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Philip Macdonald

Studies on the Photosynthetic Properties of s-triazine Sensitive
and Resistant Weeds.

by

Philip Alan Macdonald, B.Sc.

A thesis submitted to the Faculty of Graduate
Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

Department of Biology

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Ottawa, Canada

April, 1985

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ABSTRACT

Studies were conducted which compared PSII mediated processes in triazine sensitive and resistant biotypes of Chenopodium album and Amaranthus retroflexus. The activation energy of the rate limiting step of the Hill reaction to ferricyanide was similar for the two biotypes of both species. Activation energies were $7.8 \text{ K cal mol}^{-1}$ for the resistant biotype and $7.3 \text{ K cal mol}^{-1}$ for the sensitive biotype of C. album. A value of $6.9 \text{ K cal mol}^{-1}$ for the activation energy was calculated for the two biotypes of A. retroflexus. Oxygen evolution was monitored in flashing light. The resistant biotype produced damped oxygen yield sequences when compared to the sensitive biotype, with both species. This was explained in terms of the Kok scheme as arising from a higher miss parameter, and a non-zero distribution for S_3 and S_2 after dark adaptation. The process of S_3 and S_2 decay was described by a second order model in chloroplasts from the sensitive biotype of C. album. The deactivation of the S_3 and S_2 states was biphasic in resistant chloroplasts with an initial fast phase followed by a long, slow phase. The $S_0^* \rightarrow S_1$, $S_1^* \rightarrow S_2$, and $S_2^* \rightarrow S_3$ dark turnovers all proceeded more rapidly in resistant chloroplasts. The rate constant of the rate limiting step of oxygen evolution appeared to be the same for both biotypes.

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LIST OF ABBREVIATIONS AND SYMBOLS

atrazine - 2-chloro-4-ethylamino-6-(isopropylamine)
 -s-triazine

B - the secondary 2 electron acceptor of photosystem two

Chl - chlorophyll

CO₂ - carbon dioxide

CytF - cytochrome F

D - the electron donor in a deactivation reaction of the
 S states

[Do] - the concentration of the electron donor in a
 deactivation reaction of a Kok S state at
 the start of deactivation

DCMU - 3-(3,4 dichlorophenyl)-1,1-dimethyl urea

Ea - the activation energy of the rate limiting step of
 electron transport

k - the rate constant of a reaction

kg/ha - kilograms per hectare

Kda - kilodalton

metribuzin - 4-amino-6-isopropyl-3-methylthio-1,2,
4-triazin-5-one

MnIII, MnIV - two oxidation states of a manganese atom

NADP - nicotinamide adenine dinucleotide phosphate

PC - plastocyanin

PQ - plastoquinone

prometryne - 2,4-bis(isopropylamino)-6-methylthio
-s-triazine

PSI - photosystem I (one)

PSII - photosystem II (two)

Q - the primary electron acceptor of photosystem II

R - the gas constant = 1.9817 Cal. K mol⁻¹

tricine - N-tris-(hydroxymethyl) methylglycine

$S_0.....S_3$ - the four states of the Kok model

simazine - 2-chloro-4,6-bis(isopropylamino)-6-methylthio
-s-triazine

$[S_{n0}]$ - the concentration of the Kok S state at the start of
deactivation

$Y_n(\Delta t)$ - the yield on the n th flash delivered at a time t
after a preceding flash

Z - primary electron donor to photosystem II

α - proportion of misses of the Kok model

β - proportion of double hits of the Kok model

$\gamma_n(\Delta t)$ - probability that a centre will undergo a dark
rearrangement at a time t , for the n th dark
process of the Kok scheme

ϕ - phase of the modulated oxygen signal

ν - frequency of light modulation

INTRODUCTION

History and Agricultural Applications of s-triazine Herbicides

In 1952 the J.R. Geigy Research Unit synthesized the first herbicides of the s-triazine family (Esser et al., 1975). Subsequently, numerous triazine derivatives have been synthesized and screened for herbicidal action (ibid). These herbicides have proved to be very effective and are currently employed in a variety of agricultural applications. Applications include the specific control of grasses and broadleaf weeds in corn and other cereals, orchard crops, and also, weed control on industrial sites. The s-triazines are particularly useful because of their persistence in the soil. This persistence allows for weed control at preplanting, pre-emergent and post-emergent stages of a crop without the need for repeated applications or the use of a variety of herbicides (Esser et al., 1975, Ashton and Crafts 1973). The structure of s-triazine and some of its derivatives are illustrated in figure 1.

Tolerance Mechanisms

The use of herbicides relies on the tolerance of the crop to the chemical applied, and the susceptibility of the weed species. In agricultural use, the s-triazine

herbicides are applied at concentrations of 1.15 to 3.5 kg/ha (Bandeem and McClaren, 1976), at which concentrations weed species are killed, but crop plants remain unaffected.

In orchards and vinyards weed control is accomplished through the use of the s-triazine, simazine (2-chloro-4, 6-bis (ethylamino)- s-triazine). This herbicide is applied to the soil and since it has a low water solubility it is not leached from the surface layer of the soil. As a result, the deep-lying roots of vines and fruit trees are not exposed to the simazine. The weed species which have roots near the surface of the soil are exposed, absorb the herbicide and are killed.

Some plants tolerate s-triazines by chemically transforming them to related but non-toxic forms. This type of resistance is displayed by corn which can use several detoxification procedures. A chemical factor, characterized as (2,4-dihydroxy-3-keto-7-methoxy-1, 4-benzoxazine) is responsible for the detoxification of the s-triazines (Palmer and Grogan, 1965). This is accomplished by hydroxylation of the number two position of the triazine ring, with dechlorination, demethoxylation, demethylation, or demethylthiolation depending on the parent substitution (Montgomery and Freed, 1961). An additional mechanism which involves

dealkylation of the alkyl side chains of the triazine ring, followed by their subsequent oxidation, has also been identified in maize (Ashton and Crafts 1973). This system operates in conjunction with the previously described chemical factor. Cleavage of the triazine ring may also occur, however research results are not in agreement on this point (Esser et al., 1975).

Cotton appears to provide an example of a related approach to herbicide tolerance. With this crop no rapid hydrolysis mechanism has been identified, however a tolerance to the s-triazine prometryne (2,4-bis (isopropylamino)-6-methylthio-s-triazine) is apparent. Sikka and Davis (1968) have shown that this herbicide is translocated extremely slowly through the plant. As prometryne is a soil applied herbicide, the chemical is first absorbed through the root system. The slow movement of the herbicide allows for the metabolic breakdown to less toxic forms before reaching the chloroplasts of the leaves, the primary site of action.

In recent years weed biotypes have begun to appear which display resistance to the s-triazine herbicides (Holliday and Putwain, 1980). The s-triazine resistant weed biotypes differ from tolerant plants in showing resistance at concentrations 1000 times that normally used in agricultural weed control (Pfister et al., 1979). This

resistance is inherited cytoplasmically through the maternal parent (Warwick and Black, 1980a). Initial studies indicated no differences in metabolism, translocation, or morphology between the susceptible and resistant forms (Jensen et al., 1977). Recent reports suggest that differential degradation of the triazine herbicides may occur between the susceptible and resistant biotypes (Khan et al., 1984), however, these differences are not large enough to account for the resistant biotype. The resistance to triazines was found to persist in isolated chloroplast preparations from resistant strains, indicating that changes in the photosynthetic apparatus were responsible for the lack of susceptibility to the triazine herbicides (Souza-Machado et al., 1978). The resistant strains were poor competitors when grown along side the susceptible plants in the absence of the triazine herbicides (Warwick and Black 1980b). This is generally thought to result from an impaired photosynthetic capacity in the resistant biotype (see Radosevich and Holt, 1982, for a review).

Model for the Appearance of the Resistant Biotype

Despite the widespread and increasing use of herbicides in agricultural weed control, the appearance of an inheritable resistance, as seen with pesticides, rodenticides and antibiotics, is relatively rare (Gressel

and Segal, 1978). These authors generated a complex mathematical model to investigate the reasons for the occurrence of s-triazine resistance in a wide variety of weeds. The model considered the compound effects of the usually low selection pressure of herbicides, the effects of the large soil reservoir of susceptible seeds, the ability of herbicide thinned stands of weeds to produce proportionately more seeds and the lower reproductive fitness of resistant strains when they are competing with susceptible strains in the absence of herbicides. These characteristics combine to reduce the rate of enhancement of resistance. The model considered these factors and generated different scenarios of herbicide kill, seed longevity and fitness. This led to the suggestion that genetic resistance to the s-triazine herbicides by weeds has resulted from the high proportion of killing (greater pressure) exerted by these herbicides. This factor, coupled with the high persistence of the s-triazines in the soil, shortens the period of the season over which the difference in relative competitiveness can act to dampen the appearance of resistance. The herbicide resistant biotypes have all appeared in areas where s-triazine herbicides have been used exclusively in the same field, for extended periods of time (10-15 years) (Holliday and Putwain, 1980). This continued use of the same herbicide would be expected to further increase the appearance of

resistance by enhancing the selection pressure for resistant weed strains.

Site of Action of s-triazine Herbicides

Triazine herbicides are one of a large variety of chemicals whose mechanism of action is to block electron transport. The chemical groups of ureas, amides, triazines, triazanones, pyradizones, carbamates and nitrophenols, all block photosystem II dependent Hill reactions (Wright and Corbett, 1979). Competitive binding experiments by Tischer and Strotman (1979) have shown that a ^{14}C labelled triazine can be replaced by ureas, pyradizones, carbamates or nitrophenols. This led to the suggestion that all these inhibitors are acting at the same site. Further work by Tischer and Strotman (1979) indicated that mild trypsinization of chloroplasts (8-10 min., 25 C, at pH 8.0) removed the ability of chloroplasts to bind metribuzin (4-amino-6-isopropyl-3-methyl-thio-1,2,4- triazin-5-one) a triazinone derivative. This complemented the earlier work of Renger (1976) which had shown that DCMU (3-(3,4 dichlorophenyl)-1,-dimethylurea) blockage of electron transport could be removed by mild trypsinization if an appropriate electron acceptor was present. By using increasing trypsin concentrations it was demonstrated that the amount of DCMU bound decreased with increasing trypsin

concentration. Further work by Renger (1979) showed that in the presence of DCMU, at concentrations sufficient to completely inhibit photosystem II electron transport, the O_2 yield in response to a light flash first increases with incubation time, then decreases. This situation mirrors the effect of increasing trypsin incubation in the absence of DCMU. Renger speculated that the decline in O_2 yield after reaching a maximum reflects a degradation in the O_2 evolving complex by trypsin. The author suggests two possible explanations for the data; either mild trypsinization interrupt electron flow by modifying a protein component, while simultaneously increasing the accessibility to exogenous electron acceptors, such as ferricyanide without changing the binding affinity for DCMU type inhibitors, or, alternately, that trypsin digestion of a protein component changes the properties of a binding site for DCMU type inhibitors.

The work of Boger and Kunnert (1979) confirmed Renger's (1979) second hypothesis. Using isolated chloroplasts and a variety of inhibitors which act at the DCMU-binding site they attempted to follow the kinetics of release of inhibition, with increasing trypsin concentration. The different kinetics of reversal of inhibition displayed by different groups of inhibitors indicated that trypsinizing chloroplasts did not merely increase the accessibility of exogenous electron donors,

without changing the binding affinity of the inhibitor. Additionally it appeared that the DCMU binding site represented a binding domain, with different inhibitors binding in different areas of the binding site. This model is consistent with observations on chloroplasts isolated from triazine resistant weeds, which will not bind triazine herbicides but still retain the ability to bind DCMU (Pfister et al., 1979).

The preceeding evidence indicates that the binding site for the s-triazines and other herbicides which act at the DCMU binding site is a protein. The susceptibility of the protein to trypsin attack indicates that it is exposed on the outer surface of the thylakoid membrane. Before presenting evidence for the nature and location of the herbicide binding protein a review of the functioning and structural components involved in photosynthetic electron transport will be necessary.

Photosynthetic Electron Transport

Photosynthetic linear electron transport occurs between two connected systems, designated photosystem I (PSI) and the site of oxygen evolution, photosystem II (PSII). (Fig 1) Light absorbed by the pigment molecules raises these molecules to an excited state. Excitation energy is funneled via resonance transfer to a "reaction centre" which donates an electron to the primary electron

acceptor of the electron transport chain. The reaction centre regains its electron by oxidizing a donor molecule (Noble, 1970). The movement of the electron from the reaction centre to the primary acceptor of the linear electron transfer chain can ultimately lead to the reduction of NADP to be used in the reduction of CO_2 in the Calvin cycle. The primary electron donor is water. The oxidized reaction centre which has lost an electron can accept another from a donor, as in the steps leading to oxygen evolution. This phenomenon (O_2 evolution) will be discussed in more detail later.

Enzymes and light harvesting and reaction centre pigments associated with specific proteins are apparently organized into at least three distinct functional and structurally separate complexes within the chloroplast thylakoid membrane (Bricker et al., 1983). Functional activity of these complexes relies on ordered interactions of proteins, chlorophylls and lipids (Pfister and Arntzen, 1979). The three generally accepted complexes are the PSI complex which photochemically excites electrons accepted from reduced plastocyanin and reduces NADP, the cytochrome b-563-F complex, which is believed to accept electrons from plastoquinol and reduce plastocyanin, and the PSII complex which extracts electrons from water by light driven steps and reduces plastoquinone (Metz et al., 1980). Of these three complexes, it is the PSII complex

which is of the greatest relevance to this study.

Characterization of the PSII complex has been greatly aided by the recent isolation of PSII particles with highly active O_2 evolving ability (Kuwabara and Murata, 1982). Using these particles in conjunction with data from topological studies (Bricker and Serman, 1982, Zurawski et al., 1982), Bricker et al., (1983), have presented a model for the organization of the PSII complex in the thylakoid membranes of higher plants. They suggest the core contains 49 and 45 Kda polypeptides associated with the reaction centre. The reaction centre designated P680 is coupled to tightly bound chlorophyll a molecules, which act as light harvesting antennae (Amesz, 1982). Additional 34Kda, 32 KDa and cytochrome b559 polypeptides are also associated with this complex (Bricker et al., 1983). All of these peptides possess surface exposed regions which are sensitive to trypsin (ibid). Loosely associated with the reaction centre complex is an additional pigment-protein complex of 32, 25 and 14 Kda. Manganese has been suggested to interact with one or more of these polypeptides (Amesz, 1983). This complex serves a light harvesting function and can transfer energy to the core pigment molecules (Bricker et al., 1983).

Electron donation from water to P680 remains poorly understood, probably because of the complexity of the

pathway, as well as the extreme lability of the constituents (Amesz 1983, Nugent et al., 1982). The O_2 evolving complex is generally believed to be located on the inner surface of the thylakoid membrane (Renger, 1979, Blankenship and Sauer, 1974). Detergent disruption, to release polypeptides, followed by reconstitution have implicated polypeptides of varying molecular weights as the oxygen evolving enzyme. Currently a 33Kda protein has been reported to inactivate O_2 evolution upon extraction (Bricker et al., 1983, Kuwabara and Murata, 1982). From a variety of studies it appears that manganese is certainly involved in O_2 evolution (see Amesz, 1983, for a review). Electron donation to P680 is from two donors termed Z_1 , and Z_2 (Bouges-Bocquet, 1980). These donors have been suggested to be plastosemiquinones in a special environment, probably not protonated but associated with a metal cation, possibly Mg^{2+} or Ca^{2+} . Nugent et al., (1982) have suggested that another fast donor located between Z_1 and Z_2 may be operating.

A model for O_2 evolution was proposed by Kok et al. in 1970. This model describes the pattern of O_2 evolution which occurs after dark adapted chloroplasts are subjected to a series of short, saturating light flashes. It is observed that no oxygen is evolved on the first flash, little or no oxygen is evolved on the second flash and the maximum yield is produced on the third flash. The flash

yields oscillated with a period of four on subsequent flashes, gradually damping to a steady state oxygen yield.

These results were interpreted as indicating that a series of four positive charges are accumulated during 4 consecutive photoreactions, before oxygen can be evolved. The reaction scheme is:

S_0 , S_1 , S_2 , S_3 , and S_4 are the five states of the reaction centre. The absorption of a flash results in the transfer of an electron into the electron transport chain. Dark rearrangements proceed ~~after~~ a flash, turning the centre back to a photoactive state. These dark reactions are accompanied by the reoxidation of Q^- . The final step $S_3^* \rightarrow S_4 \rightarrow S_0$ proceeds spontaneously and is accompanied by the evolution of an oxygen molecule.

The pattern of flash yields can be explained by assuming that after dark deactivation 25% of the centres

are in the S_0 state and 75% in the S_1 state. Thus no oxygen will be produced until the third flash and this also gives the maximum yield. Two additional parameters are invoked to explain the gradual damping of the oscillating pattern. These are designated as misses (α), where a photochemical event does not proceed despite the absorption of a photon, and double hits (β), where two photochemical events proceed in the space of one flash. Typically the best fitting values of α and β are about 10% and 5% respectively (Forbush et al., 1971).

A recent study by Dekker et al., (1984) offers a chemical explanation for the kinetic model of Kok (1970). Using PSII particles they recorded absorbance changes at 300nm in response to 10 μ sec. xenon flashes, and correlated this with the absorbance changes of MnIII to MnIV. From their data they suggested that 3 MnIII ions are successively oxidized to MnIV and reduced simultaneously with Z after the next photoreaction. The oxidation of an MnIII to MnIV atom will occur in the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions. Three MnIV ions are then reduced to MnIII during the $S_3 \rightarrow S_4 \rightarrow S_0$ transition.

The electron acceptors of PSII have been characterized by fluorescence and absorption change measurements, and EPR spectroscopy. Fluorescence measurements have indicated the presence of two acceptors

with redox potentials of about 0 and -250 MV (Horton and Croze 1979). Direct absorption measurements show a quinone acceptor Q (Malkin 1978) and a pheophytin intermediary carrier (Klimov et al., 1977). The EPR studies of Evans et al., (1982) indicate an interaction between the quinone acceptor and an iron atom. A secondary acceptor (B), also a quinone (Pfister and Arntzen, 1979) is a two electron carrier, acting as a gating mechanism to the plastoquinone pool which interconnects PSI and PSII (Veluthrys and Ames 1974). Vermaas and Arntzen (1983) suggest that Q and B are bound at two specific regions on specific PSII proteins. The quinone at the primary electron acceptor site does not exchange readily with the plastoquinone pool, whereas the secondary acceptor exchanges readily when in the fully oxidized or reduced form. The protein complex to which the secondary acceptor binds is called the Q-/PQ oxidoreductase by these authors.

Mode of Action of s-triazine Herbicides

The s-triazine herbicides, DCMU and the other unrelated PSII inhibitors are believed to act at the level of B (Pfister and Arntzen, 1979). Using susceptible chloroplasts from Amaranthus sp. Pfister and Arntzen (1979) were able to demonstrate DCMU and atrazine inhibited electron transport between Q and B. Chlorophyll

fluorescence from the isolated chloroplasts was detected during a 2 msec. weak measuring flash administered once every two seconds. This is considered dark fluorescence as Q remains oxidized. At various times the chloroplasts were illuminated with an 8 μ sec. intense saturating flash given during the first half of the dark period between fluorescence measurements. Addition of DCMU or atrazine caused no increase in fluorescence with the weak measuring beam, but application of the 8 μ sec. saturating flash to the inhibited chloroplasts caused an increase in fluorescence to a maximal value. This was taken to indicate that the single flash entirely filled the electron pool available before the herbicide block, thus the Q pool was reduced. This experiment can be compared to similar studies on bicarbonate depleted chloroplasts in which electron transport is blocked between B and the plastoquinone pool (Govindjee and Van Rensen, 1978). Bicarbonate depleted chloroplasts require three saturating flashes to give maximal fluorescence, indicating that the Q plus B pool can store a total of three charges.

Although the PSII inhibitors act at the level of B, the binding site is a protein, as has been previously discussed. Mullet and Arntzen (1981) used 2-azido-4-ethyl amino-6 isopropylamino-s-triazine, a fluorescent label, and a triazine analog, to identify a 32 Kda polypeptide as the herbicide binding protein. A recent study by Verma

and Arntzen (1983) indicates that triazine and the other PSII inhibitors compete with the secondary acceptor for a common binding domain on a protein complex. Pfister and Arntzen (1979) and Steinback et al., (1981) have shown with resistant strains of Amaranthus sp. that a membrane protein from the thylakoids of isolated chloroplasts showed different electrophoretic mobility when compared to the corresponding protein from susceptible chloroplasts. This has been interpreted to indicate that a small modification in the amino acid sequence of the protein may have occurred. A recent study by Hirschberg and McIntosh (1983) with Amaranthus hybridus has shown that a single amino acid change in the 32 Kda herbicide binding protein may account for triazine resistance. A replacement of glycine for serine on the 32 Kda polypeptide of the resistant biotype was suggested to account for the loss of triazine binding. This was thought to result from the amino acid substitution either changing the actual triazine binding site or modifying the triazine binding site by changing the tertiary structure of the protein.

Characterizing the Resistant Biotype

The value of studies on the resistant biotype lies in the usefulness of this mutation as a probe of normal photosynthetic functioning. After having been grown under identical conditions the two biotypes of S. vulgaris weeds

displayed identical leaf morphology and chlorophyll a to chlorophyll b ratios. The rate of whole plant carbon dioxide, fixation, which relies on energy from the light driven reactions, was higher in the sensitive than in the resistant plants when monitored at the same temperature and light intensity. These differences could not be explained by differential light absorption by the leaves (Holt et al., 1981). At the same light intensity, leaves from both biotypes absorbed the same number of quanta. As differences did not occur in leaf morphology, chlorophyll a to chlorophyll b ratios or light absorbance, the differences would be expected to occur in the photosynthetic apparatus of the two strains.

Bowes et al., (1980) have demonstrated that electron transport between Q and B in resistant chloroplasts of A. retroflexus occurs 10 times more slowly than it does in sensitive chloroplasts. Vermaas and Arntzen (1983) examined the competition for binding to a common PSII region by synthetic quinones and PSII herbicides. They concluded that the native quinone B (the secondary acceptor) showed a lowered affinity for the binding site in the resistant biotype when compared to the susceptible biotype. Binding of quinone head groups was thought to remain unchanged, however, quinone side group binding is altered in the resistant biotype. The lowered affinity for B to the binding site is invoked to explain the

observation that the equilibrium concentration of reduced Q after the transfer of one electron to the acceptor side of PSII is increased in the resistant biotype. These authors suggest that a lowering of the B/B^+ midpoint redox potential occurs.

This study has been initiated to further characterize the resistance mutation. This problem has been approached through electron transport studies both in the presence and absence of herbicides. Additionally, studies on the O₂ evolving complex have been undertaken in order to determine the effect of the resistance mutation on the reactions associated with the Kok model. Finally, evidence was sought which would indicate whether the resistance mutation resulted in changes on the oxidizing side of the electron transport chain.

FIGURE 1

The structure of the s-triazine molecule.

The ring substitutions for the s-triazine derivatives described in the text are as follows:

atrazine, $R_1 = \text{Cl}$, $R_2 = \text{NH C}_2\text{H}_5$, $R_3 = \text{NHC}_2\text{H}_5$

prometryne, $R_1 = \text{SCH}_3$, $R_2 = \text{NH C}_3\text{H}_7$, $R_3 = \text{NH C}_3\text{H}_7$

simazine, $R_1 = \text{Cl}$, $R_2 = \text{NHC}_3\text{H}_7$, $R_3 = \text{NHC}_2\text{H}_5$

FIGURE 2

The photosynthetic electron transport chain showing whole chain linear electron transport from water to NADP. cyt f is cytochrome f and PC is plastocyanin. Other symbols are explained in the text. (After Williams 1977)

MATERIALS AND METHODS

Chloroplast Isolation

Susceptible and resistant biotypes of Amaranthus retroflexus and Chenopodium album were germinated in a controlled environment chamber (18 hr photoperiod, 23:1°C) in a mixture of peat. After 2 weeks the seedlings were transferred to soil then placed in a greenhouse where they were grown under a 16 hr. photoperiod at 23:1°C. In the greenhouse the plants were watered regularly with distilled water, supplemented on a weekly basis with standard Hoaglands solution.

Chloroplasts were prepared using a modified version of the technique of Cockburn et al. (1968). Leaves were harvested from 5-7 weeks old plants and if necessary the midribs were removed. Approximately 15 g of tissue was coarsely chopped into 100 ml of grinding medium containing 0.33 M sorbitol, 30 mM tricine/NaOH pH 8.2, 5 mM $MgCl_2$, 2.5% Ficoll, 0.5% BSA and 0.5 mM 2-mercaptoethanol. It was then ground for 3-5 seconds in a polytron homogenizer (Kinematica GmbH. Luzern Switzerland) at a speed control setting of six. Tricine buffer and BSA were obtained from Sigma Chemical Corp. (St. Louis Mo.), ficoll (type 400) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). After grinding, the homogenate was filtered through 8 layers of cheesecloth and one layer of cotton

wool, then centrifuged at 4000xg for 90 seconds in a refrigerated Sorvall RC2 ultracentrifuge. The pellet was gently dispersed with a camel hair brush in approximately 2 ml of standard medium A, which contained 0.33M sorbitol, 5 mM $MgCl_2$, 30mM tricine/NaOH pH 8.2, 2.5% ficoll and 0.5% BSA. If broken chloroplasts were desired, the pellet was resuspended in 10 ml of a hypotonic medium containing 100 mM NaCl, 5mM $MgCl$ and 30mM tricine/NaOH pH 8.2. The chloroplasts were left at 0°C in the dark for 3 minutes, then centrifuged at 4000xg in a Sorval RC2 refrigerated centrifuge for 90 seconds. The supernatant was discarded and the pellet resuspended in approximately 2 ml of standard medium A. The chlorophyll concentration of the preparation was assayed according to the method of Vernon (1960). The chloroplasts were stored on ice in the dark until required. All steps in the procedure were performed at approximately 4°C.

Ferrocyanide Assay

Avron and Shavit (1963) have described a sensitive, colorimetric assay for reduced ferricyanide (ferrocyanide), using O-phenanthroline as the colour developing agent. This technique was adapted to provide a direct assay for ferrocyanide produced by the Hill reaction with ferricyanide as an electron acceptor. The assay was then applied to experimentally derive Hill and

Arrhenius plots.

Seven identical 13x100mm tubes containing 100 mM NaCl, 2mM $MgCl_2$, 3mM KH_2PO_4 , 5mM NH_4Cl , 0.5mM $K_2Fe(CN)_6$ (standard medium B) and an aliquot of the chloroplast suspension to a final concentration of $5\mu g/ml$ were prepared. The final volume in each tube was 5 ml. The tubes were placed into a plexiglass box, through which water at a constant temperature ($20 \pm 0.05^\circ C$) was circulated, by a Lauda K2RD constant temperature water bath. Trichloroacetic acid was added to a final concentration of 3% to one of the tubes immediately, halting any reaction. This served as a zero time blank for the assay. The tubes were then exposed to saturating light from a 300 watt, G.E. tungsten floodlamp located 13.5 cm from the samples, at an intensity of $3.6 \times 10^4 \text{ ergs cm}^{-2} \text{ sec}^{-1}$ for 30 seconds. The reaction was then halted by the addition of 3% TCA to each tube. The tubes were then decanted into 7 centrifuge tubes and centrifuged at 1800xg for 5 minutes in a Sorval GLC2 centrifuge to remove suspended debris. A 2.1 ml aliquot from each centrifuge tube was mixed into 0.3 ml of 3 M sodium acetate pH 6.0, 0.3 ml of 1.2 M citric acid and 0.15 ml of 3% 1, 10-phenanthroline solution in absolute ethanol. Upon mixing, the colour began to develop. A period of 5 minutes was allowed for the colour to develop fully. The absorbance of the resulting coloured complex was read at 510 nm against the zero time blank in a

Varian-Cary 219 spectrophotometer. The procedure was repeated with samples exposed to the light for times of 1 minute, 1.5 minutes and 2 minutes. An appropriate light exposure time for the assay was chosen by graphing average absorbance values against time. The linear portion of the graph was assumed to represent the light exposure times at which ferricyanide concentration did not limit electron transport. A light exposure time was chosen from the linear portion of the graph and used for all further assays of Hill activity. Rates of electron transport varied with each chloroplast preparation thus each chloroplast isolation required the selection of an appropriate light exposure time.

Hill Plots

Hill plots provide a means for analyzing the inhibition of measured values of electron transport at varying concentrations of atrazine and DCMU. The binding of these molecules may occur independently or cooperatively. Independent binding of a small molecule, I to a macromolecule E, requires that the binding of an I to one site, does not influence the binding of another I to any other site on the same macromolecule. If binding is fully cooperative, the binding of the first I influences the subsequent binding of other I molecules, with the result that the remaining sites on the macromolecule fill

up immediately. If one step n-site binding is assumed, then binding properties can be defined by the Hill equation which is derived as follows:

$$KI = \frac{[E][I]^n}{[EIn]}$$

where E represents the macromolecule, I is the small molecule binding to a site on the macromolecule, and EI represents bound I. KI is the binding constant.

If V is defined as the number of molecules of I bound per molecule of E

$$2) \quad V = \frac{n[EIn]}{[EIn] + [E]}$$

From equation 1)

$$[EIn] = \frac{[E][I]^n}{KI}$$

substituting into 2)

$$V = \frac{n[E][I]^n}{KI \frac{[E][I]^n + [E]}{KI}}$$

therefore:

$$V = \frac{n[I]^n}{KI + [I]^n}$$

$$\therefore V = \frac{n[I]^n}{[I]^n + KI}$$

rearranging the result

$$V([I]^n + KI) = n[I]^n$$

$$\therefore V[I]^n + VKI = n[I]^n$$

$$3) \quad \frac{V}{n-V} = \frac{[I]^n}{KI}$$

if θ is defined as the fraction of sites occupied by I then the number of occupied sites becomes:

$$V = n\theta$$

$$\text{then} \quad \frac{V}{n-V} = \frac{\theta}{1-\theta} = \frac{[I]^n}{KI}$$

taking the log of both sides:

$$4) \quad \log \frac{\theta}{1-\theta} = n \log [I] - \log KI$$

If binding is considered to be equivalent to

inhibition then $[I]$ will be the concentration of herbicide molecules and θ can be taken as % inhibition. Plotting $\log [\% \text{ inhibition} / (100\% - \% \text{ inhibition})]$ versus the log of the herbicide concentration, should result in a linear relationship. The slope of the line will be n , the Hill coefficient.

Tischer and Strotmann (1977) have demonstrated mathematically, then verified experimentally, that Hill plots are sensitive to chlorophyll concentration. Directly assaying for ferrocyanide, with the sensitive assay previously described, permitted the use of very low chlorophyll concentrations ($5\mu\text{g/ml}$). An additional advantage of this technique was that it was possible to produce an entire range of values for the Hill plots at the same time. As rates of electron transport from isolated chloroplasts can be seen to decline over time, generating all values from chloroplasts of the same relative age should alleviate a potential source of error.

The basic ferrocyanide assay was adapted to generate Hill plots. The seven tubes were prepared as before but in addition either DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea or atrazine (2-chloro-4-ethyl-amino-6(isopropylamine)-s-triazine) was added to 5 of the tubes at a range of concentrations. (see fig. 4, fig. 5, and fig. 6) Of the two tubes to which no herbicide was added,

one served as a zero time blank for the assay, while the other served as a control providing an uninhibited rate of electron transport. The tubes were reacted and assayed in the same manner as in the basic procedure and observed rates were used to derive Hill plots for atrazine and DCMU respectively.

Arrhenius Plots

Varying the temperature at which a reaction proceeds has provided important keys to the theory of rate processes. The so-called Arrhenius plot derives from the equation which describes the variation of the rate constant k with temperature:

$$\frac{d \ln k}{dT} = -\frac{E_a}{RT^2}$$

where E_a is called the activation energy, R is the gas constant and T is the absolute temperature. Integrating the preceding equation yields:

$$\ln k = -\frac{E_a}{RT} + \ln A$$

where $\ln A$ is the constant of integration.

From this it follows that a plot of the logarithm of

the rate constant against reciprocal temperature should be a straight line. The validity of the Arrhenius equation has been confirmed in this way for a large number of experimental rate constants (Moore 1972).

In this study the activation energy for the rate limiting step of photosynthesis was determined for the two biotypes of C. album and A. retroflexus. Arrhenius plots were generated by assaying rates of ferricyanide reduction at 10, 15, 20 and 25°C.

Rates of ferricyanide reduction were determined using the ferrocyanide assay already described. The temperature was varied by presetting the water bath to the appropriate temperature, then allowing the reaction tubes to equilibrate for 5 minutes at the preset temperature before exposure to the light source. An additional tube was placed in another chamber and held at 20°C by a separate identical water bath. A light source of the same type and intensity was provided. This sample was reacted and assayed concurrently with a trial at a selected temperature. This provided an indication of the decline in activity of the chloroplast preparation during subsequent trials. It was then possible to differentiate the effect on the rate which resulted from the change in temperature from that which arose from the decline in activity of a chloroplast preparation over a working day,

and apply an appropriate correction factor.

The natural logarithm of the corrected rates was plotted as a function of the inverse of the temperature (K).

The slope of the line is defined as $-E_a/R$ where:

E_a = the activation energy (Cal. mol^{-1}) R = the gas constant = $1.9817 (\text{Cal. K}^{-1} \text{mol}^{-1})$

Flash Experiments

Flash Lamps

By measuring O_2 yields in response to a series of short, saturating flashes of light it is possible to investigate the nature of the O_2 evolving complex (see Joliot and Kok, 1975 for a review).

In these experiments short saturating flashes were obtained by discharging a $0.5 \mu\text{F}$ capacitor charged to 1800 volts through a G.E. type Ft-230 flash tube. The flash lamp was triggered by a bare 0.030 gauge copper wire (type II trigger) located on the outer surface of the flash tube. The duration of the flash was $4 \mu\text{sec}$ at $I_{\text{max}}/3$ which was short enough to separate the different photoreactions (Weiss et al., 1971). Inserting a Kodak 50% neutral density filter between the light source and the chloroplast sample resulted in less than 20% inhibition in the steady state O_2 yield from the chloroplasts. This

indicated that the lamp gave saturating flashes.

Flash Controller

The number and spacing of the flashes was determined by a flash controller designed and built by Carleton University Science Workshops. The flash controller could initiate from 1 to 99 flashes spaced from 0.1 to 9.9 seconds apart. It was also possible to follow any one of the first 10 flashes in a flash sequence by a flash spaced from 0.1 to 999.9 msec. after the previous one. Since the time required for the capacitor to recharge limits the time interval between flashes, a second identical flash lamp was required to accomodate these short time periods.

Optical Arrangement

The two flash lamps were located on opposite sides of the chloroplast sample. Light from the lamps was focused with two separate sets of lenses, then reflected onto the photosynthetic material by a first surface mirror (Fig. 3).

Polarographic Cell

Oxygen evolution in response to single flashes of light was detected by a polarographic cell, similar to that described by Joliot (1972). The cell is illustrated in Fig. 2. The body of the polarographic cell was

constructed of grey teflon, with a clear lucite window on the upper surface of the cell to allow exposure of the sample to the light flashes.

The chloroplast suspension was continuously perfused with fresh medium which flowed through the middle section of the cell. Medium could then diffuse across the lower dialysis membrane to the chloroplast sample.

The upper section of the cell contained a coiled silver wire coated electrochemically with AgCl. This Ag/AgCl electrode was the current carrying anode. The upper section of the cell was bathed in a 100 mM NaCl solution.

Measurement of the Oxygen Yield

When the platinum electrode of the polarographic cell was biased at -0.75V with respect to the reference electrode, the current flowing through the cell was proportional to the O_2 produced by the chloroplast sample on the platinum electrode. The O_2 spikes produced by the chloroplasts in response to the flashing light were recorded on a Tektronix R5031 storage oscilloscope. The height of each spike was used as a relative measure of the O_2 produced by each flash.

Experimental Procedure

In a typical experiment 0.5 ml of a 0.075 mg chl/ml chloroplast suspension enriched with ferredoxin at a concentration of 0.1 mg/mg chl was injected into the polarographic cell. A bathing medium of 100 mM NaCl, 30mM tricine/NaOH pH 8.2, 2mM $MgCl_2$, 3mM KH_2PO_4 , and 0.5 mM NADP (nicotinamide adenine dinucleotide phosphate) (standard medium C) flowed through the cell. The bathing medium was held in a glass reservoir connected to the cell by tygon tubing. The flow rate was controlled by a tap located after the cell. The chloroplast sample was held in complete darkness for 10 minutes, then exposed to a sequence of 30 flashes, spaced 0.3 seconds apart. Ambient light was excluded by the use of two metal shields which surrounded the two flash lamps. Spaces large enough to admit light were covered with black cloth. Electrical noise was reduced by careful grounding. The O_2 yields in response to the series of flashes was recorded by measuring the spikes on the screen of the oscilloscope with a ruler. All experiments were carried out at temperature of $22^\circ C \pm 1^\circ C$.

Deactivation Experiments

Forbush et al., (1971) described a procedure to determine the relaxation times of the S_3 and S_2 states of the Kok model (Kok et al., 1970) in isolated spinach chloroplasts. A similar procedure was used in this study

to follow S_3 and S_2 deactivation in chloroplasts isolated from the two biotypes of C. album. Standard medium C was the bathing medium for these experiments. All experiments took place at $22^\circ\text{C} \pm 1^\circ\text{C}$.

S_3 Deactivation

If two saturating flashes are given to chloroplasts after a 10 minute dark period, it would be expected that the majority of reaction centres would advance to the S_3 state. Any centres remaining in the S_3 state after a period Δt will evolve O_2 on the next flash (third flash). Thus, by varying Δt , it is possible to monitor the decay of the S_3 state. Chloroplasts were given two saturating flashes spaced 0.3 sec. apart after the 10 minute dark period. After a variable time Δt , a series of 30 saturating flashes spaced 0.3 sec. apart was initiated. The O_2 yield on the third flash was recorded, then normalized to the steady state O_2 yield.

S_2 Deactivation

Delivering one saturating flash to dark adapted chloroplasts should advance the centres to the S_2 state. After a variable time Δt any centres which remain in the S_2 state will evolve O_2 on the second flash following Δt (the third flash).

Chloroplasts were given a ten minute dark period,

then exposed to one saturating flash. Following a variable time Δt , a series of 30 flashes, 0.3 sec. apart were administered. The O_2 yield on the third flash was recorded and normalized to the steady state O_2 yield.

Time Course of the Forward Reaction

Flash polarography can be used to study the time course of the four forward reactions of the Kok model of O_2 evolution. The methods used in this study are similar to those used by Bouges-Bocquet (1973). Following the nomenclature developed by this author the reactions were designated Y_0 , Y_1 , Y_2 and Y_3 . The term $Y_n(\Delta t)$ refers to the probability that a centre in a state S_n^* just after a flash, will undergo dark rearrangement to a state S_{n+1} , at a time Δt , after the flash. In a typical experiment the chloroplasts were perfused with standard medium C. After 10 minutes of darkness, 30 saturating light flashes spaced 0.3 sec. apart were delivered. This was repeated until the flash yields stabilized. The rate of one of the four forward reactions was then determined.

The reaction $S_0^* \rightarrow S_1$ is defined by the expression:

$$Y_0 = \frac{Y_4(\Delta t) + Y_3(\Delta t) - Y_3(0.3 \text{ sec.})}{Y_4(0.3 \text{ sec.})}$$

where $Y_n(\Delta t)$ = the yield on the nth flash delivered Δt after a preceeding flash. $Y_n(0.3)$ = the yield on the nth flash delivered 0.30 sec. after the preceeding flash.

After the O_2 flash yields have stabilized, the sample is left in darkness for 5 minutes, then given 3 flashes of light separated by 0.3 sec. intervals. After a 6 minute dark period 30 light flashes are delivered. The first two flashes are separated by Δt , the remaining flashes are spaced 0.3 sec. apart.

The reaction $S_1^* \rightarrow S_2$ is designated γ_1 where:

$$\gamma_1 = \frac{Y_3(\Delta t)}{Y_3(0.3 \text{ sec.})}$$

Chloroplasts are given 10 minutes of darkness, then exposed to 30 flashes 0.3 sec. apart except for the first and second flashes, which were separated by Δt

The reaction $S_2^* \rightarrow S_3$ is expressed as:

$$\gamma_2 = \frac{Y_3(\Delta t)}{Y_3(0.3 \text{ sec.})}$$

After a 10 minute dark period the chloroplast sample was given 30 flashes spaced 0.3 sec. apart, except for the second and third flashes, which were separated by Δt .

For a light flash delivered at a time Δt after the preceeding flash, Δt was shorter than the recycle time of the flash lamp. It was therefore necessary to use a second flash lamp to deliver this flash.

The sequencing of the flashes and the arrangement of the lamps was as described earlier in this section. All experiments took place at $22 \pm 1^\circ \text{C}$.

Computer Fitting of Flash Yields to the Kok Model.

Experimentally obtained flash yields were fitted by computer to the Kok model of O_2 evolution. The program outputted values for $\alpha, \beta, S_0, S_1, S_2$ and S_3 . The program appears in appendix 1.

$S_3^* \rightarrow S_0$

Initially investigation of the $S_3^* \rightarrow S_0$ dark step was undertaken using the procedure outlined by Bouges-Boquet (1973). With this procedure varying the time Δt , between the third and fourth flash allows the study of Y_3 the probability of the $S_3^* \rightarrow S_0$ transition occurring in a centre. In this case the yield on the seventh flash (Y_7) detects the amount of centres in state S_0 formed by the

fourth flash. When this technique was applied to resistant type chloroplasts, varying Δt between the third and fourth flash resulted in little or no observable change in Y_7 . This apparently arose because of the rapid damping of the flash yield sequence routinely observed with resistant type chloroplasts. A different approach was therefore required for the study of this reaction.

The $S_3^* \rightarrow S_0$ step is the O_2 evolving step of the Kok scheme (Joliot and Kok 1975). The work of Bouges-Bocquet (1973) indicates that this is the rate limiting step leading to O_2 evolution. Joliot et al., (1966) have also examined the rate limiting step of O_2 evolution using the modulated polarograph. These workers have derived equations which describe the relationship between the phase lag of the modulated O_2 signal and the rate constant k of the rate limiting step of O_2 evolution.

The work of Sinclair (1984) suggests that a close relationship exists between the rate constant of the rate limiting reaction observed with the modulated polarograph and the parameter which characterizes the $S_3^* \rightarrow S_0$ transition. It follows, then, that a comparison between the respective rate constants of the two biotypes observed with the modulated polarograph should indicate whether differences exist between the parameter of the two types of C. album.

The modulated polarograph used in this study is similar to that of Joliot et al., (1966). Chloroplast samples are illuminated by a light beam, the intensity of which is varied in a sinusoidal fashion. The chloroplasts will produce waves of O_2 in response to the modulated light. The O_2 produced is detected at a platinum electrode, resulting in an oscillating flow of current. A lock-in amplifier amplified and measured the amplitude of the modulated current in a range of frequencies around that of the light modulation. Thus only the photosynthetically produced O_2 current is measured. Simultaneously with the amplitude measurements, the phase lag of the modulated current behind the frequency of light modulation, was measured by a vector voltmeter. The phase lag measurement at a variety of light modulation frequencies was used in these experiments to compare the rate constant for the rate limiting step of O_2 evolution for the two biotypes of C. album.

The modulated electrode used in these experiments has been described by Sinclair and Arnason (1976). In a typical experiment chloroplasts of either the sensitive or resistant biotype at a concentration of 0.075 mg Chl/ml enriched with ferredoxin at a concentration of 0.4 mg/mg Chl were injected into a polarographic cell, similar to that described in the flash experiments. A bathing medium of 0.33 M sorbitol, 30 mM tricine/NaOH pH 8.2, 10

mM MgCl_2 , and 1 mM NADP flowed through the cell. A perfusion system, similar to that described for the flash experiments, ensured a continuous supply of fresh medium. The chloroplasts were allowed to settle on the electrode for 10 minutes, in the dark, then illuminated with a light, which was modulated at 9, 16, 25 or 36 HZ, and had been passed through a 640 nm interference filter. Phase readings were recorded at each light modulation frequency. As the Joliot theory of the modulated polarograph (Joliot et al., 1966) does not take into account the phase lag induced by the measuring circuit, phase measurements at each light modulation frequency were recorded using a fast responding photodiode instead of chloroplasts. These readings were subtracted from those made with chloroplasts, to give a phase lag which represents the contribution of the biological material and diffusion of O_2 to the detecting electrode. All experiments were carried out at $22^\circ\text{C} \pm 1^\circ\text{C}$.

FIGURE 3

Schematic diagram of the polarographic cell.

(After Arnason 1976)

LIGHT

FIGURE 4

Schematic diagram of the flash apparatus. Details of construction contained in Arnason (1976).

RESULTS

Hill Plots

Using isolated chloroplasts from the susceptible and resistant biotypes of C. album and A. retroflexus, the effect of DCMU and atrazine on the Hill reaction, with ferricyanide as an electron acceptor, was analyzed. Addition of either atrazine or DCMU inhibited electron flow (indicated by reduced rates of ferricyanide reduction), in the susceptible biotype of both species. Similarly, DCMU inhibited electron transport in resistant type chloroplasts. Atrazine, however, had no observable effect on the resistant biotype within the range of concentrations used in this study. This is consistent with the observations of Pfister et al., (1979), who reported that within the solubility range of atrazine (approximately 2×10^{-4} M), 50% inhibition of electron transport could not be achieved with resistant type chloroplasts from Senecio vulgaris.

The measured values of electron transport inhibition at varying concentrations of DCMU and atrazine were analyzed by Hill plots to characterize the binding properties of these herbicides. Binding of small molecules to macromolecules can occur by either independent or cooperative binding. Using the Hill equation, and assuming that binding is equivalent to

inhibition, it is possible to plot $\log [\% \text{inhibition} / (100 - \% \text{inhibition})]$ versus $\log [\text{herbicide}]$ for DCMU or atrazine. Figure 4 illustrates the Hill plots obtained for the sensitive biotypes of C. album and A. retroflexus with atrazine. The slope of the line defines the Hill coefficient. For both species the value of the Hill coefficient approached unity. This indicates the independent binding of a single herbicide molecule to the receptor site. Figure 5 and figure 6 illustrate the Hill plots observed with the two biotypes of C. album and A. retroflexus respectively, with DCMU as the inhibitor. The values obtained for the Hill coefficient again approached unity, indicating independent binding of a single herbicide molecule.

Temperature Experiments

The temperature dependence of the rate limiting process of the Hill reaction, with ferricyanide as an electron acceptor, was studied, using chloroplasts isolated from the two biotypes of C. album and A. retroflexus. In the range of temperatures used in this study the Hill reaction is temperature dependent, but the isolated chloroplasts are not subject to thermal denaturation (Bishop et al., 1955). Rates of electron transport at saturating light intensity, at a variety of temperatures were calculated by assaying directly for

reduced ferricyanide. From these data it was possible to generate the Arrhenius plots.

Figure 7 illustrates the Arrhenius plots calculated for the two biotypes of *C. album*. A line of best fit, determined by regression analysis was drawn in each case. Activation energies of $7.3 \text{ K cal. mol}^{-1}$ for the sensitive biotype of *C. album* and $7.8 \text{ K cal. mol}^{-1}$ for the resistant biotype were calculated from the plots. Similar results are shown in figure 8 for the same experiment performed with chloroplasts isolated from the two biotypes of *A. retroflexus*. An activation energy of $6.9 \text{ K cal. mol}^{-1}$ was calculated for both biotypes from the Arrhenius plots.

For the PSII mediated electron transport reaction studied here, the rate determining step is probably the reoxidation of the plastoquinone pool (Veluthys and Ames, 1974). The values calculated from the Arrhenius plots would therefore be the activation energy of this process. The lack of significant curvature in the lines indicates that only one process is rate limiting at the range of temperatures used in these experiments.

Reactions Associated with the Oxygen Evolving Complex.

Flash patterns

The intention of these experiments was to determine if the herbicide resistance mutation had affected the

donor as well as the acceptor side of PSII, particularly the reactions associated with the Kok scheme of oxygen evolution. Figure 9 illustrates the typical pattern of oxygen yields obtained from the sensitive biotype of A. retroflexus when exposed to a series of 30 flashes spaced 0.3 seconds apart after a 10 minute period of dark adaptation. The yield of each flash is normalized with respect to the steady state. The appearance of the O₂ yield sequence is similar to that described for spinach chloroplasts by Kok et al., (1970), in showing an oscillating pattern with a period of four, which gradually damps to a steady state. No significant oxygen yield is detected on the first flash and only a small yield was apparent on the second flash. The flash yields were fitted to the Kok model with a computer by a least square procedure. The standard deviation between the theoretical and experimental flash yields was 0.03. This indicates an excellent correlation between the Kok model and the experimental results. The fitting procedure also yielded a value of α , the proportion of misses, of 0.21 and a value of 0.05 for the proportion of double hits (β). This corresponds to typical values reported for isolated spinach chloroplasts of 0.15 and 0.05 for misses and double hits respectively (Forbush et al., 1971).

The flash yield sequence obtained from the chloroplasts isolated from the sensitive biotype of C.

album was very similar to that observed with sensitive A. retroflexus (fig. 10). Fitting the Kok model to the experimental results resulted in values of 0.22 and 0.06 for α and β respectively. Again, the Kok model gave a good description of the results, with a standard deviation of 0.07 between the theoretical and experimental flash yields.

The pattern of oxygen yields obtained from chloroplasts isolated from the resistant biotype of A. retroflexus is illustrated in figure 11. A number of differences are immediately apparent when the flash yields from the two biotypes of A. retroflexus are compared (figures 9 and 11). Considering that the flash yields have been normalized to the steady state, it is evident that the oscillating pattern of flash yields is far more damped when observed with resistant type chloroplasts than with the sensitive biotype. With the resistant biotype the sequence of oxygen yields damps to a steady state after about 10 flashes, whereas with the sensitive biotype the pattern persists until almost 15 flashes of light have been delivered.

The Kok model predicts that after 10 minutes of dark deactivation the reaction centres should relax to an S state distribution of approximately 25% S_0 and 75% S_1 , i.e. S_2 and S_3 are unstable in the dark and will relax to the S_1

state (Kok et. al., 1970). Thus the appearance of an oxygen yield on the first flash as observed with resistant A. retroflexus (figure 11) is quite surprising, disregarding misses and double hits. The magnitude of the oxygen yield suggests that approximately 10% of the reaction centres are in the S_3 state after dark adaptation. Similarly, any oxygen evolved in the second flash should arise only from a double hit advancing a centre from the S_1 state to the S_3 state thus preparing it to evolve O_2 on the following flash. The second flash yield should be quite small as is shown in figure 9 with the sensitive biotype. The second flash yield from the resistant biotype, however, was larger than that observed with the susceptible biotype. A comparison of the normalized O_2 yields from the two biotypes reveals that the second flash yield is nearly 3 times greater in the resistant type than in the sensitive biotype. This implies either an increased proportion of double hits or a stable fraction of S_2 remains after dark deactivation.

For the purposes of fitting the Kok model to the flash yield sequence from the resistant biotype, the proportion of double hits was considered to be the same as that calculated for the sensitive biotype (0.06). The yield on the second flash, above that which would be expected with a β of 0.06, was considered to be a measure of the centres remaining in S_2 after dark

deactivation. Similarly, the observed O_2 yield on the first flash, which can only arise from a centre already in the S_3 state, was taken as a measure of the proportion of S_3 remaining after the dark period. As S_0 can only be generated in the light (Kok et al., 1970) the net result of the stability of the S_2 and S_3 states in the dark, would be to decrease the concentration of S_1 proportionately. The theoretical flash yields calculated from the best fit of the Kok model, using these assumptions, are shown in figure 11 by the open symbols. The standard deviation between the experimental and theoretical points was 0.03, with an α of 0.26 and a β of 0.06. Apparently, assuming non-zero distributions for the higher S states, the Kok model gives an accurate description of the observed results.

The proportion of misses was higher with the resistant biotype than with the sensitive type. When this is considered in conjunction with the starting distributions of the S states, the flash pattern would be expected to damp quickly to the steady state as was observed experimentally.

Figure 12 shows the flash yield sequence from the resistant biotype of C. album. The same analysis of the flash yield sequence was applied as with resistant type A. retroflexus. Fitting the Kok model resulted in a

standard deviation between the theoretical and experimental flash yields of 0.03. Values of α and β were 0.26 and 0.6 respectively. Again, the resistant biotype displays a higher proportion of misses than the susceptible type.

Deactivation of the S_2 and S_3 States of the Kok Model.

The analysis of the flash yield sequence from resistant type chloroplasts suggests that the S_2 and S_3 states are more stable in the dark than in susceptible chloroplasts. In order to test this assumption and thus further confirm the analysis of the flash patterns, the decay of the S_2 and S_3 states was monitored.

S_3 Deactivation

The evolution of O_2 on the first flash of a sequence, after 10 minutes of dark relaxation, indicates that a proportion of centres must still be in the S_3 state, even after the usual dark period. This phenomenon was noted with both resistant A. retroflexus and resistant C. album but not with the sensitive biotype of either species, thus the first flash yield did not arise as a result of an artifact or light leak.

Deactivation of the S_3 state can be examined by priming the S state clock with 2 flashes, then allowing a varied dark period before administering a series of

saturating flashes. Chloroplasts were given 2 saturating flashes spaced 0.3 seconds apart, then allowed a variable dark period Δt before administering a sequence of 30 flashes at 0.3 second intervals. During the dark period, Δt , a proportion of the reaction centres in the S_3 state will deactivate. Whatever proportion of these reaction centres remains in the S_3 state will evolve oxygen on the first flash after the dark period. If this is compared to the normal condition where a 0.3 second dark period separates the second and third flash, the kinetics of deactivation can be examined.

Figure 13 shows the time course of S_3 deactivation in the two biotypes of C. album. The open symbols represent the susceptible biotype, the closed symbols represent the resistant type. Deactivation proceeds at approximately the same rate during the early stages, with half times of about 50 seconds. With increasing time the curves diverge until at 300 sec. over 30% of the centres in resistant chloroplasts are in the S_3 state compared to about 10% in susceptible chloroplasts. At 600 sec., 10% of the centres in resistant chloroplasts are still in the S_3 state. The deactivation curve for the susceptible biotype goes to a value too small to measure. In resistant chloroplasts the deactivation reaction is clearly biphasic with a fast and slow phase. The point of inflection occurs at about 120 seconds.

S_2 Deactivation

The decay of the S_2 state was also examined. Chloroplasts were given a single saturating flash of light then left in the dark for a variable time Δt . The chloroplasts were then exposed to a series of 30 saturating flashes at 0.3 second intervals. The yield of the third flash is taken as the measure of the amount of S_2 state remaining after the variable dark-period.

Figure 14 illustrates the kinetics of S_2 deactivation observed with the sensitive and resistant biotype of C. album. The open symbols represent the sensitive biotype, the closed circles represent the resistant biotype. Again, deactivation can be seen to proceed at about the same rate during the early stages, with a half time of about 120 seconds. After 120 seconds the curves diverge until at 300 seconds, the proportion of centres remaining in the S_2 state is twice as great in the resistant type as compared to the sensitive type. At 600 seconds 34% of the centres in the resistant biotype remain in the S_2 state compared to 17% of the centres in the susceptible biotype. The S_2 deactivation process in the resistant biotype appears to be biphasic with a fast and slow phase. The point of inflection occurs at about 120 seconds.

Deactivation as a Second Order Process in the Sensitive Biotype

The process of deactivation relies on the neutralization of positive charges stored in the charge storage complex by some electron donor. Deactivation has been studied in isolated chloroplasts by Forbush et al., (1971) who reported that deactivation proceeds by complex, mixed kinetics. Sinclair and Cousineau (1982) reported that S deactivation in Chlorella sp. can be analyzed in terms of a simple second order model. In the model described by Sinclair and Cousineau, the Kok S state is considered to interact with an electron donor D as follows:

where $n = 3$ or 2

The time course of the reactions is described by:

$$\frac{d[S_n]}{dt} = -K[D][S_n] \quad (2)$$

where K is the rate constant. The solution of the differential is:

$$\ln \frac{[S_n]}{[S_n]_0} = \ln \frac{[D]}{[D]_0} + k([S_n]_0 - [D]_0)t \quad (3)$$

When the second-order model was fit to the S and S deactivation results obtained for sensitive *C. album* and alternate solution of equation 2 was applied, which is

alternate solution of equation 2 was applied, which is valid only when the concentration of the electron donor is equal to that of the S state at the start of deactivation. This solution is:

$$\frac{1}{[S_n]} = \frac{1}{[S_n]_0} + kt$$

where $[S_n]_0$ is the concentration of the S state at the start of deactivation and k is the rate constant, assuming that the concentration of the S state is equal to the concentration of the electron donor at the start of deactivation, and that the deactivation process in question obeys second order kinetics, then a plot of $1/[S_n]$ versus time will yield a straight line. This line should extrapolate to unity, as at onset of deactivation the electron donor and the S state are in equal concentrations. The slope of the line will yield the rate constant.

The fitting of the second order model to the S_2 and S_3 deactivation results obtained from sensitive C. album is shown in figure 15. The open symbols represent $1/[S_3]$, the closed symbols represent $1/[S_2]$. The slopes yielded rate constants of $0.03[S_3]^{-1}\text{sec}^{-1}$ and $0.008[S_2]^{-1}\text{sec}^{-1}$ for S_3 and S_2 deactivation respectively. The intercept of the $1/[S_3]$ plot was at 1.20. The intercept of the $1/[S_2]$ plot was at

0.76. The second order model apparently gave a good description of the kinetics of S_2 and S_3 deactivation in the sensitive biotype of C. album.

Forward Reactions of the Kok Model

The time course of the forward reactions of the Kok model were followed in the resistant and sensitive biotype of C. album. Figure 16 shows the time course of the $S_0 \rightarrow S_1$ forward step in chloroplasts isolated from the two biotypes of C. album. The curves display markedly different kinetics between the two biotypes for the $S_0 \rightarrow S_1$ transition. The half time for this forward step is 0.5 msec. for the sensitive biotype and less than 0.2 msec. for the resistant biotype. By 1.0 msec. the reaction is 80% complete in the resistant biotype. With sensitive chloroplasts the reaction is approaching 80% completion at 2.0 msec.

The time course of the $S_1 \rightarrow S_2$ forward step for sensitive and resistant C. album is shown in figure 17. The half time for the $S_1 \rightarrow S_2$ turnover in the sensitive chloroplasts is 0.35 msec., while the half for this transition in resistant chloroplasts is less than 0.1 msec. The $S_1 \rightarrow S_2$ transition occurred very quickly in the resistant biotype. After 0.6 msec. the reaction was 80% complete. With susceptible chloroplasts the reaction was 80% complete after 1.6 msec.

The time course for the $S_2^* \rightarrow S_3$ transition in the two biotypes of C. album is shown in figure 18. Although once again the transition occurred more rapidly in the resistant chloroplasts, the difference was far less pronounced. The half times for the $S_2^* \rightarrow S_3$ turnover were 0.3 msec. for the resistant biotype, and 0.4 msec. for the sensitive biotype.

The $S_3^* \rightarrow S_0$ forward step has been shown to be the rate limiting step of oxygen evolution in the Kok scheme (Bouges-Bocquet, 1973). The rate determining step of oxygen evolution can be examined in the modulated O_2 electrode by observing the effect of the modulation frequency of light on the phase lag of the oxygen signal.

Figure 19 shows a plot of the phase lag of the oxygen signal with respect to the modulated light source versus the square root of the frequency of modulation, for sensitive and resistant chloroplasts from C. album. At the 4 modulation frequencies used in this experiment, the phase lag of the oxygen signal was nearly the same whether the sensitive or resistant biotype was examined. This indicated that the rate constant of the rate limiting step was probably the same for both biotypes. Phase lag readings were attempted at higher modulation frequencies, however the oxygen current at higher frequencies was not sufficiently large to allow an accurate determination of the phase lag.

FIGURE 5

Hill plots for A. retroflexus (open squares) and C. album

(closed squares) in the presence of various concentrations of the herbicide atrazine. Inhibited rates of electron transport were calculated from a direct assay of ferricyanide reduction. The slopes of the lines yielded Hill coefficients of 1.05 ± 0.01 (S.D.) for C. album and 1.11 ± 0.01 (S.D.) for A. retroflexus. Lines were fitted by regression analysis. The regression coefficient for the line of best fit was 0.99 in both cases.

FIGURE 6

Hill plots for the susceptible (open squares) and resistant (closed squares) biotypes of C. album in the presence of various concentrations of the herbicide DCMU. Inhibited rates of electron transport were calculated from a direct assay of ferricyanide reduction. The slopes of the lines yielded Hill coefficients of 1.10 ± 0.02 (S.D.) for the resistant biotype and 1.03 ± 0.02 (S.D.) for the sensitive biotype. Lines were fitted by regression analysis. The regression coefficients for the lines of best fit were 0.97 and 0.98 for the sensitive and resistant biotypes respectively.

FIGURE 7

Hill plots for the sensitive and resistant biotypes of A. retroflexus in the presence of various concentrations of the herbicide DCMU. Inhibited rates of electron transport were calculated from a direct assay of ferricyanide reduction. The open symbols represent the sensitive biotype, the closed symbols represent the resistant biotype. The slopes of the lines yielded Hill coefficients of $1.03 \pm .03$ (S.D.) for the sensitive biotype and $1.10 \pm .03$ for the resistant biotype. Lines were fitted by regression analysis. The regression coefficients for the lines of best fit were 0.96 and 0.98 for the sensitive and resistant biotypes respectively.

FIGURE 8

Arrhenius plots for the sensitive and resistant biotype of C. album. Rates of ferricyanide reduction at a variety of temperatures were calculated by a direct assay of reduced ferricyanide. The open symbols represent the sensitive biotype, the closed symbols represent the resistant biotype. Lines were fitted by regression analysis. Activation energies of $7.3 \text{ K cal. mol}^{-1}$ for the sensitive biotype and $7.8 \text{ K cal mol}^{-1}$ for the resistant biotype were calculated from the lines of best fit. The regression coefficient for the line of best fit was 0.99 in both cases

FIGURE 9

Arrhenius plots for the sensitive and resistant biotype of A. retroflexus.

Rates of ferricyanide reduction at a variety of temperatures were calculated by a direct assay of reduced ferricyanide. The open symbols represent the sensitive biotype, the closed circles represent the resistant biotype. Lines were fitted by regression analysis. An activation energy of $6.9 \text{ K cal. mol}^{-1}$ was calculated for both biotypes from the lines of best fit. The regression coefficients for the lines of best fit were 0.98 and 0.97 for the sensitive and resistant biotypes respectively.

FIGURE 10

The flash sequence from the susceptible biotype of A. retroflexus. After a 10 minut dark period the chloroplasts were exposed to a sequence of 30 flashes spaced 0.3 seconds apart. The closed symbols represent the yield on each flash. These yields were normalized to the steady state. The open symbols represent the theoretical flash yields obtained from the best fit of the Kok model with $\alpha = 0.21$ and $\beta = 0.05$. The standard deviation of the experimental from the theoretical points was 0.03. The initial (dark) distributions for S_0 , S_1 , S_2 and S_3 were calculated to be 1:3:0:0 respectively, as determined by the best fit of the Kok model.

FIGURE 11

The flash sequence from the susceptible biotype of C. album. After a 10 minute dark period the chloroplasts were exposed to a sequence of 30 flashes spaced 0.3 seconds apart. The closed symbols are the yield on each flash. These yields were normalized to the steady state. The open symbols represent the theoretical flash yields obtained from the best fit of the Kok model with $\alpha = 0.22$ and $\beta = 0.06$. The standard deviation of the experimental from the theoretical points was 0.07. The initial (dark) distributions for S_0 , S_1 , S_2 and S_3 were calculated to be 1:3:0:0 respectively as determined by the best fit of the Kok model.

FIGURE 12

The flash sequence from the resistant biotype of A. retroflexus. After a 10 minute dark period the chloroplasts were exposed to a sequence of 30 flashes spaced 0.3 seconds apart. The closed symbols are the yield on each flash. These yields were normalized to the steady state. The open symbols represent the theoretical flash yields obtained from the best fit of the Kok model with $\alpha = 0.26$, and $\beta = 0.06$. The standard deviation of the experimental from the theoretical points was 0.03. The initial (dark) distributions for S_0 , S_1 , S_2 , and S_3 were calculated to be 1:2.54:0.36:0.11 respectively as determined by the best fit of the Kok model.

FIGURE 13

The flash sequence from the resistant biotype of C. album. After a 10 minute dark period the chloroplasts were exposed to a sequence of 30 flashes spaced 0.3 seconds apart. The closed symbols are the yield on each flash. These yields were normalized to the steady state. The open symbols represent the theoretical flash yields obtained from the best fit of the Kok model with $\alpha = 0.26$, and $\beta = .06$. The standard deviation of the experimental from the theoretical points was 0.03. The initial (dark) distributions for S_0 , S_1 , S_2 and S_3 were calculated to be 1:2.55:0.36:0.11, respectively as determined by the best fit of the Kok model.

FIGURE 14

The deactivation of the S_3 state in sensitive and resistant C. album. The open symbols represent the sensitive biotype and the closed symbols represent the resistant biotype. Chloroplasts were held in the dark for 10 minutes then exposed to two saturating flashes separated by 0.03 seconds. Following a variable time Δt the chloroplasts were exposed to an additional 30 flashes separated by 0.03 seconds. The yield on the first flash following Δt was taken as a measure of the remaining S_3 state. Curves were fitted by eye. Error bars are standard deviations.

FIGURE 15

The deactivation of the S_2 state in sensitive and resistant C. album. The open symbols represent the sensitive biotype, and the closed symbols represent the resistant biotype.

Chloroplasts were held in the dark for 10 minutes then exposed to a saturating light flash. Following a variable time Δt the chloroplasts were exposed to an additional 30 flashes separated by 0.03 seconds. The yield on the second flash following Δt was taken as a measure of the remaining S_2 state. Curves were fitted by eye. Error bars are standard deviations.

FIGURE 16

$I/[S_2]$ and $I/[S_3]$ versus time for the sensitive biotype of C. album. This figure represents an attempt to fit experimental results to a second order model where $[S_0] = [D]$. The plot should increase linearly with time and intercept the axis at unity. The open symbols represent $I/[S_3]$ and the closed symbols represent $I/[S_2]$. Lines were fitted by regression analysis. The slopes which give the rate constant were $0.03[S_3]^{-1} \text{ sec}^{-1}$ for S_3 deactivation and $0.008 [S_2]^{-1} \text{ sec}^{-1}$ for S_2 deactivation. The intercepts were 1.20 for S_3 deactivation and 0.76 for S_2 deactivation.

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FIGURE 17

Time course of the $S_0^* \rightarrow S_1$ transition. Chloroplasts were in darkness for 5 minutes then given 3 flashes of light at 0.3 second intervals. This was followed by 6 minutes of darkness then 30 flashes at 0.3 second intervals except for the first and second flashes which were separated by Δt .

$$Y_0(\Delta t) = \frac{Y_4(\Delta t) + Y_3(\Delta t) - Y_3(0.3 \text{ sec.})}{Y_4(0.3 \text{ sec.})}$$

Curves were fitted by eye.

FIGURE 18

The time course of the $S_1^* \rightarrow S_2$ transition. The chloroplasts were dark adapted for 10 minutes then given 30 flashes of light at 0.3 second intervals, except for the first and second flashes which were separated by Δt .

$$\frac{Y_1(\Delta t)}{Y_3(0.3 \text{ sec.})} = \frac{Y_3(\Delta t)}{Y_3(0.3 \text{ sec.})}$$

Curves were fitted by eye.

FIGURE 19

The time course of the $S_2^* \rightarrow S_3$

turnover. The chloroplasts were given a 10 minute dark period, followed by 30 flashes spaced 0.3 seconds apart, except for the second and third flashes which were separated by Δt .

$$\chi_2(\Delta t) = \frac{y_3(\Delta t)}{y_3(0.3 \text{ sec.})}$$

FIGURE 20

The phase lag between the oxygen current and the light modulation frequency obtained with chloroplasts from sensitive and resistant C. album. The open symbols represent the sensitive biotype, the closed symbols represent the resistant biotype.

DISCUSSION

Hill Plots

Hill plots are useful tools for examining the binding properties of small molecules interacting with macromolecules. A classic example of the application of these plots is the binding of oxygen to myosin and the binding of oxygen to hemoglobin (Marshall, 1978). In the first case the slope of the Hill plot gives a value of unity, indicating the independent binding of a single oxygen molecule per macromolecule. In the latter case the slope of the Hill plot yields a value of 4, signifying the cooperative binding of oxygen to 4 interacting sites per macromolecule. The linearity of the Hill plot for hemoglobin indicates that binding is highly cooperative. When the first oxygen binds to a hemoglobin molecule the remaining three bind so strongly it is as if all four oxygens bind at once.

The Hill plots reported here for the binding of DCMU using the sensitive and resistant biotypes of A. retroflexus and C. album yield a slope of unity, suggesting that inhibition of electron transport occurs by the independent binding of a single molecule of inhibitor. Van Rensen et al. (1978) reported a Hill coefficient of 2 for DCMU. The sensitivity of the ferrocyanide assay allowed much lower chlorophyll concentrations to be used

in the determination of the Hill coefficient reported here ($[chl] = 5 \mu g/ml$ versus $35 \mu g/ml$ in the study of Van Rensen et al., 1978). As Hill plots are highly sensitive to chlorophyll concentrations (Tischer and Strotman, 1977), the value related here should be taken as more accurate. This finding confirms earlier work done by Pfister et al. (1979) who reported a Hill coefficient of one for DCMU with sensitive and resistant biotypes of Senecio vulgaris, also using low chlorophyll concentrations ($5 \mu g/ml$).

The Hill coefficient for atrazine was also shown to be unity, using the sensitive biotypes of A. retroflexus and C. album. This is in agreement with Pfister et al. (1979) who reported a Hill coefficient of one for atrazine using sensitive S. vulgaris.

The resistant biotypes used in this study showed a slightly higher resistance to DCMU than the sensitive biotypes. In order to achieve 50% inhibition (I_{50}) in the sensitive biotype of A. retroflexus a concentration of $3.6 \times 10^{-8} M$ was required, as compared to $6.9 \times 10^{-8} M$ for the resistant biotype. Similarly, sensitive C. album required a concentration of $5 \times 10^{-8} M$ DCMU for 50% inhibition of the Hill reaction, while I_{50} occurred at a concentration of $10^{-7} M$ in the resistant biotype. Atrazine and DCMU are thought to compete for a similar binding site

(Pfister and Arntzen, 1979) on the 32 kilodalton herbicide binding protein (Mullet and Arntzen, 1981). The lowered susceptibility of the resistant biotype to DCMU may arise from the modification in the 32 kilodalton herbicide binding protein believed to confer herbicide resistance (Vermaas and Arntzen, 1983).

Temperature Experiments

Studies on the temperature dependence of the Hill reaction, with ferricyanide as an electron acceptor, were initiated by the results of Darmency and Gasquez (1982). These workers indicated that the activation energy of the Hill reaction to ferricyanide was lower in the resistant biotype of Polygonum lapathifolium compared to the sensitive type.

The activation energy of the rate limiting step of the Hill reaction to ferricyanide was calculated for the two biotypes of C. album and A. retroflexus. For C. album values of 7.3 K cal mol⁻¹. and 7.8 K cal mol⁻¹. for the sensitive and resistant biotype respectively were calculated from the Arrhenius plots. A value for the activation energy of 6.9 K cal mol⁻¹. was calculated for both biotypes of A. retroflexus from the Arrhenius plots. In all cases the Arrhenius plots showed no significant curvature as indicated by the regression coefficients. This suggests that only one process was rate limiting.

The close correlation of the values obtained for the activation energies for both biotypes, and both species, indicates that the activation energy of the same rate limiting process was investigated in all cases. For the PSII mediated electron transport reaction studied here, the rate determining step is probably the reoxidation of the plastoquinone pool (Veluthys and Ames, 1974). The values calculated here are probably the activation energy for this process.

For the species used in this study, there were no significant differences in the activation energies for the process studied between either the two biotypes or the two species. The conflict between the results found here, and the work of Darmency and Gasquez (1982) may simply represent differences between species. Work by Gasquez et al. (1981) showed that the resistant biotype of P. lapathifolium was favoured at lower temperatures. Resistant plants flowered earlier and grew faster when compared to the sensitive plants. This led Darmency and Gasquez (1982) to suggest that there was a correlation between chilling resistance and the herbicide resistance quality. As yet there have been no other studies which suggest that this is true. The chilling resistance may have been unique to the plants used by these workers and may explain the lower activation energy discovered for the resistant type. Alternately, differences in experimental

protocol may explain the conflicting findings of this study and that of Darmency and Gasquez (1982). Their results represent rates from four different chloroplast isolations at each temperature. Oxygen evolution was monitored with a platinum electrode and corrections made for the electrode sensitivity to temperature. From their data points, 2 parallel lines of best fit were determined by regression analysis, for the resistant biotype, and the activation energies calculated from the slope.

For the Arrhenius plots shown in figures 7 and 8 the data points represent results from a single preparation. The errors are standard deviations of 5 trials at each temperature. Only one line of best fit was determined in each case, with regression coefficients ranging from 0.97 to 0.99. Rather than monitoring oxygen production at different temperatures with a polarograph, reduced ferricyanide was assayed directly at room temperature.

The procedure used to determine the activation energies in this study yielded points which gave an excellent fit for the Arrhenius plots. Differences may exist in activation energies for the rate limiting step of the Hill reaction to ferricyanide between the two biotypes of P. lapathifolium but the studies reported here indicate that this is not generally applicable to the resistant biotype.

Flash Patterns

The flash yield sequences observed with sensitive and resistant chloroplasts from C. album and A. retroflexus yielded some important and interesting results. The oxygen yield sequence from the sensitive biotypes from both species appeared very similar to that normally obtained with spinach chloroplasts (fig. 9 and fig. 10). The proportion of misses was somewhat higher with the weed chloroplasts than with spinach, however, the appearance of the oscillating flash yields was very similar and the Kok model gave an excellent description of the experimental observations. The damped oscillations of the flash yield sequence observed with the resistant chloroplasts of both species, differed sharply from that observed with spinach (fig. 11 and fig. 12). The observed damping of the flash yield sequence obtained from resistant chloroplasts is predicted, at least partially, by the higher proportion of misses obtained on fitting the Kok model to the resistant biotypes. The underlying reason for the higher miss parameter probably derives from the nature of the resistance mutation. Bowes et al. (1980) reported a 10 fold smaller rate constant for the Q^- to B electron transfer reaction in resistant chloroplasts from A. retroflexus. Similarly, Pfister and Arntzen (1979) have demonstrated a slower rate of Q^- reoxidation after a saturating flash, with chloroplasts from resistant C.

album. They found that a portion of Q^- in resistant chloroplasts was stable for many seconds. The slowed electron transfer between Q^- and B results in an increased probability of an electron, shared between Q and B, being present at Q^- when a flash is delivered. If Q is present as Q^- , the reaction centre cannot accept a flash, as electron transfer is blocked, until Q^- is reoxidized. This would result in a larger proportion of closed reaction centres with resistant chloroplasts at any one time. The net result would be an increase in the miss parameter.

The other contribution to the damping of the flash yield sequence observed with resistant chloroplasts arose from the starting S state distribution. Assuming non-zero distributions for the S_2 and S_3 states the Kok model gave an excellent description of the experimental results with a standard deviation of 0.03 between the experimental and theoretical points.

Double hits arise primarily from S-state recoveries which occur during the long tail of the Xenon flash used in these experiments (Joliot and Kok, 1975). Although double hits may arise from intrinsic differences between species, or biotypes, β would be expected to be a constant parameter, arising from the experimental design. Values for β , calculated from the computer fitting procedure, were very similar for both species and both biotypes. For

resistant and sensitive C. album, and resistant A. retroflexus, β was calculated as 0.06 (6% double hits). For sensitive A. retroflexus, β was given as 0.05. These values correlate well with the 5% double hits reported by Kok et al. (1970) with spinach chloroplasts, using a similar light source. The consistent value, and the excellent fits obtained with the fitting program, indicate that the assumptions about the starting concentrations of S_2 and S_3 states at the end of the dark period were valid.

Deactivation of the Kok S States

S_3 Deactivation.

The process of deactivation is not well understood. A common explanation is that the S states are reduced by some endogenous electron donor. One donor which has been suggested for S_3 deactivation is the reduced form of the secondary electron acceptor, B (Veluthys and Ames, 1974). The secondary acceptor may be the donor D, indicated by the fitting of the second order model to the S_3 deactivation results from sensitive chloroplasts of C. album, (fig.15). In this case the donor and the S state are in equal concentrations at the start of deactivation. The reduced form of B would be created by the two priming flashes given to the dark adapted chloroplasts in a deactivation experiment. Thus B would be a possible electron donor.

In contrast to the simple second order kinetics for S_3 deactivation shown by the susceptible biotype, S_3 deactivation in the resistant biotype proceeds by far more complex kinetics. The decay of S_3 was similar in the two biotypes up to 120 seconds. At times greater than 120 seconds a slow phase of deactivation is evident with the resistant chloroplasts. It appears that during the early phase of deactivation, S_3 decay proceeds via the same route in both sensitive and resistant chloroplasts. At longer times, however, another process is involved in S_3 deactivation with the resistant biotype. This may represent either reduction of the S_3 state by another donor, or the gradual consumption of the donor observed in the early phase.

S_2 Deactivation

The kinetics of S_2 deactivation observed with susceptible C. album was similar to the kinetics observed for S_3 decay with this biotype. A simple second order model gave a good description of the experimental results. Again, the concentration of the electron donor was equivalent to the concentration of the S state at the onset of deactivation. A number of authors have suggested that the electron donor for S_2 deactivation is Q^- (C.F. Bouges-Bocquet et al., 1973, Vermaas et al., 1984). The electron donation is suggested to occur via back electron

flow from Q^- , through the reaction centre P680, to the S_2 state (Joliot and Kok, 1975). A simple second order model gave a good description of the experimental results.

Identifying Q as the electron donor for S_2 deactivation contradicts the results obtained for S_2 decay with C. album resistant chloroplasts. Considering the slowed electron transport from Q^- to B observed by Bowes et al., (1980) with resistant chloroplasts from *A retroflexus* a higher equilibrium concentration of Q^- should result. This is expected to create a high probability of back reactions to the donor side of PSII, resulting in much faster S_2 deactivation in resistant chloroplasts. As figure 14 illustrates, after an initial phase where deactivation proceeds at the same rate in the two biotypes, deactivation proceeds more slowly in the resistant biotype. This suggests that with resistant chloroplasts, either Q is not the electron donor or in resistant chloroplasts this pathway has been disabled.

The slow phase of S_2 deactivation has also been reported by Vermaas et al. (1984) to occur with resistant chloroplasts from Brassica napus. These authors, however, did not speculate on the cause of the slow phase of S_2 decay.

Forward Reactions of the Kok Model

The dark transitions observed with chloroplasts from the sensitive biotype of C. album, were similar to those observed with isolated spinach chloroplasts by Bouges-Bocquet (1973). However, the time course of these reactions was significantly changed when observed with chloroplasts from the resistant biotype. The $S_0^* \rightarrow S_1$, and $S_1^* \rightarrow S_2^*$ transitions occurred significantly faster in resistant chloroplasts. The $S_2^* \rightarrow S_3$ transition also occurred more quickly in resistant chloroplasts, but did not display the same rapid rise as the other forward steps examined.

From measurements of fluorescence decay, following a single flash, Kok et al. (1970) proposed that dark $S_n^* \rightarrow S_{n+1}$ turnovers are limited by the reoxidation rates of Q^- the primary electron acceptor of PSII. Bowes et al. (1980) have followed the time course of Q^- reoxidation in dark adapted susceptible and resistant chloroplasts of A. retroflexus following a single saturating flash. The rate of Q^- reoxidation observed with resistant chloroplasts was greater than 10 fold slower compared to the rate observed with sensitive chloroplasts. Additionally, binary oscillations in the rate of Q^- reoxidation corresponding to the $Q^-B \rightarrow QB^-$, and $Q^-B^- \rightarrow QB^{2-}$ were shown by these workers to be of opposite phase. This means that in sensitive chloroplasts, the $Q^-B \rightarrow QB^-$ transition which occurs on the first flash after dark adaptation proceeds more quickly

than the $Q^-B^- \rightarrow QB^{2-}$ transition which occurs on the second flash. In resistant the relationship is reversed.

The work of Kok et al. (1970) and Bowes et al. (1980) suggests that the $S_0^* \rightarrow S_1$, and $S_2^* \rightarrow S_3$ transitions should proceed far more slowly in the resistant biotype. This contradicts the results reported here, for the $S_0^* \rightarrow S_1$ and $S_1^* \rightarrow S_2$ transitions observed with resistant chloroplasts. Unfortunately there is no data which reconciles these opposing results.

Holt et al. (1981,1983) have performed investigations on the reactions associated with the Kok model, using chloroplasts isolated from the susceptible and resistant biotype of S. vulgaris. Some of this groups' findings contradict the results reported here, however, there were important differences in experimental procedure between the two studies. These workers do not report the use of an electron acceptor in the chloroplast bathing medium when monitoring flash yields. The absence of an electron acceptor would result in the intersystem intermediates of the electron transport chain becoming successively more reduced. This strong reducing environment would result in rapid deactivation of the S states. This may explain the much faster S_2 deactivation Holt et al. (1983) report for resistant chloroplasts of S. vulgaris compared to the sensitive biotype ($t_{1/2} = 15$ sec. vs. $t_{1/2} = 40$ sec.).

The stable fraction of S_2 has been reported here and by Vermaas et al. (1984).

Holt et al. (1983) found that S_3 decay proceeds at the same rate in chloroplasts from the two biotypes of S. vulgaris. They have monitored S_3 deactivation only to 100 seconds. At times less than 120 seconds S_3 deactivation proceeded at the same rate in chloroplasts from C. album. Perhaps if these groups had extended their measurements to longer times they would have observed a slow phase of deactivation with resistant chloroplasts as well.

The study by these workers of the dark reactions of the Kok scheme did not include the $S_3^* \rightarrow S_0$ transition. This probably indicates that they also experienced difficulties accurately monitoring this step. The $S_0^* \rightarrow S_1$ and $S_1^* \rightarrow S_2$ transitions were found to proceed slower in resistant chloroplasts as predicted by the work of Bowes et al. (1980). As stated earlier the $S_0^* \rightarrow S_1$ and $S_1^* \rightarrow S_2$ transitions were found to proceed far more quickly in resistant chloroplasts from C. album. Holt et al. (1983) reported that the $S_2^* \rightarrow S_3$ turnover proceeds somewhat more quickly in resistant chloroplasts of S. vulgaris in agreement with the results obtained with resistant chloroplasts of C. album for this study. They did not speculate on why the $S_2^* \rightarrow S_3$ transition proceeded more quickly with the resistant biotype. Apparently if the

differences between the biotypes for the S state transitions are to be understood, then more experimentation will be required. This may help explain the differing results obtained for S state turnovers with the resistant biotype in this study and that of Holt et al. (1983).

This study has focused on an examination of the differences in photosynthetic performance between the herbicide sensitive and resistant biotypes. The properties of the photosynthetic light reactions shown by the resistant biotype have not been demonstrated in normal systems and as such they provide a useful probe of normal photosynthetic functioning. There are many aspects of the light driven reactions which are still not well understood. The herbicide resistance mutation may prove to be useful in clarifying some of them.

Summary

- 1) The Hill coefficient for both atrazine and DCMU is unity indicating the independent binding of a single molecule.
- 2) The activation energy for the rate limiting step of the Hill reaction with ferricyanide as an electron acceptor is 7.3 K cal mol⁻¹ for the sensitive biotype of C. album, 7.8 K cal mol⁻¹ for the resistant biotype, and 6.9 K cal mol⁻¹ for both biotypes of A. retroflexus.

3) The miss parameter of the Kok scheme of O_2 evolution is higher in the resistant biotypes of A. retroflexus and C. album than in the corresponding susceptible biotype.

4) The dark distribution of the Kok S states in resistant biotypes of C. album and A. retroflexus is 1:2.54:0.36:0.11 as compared to 1:3:0:0 for the corresponding sensitive biotypes.

5) The decay of the S_3 and S_2 states of the Kok model is a second order process in susceptible chloroplasts of C. album.

6) The decay of the S_3 and S_2 states in resistant chloroplasts of C. album is biphasic with a slow phase which persists for times greater than ten minutes.

7) The $S_0^* \rightarrow S_1$, $S_1^* \rightarrow S_2$, and $S_2^* \rightarrow S_3$ transitions are faster in the resistant biotype of C. album, than in the susceptible biotype.

8) The rate constant of the rate determining step of O_2 evolution is the same in the two biotypes of C. album.

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Appendix I

This program fits the experimentally derived flash yields to the Kok model, using a least squares procedure. Values of α , β and the $S_0:S_1$ ratio are calculated. This program was adopted from a version originally designed by Arnason (1976). A list explaining some of the fortran variables used in the program follows:

NCRV the number of experiments to be analysed

P(I) the oxygen yield from the Ith flash

NF the total number of flashes in the sequence

A the proportion of misses, α

B the proportion of double hits, β

SR the ratio of S_1/S_0 after a dark period

S(1,1) the value of S_0 after a dark period

S(2,1) the value of S_1 after a dark period

S(3,1) the value of S_2 after a dark period

S(4,1) the value of S_3 after a dark period

S(1,2) the value of S_0 after the first flash

DA the increment of a

DB the increment of B

DSR the increment of SR

Y(I) the normalized flash yields

TY(1) the theoretical flash yields predicted by the Kok
model.

```

      DIMENSION S(4,400), I(400), Y(400), IY(400), SQ(400)
      I, ICOM(20)
      REAL H
      READ(5, 1) NCRV
      1  FORMAT(6)
      DO 12 J=1, NCRV
      READ(5, 13) ICOM
      13  FORMAT(2044)
      PRINT(6, 14) ICOM
      14  FORMAT(1H, 204)
      READ(5, 15) HF, A, B, SR, S(3,1), S(4,1)
      15  FORMAT(6)
      READ(5, 17) (P(I), I=1, HF)
      17  FORMAT(6)
      S(1,1)=(4.0-S(3,1)-S(4,1))/(1+SR)
      S(2,1)=SR*S(1,1)
      OUTPUT(6) A, B, SR, S(1,1), S(2,1), S(3,1), S(4,1)
      I=10
      SM=0.
      18  SM=SM+P(I)
      I=I+1
      IF (I-NF) 18,18,19
      19  I=I-1
      M=SM/(NF-9)
      DO 20 I=1, NF
      Y(I)=P(I)/H
      20  DO 65 J=1, 2
      READ(5, 47) I0, I0B, DSR
      67  FORMAT(36)
      OUTPUT(6) I0, I0B, DSR
      DO 56 J2=1, 2
      DO 54 L=1, 3
      K=1
      SQ(K)=SUMSQ(Y, TY, A, B, S, NF)
      LDUM=0.
      33  K=K+1
      90  IF (L-1) 60, 80, 21
      80  A=A+D0A
      60  TO 23
      21  IF (L-2) 22, 22, 24
      22  R=8+D0B
      60  TO 23
      24  IF (L-3) 26, 26, 32
      26  SR=SR+D0SR
      S(1,1)=(4.0-S(3,1)-B(4,1))/(1+SR)
      S(2,1)=SR*S(1,1)
      60  TO 23
      37  IF (LDUM-2) 91, 54, 34
      23  IF (LDUM-2) 91, 54, 34
      91  SQ(K)=SUMSQ(Y, TY, A, B, S, NF)
      IF (SQ(K)-SQ(K-1)) 33, 34, 34
      34  LDUM=0
      47  IF (L-1) 35, 35, 36
      35  A=A+D0A
      IF (A) 71, 71, 72
      71  A=0.
      72  CONTINUE
      60  TO 37

```

```

15 IF(L-2) 50,55,38
50 R=N OR
50 IF (B) 73,73,74
73 CONTINUE
74 CONTINUE
75 IF (I-3) 39,39,46
39 SR=SR+USR
75 IF (SR) 75,75,76
75 PRIN(6, 500)
500 FORMAT(5HSE=0 )
76 STOP
76 CONTINUE
S(1,1)=(4.0-S(1,1)-R(4,1))/(1.1)
S(2,1)=SR(S(1,1))
GO TO 37
45 STOP
37 CONTINUE
IF (LDUR-1) 50,51,54
50 I (K-2) 43,48,49
48 LDUR=1
K=1
S(0,K)=SUMSQ(Y, TY, A, B, S, N)
52 K=K+1
GO TO 47
51 S(0,K)=SUMSQ(Y, TY, A, B, S, N)
IF (S(0,K)-SR(K-1)) 52,53,53
53 LDUR=2
GO TO 49
49 CONTINUE
54 CONTINUE
66 CONTINUE
OUTFUT(6) A,B,S(1,1),S(2,1),S(3,1), (A,1),S(0,1)
PRIN(6, 60)
FORMAT(25H 1 Y(1) TY(1) )
60 PRIN(6, 81) (1, Y(1), TY(1), 1-1,NF)
61 FORMAT(1H 12, 2F10.3)
12 CONTINUE
END
FUNCTION SUMSQ(Y, TY, A, B, S, N)
DIMENSION S(4,100), ((100), 1) (100)
N=1
N=N+1
S(1,N)=(1-A-N)*S(4,(N-1))+(B*(3*(N-1)))+(A*S(1,(N-1))
S(2,N)=(1-A-B)*S(1,(N-1))+(A*S(4,(N-1)))+(A*S(2,(N-1))
S(3,N)=(1-A-B)*S(2,(N-1))+(B*S(1,(N-1)))+(A*S(3,(N-1))
S(4,N)=(1-A-B)*S(3,(N-1))+(A*S(2,(N-1)))+(A*S(4,(N-1))
TY(N)=(1-A-B)*S(4,(N-1))+(B*S(3,(N-1)))/(1-A)
IF (N-NF) 2,3,3
3 SUMSQ=0.
TY(1)=0.
N=1
N=N+1
SUMSQ=SUMSQ(1, TY(N)-Y(N))1*2
IF (N-NF) 4,5,5
5 CONTINUE
RETURN
END

```

END

2 1 0 2 8 6

FIN