A Fascination with Enzymes: 
The Journey Not the Arrival Matters

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Paul Talalay

From the Lewis B. and Dorothy Cullman Cancer Chemoprevention Center, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

My journey of medical discovery spans more than 50 years: first at the University of Chicago (1944–1948 and 1950–1963) and then at Johns Hopkins (since 1963). Although enzymes have been the central theme in all our studies and the thread that has connected them, the control of cancer has always been the ultimate goal.

Coming to America: Massachusetts Institute of Technology

I arrived in New York from war-torn England on July 8, 1940, soon after the evacuation of the Allied Expeditionary Force from Dunkirk and after a harrowing 10-day voyage across the U-boat-infested Atlantic. It was a beautiful sunny morning and my first view of the crystal clear Manhattan skyline that dwarfed the Statue of Liberty still remains, 65 years later, an indelible memory symbolic of peace, freedom, and a better future. It was the second major disruption in the life of our family. On March 31, 1933, my 10th birthday, soon after Hitler came to power, my Russian father made the courageous and visionary decision that we should immediately leave Germany where we were living and where I was born. On the next day, the “Boycott” of the Jewish department stores on the Kuhrfürstendamm in Berlin led to their looting and burning. After further wanderings, we found our way to England where we received a warm welcome. I learned English and went to school regularly for the first time. I was fortunate to go to Bedford School where the excellent teaching in science, and especially biology, directed my interests toward a career in medicine.

In the fall of 1940, I entered MIT as an undergraduate majoring in biology. A remarkable lecture and laboratory course in enzymology taught by Irwin W. Sizer sparked a passion for enzymes that has never left me. It was one of the first courses in enzymology taught anywhere. My laboratory project required the purification of liver catalase according to the method of Sumner and Dounce (1). It is staggering now to look back on the primitive state of protein purification in those days.

Much of my four undergraduate years at MIT was spent in an electron microscopy laboratory studying biological ultrastructure under the direction of Francis O. Schmitt. I started out as a dishwasher in the laboratory but before long was analyzing the electron micrographs and became involved in a war-time project on the ultrastructure of natural and synthetic rubber fibers, described in the first paper in which I was an author (2). When I was leaving MIT for the medical school of the University of Chicago, Schmitt suggested that I might explore opportunities for research with the urologist Charles Huggins. I recall Schmitt’s prophetic but seemingly casual remark: “You might do much worse than work with Huggins.” I could not have known then that Charles Huggins would become the dominant force that molded my professional career and indeed much of my life. Huggins, whose laboratory motto was “Discovery is our Business” (which I have adopted), introduced me to the joys of scientific discovery and nurtured my belief that I too would be able to make contributions to our joint “business.”
I learned an unforgettable lesson: encouragement not intimidation is the tool for identifying and developing scientific talent and potential in the young (Fig. 1).

The First Chicago Period: “Chromogenic Substrates”

In 1944 I started medical school at the University of Chicago and began working in the laboratory of Charles Huggins in the Department of Surgery. He asked me to develop simpler methods than those that were then available for measuring serum acid and alkaline phosphatases. Huggins was the Professor and Chief of Urology. There was an atmosphere of intense excitement in his laboratory. With R. E. Stevens and Clarence V. Hodges, Huggins had recently published two classic papers (3, 4): “Studies on Prostatic Cancer I and II,” which demonstrated the effects of castration, of estrogens, and of androgens on serum phosphatase levels in patients with extensive metastatic carcinoma of the prostate. They reported large clinical improvements in a significant number of these patients after orchiectomy and estrogen treatment. As a freshman medical student I was profoundly moved when I saw the remissions of the disease in some of these desperately ill patients. It was the first time that cancer had been shown not to be a wholly “autonomous disease” but sustained, as Huggins put it, “by hormones of the body that were normal in amounts and kind” (5). These discoveries were a major scientific breakthrough. I was inspired by the revelation that even advanced and disseminated cancer could be controlled, and from that time on my research interests never strayed far from the cancer problem. Charles Huggins’ contributions were finally recognized by award of the Nobel Prize in Physiology or Medicine in 1966 “for his discoveries concerning hormonal treatment of prostatic cancer.”

Huggins was convinced that assays of serum phosphatase activities had made his discoveries possible, and he continually advocated the use of highly quantitative methods for assessing disease status in clinical research. The phosphatases were “biomarkers” for the status of prostate cancer. Simpler methods were needed to measure their activities, and he suggested that the phosphate ester of phenolphthalein might be a suitable new colorless substrate that upon hydrolysis by phosphatases would give rise to colored phenolphthalein. The synthesis was relatively simple, and when Huggins first saw the red color resulting from hydrolysis of phenolphthalein phosphate by alkaline phosphatase, he immediately offered me a job in his laboratory at $50 per month, provided I quit working in the Admitting Office where I received $8 each Sunday for a 15-hour day. It was an easy decision. Our method was published in the Journal of Biological Chemistry in 1945 (6).

The phenolphthalein ester presented one technical problem: it was a diphosphate and hydrolysis led to two products with different spectral characteristics, thus complicating the
kinetic behavior. Shortly thereafter, Oliver Lowry introduced \( p \)-nitrophenyl phosphate as a phosphatase substrate; its kinetics of hydrolysis was simple, and it became and remains the universally used substrate for measuring phosphatase activities.

Huggins was fascinated by the enzymatic conversions of colorless substrates to colored products and coined the term “chromogenic substrates,” which was first used in our second paper describing the assay of \( \beta \)-glucuronidase (7). For this study, I isolated phenolphthalein monoglucuronide from the urine of rabbits dosed with phenolphthalein. This glucuronide was rapidly hydrolyzed by \( \beta \)-glucuronidase, its kinetic behavior was simple, and it has become the standard substrate for measuring \( \beta \)-glucuronidase activity. Chromogenic substrates have achieved widespread acceptance and now comprise countless examples.

**The Second Chicago Period: Pseudomonas testosteroni and Enzymatic Transformations of Steroids**

I transferred from Chicago to the Yale Medical School in 1946 so that I could spend more time with my family in New Haven where my father had been diagnosed with serious cardiovascular disease. After receiving the M.D. degree from Yale in 1948, I spent two very exciting years as a surgical House Officer at the Massachusetts General Hospital (1948–1950), a training period that played a major role in molding my scientific style. I returned to the University of Chicago on July 1, 1950 as an American Cancer Society-Damon Runyon Fund Postdoctoral Fellow and later that year was promoted to Assistant Professor in Charles Huggins’ research group, which in 1951 became the Ben May Laboratory for Cancer Research. I wanted to work on steroid hormones to understand their mode of action and especially how androgens sustained and estrogens inhibited cancers of the prostate. At that time knowledge of the chemistry and stereochemistry of steroids was quite advanced, and there was a wealth of information on the structures of the many steroid metabolites found in the urine, but surprisingly not a single enzymatic transformation of steroids had been described.

The microbiologist Edward Adelberg suggested the possibility of advancing understanding of steroid enzymology by isolating bacterial auxotrophs that could grow on steroids as their sole source of carbon and energy. Thus I spent a most pleasant August 1951 at the Department of Microbiology of the University of California in Berkeley. From soil beneath a rosebush on the Berkeley campus I isolated a motile *Pseudomonas* that could grow on testosterone-containing medium as its only source of carbon and energy (8). It was named *P. testosteroni*. Manometric determinations of oxygen consumption revealed that under conditions in which carbon assimilation was blocked, *P. testosteroni* completely oxidized all 19 carbon atoms of testosterone to \( \text{CO}_2 \) by enzymes that were induced by steroids (Fig. 2).
In our initial 1952 paper (8) in *Nature* describing *P. testosteroni*, we noted that centrifuged cell-free extracts of the steroid-induced organism catalyzed the NADH-dependent (then known as DPNH, reduced diphosphopyridine nucleotide) reduction of the 17-ketone function of 4-androstene-3,17-dione to the 17β-hydroxyl group of testosterone. We even envisaged the analytical value of using this enzyme to estimate steroids by monitoring the oxidoreduction of NAD/NADH spectroscopically.

**Hydroxysteroid Dehydrogenases (HSD)**—*P. testosteroni* contained a family of enzymes catalyzing the nicotinamide nucleotide-dependent, reversible, and stereospecific interconversions of specific hydroxyl and carbonyl groups on the steroid skeleton and side chain. These enzymes were designated hydroxysteroid dehydrogenases (HSDs), with the appropriate positional and steric prefixes to specify the positions of the carbon atoms that were oxidized and the steric configurations of the reactive hydroxyl groups (Reaction 1).

![Reaction 1](image)

The first two HSDs that were purified and characterized were a 3β-, 17β-, and 3α-hydroxysteroid dehydrogenase (9–12). Metabolic transformations of steroids could now be understood in enzymatic terms, and the specificity of interactions of steroids with complementary proteins could be elucidated. We believed then (erroneously) that detailed studies on the affinities and kinetics of the interactions of these enzymes or their mammalian counterparts with steroids might shed light on the biological specificity of steroids, their interactions with complementary intracellular proteins, and possibly their mode of action. In these early experiments on HSDs I was fortunate to have the highly skilled technical assistance of Marie M. Dobson and Philip I. Marcus. Philip went on to complete a Ph.D. and is now an internationally recognized virologist.

The important discovery of the estrogen receptor by my colleague Elwood Jensen diverted attention from our notion that steroid-transforming enzymes would provide insight into the mechanism of action of steroid hormones. Nevertheless, the availability of highly purified HSDs led to a number of new developments as discussed in the following paragraphs.

**Stereospecificity of Hydrogen Transfer from Nicotinamide Nucleotides**—Isolation of the first HSDs coincided with the classic demonstration by B. Vennesland and F. H. Westheimer and their colleagues at the University of Chicago that nicotinamide nucleotide-linked dehydrogenases promoted the direct and stereospecific transfer of a presumed hydride group between the 4-position of the dihydronicotinamide and the substrate. Birgit Vennesland, Frank Loewus, and H. Richard Levy determined the stereospecificity of hydrogen transfer catalyzed by our 17β-HSD (13). It was known then that alcohol, malic, and lactate dehydrogenases from a variety of sources all utilized the same diastereomeric hydrogen at position 4 of the dihydronicotinamide ring of NADH (later identified as having the 4S configuration by J. W. Cornforth). When 4-androstene-3,17-dione was converted to testosterone by 17β-HSD in the presence of deuterium-labeled NADD (prepared enzymatically from 2-deuteroethanol), surprisingly no deuterium was found in the testosterone. Although several possible explanations were considered, we established that 17β-HSD utilized the hydrogen on the opposite side (4R) from the previously studied dehydrogenases. This finding led to the realization that NAD(P)-linked dehydrogenases belong to two large classes that transfer, respectively, the 4S- or the 4R-hydrogen to and from NAD(P)H (13); 17β-HSD was the first representative of the family of enzymes that transferred the 4R-hydrogen.

**Equilibria of Oxidoreductions: Axial and Equatorial Conformations**—Direct measurements of the equilibria of the interconversions of hydroxy- and ketosteroids in relation to the configuration and conformation of their hydroxyl groups were made with purified HSDs. These reactions were easily reversible, and the equilibria could be shifted completely in the direction of oxidation or reduction. Accurate determination of the equilibrium constants of the oxidations of a variety of equatorial and axial hydroxyl substituents in solution provided the free energy differences between axial and equatorial conformations (14) and were in full agreement with the predictions of conformation theory.

**Analytical Applications of Hydroxysteroid Dehydrogenases**—The availability of purified HSDs of known positional and steric specificities led logically to their use for identification and
quantification of steroids and groups of steroids on the basis of the stereospecific oxidoreduction of specific hydroxyl groups on the steroid skeleton and side chain and spectroscopic or fluorimetric determination of the accompanying changes in reduced nicotinamide nucleotides. This work continued for many years, beginning with the studies by Barbara Hurlock in the 1950s and subsequently by Donna Payne and Mikio Shikita. We attained sufficient analytic sensitivity to quantify steroids not only in urine but also in plasma. Ultimately, by use of the enzymatic cycling methods for nicotinamide nucleotides developed by Oliver Lowry, low picomole levels of steroids were estimated reliably (15). In the most recent contribution, a single step enzymatic assay was developed for 17-oxy- and 17-hydroxysteroids, based on their catalytic participation in the transfer of hydrogen from NADH to thionicotinamide-NAD by the transhydrogenase function of HSDs (see below) (16).

Although the principal value of these enzymatic methods was for the quantitative micro-analyses of steroids, this was by no means their only usefulness. The HSDs were shown to be very valuable reagents for determining the steric configuration, the chemical and stereochemical purity of steroids, and for the resolution of synthetic (±)-racemic steroids, as well as for the small scale stereospecific synthesis of steroids for chromatographic reference purposes.

Transhydrogenase Functions of Hydroxysteroid Dehydrogenases—One of the interesting (and enigmatic) properties of HSDs from animal tissues is their almost universal dual nucleotide specificity in contrast to the microbial enzymes, which were mostly NAD-dependent. I was aware of this but did not then (nor do I now) know how to assess its significance. One scientific excursion provided a scientific rationale although its ultimate importance remains in doubt.

In the mid-1950s, Claude A. Villee of Harvard reported that in the presence of isocitrate the reduction of NAD (but not of NADP) catalyzed by crude extracts of human placenta was accelerated by low concentrations of certain steroids, especially estradiol. At that time almost no credible effects of physiological concentrations of steroids on cell-free systems in vitro had been reported. The observed phenomenon was attributed to the presence of a steroid-stimulated NAD-dependent isocitrate dehydrogenase (ICD), which differed in this respect from the analogous and very much more active NADP-dependent ICD present in the same preparations. My colleague and dear friend H. Guy Williams-Ashman was intrigued by these findings. One day in the Fall of 1957, we were having lunch at the Quadrangle Club at the University of Chicago, and Guy scribbled on a paper napkin an imaginative explanation based on our knowledge that placental supernatant fractions also contained a 17β-HSD (studied by Lewis Engel) that interconverted estradiol and estrone and could utilize both nicotinamide nucleotides. That afternoon, we obtained a placenta from the Chicago Lying-in-Hospital (there were no concerns in those days about IRB approval or the hazards of human tissues) and showed that the stimulatory effect of estradiol on the NAD-linked ICD reaction was lost upon fractionation of placental supernatant fractions but could be restored by adding catalytic quantities of NADP. The apparent stimulation of the NAD-dependent ICD could be accounted for by the coupling of a “normal” steroid-insensitive NADP-specific ICD with the action of a soluble transhydrogenase which alone was steroid-dependent, as shown in Reaction 2.

\[
\begin{align*}
\text{Isocitrate} + \text{NADP}^+ & \rightarrow \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADPH} + H^+ \\
\text{NADPH} + \text{NAD}^+ & \rightarrow \text{NADP}^+ + \text{NAD} \\
\text{Isocitrate} + \text{NAD}^+ & \rightarrow \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADH} + H^+
\end{align*}
\]

Reaction 2

We confirmed our suspicion that the steroid-dependent nicotinamide nucleotide transhydrogenase was indeed the 17β-HSD. The stimulatory effect of estradiol was unrelated to isocitrate and could be demonstrated equally well with any NADPH-generating system. We then established that the nicotinamide nucleotide transhydrogenase activity was promoted by the HSD, as shown in Scheme 1. These studies were published in two papers in Proceedings of the National Academy of Sciences of the U. S. A. in 1958 (17, 18). Our demonstration that oxidoreductions of steroid hormones by HSDs could promote reversible transfer of hydride groups between NAD(H) and NADP(H) was slowly accepted by others. Williams-Ashman and I hypothesized that these reactions could exercise cellular regulatory roles in adjusting the balance between NADH-dependent energy-generating oxidative reactions and NADPH-requiring cellular synthetic processes. There seemed to us to be a certain simple elegance to this proposal, but it received a less than enthusiastic reception by the scientific community. We envisaged a type of coenzymatic function of steroids that regulated the flow of reducing
equivalents between the two large intracellular nicotinamide nucleotide pools. However, difficulties in obtaining experimental support for the importance of this process in vivo weakened our suggestion for its potential regulatory significance.

Other Microbiological Transformations of Steroids—H. Richard Levy (postdoctoral fellow) and S. J. Davidson (graduate student) identified three flavoproteins involved in the introduction of the $\Delta^1$, $\Delta^4$-$\Delta^5$, and $\Delta^4$-$\Delta^5$-olefins into steroid Ring A (19–21). In another study, Richard Prairie, a graduate student, showed that the mechanism of lactonization of Ring D of testosterone to form testolactone involved oxidation of the 17$\beta$-hydroxyl group by an NAD-dependent 17$\beta$-HSD followed by action of an NADPH- and oxygen-requiring lactonase. Isotope incorporation and exchange experiments with $^{18}$O$_2$ and H$_2$O$_{18}$O established that the oxygen atom linking C-13 and C-17 in the six-membered D ring of the steroid lactone was derived from molecular oxygen. The lactonase mechanism was therefore formally similar to the Baeyer-Villiger oxidation of cyclic ketones to lactones by peracids (22).

At about this time, I left the University of Chicago and became the Director of the Department of Pharmacology and Experimental Therapeutics at the Medical School of Johns Hopkins University.

Studies on S-Adenosylmethionine

How the Microbial Degradation of Steroid Hormones Led to Inhibitors of the Synthesis of S-Adenosyl-L-methionine—Although we were intrigued by the undoubtedly intricate enzymatic pathways by which $P$. testosteroni could functionalize and completely oxidize all of the carbon atoms of testosterone (C$_{19}$H$_{28}$O$_2$) to carbon dioxide and water, we decided not to pursue this issue because it appeared to be only remotely relevant to the problems of medicine. Our limited studies focused on the enzymatic cleavage of steroid Ring A. Soluble extracts of steroid-induced $P$. testosteroni converted the A ring of 4-[14C]androstene-3,17-dione to an unusual 6-carbon keto acid, 2-oxo-cis-4-hexenoic acid, which was rapidly aminated to 2-amino-cis-4-hexenoic acid (23, 24). (Fig. 3). Interestingly, much earlier, William Shive of the University of Texas (25) had observed that these conformationally restricted amino acids antagonized methionine-dependent bacterial growth, presumably because –CH=CH– and –S– groups are isosteric.

Therefore, we synthesized both the cis and trans isomers of the 6-carbon crotylglycines as well as their precursor oxo-acids. The potential of developing antitumor agents prompted us to undertake an extensive series of studies on conformationally restricted analogues of methionine as inhibitors of the methionine adenosyltransferase reaction. We selected adenosyltransferase preparations obtained from mammalian liver, bakers' yeast, and Escherichia coli as target enzymes. Postdoctoral fellows J. B. Lombardini, A. W. Coulter, Janice R. Sufrin, and T.-C. (David) Chou were my principal collaborators in this problem (26, 27). A large number of conformationally rigidified unsaturated and cyclic analogues were synthesized (e.g. 2-amino-4-hexynoic acid and 1-amino-3-methylcyclopentanecarboxylic acid). Unfortunately, several years of work did not uncover potent or species-selective enzyme inhibitors. The results were not promising, and we abandoned work on this project. Nevertheless, it was shown that inhibitors could depress levels of adenosylmethionine in tissues, and it provided an opportunity to undertake detailed kinetic studies of the enzymes synthesizing S-adenosyl-L-methionine (SAM).

In the course of these studies, we needed to quantify the combined effects of two or more inhibitors and to determine whether the effects were additive, synergistic, or antagonistic. David Chou undertook a formal analysis of these questions, and they developed into a major theme of his subsequent scientific life. Chou showed that the inhibition kinetics of two or more
reversible (but mutually exclusive) inhibitors (competitive, noncompetitive, or uncompetitive) could be described, irrespective of mechanism (sequential or ping-pong), by exceptionally simple equations that provided a rigorous definition for the summation, synergism, or antagonism of two or more inhibitors. Moreover, knowledge of the kinetic constants ($K_m$ and $V_{max}$) for substrates and inhibitors was not required for this analysis (28). Chou then generalized these considerations to analyze the quantitative effects of a wide variety of inhibitors in cell, animal, and human systems. He developed a simple generalized relation, which he designated the Median Effect Equation, and computerized its use. This equation has become the standard tool for quantifying the effects of multiple drugs in systems varying in complexity from isolated enzymes to human diseases; it has been used to analyze thousands of chemotherapy studies worldwide (29, 30).

**Absolute Configuration of the Sulfonium Center of S-Adenosylmethionine—**By 1973, I had devoted 10 years to directing the Department of Pharmacology and Experimental Therapeutics at Johns Hopkins. It had been a satisfying but challenging experience. A number of outstanding faculty members had been appointed, including among others: Donald S. Coffey, Pedro Cuatrecasas, Cecil H. Robinson, and Solomon H. Snyder. Mackenzie Walser, a distinguished renal physiologist, was already a member of the faculty when I arrived. These individuals enjoyed international scientific reputations. A Ph.D. program in pharmacology was in place for the first time, the scope of our teaching had been expanded, and a mass spectrometry center had been established under the direction of Catherine C. Fenselau. However, my own research lagged. I was 50 years old, and the departmental and institutional demands of the directorship had left their mark on my science.

With support from a Guggenheim Fellowship, I took sabbatical leave to work with Professor John Warcup (Kappa) Cornforth, who was then head of the Milstead Laboratory for Chemical Enzymology at Shell Research in Sittingbourne, Kent. Australian by birth and trained under Robert Robinson at Oxford, Cornforth had spent the war as part of the British team that elucidated the structure of penicillin. A series of subsequent classical and very elegant studies on the stereochemistry of the biosynthesis of cholesterol and fatty acids (with George Popjak) were recognized by award of the Nobel Prize in Chemistry in 1975 for “work on the stereochemistry of enzyme-catalyzed reactions.”

We spent a very happy year living in a tiny village in Kent (Bredgar) where my English wife Pamela enjoyed being “home” again, and my three daughters benefited from very successful exposures to British education. Kappa and his wife Rita (also a chemist) were most gracious hosts. It all seemed an idyllic time for us, but I could not have predicted at the outset that the sabbatical year would change the course of my professional career.

Cornforth suggested that I determine the absolute configuration of the sulfonium center of $S$-adenosyl-$L$-methionine (SAM). This compound was isolated and its structure was elucidated by Giulio Cantoni, who began his work on transmethylation mechanisms at Case Western Reserve University in Cleveland and has spent all of his subsequent scientific career at the NIH. Cantoni showed that the $(-)$-diastereomer participated preferentially in nearly all of its donor reactions. The molecule had defied numerous efforts at crystallization, and Cornforth suggested that the problem be solved by chemical degradation. He proposed a degradation scheme comprising the cleavage of the labile adenosylribose linkage by base, followed by periodate oxidation of the ribose moiety to provide (it was hoped) only one of the diastereomers of $S$-carboxymethyl-$L$-methionine (Fig. 4). The project was risky and technically demanding, because it was uncertain whether chirality would be preserved during the degradation and
whether the diastereomeric products could be obtained in adequate quantities for crystallization and establishment of configuration by x-ray crystallography. Fortunately, my professional future did not depend on the success of the outcome since grant-giving bodies were not exactly rushing to support an esoteric project of this type. Moreover, it was anticipated that the results could be summarized completely by a single letter: S or R. Earlier, W. H. Stein and S. Moore had separated the two diastereomers of S-carboxymethyl-L-methionine on an analytical scale by ion exchange chromatography, but we were not able to obtain adequate quantities for crystallization and chemical work by these methods. Cornforth recommended a more classical approach: separation by fractional crystallization. The polyiodide salts crystallized beautifully. The black crystals of the diastereomers had distinctive morphologies, and the shiny pyramids of the less soluble diastereomer could be separated mechanically from the long needles of the more soluble diastereomer (Fig. 4). Even my wife Pamela (a Ph.D. in Biochemistry from Cambridge) honed her experimental skills by sorting the crystals when I was away. As it turned out, the more soluble and more difficult to prepare diastereomer had the structure corresponding to the natural SAM. On hearing this, Cornforth remarked: “the desired isomer is always in the mother liquor.”

The x-ray crystal structure was elucidated by Jenny P. Glusker and H. L. Carrell at Fox-Chase in Philadelphia on a more stable crystalline (2,4,6-trinitrobenzenesulfonate) derivative, which I prepared from one of the diastereomeric polyiodides. It took more than 3 years (long after I returned to Baltimore) to complete this project with the technical help of Scott Reichard, who subsequently went to medical school. The complete story was published in a single paper (31). It was the first report of the steric configuration of a naturally occurring sulfonium center: S.

On completion of the project, Cornforth observed: “You see, Paul, the classical methods are always more fun.” He also offered an unsolicited assessment of my performance in his laboratory. In a Festschrift on the occasion of his 75th birthday, Cornforth wrote: “I think (Paul Talalay) enjoyed the bench work at Milstead like a boy on holiday, even the laborious initial separation of the A and B polyiodides, by picking out individual crystals.” It was true. I had not realized how very much I enjoyed working at the bench. Just before Christmas 1973, I flew to Baltimore to inform Dr. Russell H. Morgan, the Dean of the Medical Faculty at Johns Hopkins, that I wished to relinquish the directorship of pharmacology to devote myself more fully to research. Morgan responded perceptively, graciously, and correctly: “You don’t want me to talk you out of this decision, do you?” He created the Laboratory of Molecular Pharmacology for me, provided a budget, and recommended that I be named the first John Jacob Abel Distinguished Service Professor, a title that I continue to hold today. These generous administrative arrangements made it possible for me to remain in the same department that I had directed and to devote the last 25 years to devising strategies for chemoprotection against the risk of cancer.

\[ \Delta^5 \text{-3-Ketosteroid Isomerase (KSI): Diffusion-controlled Carbon-Hydrogen Bond Cleavage and Intramolecular Proton Transfer} \]

Undoubtedly my longest and my most passionate enzymological love affair has been with \( \Delta^5 \text{-3-ketosteroid isomerase (KSI). In the 50 years between its discovery (16) and the recent description of its intimate catalytic mechanism, we published more than 35 papers on this} \]
steroid-induced enzyme from *P. testosteroni*. It provided a wonderful education for me and a satisfying exercise in mechanistic enzymology.

Many natural 3β-hydroxysteroids are also Δ⁵-olefins (e.g. cholesterol, dehydroepiandrosterone). Nicotinamide nucleotide-dependent oxidations of these steroids to the 3-ketosteroid invariably give rise to Δ⁴-3-ketosteroids. Although the double bond migration into conjugation occurs spontaneously and is acid- and base-catalyzed, we reported in a brief Communication (32): “We wish to present evidence for the existence of a widely distributed steroid isomerase, an enzyme which is distinct from the oxidizing enzyme, and which catalyzes the migration of the double bond from Δ⁵ to the Δ⁴ position of 3-ketosteroids,” and suggested the name ketosteroid isomerase (KSI) (Fig. 5).

Frank Kawahara (a graduate student) developed an unorthodox method for the purification and crystallization of KSI. “Crystalline Δ⁵-3-Ketosteroid Isomerase” was published as the first Preliminary Communication in this Journal (33). KSI enhanced the catalytic rate about 10¹¹-fold, approaching the diffusion-controlled maximum. The isomerization occurred at comparable rates in H₂O and D₂O and when conducted in media enriched with ³H₂O or D₂O, resulted in little incorporation of the label into the product, unlike acid- or base-catalyzed isomerizations. We concluded that: “the enzymic mechanism suggests that there is a direct transfer of a proton from position 4 to 6 on the enzyme surface without exchange with the medium.” With the use of appropriately deuterium-labeled steroids, Howard Ringold (34) established unequivocally that the isomerase reaction involved the direct intramolecular (suprafacial, diaxial) transfer of the 4β-proton to the 6β-position and observed a large primary kinetic isotope effect in the C-4β-D cleavage.

By good fortune, the 125-amino acid peptide chain of KSI contained neither cysteine nor tryptophan, and the 3 tyrosine and 8 phenylalanine residues provided a unique UV spectrum in which the absorbance bands of these residues were easily identifiable (35, 36). Considerable insight into the mechanism of KSI was therefore obtainable from absorption and fluorescence spectra of enzyme-steroid complexes, as well as from hydrogen isotope exchange between steroids and the medium (36). Although the catalytic mechanism of isomerization did not involve participation of protons of the medium, high concentrations of KSI catalyzed the exchange of protons of the medium with the hydrogen atoms of the steroid reaction products. The finding that the incorporated isotope could be removed completely under enolizing conditions suggested that the reaction involved an enolic intermediate. More direct evidence for this view was provided by spectroscopy. For instance, on binding to KSI, the typical absorption of Δ⁴-3-ketosteroids was displaced to longer wavelengths, and binding of phenolic steroid competitive inhibitors (17β-estradiol and 17β-dihydroequilenin) produced large UV spectral (and for the equilenin also fluorescent) changes compatible with the formation of phenolate anions. The logical inference was that the KSI reaction involved enol or enolate intermediates.

Crystalline KSI of *P. testosteroni* was completely sequenced by Ann M. Benson, a postdoctoral colleague, by classical methods involving isolation of tryptic and chymotryptic peptides.
and their sequencing from the N terminus by Edman degradation and the C terminus by hydrazinolysis and carboxypeptidases (37). This task, which required more than 2 years, can now be accomplished in a few days. Ann Benson was my long time colleague. She brought protein sequencing expertise to our laboratory and went on to make many other important contributions.

Research on KSI remained somewhat dormant until 1987 when a highly successful collaboration was initiated with my colleague Albert S. Mildvan, Professor of Biological Chemistry at Hopkins, who had a lifelong dedication to mechanistic enzymology and wanted to apply NMR techniques to KSI. We were fortunate to interest a number of colleagues, students, and postdoctoral fellows (including Athan Kuliopulos, Liang Xue, Qinjian Zhao, and Yaw-kuen Li) in this project.

The KSI gene of *P. testosteroni* was isolated, sequenced, and overexpressed (38). Mutations of Tyr-14 to phenylalanine (Y14F) reduced $k_{\text{cat}}$ by $10^{4.7}$, whereas replacement of Asp-38 by asparagines (D38N) reduced $k_{\text{cat}}$ by $10^{5.6}$. In contrast, substitution of Tyr-55 by phenylalanine (Y55F) reduced $k_{\text{cat}}$ by only a factor of 4. Thus, the two former mutations abolished the catalytic activity of KSI whereas mutation of Tyr-55 had only a minor effect (39, 40).

Extensive studies on the combined kinetic deuterium substrate and solvent isotope effects suggested that the reaction involved acid-base catalysis and led to the formulation of the following mechanistic model. The isomerase reaction occurs in two steps (not in a concerted manner) via a dienolic intermediate(s). In the rate-limiting first step, Tyr-14 acts as a general acid that protonates (by creating a low barrier hydrogen bond) (41) the carbonyl group of the steroid substrate, whereas Asp-38 is the base that accepts the 4β-H and transfers it to the 6β-position. Asp-99 assists in polarizing the steroid carbonyl group.

**FIG. 6. Catalytic mechanism of the Δ⁵-3-ketosteroid isomerase reaction.** Tyr-14 protonates the 3-carbonyl group of the steroid, and Asp-38 accepts the 4β-H and transfers it to the 6β-position. Asp-99 assists in polarizing the steroid carbonyl group.

Inactivation of Δ⁵-3-Ketosteroid Isomerase by Mechanism-based (Suicide) Inhibitors—During studies on the biosynthesis of long chain, unsaturated fatty acids in *E. coli*, Konrad Bloch uncovered a new principle in the design of highly selective enzyme inhibitors, which he summarized as follows: “By catalyzing the transformation of a relatively unreactive substrate analogue to an extremely reactive active-site-directed reagent, the enzyme causes its own destruction.” The specificity of inhibition depends on the presence in the substrate analogue of latent functional groups that are specifically unmasked by the enzyme, thereby generating an affinity label at the desired site. Such reagents are also known as suicide substrates and mechanism-based or enzyme-activated inhibitors.

My colleague and friend Cecil H. Robinson recognized the basic similarities (of proton transfer) between the reaction promoted by the fatty acid synthetase and KSI, and made the very imaginative suggestion that A:B-seco-3-ketosteroids in which an acetylenic function replaced the C-5 to C-6 bond might be converted by the normal C-4β-proton-abstracting function of the enzyme to highly reactive α,β-unsaturated allenes susceptible to attack by nucleophilic residues of the enzyme. These predictions were fully borne out when Robinson, with his associates F. H. Batzold and D. F. Covey, synthesized a series of acetylenic seco-steroids and showed that they were rapidly converted by KSI to the conjugated allenic ketones, which inactivated the enzyme rapidly and irreversibly (45–48).
Identification of the site, chemical nature, and location of attachment of these suicide seco-steroid substrates to KSI turned out to be a complex problem, which was finally solved by the enormously dedicated efforts of Trevor M. Penning, then a postdoctoral fellow and now Professor of Pharmacology at the University of Pennsylvania. Penning found that the linkage was labile to both mild acids and bases and that when the steroid was released, both the steroid and the protein underwent subtle chemical modifications. I cannot here describe the findings in detail and the way in which they were uncovered. It will suffice to say that the linkage was shown to be an enol ester linked to the amide group of Asn-57, which in the inactivated enzyme formed an enol-imidate with the enzymatically generated steroid allene (49) as shown in Fig. 7. Unfortunately, Asn-57 does not appear to play a significant role in the catalytic process.

Much of our work on KSI was continuously supported by a single NIH Grant (AM07422), which survived reviews for 33 years, more than half the life of the NIH granting system.

Mammalian Δ⁵-3-Ketosteroid Isomerases—In a brief scientific diversion, Ann Benson searched for KSI activity in animal tissues. Supernatant fractions of beef liver displayed modest KSI activity, which was easily lost but could be restored by addition of glutathione. Purification disclosed that this KSI activity was very abundant and was promoted by glutathione S-transferases (GSTs). This identity was corroborated by chromatography and immunoprecipitation in collaboration with James H. Keen and William B. Jakoby of the NIH, who were then studying the multiplicity of GSTs (50). Δ⁵-Androstene-3,17-dione became another useful substrate for characterizing GSTs. My good friend Professor Bengt Mannervik in Uppsala clarified the detailed mechanism of the GST-dependent KSI reaction and even identified GSTs for which Δ⁵-androstene-3,17-dione was the preferred substrate (51). Through this scientific excursion we gained familiarity with GSTs, which proved invaluable in our later studies on chemoprotection against cancer.

Chemoprotection against Cancer: How Do Edible Plants Reduce the Risk?

For the last 25 years our laboratory has focused on developing strategies for reducing the risk of cancer. This interdisciplinary research program spans chemistry, growing of plants, molecular biology, animal disease models, and human studies. Our studies started with little hope or promise and were shunned as risky, if not foolhardy, by many members of the scientific community. Although this program consumes nearly all our current energies, it is summarized here only briefly because this work is very much in progress and has been recently reviewed comprehensively (52–55).

BHA and Glutathione Transferases—In 1978 my colleague and friend, the late Ernest Bueding, a biochemical parasitologist, was concerned with the question of how to avert the mutagenicity and carcinogenicity of his newly discovered agents that cured schistosomiasis. We were familiar with the then recent observations by Lee Wattenberg at the University of
Minnesota, who had shown that BHA (tert-butyl-4-hydroxyanisole) and other phenolic anti-
oxidants that are widely used as preservative food additives could substantially reduce the
tumorigenicity of several types of carcinogens in various animal organs. In one of our first
experiments we showed that feeding BHA dramatically reduced the mutagenic activity in the
urine of mice that received benzo[a]pyrene, strongly suggesting that the protective mechanism
involved major alterations in carcinogen metabolism. Fortuitously, as indicated above, assays
for GSTs were then routine in our laboratory, and we found marked elevations of the specific
activities of GSTs and of GSH levels in the livers and other organs of the BHA-fed animals
(56–58). These findings led us to propose that these phase 2 elevations were responsible for
protection against carcinogens. In 1978 we wrote: “The most plausible mechanism for protective
effects of antioxidants invokes alterations of the balance between metabolic activation and
inactivation of carcinogens.” The concept that induction of phase 2 genes is an effective and
sufficient strategy for achieving protection against carcinogenesis is now widely accepted. The
observations of Wattenberg were of profound importance because they showed for the first
time that widely consumed dietary components, presumed to be of low toxicity, were able to
block carcinogenesis.

Cloning of an abundant GST (mu class) in collaboration with John Morrow and his associ-
ates (59) demonstrated that BHA treatment raised the level of the mRNA transcripts for this
enzyme substantially and increased its rate of synthesis in liver slices. The conclusion that
induction resulted from enhanced gene transcription has been confirmed for many other phase 2
enzymes.

NADPH:Quinone Acceptor Oxidoreductase (Quinone Reductase; NQO1)—A very useful ad-
vance was the observation made with Ann Benson (60) that an enzyme discovered by Lars
Ernster in Stockholm and that became known by a bewildering number of synonmys (e.g. DT
diaphorase, NA(P)H:quinone reductase, quinone reductase, menadione reductase) and is now
commonly called nicotinamide quinone oxidoreductase 1 (NQO1) was also induced by BHA in
mouse organs, especially in the epithelia of the gastrointestinal and urinary tracts, where
potential exposure to dietary carcinogens and mutagens is likely to be most intense. This
FAD-dependent enzyme, which utilizes NADH and NADPH with comparable efficiencies and
catalyzes the obligatory two-electron reduction of quinones to hydroquinones, has emerged as
a fundamental prototype of the phase 2 response. NQO1 is of central importance in protecting
cells against oxidative cycling (and the generation of reactive oxygen species) and GSH
depletion by quinones (61) and has provided a highly useful index for phase 2 gene status of
cells (62).

Mary De Long (a Research Associate), Hans Prochaska (an M.D.-Ph.D. student), and
Annette Santamaria (a technical assistant) were the first to demonstrate phase 2 gene
induction in cultured cells by a wide variety of chemical types of inducers (63–65). The very
useful and widely adopted microtiter plate system that they developed involved quantifying
the elevation of the specific activity of NQO1 in murine hepatoma 1c1c7 cells. This enzyme was
relatively easy to measure and showed a large and highly reproducible response range, and the
cells were robust and easy to manage. Moreover, mutants of Hepa1c1c7 were available that
were deficient in expression or function of certain cytochromes P450. This made it possible to
distinguish two classes of inducers of the phase 2 response, those that were dependent on and
also induced cytochrome P450 function (mostly large planar aromatics), which we designated
as bifunctional inducers, and those that can induce in the absence of cytochrome P450
function, as monofunctional inducers (66). A major implication of this distinction is that
monofunctional inducers are more desirable as chemoprotectors since cytochromes P450 may
be involved in carcinogen activation, whereas phase 2 enzymes only very rarely activate
procarcinogens to carcinogens.

At the same time, we undertook structural and kinetic studies on NQO1. Hans Prochaska
showed his scientific versatility by developing a highly efficient single step affinity chroma-
tography for purifying NQO1 from rat liver and crystallized this enzyme for the first time (67).
He also was the first to purify and crystallize two closely related NQO1 isoforms from mouse
liver. We introduced our colleague L. Mario Amzel, a crystallographer and Professor of
Biophysics and Biophysical Chemistry, to the world of quinone reductases. Rongbao Li (a
graduate student) with Amzel and Mario Bianchett solved the structure of rat NQO1 (68).
Qinjiang Zhao (postdoctoral fellow and an important contributor to the KSI problem) and my
long time associate David Holtzclaw isolated, cloned, and overexpressed the closely related
The crystal structure of NQO2, an FAD-containing metalloflavoprotein, was elucidated by Christine Foster working jointly in Amzel and our laboratories (69). Amzel and Bianchett have since made important contributions to the structure of human NQO1 and especially how it is adapted to the unmasking of quinone groups of chemotherapeutic alkylating agents such as mitomycins, anthracyclines, and aziridylbenzoquinones (70).

**Phase 2 Inducer Potency of Plant Extracts: Isolation of Sulforaphane**—With the availability of a relatively simple microtiter plate method for quantifying inducer activity, we asked whether the widely recognized lower incidence of malignancies in high fruit and vegetable consumers might be attributable, even in part, to the presence of protective phase 2 enzyme inducers in these plants. Hans Prochaska attacked this problem with enthusiasm. Organic solvent extracts of a broad selection of fruits and vegetables from local markets disclosed not only that inducer activity was present but also that it varied enormously among plant families (65). The *Cruciferae*, and especially those belonging to the *Brassica* genus (e.g. cauliflower, kale, broccoli, cabbage) were the richest sources of inducer activity. Yuesheng Zhang, who obtained his Ph.D. degree in our department, isolated the active inducer principle from broccoli. Extensive studies with organically grown vegetables reassured us that the inducer activity was not due to contamination by pesticides or insecticides. We found a reliable source of SAGA broccoli, grown in Caribou, Maine, and shipped to us frozen. Repeated chromatographies monitored by NQO1 activity assays revealed that the inducer activity was attributable to a single compound and finally led Zhang to isolate a few milligrams of a liquid from 1 pound of broccoli. We had no preconceived notions as to its identity. Professor Gary H. Posner of the Department of Chemistry of Johns Hopkins University rapidly showed that it was an isothiocyanate (mustard oil), 1-isothiocyanato-4-(methylsulfinyl)butane (Fig. 8) (71). Sulforaphane had already been isolated many years earlier by Czech workers, based on its rather weak antibiotic activity, from a widely distributed cruciferous weed known as hoary cress and from savoy and red cabbage. Posner synthesized gram quantities of sulforaphane and also a number of its analogues, varying in the length of the methylene chain and the state of oxidation of the sulfur of the methylthio group. Bioassays revealed that sulforaphane was the most potent naturally occurring phase 2 inducer known.

Availability of gram quantities of sulforaphane made it possible to evaluate (in collaboration with my long time colleague Thomas W. Kensler) the ability of this compound to suppress tumor formation in the Huggins single-dose DMBA rat mammary cancer model. It was extremely gratifying that sulforaphane inhibited the incidence and multiplicity of mammary tumors and also delayed their development (72). This observation provided strong confirmation for the principle that phase 2 gene induction resulted in protection against carcinogenesis.

Gary Posner undertook a methodical analysis of the structure-inducer activity relation among a large series of isothiocyanate analogues that he synthesized. He designed a series of conformationally restricted (norbornyl) isothiocyanates and provided guidelines for predicting their inducer potencies. The most potent of the analogues were also inhibitors of tumor formation in the mammary tumor model (73).

The finding that broccoli contained a very potent cancer chemoprotective agent generated considerable and unanticipated media attention, including a story on the front page of *The New York Times*, inclusion among *Historic Documents of 1992* (Congressional Record), and selection as one of the 100 major discoveries of the last century by *Popular Mechanics*. This hyperbole was undoubtedly fueled by George H. W. Bush, then President of the United States, who had publicly declared his dislike for broccoli, claiming that his mother had made him eat broccoli but that now that he was President of the United States he was no longer obliged to do so.
We realized that plants were potentially very rich sources of anticarcinogenic phase 2 inducers and that we did not have the facilities or expertise to undertake their study. To remedy this deficiency we founded the Brassica Cancer Chemoprotection Laboratory in old but serviceable space in the Blalock Building of the Johns Hopkins Hospital. By very good fortune, Jed W. Fahey, a Hopkins graduate who was trained as a plant physiologist, joined our laboratory in 1994. His knowledge and contributions have been an essential part of developing plants to combat cancer. Fahey soon showed that sulforaphane was actually an artifact of isolation; the intact plants contain almost exclusively the glucosinolate precursor of sulforaphane, known as glucoraphanin. Upon plant injury, food preparation, or eating, the intracellularly segregated enzyme myrosinase is released and hydrolyzes the water-soluble and stable glucoraphanin to the highly reactive sulforaphane, which is the ultimate inducer (74).

Analysis of a selection of randomly selected broccoli heads from supermarkets disclosed huge variations in inducer potential that were unrelated to their appearance. Jed Fahey (74) then grew broccoli sprouts from seeds. Nearly 100 varieties of broccoli seeds are commercially available, and whereas they also vary greatly in their glucoraphanin content, the concentration of this glucosinolate in seeds and 3-day-old sprouts grown from them was always much higher than that in market stage broccoli heads. Indeed it became possible to grow sprouts that contained 20 times higher concentrations of glucoraphanin than many commercial broccolis. Three-day-old broccoli sprouts are well tolerated by humans. The repeated administration of calibrated doses of glucoraphanin or sulforaphane in the form of broccoli sprout extracts produced no significant toxicity in Phase 1 safety and tolerance tests in volunteers. Broccoli sprouts are a food and therefore an ideal vehicle for administration of glucoraphanin or sulforaphane (after myrosinase hydrolysis) to humans without elaborate regulatory requirements. A number of clinical trials of broccoli sprouts in high risk cancer-prone populations are in progress.

One serendipitous finding made by Fahey and his collaborators (75) is that sulforaphane is bactericidal for Helicobacter pylori, an infection that causes peptic ulcers and is associated with great increases in the risk for gastric cancer. The value of broccoli sprouts for treatment of Helicobacter infections is also being evaluated.

Chemistry of Inducers—Elucidation of the structural requirements for inducer activity led to two important advances: (i) it identified inducers of dietary origin that were suitable for chemoprotective studies in humans and (ii) it played a major role in providing insight into the mechanism whereby inducers signal the transcription of phase 2 genes.

Mechanistic understanding of the up-regulation of the phase 2 response required better information on the relation of structure to inducer potency. The first step was the finding that inducer potency of various BHA analogues was not highly dependent on structure (76, 77). Thus a number of synthetic dialkyl ethers of tert-butylhydroquinone were weaker phase 2 inducers than BHA itself. Indeed, the unsubstituted tert-butylhydroquinone and even hydroquinone itself were the most potent inducers. We concluded that BHA was metabolized to more potent intermediates and that involvement of a structurally complementary “receptor” in the signaling process was unlikely. Although this finding provided only a modest advance in our understanding of how structure affected inducer activity, Hans Prochaska, who had synthesized and tested all of the alkyl analogues of BHA, was not discouraged. He argued that all the compounds tested up to this time were substituted 1,4-diphenols and weaker phase 2 inducers than BHA itself. Indeed, the unsubstituted tert-butylhydroquinone and even hydroquinone itself were the most potent inducers. We concluded that BHA was metabolized to more potent intermediates and that involvement of a structurally complementary “receptor” in the signaling process was unlikely. Although this finding provided only a modest advance in our understanding of how structure affected inducer activity, Hans Prochaska, who had synthesized and tested all of the alkyl analogues of BHA, was not discouraged. He argued that all the compounds tested up to this time were substituted 1,4-diphenols and suggested that 1,2-diphenol (catechols) and 1,3-diphenol (resorcinol) derivatives should be tested to determine whether oxidative lability (to which hydroquinones and catechols but not resorcinol derivatives were susceptible) controlled inducer potency. Evaluation of a substantial number of analogues led to the conclusion that only oxidizable diphenols (and also the corresponding phenylenediamines) were inducers, whereas resorcinols were invariably inactive. These findings showed that oxidative lability was critical for inducer activity (78) but did not answer the question of whether the oxidizable diphenols themselves or their quinone oxidation products were the ultimate inducer species. Insight into this question was obtained unexpectedly by dissection of the structure of a series of coumarins. We concluded that inducers of this chemical family contained previously unrecognized structural features. They were Michael reaction acceptors (79), and their inducer potencies were closely correlated with their Michael reactivities. Quinones are potent Michael acceptors, and we inferred that the above mentioned oxidizable diphenols exerted their inducer activities through conversion to quinones. This
permitted prediction of which structures were likely to be inducers, and many new synthetic and naturally occurring inducers have since been identified on this basis (80–82).

More than 10 different chemical classes of inducers have now been recognized including isothiocyanates and their thiol addition products, dithiocarbamates, as well as 1,2-dithiole-3-thiones, trivalent arsenic derivatives (e.g. phenyl arsionoxide), heavy metals, certain conjugated cyclic and acyclic polyenes (including porphyrins, chlorophyllins, and chlorophyll), and vicinal dimercaptans. Notably, these inducers have few structural similarities. They are mostly electrophiles, and all can react chemically with thiol groups by alkylation, oxidation, or reduction, suggesting to us that the intracellular sensor for inducers is likely to contain very highly reactive (cysteine) thiols. This suggestion was bolstered by the following: (a) the potencies of inducers paralleled their reactivity with thiols (the inducer potencies of Michael reaction acceptors are related to their reaction rates as electrophiles); (b) the very high inducer potency of trivalent arsenicals (e.g. phenyl arsionoxide) suggested the involvement of two vicinal thiols, possibly resulting in the formation of relatively stable cyclic thioarsenites; and (c) the potency order of divalent cations (Hg²⁺ > Cd²⁺ > Zn²⁺) corresponded exactly to their thiol reactivity (83). Interestingly, inducers can modify thiol groups by a variety of mechanisms including: (i) alkylation (Michael reaction acceptors, isothiocyanates, quinones); (ii) oxidation (e.g. peroxides and hydroperoxides); and (iii) direct reaction with thiol/disulfide linkages (e.g. vicinal dithiols such as 1,2-dimercaptopropanol, lipoic acid). These rather promiscuous response mechanisms provide plasticity for cellular responses to a variety of electrophilic and oxidant stressors.

The molecular mechanisms by which a large number of inducers of different chemical types up-regulate the phase 2 response were analyzed by Tory Prestera (an M.D.-Ph.D. student). He transiently transfected hepatoma cells with a construct containing the promoter region of the mouse glutathione transferase Ya gene linked to a growth hormone promoter gene. In this system there was a very close correlation between the potencies of inducers in elevating growth hormone expression and in inducing the phase 2 response over a range of 4 orders of magnitude of concentration (84, 85). These experiments provided strong evidence that all classes of inducers stimulate the same regulatory element (the antioxidant response element (ARE) described below). We proposed that this cellular response to electrophile and oxidative stress be designated the “electrophile counterattack response.” What, then, is the identity of the sensor for inducers?

The Quest for the Elusive Sensor for Phase 2 Inducers and the Molecular Mechanisms of Induction—Our initial efforts to identify the sensor containing highly reactive thiol groups that recognize and react with inducers involved use of tritiated dexamethasone mesylate, an alkylating agent that had been synthesized as a ligand for the corticosteroid receptor. This steroid mesylate was a moderately potent inducer, but we were unable to identify the sensor, although we isolated a number of more abundant proteins endowed with highly reactive cysteine residues (55). Identification of the sensor finally became possible with the discovery of Keap1 by Yamamoto and colleagues (86). Murine Keap1 is a multidomain 624-amino acid protein that includes 25 cysteine residues, many of which are intrinsically highly reactive because they are flanked by basic residues which results in substantial lowering of their $pK_a$ values.

Three cellular components are centrally involved in phase 2 gene regulation: (i) antioxidant response elements (AREs), cis-acting promoters of transcription that are present in the upstream regions of many phase 2 genes and have the consensus sequence: TGA(G/C)NNNGC, first described by Cecil B. Pickett; (ii) Nrf2 (nuclear factor-erythroid 2-related factor 2), a basic leucine zipper transcription factor that is itself inducible, binds in heterodimeric combination with other transcription factors to the ARE promoters, and signals enhanced transcription of phase 2 genes; and (iii) Keap1, a Kelch family multidomain repressor protein that is normally localized in the cytoplasm where it is tethered to the actin cytoskeleton. Keap1 binds to Nrf2 very tightly and is thereby largely retained in the cytoplasm so that its activity is repressed. Inducers disrupt the Keap1-Nrf2 complex, thereby releasing Nrf2 for translocation to the nucleus and activation of the transcription of phase 2 genes (Fig. 9).

Cloned and overexpressed mouse Keap1 was purified by Albena Dinkova-Kostova (research associate) and David Holtzclaw. When pure Keap1 was exposed for a limited time to $[^3H]$dexamethasone mesylate, 4 of its 25 cysteine residues became labeled and were identified as Cys-257, Cys-273, Cys-288, and Cys-297 by MALDI-TOF mass spectrometry. All of these
Cysteine residues are located in the cysteine-rich intervening region (IVR) of Keap1 that links the BTB (broad complex) dimerization domain with the double glycine (DGR) or Kelch domain, which is involved in binding actin and Nrf2. Albena Dinkova-Kostova and David Holtzclaw also showed that complexes of pure Keap1 with the critical Neh2 segment of Nrf2 were disrupted by addition of inducers and that covalent interaction of Keap1 with various inducers could be demonstrated directly by ultraviolet spectroscopy. These findings finally provided very strong and direct evidence that certain cysteine residues of Keap1 were indeed the intracellular sensors for phase 2 inducers (87). With the knowledge that the highly reactive cysteines were located in the IVR of Keap1, mutation experiments were carried out by Nobunao Wakabayashi, a visiting scientist from Tsukuba University in collaboration with Dinkova-Kostova and Holtzclaw (88). Single and multiple mutations of the four aforementioned cysteine residues established that substitution of either Cys-273 or Cys-288 but not of any other cysteine residues abrogated the capacity of Keap1 to repress the activity of Nrf2 (88).

The question of what is responsible for the especially high reactivity of the cysteine residues that function as sensor for inducers has now turned in a new direction. My colleagues have established that Keap1 is a zinc-containing metalloprotein and that upon binding of inducers, the tightly bound zinc is displaced from the protein. It thus appears that Keap1 is regulated by inducers through the participation of a zinc-sulfhydryl switching mechanism (89). Work on the fascinating mechanism of Keap1 and its implications for chemoprotection against cancer is in full swing.

Some Final Thoughts

Any merit to which these discoveries might be entitled is due entirely to a remarkable group of students, postdoctoral fellows, and other collaborators who have been my laboratory family for more than 50 years and who have enriched my life immensely. Many have become lifelong friends. It has been a joy to witness their scientific development and to draw pleasure from their accomplishments. Unfortunately, it has not been possible to acknowledge explicitly all who have played a part in our scientific journey. In writing these Reflections, I was forced to consider whether they might provide any personal enlightenment about the nature of the process that Peyton Rous called “finding out.” Since I have always considered science to be an artistic endeavor, it seemed highly unlikely that there are any rules for success. There is perhaps one conclusion to which my experiences lead. Good fortune plays a major role in discovery. Much as we might try, there is no “road map” to discovery. All “road maps” are based on mistaken notions and generally lead to failure. They cannot anticipate serendipity.

I am deeply honored to have been asked to write these “Reflections” as we celebrate the 100th birthday of The Journal of Biological Chemistry. It seems especially appropriate that this essay originates from the Department of Pharmacology at Johns Hopkins where the Journal was founded in 1905 and edited by John Jacob Abel, the first professor and acknowledged “father” of American pharmacology, and by Christian A. Herter.

I was privileged to serve as an Associate Editor of the Journal for 5 years (1962–1966) under John T. Edsall at a time when the Editorial Board consisted of only 22 members. Our annual board meetings were held each Spring during the Federation (FASEB) meetings in Atlantic City. Edsall read all the manuscripts, annotated them with his thick black pencil, and always
signed the decision letters. A man of great dignity, Edsall was one of the most beloved Editors and taught all of us so much about the scholarship and ethics of scientific communication.

We regularly pay homage to the JBC in our department by asking our graduate students who was the author of the first paper in the Journal. If the reader needs a little help, the answer may be found in the list of references (90).

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I cannot end without acknowledging that my scientific efforts would not have been possible without the love and support of my wife (and friend) Pamela and our four children. “Acknowledgment” is hardly the appropriate word to describe their role in my life.

Address correspondence to: ptalalay@jhmi.edu.

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