhart demonstrated that the inhibitor is the ultimate end product cytidine triphosphate (CTP), which has no structural similarity to the substrates aspartate or carbamyl phosphate (13). An indication of a regulatory site distinct from the catalytic site was that ATP activates the enzyme in contrast to the inhibitory CTP (Fig. 1). ATP, which is not a substrate, evidently cannot bind to the active site because this would have to be inhibitory, and therefore it must bind to a different, regulatory site.

The key came from an unusual observation. Gerhart kept getting variable results of inhibition by CTP, although the pure enzyme always had high catalytic activity. When we examined his data closely we noticed that inhibition was strong at the beginning of each week and decreased thereafter. His procedure was that each Monday he thawed an aliquot of the deep-frozen enzyme and stored it in the refrigerator for later use. Hypothesizing that the enzyme must change its properties during this cold storage, he warmed it systematically and found that brief exposure to 65 °C abolished inhibition by CTP but not catalytic activity. Thus,
we dissociated sites that we named regulatory as distinct from functional ones (14). Gerhart went on to separate the regulatory and catalytic subunits of the enzyme, later investigated in detail by physical chemistry and x-ray diffraction.

At the same time, Jean-Pierre Changeux in the laboratory of Monod investigated the mechanism of feedback inhibition of the isoleucine-valine pathway discovered by Ed Umbarger, and from kinetic studies concluded that there are inhibitory sites in addition to catalytic ones. These may be the first molecular demonstrations of regulation of protein function by a small molecule. Monod conceived the generalization of allostery, which he called “the second secret of life.” The two types of binding sites on proteins, one functional and the other regulatory, permit regulation of any biological reaction by a process in which a regulatory molecule need have no structural similarity to the molecules acted upon (15). He combined three lines of research to create the allosteric concept: (i) feedback inhibition with regulatory sites; (ii) control of gene expression (see below); and (iii) cooperative binding of oxygen to the subunits of hemoglobin (16).

Another major development arising from feedback inhibition is the finding that enzymes often function in complexes with other proteins rather than as single proteins, which was then the biochemical concept. An early example is Prem Reddy’s report that DNA synthesis is not catalyzed by its polymerase acting alone but by a multienzyme complex that we named “replitase” (17), a finding that initially met with considerable opposition. It should not have been surprising because proteins are synthesized by very large multiprotein complexes, ribosomes, and we now know that complexes consisting of RNA polymerase plus regulatory transcription factors synthesize RNA. Reports of feedback inhibition, regulatory subunits, allosteric sites, and multiprotein complexes now abound in the literature.

**Regulation of Enzyme Expression by Repression**

In addition to regulation of enzyme activity as outlined above, there evidently was another major regulation that determined amounts of enzymes. Enzyme activities were known to “adapt,” to change dramatically, as a function of the nutrients provided to bacteria. This mechanism would provide a coarse control of metabolic regulation relative to fine regulation by feedback inhibition. I began to investigate such regulatory mechanisms for enzyme synthesis early in the 1950s (10, 18). When in 1957–1958 I had the opportunity to take a sabbatical leave I decided to go to the laboratory in Paris of Jacques Monod, the outstanding investigator of this problem. He studied the dramatic changes of β-galactosidase activity in *E. coli* as a function of availability of β-galactosides and other carbon sources.

Monod, Francois Jacob, and I discovered the general molecular mechanism of this process. It is by action of a protein we named the repressor that specifically blocks gene expression,
which is released when a low molecular weight inducer molecule binds to it. Specifically, expression of the β-galactosidase gene, and two adjacent genes, is inhibited by a repressor molecule that binds to an upstream operator sequence of the bacterial DNA. This negative regulation is released by binding of a β-galactoside to an allosteric site of the repressor (19). This has often been reviewed (15, 20, 21). This research is the basis for current concepts of the major mechanism for regulating gene expressions in both prokaryotes and eukaryotes.

Our investigations of gene expression provided one of the origins for discovery of messenger RNA. Since the enzyme probably is not made directly on DNA, we proposed from kinetics an unstable intermediate between gene and enzyme (22). This was soon thereafter shown by others to be an RNA. In accord with an unstable intermediate, we then demonstrated that the enzyme begins to be produced about a minute after its gene is activated and quickly ceases after the inducer is removed (23). Monica Riley demonstrated that destruction of the gene by radioactive decay of incorporated 32P causes cessation of enzyme synthesis (24).

Membrane Changes of Cancer Cells

I moved to Princeton in 1961 to become the first Chairman of the Biochemical Sciences Department. Techniques by this time had progressed sufficiently to make tissue culture of mammalian cells feasible for investigators in general. In 1963 an opportunity to participate in a cancer meeting in South America reinitiated my thinking about cancer. I needed a topic and so speculated that cancer and normal cells differ in surface functions that regulate growth by interacting with the extracellular environment (25). However, little was then known about growth factors, as supplied in serum, or of their receptors, so I turned to a surface-related activity that I had investigated in relation to β-galactosidase induction, transport of small molecules across the membrane into E. coli. These experiments had provided a valuable lesson. Monod and Jacob proposed the operon model: that adjacent genes, in particular for β-galactosidase and galactoside transport (permease), are co-induced by galactosides. I objected that the galactinol induces the transport system but not the enzyme. The solution to this dilemma is that this sugar is an α-galactoside, which induces a different permease that tests positive in the assay for β-galactoside permease, and so the operon hypothesis was not contradicted (26).

Molecular mechanisms of transport were then unknown. To learn about them, Jacques Dreyfuss and I investigated sulfate ion uptake (27) and identified a novel class of transport-related proteins. A mutant of Salmonella typhimurium that could not grow on sulfate did not accumulate the ion and so was defective in its transport, but we noted that a very small amount of sulfate was associated with these bacteria. We hypothesized that this sulfate is bound to a protein located outside the cell membrane, and our experiments demonstrated a small protein located between cell wall and membrane to which sulfate binds firmly. Its synthesis is repressed by sulfate, so by derepression in cells grown with an organic sulfur source and followed by a selective release technique we were able to obtain crystals after only 4-fold purification. This sulfate binding protein is one of the first transport proteins to be purified and the first of the “binding proteins” that are involved in active transport and chemotaxis (28).

We then turned to investigating transport into normal and cancer cells. In a series of studies we showed that their transport of small molecules differs and is highly regulated, being altered by viral transformation, cell-cell contact, and serum addition, and it changes through the cell cycle (29). At this time Max Burger and Allan Goldberg joined my laboratory and investigated carbohydrate differences on cancer versus normal cell surfaces (30), and Dennis Cunningham investigated phospholipid turnover (31). To begin studies of growth regulation by externally supplied growth factors we compared their requirements by normal and transformed cells (32).

Disregulation of the Cell Cycle in Cancer

In 1972–1973 my late wife Ruth Sager and I took a sabbatical with Sir Michael Stoker at the Imperial Cancer Research Fund Laboratory in London. Our objectives were to learn about cancer, especially applications of tissue culture. We worked long hours, surprising our colleagues by sometimes returning to the laboratory after dinner; to make up for this, we made several exciting and informative trips around Europe.

I soon decided that the hallmark of cancer on which I should focus was deregulated cell proliferation, and Ruth chose genetic defects for her subject of investigation, an area in which she later made major contributions. The process of cell proliferation is organized as the cell
cycle, which provided a good starting point for me because I previously studied cell cycle events in synchronized bacteria (33, 34). My initial question was where in the cycle growth regulation is exerted. I discovered that regulation for normal cells is exerted in late G1 phase, at about 2 h prior to initiation of DNA synthesis. This is in contrast to prior proposals that growth control is exerted prior to cell division. Thus, a wholly different set of molecular events came into consideration, molecules involved in which were soon identified. I named the time of this process the restriction point, a term that survives today (35). Lee Hartwell at this time applied his cycle-regulating yeast mutants to demonstrate that growth of yeast is similarly regulated in G1 at “start.” The restriction point and start are the first demonstrations of what Hartwell later named “checkpoints.”

Importantly, I showed that these restriction point requirements are relaxed in cancer cells, providing a basis for the greater proliferative capacity of cancer (36). This research on restriction point control and its relevance to cancer have been summarized (37). We proceeded to investigate related molecular events in G1. Expression of the oncogenic protein Myc was changed in cancer cells (38); we showed that transit through G1 is influenced by serum supply, growth factors, and nutrients, and actin and other proteins are synthesized sequentially after cells enter the cycle (39). Rapid protein synthesis was needed to enter S phase, especially by normal as compared with cancer cells, which suggests the requirement for growth control of a protein with a short half-life. Indeed, we discovered only one protein (p68) of many detected as a spot on two-dimensional gels that had the three required characteristics. It increased in G1, was unstable, and more was present in cancer cells (40). Henry Yang and I demonstrated one of the first changes of protein phosphorylation during G1, which differed between normal and transformed cells (41).

Ruth and I moved to the Dana-Farber Cancer Institute and Harvard in 1975. I investigated the then unknown post-restriction point events at the end of G1 that initiate onset of DNA synthesis. My laboratory developed the appearance at the end of G1 of thymidine kinase as an alternative marker for S phase initiation, one that is more subject to molecular investigation than is DNA synthesis (42). Prem Reddy and I showed that several enzymes involved in DNA synthesis are produced at the G1/S interface and translocate into the nucleus where they form a “repliase” multiprotein complex for DNA synthesis (17). It contains E2F, retinoblastoma-like protein, and Cdc2 kinase. It binds to the mouse thymidine kinase gene promoter (43). With the discovery of cycle-dependent kinases (Cdks) and cyclins (providing another example of interacting catalytic and regulatory proteins) we asked whether one of these molecules is the restriction point protein. We concluded that cyclin E is the most promising candidate (44). Furthermore, we found that cyclin E is over expressed in cancer, and it potentially provides a molecular marker for cancer (45).

End Notes: Applications to Cancer

By 1990 research on regulation of the cell cycle was so plentiful and in such good hands that I decided to apply the basic knowledge I had gained to the study of cancer. On the one hand I tried to find methods for detecting cancer earlier and on the other searched for novel agents to treat it more effectively. This recent research will only be touched upon here.

Gene Expression and Differential Display: Cancer Detection—I wanted to discover molecular changes that underlie cancer. These could indicate mechanisms of transformation and furthermore could provide tools for cancer detection and therapy. Peng Liang and I invented the differential display technique for detecting the subset of mRNAs that are present in a cell and with it could discover changes in deregulated gene expressions in cancer (46). This method is based on synthesizing short cDNAs from 3′ ends of many mRNAs and then displaying them on sequencing gels for side-by-side comparisons of the products from normal and cancer cells. It has been applied extensively to discover changes of gene expression; there are now about 2000 citations of it (47). Examples from my laboratory include discovering a gene whose expression changes downstream of ras oncogene activity (48) and another that defectively regulates mitosis in cancers (49).

The concept of expression genetics (also named functional genomics) has been excellently summarized (50). Katherine Martin and Ruth Sager systematically applied differential display to discover hundreds of genes whose expressions are defectively regulated in breast cancers. Their research has continued in my laboratory, demonstrating that selected markers readily distinguished estrogen receptor positive from negative human breast cancers and
other properties (51). Then we determined whether these markers permit cancer detection in small samples of patient blood. We had shown that this approach is feasible (52). Sensitivity of the assay is sufficient to detect solid tumor cells disseminated in 3 cc of blood samples from patients (53). Expression-based blood assays, as developed with the screening approach described here, have the potential to detect and classify solid tumor cells originating from virtually any primary site in the body. Earlier detection should be effective in reducing cancer mortality, especially as better therapies are developed.

**Novel Chemotherapies**—Based on cell cycle control studies, long ago I proposed a modification of chemotherapy based upon protecting normal cells from drug-induced death, thereby increasing the therapeutic index (54). This concept has recently been developed further (55). As another checkpoint-based approach, Ching Lau and I discovered that a caffeine derivative selectively makes DNA-damaged cancer cells pass through their G2 checkpoint, causing chromosome fragmentation and death (56). This effect was specific for killing cancer cells put into mice (57). These findings led to clinical trials, unfortunately unsuccessful because of nausea and vomiting.

We are now developing several potential anticancer therapies. A natural product β-lapachone (58) combined with taxol is remarkably effective against tumors implanted into mice (59). We also reported anti-AIDS effects of β-lapachone and two other compounds (60). An important novel chemotherapy is based upon specifically causing programmed cell death (apoptosis) of cancer cells, as demonstrated with β-lapachone by Chiang Li (61). As another chemotherapeutic approach, Debajit Biswas demonstrated that the kinase C inhibitor Go6976 specifically causes apoptosis of estrogen receptor negative (ER−) breast cancer cells and the disappearance of tumors from their implantation in mice (62). This drug blocks activation of transcription factor NF-κB, which is elevated by epidermal growth factor in many ER− cancers. NF-κB is anti-apoptotic, and Go6976 recreates the apoptotic capacity of these cells, a novel demonstration of a chemotherapeutic principle.

In conclusion, my scientific path has meandered, not following any central direct pathway, if such a pathway exists, but rather it led along byways and across unexplored terrain toward my goal of learning about the defects of molecular regulation that underlie cancer. My wish is that these results will prove to be useful.

Dedicated to my late wife, Ruth Sager.

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