Research on Biochemistry of Herbicides: An Historical Overview

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Z. Naturforsch. **48c**, 121–131 (1993); received October 10, 1992

Herbicide Targets, Photosynthesis, Amino Acid Biosynthesis, Lipid Biosynthesis, Diphenyl Ethers

Otto Warburg, the father of cellular bioenergetics, seems to have been the first investigator to report on inhibition of a plant biochemical reaction by a progenitor of a selective herbicide. The year was 1920 and the compound was phenylurethane (ethyl N-phenylcarbamate or EPC). Warburg found that it strongly inhibited photosynthesis in *Chlorella*. EPC did not develop into a commercial herbicide, but the isopropyl derivatives (propham and chlorpropham) which were introduced in the late 1940s became selective herbicides. The phenylureas (monuron and diuron) were introduced in the early 1950s and shortly thereafter, interference with the Hill reaction by both phenylureas and phenylcarbamates was reported. During the latter part of the 1950s, into the 1960s, and even now, additional herbicidal chemistry was and is being announced that interferes with the Hill reaction. Duysens, in 1963, identified the site of action of diuron, *i.e.*, on the acceptor side of PS II. Corwin Hansch, in 1966 introduced the SAR or QSAR concept in which inhibitory action of Hill inhibitors was related to various chemical and physical parameters.

Because of differential responses to partial, thylakoid-associated reactions, the Hill inhibitors were subsequently divided into two groups: pure electron transport inhibitors (phenylureas, *s*-triazines, triazinones, and uracils) and inhibitory uncouplers (acylanilides, dinitrophenols, benzimidazoles, dinitroanilines, and benzonitriles). The inhibitory uncouplers (dinoseb-types), unlike the diuron-types, uncoupled photophosphorylation by interacting with the coupling factor complexes in both chloroplasts and intact mitochondria. Additionally, the bipyridyliums were shown to be reduced by PS I, hence, diverted electrons from the native acceptor.

Field observations of triazine resistance were reported in 1970 and resistance was subsequently demonstrated at the thylakoid level. Application of the techniques of genetic engineering and biotechnology resulted in identification of the 32 kDa herbicide-binding protein and determination of its amino acid sequence. Crystallization and X-ray examination of the photosynthetic reaction center from *Rhodopseudomonas* by Michel *et al.* in the mid-1980s provided new models to account for interactions of herbicides with the D-1 protein.

During the 1980s, herbicides were identified that interfered with biochemical machinery in chloroplasts that is not involved in electron transport and light harvesting: inhibition of lipid biosynthesis by aryloxyphenoxypropionates and cyclohexanediones, aromatic amino acid biosynthesis by glyphosate, branched chain amino acid biosynthesis by sulfonylureas and imidazolinones, carotenoid biosynthesis by pyridazinones, and porphyrin biosynthesis by diphenylethers and oxadiazoles. The current status of research in most, if not all, of the above areas was reported through oral and poster presentations at this Omiya Symposium.

Introduction

Whereas the target sites of the majority of the major commercial families of herbicides is now known, this was not true in 1980. At that time, the interactions of herbicides with PS II were the best known. Developments have progressed dramatically since then. Where, when, and how did biochemical research with herbicides develop? These are the questions that I hope to chronologically identify in this contribution.

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/93/0300–0121 $ 01.30/0

Inhibitors of many different types have been used to elucidate almost all of the biochemical pathways associated with animal and plant metabolism. Herbicides and research related to their biochemical mechanisms of action have helped to unravel details of several of the biochemical pathways in plants. Limitations of space will prevent detailed documentation of observations and reports. For some observations, reports were published within a year or two of each other by different groups of investigators who had worked independently. Hence, it is difficult to impartially identify which laboratory did what and when in specific terms. Of primary importance, however,

are the contributions that have been made to our storehouse of knowledge on the biochemistry of plants through site-of-action studies with herbicides by a large number of researchers. I have primarily considered observations made with herbicides until about the mid-1980s and have included contributions made at the molecular level (primary responses) as well as the extrapolation of these observations to the expression of toxicity (lethality) at the whole plant level (secondary responses).

Historically, effects of various chemicals on photosynthesis and respiration have been studied since around 1878 when chloroform was described as reversibly inhibiting photosynthesis at concentrations that did not affect respiration [1]. Early studies included alkaloids (quinine, strychnine, morphine), diethyl ether, alcohols, cyanide, hydroxylamine, dinitrophenol, various gases (hydrogen sulfide, carbon monoxide, sulfur dioxide, and nitrous oxides), sodium azide, *o*-phenanthroline, and urethanes. Inhibitors such as cyanide, hydroxylamine, and hydrogen sulfide were referred to as catalyst poisons and as such were considered to form a complex with metal atoms associated with enzyme prosthetic groups. Others, including dinitrophenol were considered to probably react with specific groups on catalytically active proteins [1]. Effects of most of the compounds had previously been explored on animal systems. Hence, information was available on the general types of physiological alterations that were induced. This was especially true for the dinitrophenols and the urethanes.

Chloroform and ether were originally classified as narcotics and as such were considered to act by blocking active surfaces. In animal studies with homologous series of organic compounds, the efficiency of narcotization was known to increase with the length of the carbon chain, and thus to parallel surface activity. Urethane (ethyl carbamate or the ethyl ester of carbamic acid) and phenylurethane (ethyl ester of N-phenylcarbamic acid, EPC) also were classified as narcotics. Urethane and phenylurethane had been used in animal studies and veterinary medicine as anesthetics, and had been shown to possess antineoplastic properties.

Warburg, whom many consider to be the father of cellular bioenergetics, introduced plant scientists to urethane and phenylurethane. He had obtained inhibition of photosynthesis and respiration in *Chlorella* cells by a series of ethyl esters of alkyl and aryl carbamates [2]. The I_{50} concentrations of the test compounds for inhibition of respiration were approximately three times that required to inhibit photosynthesis. During the 1930s and 1940s, many investigators subsequently included the urethanes as inhibitors in their studies on photosynthesis, much like diuron and atrazine have been used since the late 1950s.

Herbicides – The Early Years

Effects of phenylurethane on plant growth and development was first described by Friesen in 1929 [3] who reported on the morphological aberrations produced on wheat and oat seedlings. The effects noted included a delay of germination, development of cellular enlargement, and interference with pigmentation, *i.e.*, the seedlings had a blue-green color. Ten years later, Lefèvre [4] compared the antimitotic activity of phenylurethane to that of colchicine in wheat seedlings.

In the interval between the two observations, IAA was isolated and identified in 1934 [5], but had been synthesized in 1886 by German chemists. In the late 1930s and early 1940s, scientists performed a large number of experiments in both Europe and the U.S. in an attempt to identify the mechanisms of action of auxins and compounds that possessed auxin-like activity. The compounds were applied to a wide range of plant species and large numbers of physiological and morphological effects were observed. Findings from much of this research were either not published or publication was delayed because of the events that surrounded World War II. Templeman and Sexton [6, 7], who were associated with ICI at Jealott's Hill in the U.K., reported on field observations conducted in 1940 and 1941 with phenoxyacetic acids. The compounds were toxic to dicotyledonous, but not to monocotyledonous plants. The formative effects induced by the phenoxyacetic acids had been reported earlier by Zimmerman and Hitchcock [8]. Eventually, 2,4-D was developed for use in the U.S. and MCPA for use in Europe. The discovery of the phenoxyalkanoates revolutionized agricultural practices. Success achieved in their use probably stimulated industry to invest in research that led to the discovery of the large variety of herbici-

dal chemicals that are now available. A history of the development and use of the phenoxies in the U.S. has been compiled [9] and formative effects have been summarized [10].

Templeman and Sexton [11, 12] also reported on field observations made in 1940 with some 50 esters of arylcarbamates and thiocarbamates. The compounds were toxic to monocotyledonous, but not to dicotyledonous species. The isopropyl ester (propham) was reported as being three times more active than the ethyl ester (phenylurethane). Propham was first synthesized in 1885.

The phenylureas were introduced by DuPont with a report on the herbicidal properties of monuron [13] and the *s*-triazines were introduced as herbicides by Geigy in 1955 and 1956 [14, 15]. However, simazine had been synthesized in 1885 by German chemists.

Except for the phenoxyacetic acids, N-phenylcarbamates, phenylureas, and *s*-triazines, have played major roles in the unraveling of the intricacies of the plant's photochemical reactions.

Electron Transport Inhibition

The observation by Robin Hill in 1937 [16] that illuminated chloroplasts evolved oxygen in the presence of a suitable electron acceptor was to become an important assay for herbicide researchers. However, it was not until 1949 that Macdowall [17] reported inhibition of the Hill reaction by phenylurethane ($I_{50} = 2$ mM) and DNP ($I_{50} = 0.63$ mM).

Wessels and van der Veen [18], who were associated with Philips Research Laboratories in The Netherlands, subsequently compared the inhibitory potency of phenylurethane, 10 additional arylcarbamates and 14 phenylureas including fenuron, monuron (CMU), and diuron (DCMU) on the Hill reaction. Based on a comparison of I_{50} values, monuron and diuron were listed as being 125 and 2500 times more active, respectively, than phenylurethane. The authors also reported that monuron did not inhibit the respiration of leaf discs at concentrations that completely inhibited photosynthesis. The striking inhibition of the Hill reaction at such low concentrations by both the phenylcarbamates and phenylureas led the authors to postulate that the compounds acted as site-specific inhibitors rather than as narcotics. There were two other reports in the 1956 literature relative to inhibition of the Hill reaction by monuron [19, 20].

In 1954, Arnon *et al.* [21] described the synthesis of ATP (photophosphorylation) by illuminated chloroplasts. Soon thereafter, monuron was found to inhibit both whole chain electron transfer and phosphorylation mediated by ferricyanide, but did not inhibit PMS-mediated cyclic phosphorylation [22].

Beginning with 1959, many investigators not only repeated the Hill inhibition studies of Wessels and van der Veen [18] with N-phenylcarbamates and phenylureas, but reported inhibition of the Hill reaction by new families of herbicides including the *s*-triazines, acylanilides, uracils, benzonitriles, imidazoles, benzimidazoles, triazinones, and pyridazinones [see references in 23–25]. The action of additional herbicidal inhibitors of the Hill reaction is still being reported at frequent intervals including at this meeting.

It is interesting that most of the early papers in which the actions of candidate herbicides were evaluated against the Hill reaction were authored by individuals associated with, or in collaboration with, the chemical industry in Europe. None of the earlier publications on inhibition of Hill activity seem to have been authored by investigators connected with the U.S. chemical industry.

I continue to be puzzled by this. The climate in the U.S. during the late 1950s and early 1960s did not seem to favor structure/activity and mechanism of action studies. It is my feeling that there was considerable frustration over the disappointment associated with the extensive research that had been conducted on the mechanism of action of 2,4-D and its derivatives during the 1940s and early 1950s. The phenoxies had been shown to affect a large number of plant processes including photosynthesis, water relations, stomatal behavior, nutrient ion absorption and accumulation, respiration, carbohydrate metabolism, translocation, and interference with the action of IAA. Studies with isolated enzyme systems also had not produced fruitful results. No clear-cut picture had emerged on the mechanism or site of action of 2,4-D, or could be gleaned from the many hundreds of papers. Consequently, the relevance of the early structure/activity Hill inhibition studies was initially questioned.

In the early 1960s, investigators began reporting

that soil-applied herbicidal Hill inhibitors inhibited whole plant photosynthesis [26, 27]. The whole plant studies became possible with the availability of sensitive infrared analyzers that had initially been developed during World War II to monitor CO_2 levels in the confined atmosphere of submarines. The correlation between responses of isolated chloroplasts to herbicides and inhibition of photosynthesis of intact plants documented the relevance of the earlier Hill studies.

During the late 1950s, results from a number of different types of experiments suggested that two distinguishable light reactions were involved in photosynthesis. Hill and Bendall [28] proposed that the two light reactions operated in series and presented the now familiar Z scheme which has undergone a number of modifications over the years.

The site of action of diuron was placed on the acceptor side of PS II by Duysens [29]. He had found that diuron did not inhibit the reduction of a postulated electron acceptor from light reaction II, but did prevent the reoxidation of the reduced acceptor by light reaction I.

Correlations between chemical and physical properties of inhibitors with biological activity had earlier received attention from Corwin Hansch, a chemist located at Pomona College in California. He had initially studied the relation between stimulation of elongation, as measured in the *Avena* coleoptile straight growth test, to the chemical and physical properties of phenylacetic, phenoxyacetic, and benzoic acids [30]. Following studies with the growth regulators, Hansch had turned his attention to other biological systems and was fine-tuning his multiple regression analyses. The techniques were subsequently applied to an analysis of the inhibition of the Hill reaction by N-phenylcarbamates, phenylureas, and anilides [31].

The opportunity for researchers to get together to exchange information on interactions between chloroplasts and herbicides had its origin at the International Congress of Photosynthesis Research that was held in the Black Forest area of Germany at Freudenstadt, June 6–8, 1968. That meeting subsequently became the first of the International Photosynthesis Congresses. Trebst was on the organizing committee for the Congress and had arranged a special section entitled, "Action mechanisms of herbicides in photosynthesis". The 12 papers were subsequently published in the Proceedings [32] and dealt with various Hill inhibitors and QSAR analyses. The next gathering of researchers was held in Konstanz, Germany (1979), independently of the Photosynthesis Congresses. Subsequent satellite meetings were scheduled in conjunction with the Photosynthesis Congresses and were held in Wageningen, The Netherlands (1983), Lake Placid, N.Y. (1986), Monheim, Germany (1989), and now in Omiya, Japan (1992). Thanks to the efforts of Peter Böger and the financial contributions of various industrial sponsors, the papers from these meetings have been compiled and published in separate issues of Zeitschrift für Naturforschung.

During the late 1960s and 1970s there was considerable interest in structure-activity relations, *i.e.*, studies in which inhibitory action against the Hill reaction were correlated with various chemical, physical, and steric properties of the inhibitors, mostly by scientists outside of the U.S. These interests continue through to the present time. One of the objectives of the studies was to describe the molecular architecture of the PS II binding site. However, investigators still had not specifically identified the location of the binding site.

Impact of Triazine Resistance

A step toward reaching the objective of defining the binding site unknowingly started with the observation that the triazines were no longer able to control the growth of previously susceptible weeds beginning with an observation published by Ryan in 1970 [33]. Subsequently, chloroplasts isolated from susceptible and resistant biotypes were shown to respond similarly to intact plants, *i.e.*, the Hill reaction of chloroplasts isolated from a susceptible biotype was inhibited by atrazine, but not that of chloroplasts isolated from the resistant biotype [34].

Machado of Guelph, Canada, in traditional crossing experiments with R and S rape biotypes provided evidence for the maternal inheritance of resistance [35]. Investigators were beginning to realize that it should be possible to develop triazine-resistant plants. This was at the time that the fields of genetic engineering and biotechnology were emerging. Techniques and tools from the new

technology were applied to the PS II herbicide-binding site and the resistance problem.

Trypsinization was shown to remove the diuron-binding site, which indicated that the site was proteinaceous [36, 37]. Strotman [38] suggested that Hill inhibitors competed for a common binding site. Pfister and Arntzen [39] and Trebst and Draber [40] postulated that Hill inhibitors had overlapping binding sites that were associated with a common binding domain. Photoaffinity labeled [^{14}C]azidoatrazine was shown to bind to a 32–34 kDa thylakoid protein [41].

In the interim, Q_B had been identified as a secondary acceptor of electrons from PS II and the site of action of diuron was placed between Q_B and the PQ pool [42, 43]. Diuron and the triazines were subsequently shown to compete with PQ for binding to the secondary acceptor of PS II [44–46], *i.e.*, the herbicide-binding 32 kDa protein.

The primary sequence of the *psb*A gene was obtained [47] and the amino acid sequence of the 32 kDa protein was identified. Studies with bacterial photosystems identified sequence homology between the L and M peptides of the bacterial system and the D1 and D2 PS II proteins of higher plants [48–50]. Cloning of the *psb*A gene showed that triazine resistance correlated with replacement of serine of the susceptible biotype by glycine in the resistant biotype at position 264 of the Q_B protein [51–53].

The reaction center of *Rhodopseudomonas viridis* was subsequently crystallized and its X-ray structure determined [54, 55]. The individuals responsible for this research Hartmut Michel, Johann Deisenhofer, and Robert Huber were awarded the 1988 Nobel Prize in Chemistry. Trebst continued to refine and describe the herbicide binding niche on the Q_B protein based on the structural analyses [56, 57].

By the mid 1980s, the following was known about the 32 kDa Q_B protein which some investigators called the "herbicide-binding protein": it was maternally inherited; turned over rapidly in the light; was encoded by the D-1 photogene (*psb*A gene); the amino acid sequence was known; there was strong sequence similarity with the 14 kDa L subunit of the bacterial photosystem; it consisted of 353 amino acids; there were five transmembrane helical spans with two parallel helices; plastoquinone was the prosthetic group; inhibitory herbicides displaced PQ, bound non-covalently, and blocked the rapid turnover; and single amino acid substitutions affected binding affinity of herbicides (resistance).

Bipyridiliums

The bipyridiliums (diquat and paraquat) constitute another group of compounds that have contributed over the years to the elucidation of the chloroplast electron transport pathway. Brian *et al.* [58] of ICI published on observations made in 1955 about the phytotoxic properties of the bipyridyliums. Other investigators became aware of the unique properties of the bipyridyliums (viologens) during the mid to late 1950s. For example, Jagendorf and Avron [59] found that methyl and benzyl viologen catalyzed photophosphorylation by isolated chloroplasts.

In 1960, Homer and associates reported that the viologens formed free radicals upon single electron reduction in illuminated green tissue [60]. However, Michaelis and Hill [61] had described the redox properties of the viologens in 1933 some 27 years earlier. Mediation of cyclic phosphorylation by diquat was reported by Davenport [62]. Acceptance of an electron by the bipyridyliums from PS I was shown to give rise to a reduced free radical which can react with molecular oxygen to form a superoxide radical [63, 64]. Dismutation of the superoxide radical leads to formation of hydrogen peroxide and hydroxyl free radicals. The free radicals can initiate lipid peroxidation of membrane unsaturated fatty acids which results in the loss of membrane integrity and structure.

Nitrophenols and Uncoupling

The nitrophenols also have contributed to our understanding of the plant's photochemistry. During World War I, workers in ammunition factories where phenols were used in the manufacture of explosives, were observed to lose weight. Subsequently, in the early 1930s, 2,4-DNP was widely used as a weight-reducing agent. A French patent was obtained for the use of nitrophenols as selective herbicides in 1932 and a British patent was issued in 1935 [65]. DNOC was developed for use in Europe and dinoseb (DNBP) for use in the U.S.

Nitrophenols had been known to stimulate respiration of man, insects, yeast, and higher plant tissue [66]. The uncoupling action of nitrophenols,

i.e., stimulation of oxygen uptake and inhibition of phosphorylation was described by Loomis and Lipmann [67] for 2,4-DNP and Clowes *et al.* [68] for 2,4-DNP and DNOC, among other investigators. Macdowall [17] and Wessels [18] had shown that DNP also was a Hill inhibitor. Phenolic and some non-phenolic herbicides were subsequently shown to inhibit both cyclic and noncyclic photophosphorylation. In thylakoids, the phenolics bind reversibly and noncovalently, and competitively displace diuron-type inhibitors from the Q_B-binding site. However, the Hill reaction of chloroplasts isolated from triazine-resistant biotypes is more sensitive to the phenolics than chloroplasts isolated from sensitive biotypes. Oettmeier and his colleagues [70, 71] have conducted extensive studies with photoaffinity labeled phenolics and reported nonspecific binding to polypeptides in the 40 to 53 kDa range.

In parallel studies conducted by investigators with isolated plant and mammalian mitochondria, the phenolics also were shown to uncouple oxidative phosphorylation and to inhibit electron transport [69 and references therein].

Classification of Inhibitors

Herbicides that inhibit the photochemical reactions of isolated chloroplasts had been routinely called inhibitors of the Hill reaction. This had been done primarily for convenience and because for many years their action was evaluated under nonphosphorylating conditions, frequently with ferricyanide as the electron acceptor. By 1976, the herbicides that interferred with electron transport and phosphorylation in both chloroplasts and mitochondria could be separated into five classes: (a) electron transport inhibitors, (b) uncouplers, (c) energy transfer inhibitors, (d) inhibitory uncouplers (multiple types of inhibition), and (e) electron acceptors [23, 25, 72].

Electron transport inhibitors include the Hill inhibitors most of which are now known to bind to the Q_B protein. However, some such as the plastoquinone analog DBMIB interact either at the reducing or oxidizing side of the cytochrome b_6/f complex. Uncouplers dissociate electron transport from ATP formation by interacting with the CF_0/CF_1 coupling factor complex. Perfluidone is the only herbicide reported to function as a pure uncoupler of photophosphorylation. Energy transfer inhibitors interact with the coupling factor complex. There is no evidence that any herbicides are pure energy transfer inhibitors. However, there are suggestions that some of the diphenylethers interact as energy transfer inhibitors [73], but they also are electron transport inhibitors. The term inhibitory uncouplers was coined to identify those herbicides that act both as electron transport inhibitors and uncouplers. The bipyridyliums intercept electron flow from PS I and are classified as electron acceptors. Some investigators have conducted parallel studies with herbicides on isolated mitochondria. Results from the comparative studies have assisted in differentiation of the action of the electron transport inhibitors.

The pure electron transport inhibitors also have been called diuron-types and include the chlorinated phenylureas, pyridazinones, *s*-triazines, triazinones, uracils, and ureacarbamates. By inhibiting electron transport, they prevent the formation of a proton gradient across the thylakoid membrane that is required for the synthesis of ATP. In a coupled system, the I_{50} for inhibition of photophosphorylation by pure electron transport inhibitors approximately equals the I_{50} for inhibition of electron transport. However, with inhibitory uncouplers, in a coupled system, the I_{50} for inhibition of photophosphorylation is lower than the I_{50} for inhibition of electron transport. The inhibitory uncouplers, but not the pure electron transport inhibitors, also inhibit cyclic photophosphorylation. Inhibitory uncouplers were subsequently divided into two groups: dinoseb types and dicryl types. Included as dinoseb types were dinitrophenols, benzimidazoles, benzonitriles, bromophenoxim, and thiadiazoles. Acylanilides, dinitroanilines, diphenylethers, bis-carbamates, and perfluidone were classified as dicryl types [74, 75]. Dinoseb types, most of which contain dissociable protons, discharge ΔpH at low concentrations and collapse $\Delta\Psi$ at higher concentrations. Collapse of ΔpH can be attributed to the protonophonic (proton shuttling) action of the herbicides. However, collapse of $\Delta\Psi$ may be caused by alterations induced to the structure, integrity, and permeability of the thylakoid membrane. The non-ionic dicryl types of inhibitory uncouplers collapse $\Delta\Psi$ at concentrations that are somewhat lower than those required for the collapse of ΔpH [74].

With isolated mitochondria, the inhibitory uncouplers uncouple phosphorylation at low molar concentrations and inhibit electron transport at higher concentrations [69, 75]. In mitochondria, as in thylakoids, the inhibitory uncouplers also collapse the transmembrane potential. The dinoseb types uncouple phosphorylation and collapse $\Delta\Psi$ to the Donnan level before oxygen utilization is inhibited. In the dicryl types, collapse of $\Delta\Psi$ is paralleled by uncoupling of phosphorylation and inhibition of oxygen utilization [75]. The diuron types do not inhibit mitochondrial electron transport [25, 69, 72]. Mitochondrial studies have not attracted the attention of many investigators because there does not seem to be a specific target site.

Trebst [56, 57] also has divided the Hill inhibitors into diuron and phenolic types based on QSAR analyses and other criteria [76]. Binding orientation of the diuron types is toward serine-264 and that of the phenolic types is toward histidine-215 of the Q_B protein.

Induction of Chlorosis

It is now possible to describe the sequence of events that follow inhibition of the Hill reaction by the diuron-type herbicides that lead to phytotoxicity. Light was known to be required for the expression of toxicity, *i.e.*, there were no signs of toxicity in plants maintained in the dark. The phytotoxic response increased with an increase in light intensity and toxicity was associated with wavelengths of light absorbed by the chloroplast pigments. Some investigators suggested that the plants starved to death. However, plants maintained in the dark did not show toxic symptoms. Hilton [77] demonstrated that the feeding of carbohydrates (the products of photosynthesis) through the leaves of barley plants treated with simazine, at the two-leaf stage, circumvented the appearance of toxicity. It was subsequently shown that only the diuron-type Hill inhibitors responded in this way. Apparently glycolysis, oxidative phosphorylation, and possibly cyclic photophosphorylation can generate sufficient energy in the form of ATP to prevent the appearance of phytotoxic systems. However, carbohydrate circumventions could not be demonstrated with inhibitory uncouplers which completely shut down the plant's ATP generating machinery.

Starting in 1963, reports on the effects of Hill inhibitors on the ultrastructure of foliar tissue of treated plants began appearing in the literature. Following treatment with Hill inhibitors such as atrazine, the initial disturbance was reflected as disappearance of starch from the stroma, a swelling of the intergranal thylakoids, followed by swelling of the granal thylakoids, and a general disruption of the entire lamellar system [78]. Subsequently, the tonoplast and chloroplast envelopes ruptured. Effects on the thylakoids were reported to occur as early as 2 h following application of atrazine to the root environment of barnyard grass seedlings, and mixing of plastid and cytoplasmic contents was detected in 4 h [79]. External symptoms of injury are usually not apparent until several days after treatment with Hill inhibitors. Hence, there is a complete destruction of the internal morphology of leaves before the appearance of external symptoms. Effects are produced only in the light. Chloroplasts of plants maintained in the dark following herbicide treatment resembled, in all respects, chloroplasts from untreated, dark-control plants.

The structural alterations were subsequently ascribed to what happens when Q_A is left in a reduced state, *i.e.*, when reoxidation is blocked by a Hill inhibitor. Excitation energy associated with Q_A leads to the generation of triplet chlorophyll. The transfer of energy from triplet chlorophyll to oxygen results in the formation of singlet oxygen. Carotenoids can quench both triplet chlorophyll and singlet oxygen. However, in the absence of carotenoids or when the protective mechanism becomes overloaded, the free radicals (triplet chlorophyll and singlet oxygen) can initiate lipid peroxidation of the unsaturated membrane fatty acids and chlorophyll destruction [80, 81]. The result would be a loss of membrane integrity such as reflected in the electron micrographs, and bleaching.

Carotenoids play a pivotal role as free radical scavengers and as protectants of chlorophyll destruction and bleaching. Other herbicides directly affect the biosynthesis of carotenoids and limit the availability of scavengers to provide protection from photooxidation (bleaching herbicides). One of the first compounds shown to function in this way was the pyridazinone SAN 6706 [82]. In addition to the pyridazinones, other compounds that interfere with carotenoid biosynthesis include

fluridone, difunon, dichlormate, and aminotriazole, with extensive studies having been conducted in this area by Böger and his associates ([83, 84], and references therein).

Herbicides – Recent Developments

Diphenylethers

The diphenylethers also induce chlorosis, but through a different mechanism. The first diphenylether studied was nitrofen, the phytotoxicity of which was shown to be light promoted by Matsunaka [85]. In addition to the diphenylethers, the oxadiazoles also cause photobleaching. Both groups of herbicides cause the accumulation of large amounts of protoporphyrin IX which is highly photodynamic [86, 87], *i.e.*, singlet oxygen is generated from protoporphyrin IX by light. If the singlet oxygen is not quenched, lipid peroxidation can be initiated. These compounds are sometimes referred to as peroxidizing herbicides. Protoporphyrinogen oxidase was subsequently shown to be the molecular target for diphenylether herbicides [88].

Free radicals are generated by normal metabolism in plants in the absence of inhibitors: excess energy absorbed by Q_A in chloroplasts, by the mitochondrial electron transport chain in association with the ubiquinone pool, in the endoplasmic reticulum-associated cytochrome P-450 pathway, and the action of lipoxygenases. Free radicals can be quenched by a number of scavengers, in addition to carotenoids, including ascorbic acid, glutathione, α-tocopherol, superoxide dismutases, catalases, and peroxidases. Damage is induced in cells when the scavenging system becomes overloaded.

Inhibitors of amino acid biosynthesis

Glyphosate, a broad spectrum herbicide, was introduced by Monsanto in 1971. Jaworski [89], a Monsanto scientist, suggested that glyphosate inhibited the biosynthesis of the aromatic amino acids (tryptophan, phenylalanine, and tyrosine) based on alleviation studies with *Lemna*. Subsequently, in 1980, Amrhein and his associates [90, 91] found that glyphosate was a highly specific inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase). Inhibition of this enzyme results in the accumulation of high levels of shikimic acid. In addition to the aromatic amino acids, the shikimate pathway provides precursors associated with the biosynthesis of lignin, flavonoids, and a number of secondary metabolites. Monsanto scientists have continued to make important contributions relative to the genetics of glyphosate resistance, the molecular biology of the shikimate pathway, and on the structures and mechanisms associated with the individual enzymes of the pathway. One of the objectives of the research has been to develop glyphosate-tolerant plants.

The sulfonylureas were introduced in 1980 by DuPont [92] and the imidazolinones in 1983 by American Cyanamide [93]. Alleviation studies identified that both groups of herbicides interfered with the biosynthesis of branched-chain amino acids (leucine, isoleucine, and valine). Both herbicides were subsequently shown to inhibit the activity of acetolactate synthetase, also referred to as acetohydroxyacid synthase (ALS or AHAS) [94, 95]. The sulfonylureas are potent inhibitors of ALS activity with I_{50} values in the μmolar range having been reported. The imidazolinones are somewhat weaker inhibitors of ALS activity producing I_{50} values in the mmolar range. Scientists associated with DuPont and American Cyanamide have provided noteworthy contributions on the basic mechanisms of action of the compounds as well as the associated genetics and the development of herbicide-resistant plants through genetic engineering and by cell culture selections.

Both ALS and EPSP synthase are nuclear encoded, but are located in plastids. The aromatic and branched chain amino acids are "essential" amino acids, meaning that animals do not possess the biochemical machinery for their synthesis. Instead, animals obtain these "essential" amino acids from plant or microbial sources through their dietary intake.

Lipid biosynthesis inhibitors

The herbicidal activity of aryloxyphenoxypropionates was reported by Hoechst AG in 1971 [96]. The compounds are toxic to graminaceous plants, but are tolerated by dicotyledonous and some monocotyledonous species. The cyclohexanediones (alloxydim and sethoxydim) were introduced by Nippon Soda in 1977 and 1978 [97, 98]. Both groups of herbicides are used as postemergence grass herbicides in broadleaf crops. The

herbicides have been shown to suppress *de novo* synthesis of fatty acids in sensitive species by inhibiting the activity of plastid-associated acetyl-CoA carboxylase (ACCase). Contributions to understanding the mechanism of action of both families of herbicides have been provided by Hoppe and his associates [99], Lichtenthaler and his group [100, 101], and others including scientists associated with Dow, Chevron, and ICI [102–105].

Concluding Comments

The 1980s can be referred to as the decade of the chloroplast. The decade started with PS II as being the only herbicide target site that investigators had identified. The 1980s witnessed the introduction and elucidation of target sites for the sulfonylureas and imidazolinones, glyphosate, aryloxyphenoxypropionates, cyclohexanediones, diphenylethers, and oxadiazoles. The target sites for all are located within the chloroplast. Not only have target sites been identified, but chronic toxicity can be elucidated by extrapolating along the sequence of events that lead to phytotoxicity. For many herbicides (Hill inhibitors, inhibitors of carotenoid biosynthesis, bipyridiliums, diphenylethers, and oxadiazoles), inhibition at the target site, directly, or indirectly, results in the formation of free radicals that leads to lipid peroxidation and membrane disorganization. The suppression of oxidative and photoproduction of ATP, and the alterations to membrane permeability and integrity induced by inhibitory uncouplers also contribute to chronic toxicity.

Many of the target site enzymes are nuclear encoded, but localized in the chloroplasts of higher plants. Genes that encode the enzymes have been cloned, modified, and expressed in transgenic plants to confer herbicide tolerance. The possibility of developing herbicide-resistant crops has stimulated a great deal of work on the genetics of herbicide resistance. This research has also furthered our understanding of herbicide action. Unlike the situation with PS II inhibitors, it is encouraging to note that scientists associated with the chemical industry in the U.S. have studied the mechanisms of action of some of the newer chemistry in conjunction with the development of field practices, and have published their findings.

For a more complete and detailed coverage, and the current status, of interactions between herbicides and the biochemical pathways discussed herein, as well as other topics, the reader is referred to recently published texts [106–109] and to the contributions published in this issue of the journal.

Acknowledgements

Cooperative investigations of the U.S. Department of Agriculture, Agricultural Research Service, and the North Carolina Agricultural Research Service, Raleigh, North Carolina 27695.

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[1] E. I. Rabinowitch, Photosynthesis, **Vol. I,** pp. 300–325, Interscience, New York 1945.
[2] O. Warburg, Biochem. Z. **103,** 188–217 (1920).
[3] G. Friesen, Planta **8,** 666–679 (1929).
[4] J. Lefèvre, C. R. Sci. Soc. Biol. Paris **208,** 301–304 (1939).
[5] F. Kögl, H. Erxleben, and A. J. Haagen-Smit, Hoppe-Seyler's Z. Physiol. Chem. **225,** 215–229 (1934).
[6] R. E. Slade, W. G. Templeman, and W. A. Sexton, Nature **155,** 497–498 (1945).
[7] W. G. Templeman and W. A. Sexton, Proc. Roy. Soc. (London) **133B,** 300–313 (1946).
[8] P. W. Zimmerman and A. E. Hitchcock, Contribs. Boyce Thompson Inst. **12,** 321–343 (1942).
[9] G. E. Peterson, Agric. Hist. **41,** 243–253 (1967).
[10] P. W. Zimmerman and A. E. Hitchcock, Annu. Rev. Biochem. **17,** 601–626 (1948).
[11] W. G. Templeman and W. A. Sexton, Nature **156,** 630 (1945).
[12] W. G. Templeman and W. A. Sexton, Proc. Roy. Soc. (London) **133B,** 480–485 (1946).
[13] H. C. Bucha and C. W. Todd, Science **114,** 493–494 (1951).
[14] A. Gast, E. Knüsli, and H. Gysin, Experientia **11,** 107–108 (1955).
[15] A. Gast, E. Knüsli, and H. Gysin, Experientia **12,** 146–148 (1956).
[16] R. Hill, Nature **139,** 881–882 (1937).
[17] F. D. H. Macdowall, Plant Physiol. **24,** 462–480 (1949).
[18] J. S. C. Wessels and R. van der Veen, Biochim. Biophys. Acta **19,** 548–549 (1956).
[19] A. R. Cooke, Weeds **4,** 397–398 (1956).
[20] J. D. Spikes, Plant Physiol. Suppl. **31,** xxxii (1956).
[21] D. I. Arnon, F. R. Whatley, and M. B. Allen, J. Am. Chem. Soc. **76,** 6324–6329 (1954).
[22] A. T. Jagendorf, Brookhaven Symp. in Biol. **11,** 236–257 (1959).
[23] D. E. Moreland, Annu. Rev. Plant Physiol. **18,** 365–386 (1967).
[24] D. E. Moreland, in: Progress in Photosynthesis Research (H. Metzner, ed.), **Vol. III,** pp. 1693–1711, Int. Union Biol. Sci., Tübingen 1969.
[25] D. E. Moreland, Annu. Rev. Plant Physiol. **31,** 597–638 (1980).
[26] J. L. P. van Oorschot and M. Belksma, Weed Res. **1,** 245–257 (1961).
[27] J. L. P. van Oorschot, Weed Res. **5,** 84–97 (1965).
[28] R. Hill and F. Bendall, Nature **186,** 136–137 (1960).
[29] L. N. M. Duysens, in: Photosynthetic Mechanisms of Green Plants, Natl. Acad. Sci., Natl. Res. Council Publ. **1145,** 1–17 (1963).
[30] R. M. Muir and C. Hansch, Plant Physiol. **26,** 369–374 (1951).
[31] C. Hansch and E. W. Deutsch, Biochim. Biophys. Acta **112,** 381–391 (1966).
[32] H. Metzner (ed.), in: Progress in Photosynthesis Research, **Vol. III,** pp. 1685–1807, Int. Union Biol. Sci., Tübingen 1969.
[33] G. F. Ryan, Weed Sci. **18,** 614–616 (1970).
[34] S. R. Radosevich and O. T. Devilliers, Weed Sci. **24,** 229–232 (1976).
[35] V. Souza Machado, J. D. Bandeen, G. R. Stephenson, and P. Lavigne, Can. J. Plant Sci. **58,** 977–981 (1978).
[36] G. Renger, Biochim. Biophys. Acta **440,** 287–300 (1976).
[37] G. Regitz and I. Ohad, J. Biol. Chem. **251,** 247–252 (1976).
[38] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113–125 (1977).
[39] K. Pfister and C. J. Arntzen, Z. Naturforsch. **34c,** 996–1009 (1979).
[40] A. Trebst and W. Draber, in: Advances in Pesticide Science (H. Geissbühler, ed.), Part 2, pp. 223–234, Pergamon Press, Oxford 1979.
[41] K. Pfister, K. E. Steinback, G. Gardner, and C. J. Arntzen, Proc. Natl. Acad. Sci. U.S.A. **78,** 981–985 (1981).
[42] B. Bouges-Bocquet, Biochim. Biophys. Acta **314,** 250–256 (1973).
[43] B. R. Velthuys and J. Amesz, Biochim. Biophys. Acta **333,** 85–94 (1974).
[44] B. Velthuys, FEBS Lett. **126,** 277–281 (1981).
[45] C. A. Wraight, Isr. J. Chem. **21,** 349–354 (1981).
[46] J. Lavergne, Biochim. Biophys. Acta **682,** 345–353 (1982).
[47] G. Zurawski, H. J. Bohnert, P. R. Whitfield, and W. Bottomley, Proc. Natl. Acad. Sci. U.S.A. **79,** 7699–7703 (1982).
[48] D. C. Youman, E. J. Bylina, M. Alberti, H. Begusch, and J. E. Hearst, Cell **37,** 949–957 (1984).
[49] J. C. Williams, L. A. Steiner, R. C. Ogden, M. I. Simon, and G. Feher, Proc. Natl. Acad. Sci. U.S.A. **80,** 6505–6509 (1983).
[50] J. C. Williams, L. A. Steiner, G. Feher, and M. I. Simon, Proc. Natl. Acad. Sci. U.S.A. **81,** 7303–7307 (1984).
[51] J. Hirschberg and L. McIntosh, Science **222,** 1346–1349 (1983).
[52] J. Hirschberg, A. Bleeker, D. J. Kyle, L. McIntosh, and C. J. Arntzen, Z. Naturforsch. **39c,** 412–420 (1984).
[53] P. Goloubinoff, M. Edelman, and R. B. Hallick, Nucleic Acids Res. **12,** 9489–9496 (1984).
[54] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, J. Mol. Biol. **180,** 385–398 (1984).
[55] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, Nature **318,** 618–624 (1985).
[56] A. Trebst, Z. Naturforsch. **41c,** 240–245 (1986).
[57] A. Trebst, Z. Naturforsch. **42c,** 742–750 (1987).
[58] R. C. Brian, R. F. Homer, J. Stubbs, and R. L. Jones, Nature **181,** 446–447 (1958).
[59] A. T. Jagendorf and M. Avron, J. Biol. Chem. **231,** 277–290 (1958).
[60] R. F. Homer, G. C. Mees, and T. E. Tomlinson, J. Sci. Food Agric. **11,** 309–315 (1960).
[61] L. Michaelis and E. S. Hill, J. Gen. Physiol. **16,** 859–873 (1933).
[62] H. E. Davenport, Proc. Roy. Soc. (London) **157B,** 332–345 (1963).
[63] A. D. Dodge, Endeavor **30,** 130–135 (1971).
[64] J. A. Farrington, M. Ebert, E. J. Land, and K. Fletcher, Biochim. Biophys. Acta **314,** 372–381 (1973).
[65] G. Truffant and I. Pastac, British Patent 424,295, May 29th (1935).

[66] E. W. Simon, Biol. Rev. **28,** 453–479 (1953).
[67] W. F. Loomis and F. Lipmann, J. Biol. Chem. **173,** 807–808 (1948).
[68] G. H. A. Clowes, A. K. Keltch, C. F. Strittmatter, and C. P. Walters, J. Gen. Physiol. **33,** 555–561 (1950).
[69] D. E. Moreland, in: Weed Physiology (S. O. Duke, ed.), **Vol. II,** pp. 37–61, CRC Press, Inc., Boca Raton 1985.
[70] W. Oettmeier, K. Masson, and U. Johanningmeier, FEBS Lett. **118,** 267–270 (1980).
[71] W. Oettmeier, K. Masson, and U. Johanningmeier, Biochim. Biophys. Acta **679,** 376–383 (1982).
[72] D. E. Moreland and J. L. Hilton, in: Herbicides: Physiology, Biochemistry, Ecology (L. J. Audus, ed.), **Vol. 1,** pp. 493–523, Academic Press, London 1976.
[73] B. Huchzermeyer, Z. Naturforsch. **37c,** 787–792 (1982).
[74] D. E. Moreland and W. P. Novitzky, Z. Naturforsch. **42c,** 718–726 (1987).
[75] D. E. Moreland and W. P. Novitzky, Pestic. Biochem. Physiol. **31,** 247–260 (1988).
[76] W. Oettmeier and A. Trebst, in: The Oxygen-Evolving System of Photosynthesis (Y. Inoue, A. R. Crofts, Govindjee, N. Murata, G. Renger, and K. Satoh, eds.), pp. 411–420, Academic Press, Tokyo 1983.
[77] D. E. Moreland, W. A. Gentner, J. L. Hilton, and K. L. Hill, Plant Physiol. **34,** 432–435 (1959).
[78] F. M. Ashton, E. M. Gifford, and T. Bisalputra, Bot. Gaz. **124,** 336–343 (1963).
[79] E. R. Hill, E. C. Patala, and J. Vengris, Weed Sci. **16,** 377–380 (1968).
[80] K. E. Pallett and A. D. Dodge, Proc. 13th Brit. Crop Prot. Conf. **1,** 235–240 (1976).
[81] S. M. Ridley, Plant Physiol. **59,** 724–732 (1977).
[82] P. G. Bartels and C. McCullough, Biochem. Biophys. Res. Commun. **48,** 16–22 (1972).
[83] G. Sandmann and P. Böger, in: Biochemical Responses Induced by Herbicides (D. E. Moreland, J. B. St. John, and F. D. Hess, eds.), Symp. Series Amer. Chem. Soc. No. 181, pp. 111–130, Washington, D.C. 1982.
[84] G. Sandmann, I. A. Clarke, P. M. Bramley, and P. Böger, Z. Naturforsch. **39c,** 443–449 (1984).
[85] S. Matsunaka, J. Agric. Food Chem. **17,** 171–175 (1969).
[86] J. Lydon and S. O. Duke, Pestic. Biochem. Physiol. **31,** 74–83 (1988).
[87] M. Matringe and R. Scalla, Plant Physiol. **86,** 619–622 (1988).
[88] M. Matringe, J.-M. Camadro, P. Labbe, and R. Scalla, Biochem. J. **260,** 231–235 (1989).
[89] E. Jaworski, J. Agric. Food Chem. **20,** 1195–1198 (1972).
[90] N. Amrhein, J. Schab, and H. C. Steinrücken, Naturwissenschaften **67,** 356–357 (1980).
[91] H. C. Steinrücken and N. Amrhein, Biochem. Biophys. Res. Commun. **94,** 1207–1212 (1980).
[92] H. L. Palm, J. D. Riggleman, and D. A. Allison, Proc. Brit. Crop Prot. Conf. – Weeds **1,** 1–6 (1980).
[93] P. L. Orwick, P. A. Marc, K. Umeba, M. Los, D. R. Ciarlante, Proc. South. Weed Sci. Soc. **36,** 90 (1983).
[94] T. B. Ray, Plant Physiol. **75,** 827–831 (1984).
[95] D. L. Shaner, P. C. Anderson, and M. A. Stidham, Plant Physiol. **76,** 545–546 (1984).
[96] H. J. Nestler, in: Chemie der Pflanzenschutz- und Schädlingsbekämpfungsmittel (R. Wegler, ed.), pp. 1–25, Springer Verlag, Berlin 1982.
[97] I. Iwataki and Y. Hirono, in: Advances in Pesticide Science (H. Geissbühler, G. T. Brooks, and P. C. Kearney, eds.), Part 2, pp. 235–243, Pergamon Press, Oxford 1979.
[98] H. Ishikawa, S. Yamada, H. Hosaka, T. Kawana, S. Okunuki, and K. Kohara, J. Pestic. Sci. **10,** 187–193 (1985).
[99] H. H. Hoppe and H. Zacher, Pestic. Biochem. Physiol. **24,** 298–305 (1985).
[100] M. Focke and H. K. Lichtenthaler, Z. Naturforsch. **42c,** 1361–1363 (1987).
[101] K. Kobek, H. Focke, and H. K. Lichtenthaler, Z. Naturforsch. **43c,** 47–53 (1988).
[102] J. D. Burton, J. W. Gronwald, D. A. Somers, J. A. Connelly, B. G. Gengenbach, and D. L. Wyse, Biochem. Biophys. Res. Commun. **148,** 1039–1044 (1987).
[103] J. Secor and C. Cseke, Plant Physiol. **86,** 10–12 (1988).
[104] A. R. Redina and J. M. Felts, Plant Physiol. **86,** 983–986 (1988).
[105] K. A. Walker, S. M. Ridley, T. Lewis, and J. L. Harwood, Biochem. J. **254,** 307–310 (1988).
[106] P. Böger and G. Sandmann (eds.), Target Sites of Herbicide Action, 295 pp., CRC Press, Inc., Boca Raton 1989.
[107] A. D. Dodge (ed.), Herbicides and Plant Metabolism, Society for Experimental Biology, Seminar Series 38, 277 pp., Cambridge University Press, Cambridge 1989.
[108] R. C. Kirkwood (ed.), Target Sites for Herbicide Action, 339 pp., Plenum Press, New York 1991.
[109] N. R. Baker and M. P. Percival (eds.), Topics in Photosynthesis, **Vol. 10,** Herbicides, 382 pp., Elsevier, Amsterdam 1991.