STUDIES ON THE VARIETAL SUSCEPTIBILITY IN WINTER WHEAT TO SUBSTITUTED PHENYLUREA HERBICIDES

A Thesis submitted by

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Finally, I wish to give special thanks to my parents, without whose financial support and encouragement this thesis would not have been possible.

ABSTRACT

The studies reported in the present thesis have been concerned with the biochemistry of the varietal susceptibility of winter wheat (Triticum aestivum L.) cultivars to the substituted phenylurea herbicide, metoxuron(N'-(3-chloro-4-methoxyphenyl)-N,N-dimethylurea).

The introduction includes an historical account of the substituted phenylureas and reviews of the relationships between structure and phytotoxicity and mechanism of action. In addition selective properties of phenylureas are discussed together with their metabolic fate not only in plants but also in microorganisms and mammalian systems. Present concepts of the photosynthetic process are also outlined with special emphasis on those aspects directly pertinent to the mode of action of the substituted phenylureas.

Studies of Hill activity of isolated chloroplasts incubated with metoxuron showed a similar inhibition of DCPIP and potassium ferricyanide photoreductions in both resistant (Capelle Desprez, Cama) and susceptible (Maris Nimrod, Maris Huntsman) plants. Examination of the Hill activity of chloroplasts isolated from herbicide-treated plants showed a greater inhibition of DCPIP and ferricyanide photoreductions in susceptible compared with resistant varieties. Whereas a severe inhibition of Hill activity in susceptible varieties was followed by scorching and eventual death of the plant, tolerant cultivars recovered from an initial slight visible injury in parallel with a return of Hill activity to normal levels.

A reduction in the proportion of ethanol soluble material of leaves was also observed 24 h following treatment of wheat plants with metoxuron, the susceptible varieties showing the greater decrease. The distinction between resistant and susceptible varieties was more apparent when plants that had contacted the herbicide for longer periods were investigated. These results were also reflected in a greater inhibition of $^{14}CO_2$ fixation by metoxuron-treated leaves of susceptible plants compared to similarly treated resistant strains.

Studies of absorption and translocation of (methoxy-^{14}C)-metoxuron in resistant and susceptible wheat varieties, indicated that a differential rate of herbicide uptake may represent a factor which contributes to the observed difference in varietal response. A subcellular organelle localisation study of absorbed (methoxy-^{14}C)-metoxuron indicated that on the basis of protein content the greatest amounts of radioactivity were associated with the chloroplast fraction. In addition, chloroplasts from susceptible varieties contained a quantity of metoxuron several fold greater than those of resistant plants.

Metabolism studies made using (methoxy-^{14}C)-metoxuron indicated that a major degradative pathway in wheat involves a two-step N-dealkylation followed by hydrolysis of the ureido group to give the corresponding aniline derivative. The various metabolites were separated by thin-layer chromatography and their identity was revealed by a comparison of their R_f values with those of known synthesised standards. Time course studies indicated that metoxuron metabolites represented a greater proportion of the radioactivity of the total methanol extract from

resistant plants than from susceptible types. The metabolic route outlined was shown to result in an effective detoxication of metoxuron in in vitro assays of Hill reaction activity where the N-monodesmethylated derivative was found to be some 80% as inhibitory as metoxuron and the N-bisdesmethyl and aniline derivatives were ineffective as photosynthetic inhibitors.

Experiments made using leaf discs contained in Warburg flasks in the presence of a CO_2 trap demonstrated a loss of $^{14}CO_2$ from (methoxy-^{14}C)-metoxuron indicating that cleavage of the methoxy substituent of the aromatic ring represents a significant additional step in the metabolism of the herbicide. Cleavage of the methoxy group occurred at an appreciably greater rate in leaf discs of resistant compared to susceptible plants.

Preliminary experiments would indicate that metabolism of metoxuron by wheat leaves is achieved by the microsomal fraction of the cells which contains an N-demethylase activity requiring molecular oxygen and reduced pyridine nucleotide as co-factors. This activity was most apparent in the microsomal fraction prepared from leaves of resistant varieties.

The various experimental results are discussed in terms of their significance in accounting for the differences in response to metoxuron of the wheat varieties studied.

ABBREVIATIONS

The abbreviations used in this thesis are those listed in the Biochemical Journal (1976) 153, 1-21 (revised, 1978, 169, 1-27) with the following additions :

CCP	carbonylcyanide phenylhydrazone
CMU	N'-(3-chloro-4-phenyl)-N,N-dimethylurea
DAD	diaminodurene(2,3,4,5-tetramethyl-p-phenylenediamine)
DBMIB	2,5-dibromo-3-methyl-6-isopropylbenzoquinone
DCMU	N'-(3,4-dichlorophenyl)-N,N-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
DPC	diphenylcarbazide
Fd	ferredoxin
Fe-S	bound iron-sulphur protein
Fp	ferredoxin-NADP reductase (flavoprotein)
FRS	ferredoxin reducing substance
HEPES	N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid
MES	2-(N-morpholino)-ethane sulphonic acid
PC	plastocyanin
PD	p-phenylene diamine
PMS	phenazine methosulphate
PQ	plastoquinone
PPO	2,5-diphenyloxazole
PS1	photosystem 1
PS2	photosystem 2
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
Tricine	N-tris(hydroxymethyl)methyl glycine

CONTENTS

I N T R O D U C T I O N

The Substituted Urea Herbicides

The Advent of the Herbicide Era

Many of the most spectacular and certainly the most useful biological discoveries of this century have been concerned with the development of toxic chemicals for the control of disease or the destruction of pests. To the agriculturalist, probably the most important have been the discoveries relating to the use of chemicals for the control of competitive weeds.

Chemical weedkillers, or herbicides, are now accepted tools in plant husbandry and their study has become a new branch of biochemical and agricultural science. Progress in the subject has been extremely rapid, the number of different herbicides and their formulations having increased at such a rate so as to often render it difficult for the agriculturalist to keep abreast of the changing situation. However, there is still a great need for further scientific knowledge to serve as a basis for this new technology, many of the very effective herbicides in current use having been discovered almost by accident.

Herbicides have played an increasingly important role in agriculture and horticulture ever since it was shown that 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) could be used as selective herbicides against dicotyledonous weeds in monocotyledonous crops (Zimmerman & Hitchcock, 1942). This early observation stimulated an active search for other herbicidal compounds with the resultant discovery and development of the herbicidal properties of the substituted ureas by Thompson, Swanson & Norman (1946) shortly after the end of the second world war. Then in 1951, Bucha & Todd described the herbicidal properties of the substituted

phenylurea,N'-(p-chlorophenyl)-N,N-dimethylurea (monuron, CMU) stressing its efficacy for the control of annual and perennial grasses. Since this time many chemicals of varied properties, such as uracils, s-triazines, benzoic acid derivatives, carbamates, phenols, phenoxy aliphatic acids and others have found their way into the field of chemical weed control. Despite 25 years of research our understanding of the behaviour, action and fate of monuron and related compounds within the plant is still not complete. Our knowledge of the mode of action of many other herbicides that have been discovered since about 1950 is little better.

The usefulness of herbicides depends not so much on general toxicity as on an ability to destroy the weed without harming the crop. Most agrochemical industries now market compounds with specific recommendations for the selective control of one (or more) species growing amidst another. Such herbicide selectivity has proved valuable for example in the control of the wild oat (Avena fatua L.) growing in cereal crops, probably representing the major weed problem in the arable cropping system today. General weed killers which lack selectivity have a restricted use. The science of selective toxicity must, however, be based on an understanding of the toxic action itself, and this requires a thorough knowledge of the normal growth process of the plant.

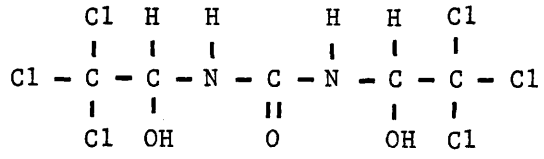
Although man's dependence on herbicides seems certain to continue in the foreseeable future and the testing and selection of new compounds is accordingly progressing at an accelerating rate, the discovery that certain weed control chemicals persist for years in the environment has

resulted in all herbicides being viewed with suspicion by people concerned in preserving the world from broad scale pollution.

Studies on the toxicity and persistence of insecticides has therefore now been extended to include compounds from many of the different groups of herbicides, notably the substituted phenylureas, uracils, s-triazines and derivatives of benzoic acid. At the same time it is acknowledged however that a degree of persistence is an essential property for herbicide effectiveness particularly in the case of soil applications. In the development of a new herbicide therefore, factors such as behaviour and persistence in the soil and toxicity toward non-target organisms have to be considered in addition to phytotoxicity and species specificity. The permissible range of activity will therefore be determined with reference to such factors as crop tolerance, soil degradation, retention against leaching and danger to wild life. Soil-active herbicides for use on non-agricultural areas such as road verges, ditch banks, fire breaks and commercial sites must of necessity be persistent for reasons of economy. The likelihood of such chemicals entering human food sources would seem to be extremely remote.

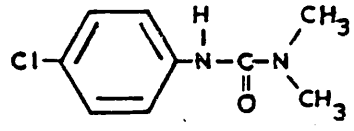
THE SUBSTITUTED PHENYLUREAS

In 1946, Thompson et al conducted a survey of some 1100 compounds for growth inhibitory activities. The compounds screened included several substituted urea compounds, dichloralurea (DCU) being the first of these to show potential for weed control. DCU was



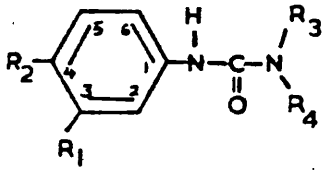
N,N'-bis(2,2,2,-trichloro-1-hydroxyethyl) urea (dichloralurea or DCU)

recommended as a pre-emergence treatment, toxic to grasses and selective in certain broad leaf plants, but has since been superseded by other herbicides and is not currently registered for use in crops (Ashton & Crafts, 1973). E.I. du Pont de Nemours and Co. (1950) were the first to observe the herbicidal properties of the substituted phenylureas and it was Bucha & Todd of the same Company who first reported in 1951 the particularly important compound, monuron.



N'-(p-chlorophenyl)-N,N-dimethylurea (Monuron, CMU)

Since these early discoveries thousands of urea derivatives have been screened for possible herbicidal activity (Abel, 1957), all effective compounds apparently conforming to the general formula



in which the substituents are commonly halogen groups at R_1 and R_2 and alkyl or alkoxy groups at R_3 and R_4, though other substitutions do exist.

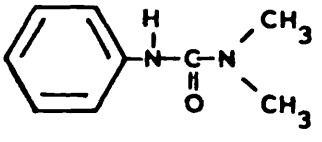
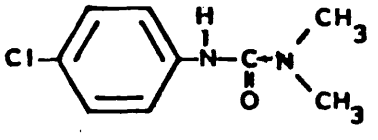
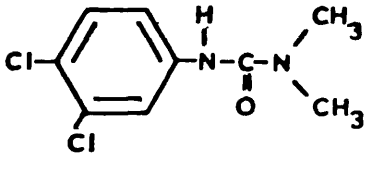
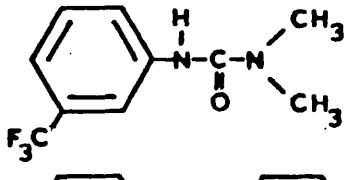
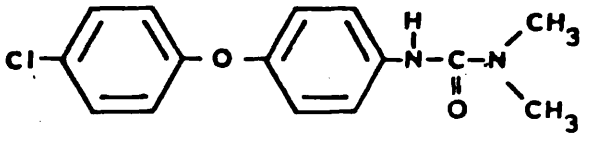
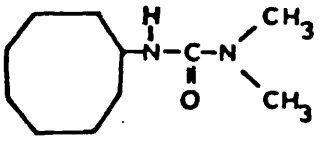
Since their discovery the substituted phenylureas have grown into one of the most prominent and diversified groups of herbicides. Even now the addition of new types of molecules to this group continues at the rate of two or three compounds each year which reach the late development or marketing stage (Geissbühler & Voss, 1971). The first important phenylureas, exemplified by monuron, were chlorine-substituted dimethyl derivatives with a high order of inherent phytotoxicity and consequently found use mainly as soil sterilants (Crafts, 1961; Geissbühler, 1969). They effect control of most annual grasses and broad leaved weeds where sufficient moisture is available (Crafts & Robbins, 1962). The compounds developed in more recent years

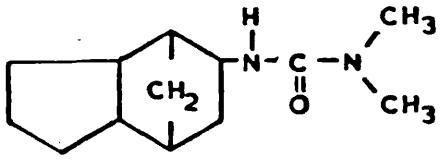
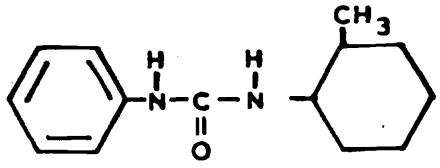
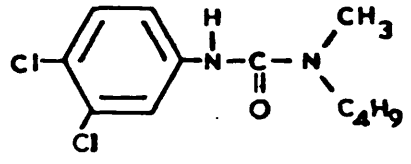
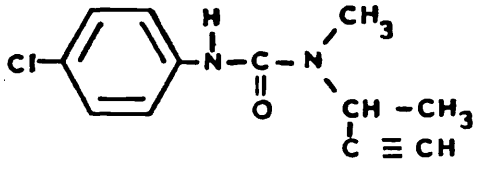
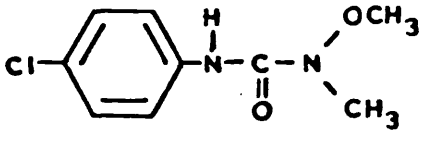
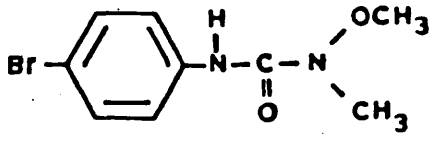
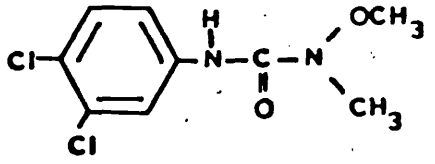
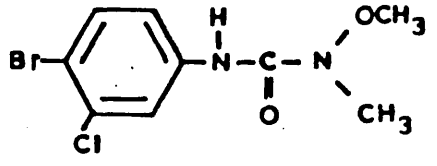
have become increasingly more complex in terms of ring substituents, ring structure and alkyl moieties (see Table 1) with resultant modifications to phytotoxicity and improvement in selectivity in many cases (Good, 1961; Moreland & Hill, 1963). Over the last 15 years the urea herbicides have therefore gradually evolved from compounds primarily used as non-selective soil sterilants into more intricate structures capable of exercising extensive selective control of weeds in many crop situations.

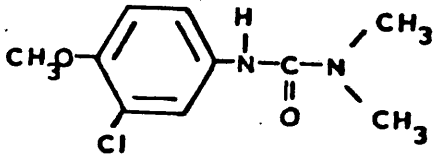
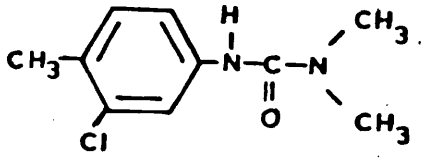
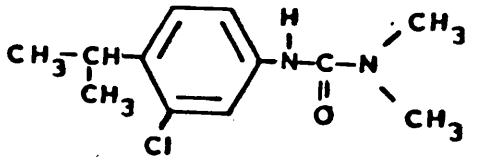
Relationship between structure and herbicidal activity

The discovery that substituted phenylureas are potent inhibitors of the Hill reaction of isolated chloroplasts (Cooke, 1956; Wessels & Van der Veen, 1956) provided an explanation for the effectiveness of these compounds as herbicides. These findings were confirmed and extended by Bishop (1958) who showed that in general herbicidal activity correlated with the effectiveness of the phenylurea in inhibiting photosynthesis in isolated chloroplasts. Numerous studies have since been undertaken in an attempt to correlate the structures of the substituted phenylureas with their potency as inhibitors of the Hill reaction. In a study of the structure/activity relationship of substituted phenylureas, Good (1961) reported that unsubstituted phenylureas such as fenuron (Table 1) were not nearly as effective as many of the substituted compounds, derivatives of 4-chlorophenylurea exhibiting a far wider range of activities. Amongst the 4-chlorophenylurea derivatives monuron was at least 10 fold more potent than its nearest competitor. Whereas derivatives of 3-chlorophenylurea such as

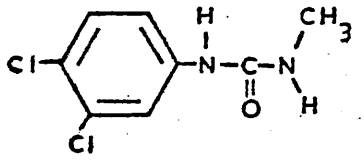
TABLE 1: . Substituted urea herbicides in general use

Structural formula	Chemical designation	Common name
	N'-phenyl-N,N-dimethyl urea	Fenuron
	N'-(p-chlorophenyl)-N,N-dimethylurea	Monuron
	N'-(3,4-dichlorophenyl)-N,N-dimethylurea	Diuron
	N'-(m-Trifluoromethyl-phenyl)-N,N-dimethylurea	Fluometuron
	N'-(4-(p-chlorophenoxy)-phenyl)-N,N-dimethylurea	Chloroxuron
	N'-cyclooctyl-N,N-dimethylurea	Cycluron

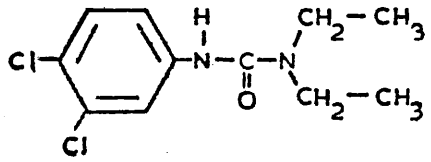
Structural formula	Chemical designation	Common name
	N'-(hexahydro-4,7-methanoindan-5-yl)-N,N-dimethylurea	Norea
	N'-phenyl-N-(2-methylcyclohexyl)urea	Siduron
	N'-(3,4-dichlorophenyl)-N-butyl-N-methylurea	Neburon
	N'-(p-chlorophenyl)-N-methyl-N-(1-methyl-2-propynyl)urea	Buturon
	N'-(p-chlorophenyl)-N-methoxy-N-methylurea	Monolinuron
	N'-(p-bromophenyl)-N-methoxy-N-methylurea	Metobromuron
	N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea	Linuron
	N'-(3-chloro-4-bromophenyl)-N-methoxy-N-methylurea	Chlorbromuron

Structural formula	Chemical designation	Common name
	N'-(3-chloro-4-methoxy-phenyl)-N,N-dimethylurea	Metoxuron
	N'-(3-chloro-4-methyl-phenyl)-N,N-dimethylurea	Chlortoluron
	N'-(3-chloro-4-isopropyl-phenyl)-N,N-dimethylurea	Isoproturon

the 3-chloro analogue of monuron showed a good level of activity, derivatives of 2-chlorophenylurea were, without exception, feeble inhibitors. Derivatives of the disubstituted compound, 3,4-chlorophenylurea, proved to be the most active inhibitors investigated by Good, the important herbicide diuron (DCMU) being the most inhibitory substance encountered. However, other 3,4-dichlorophenylureas such as the corresponding monomethylurea and 3,4-dichlorophenyl diethylurea were also very good inhibitors. Polar groups such as those of

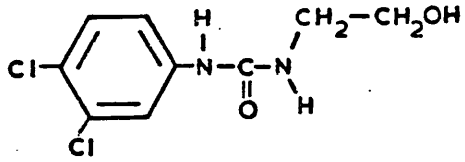


N'-(3,4-dichlorophenyl)-N-methylurea (monomethyl diuron)

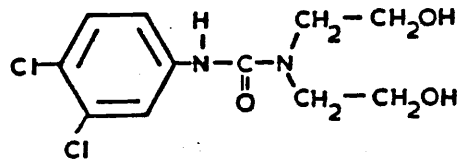


N'-(3,4-dichlorophenyl)-N,N-diethylurea

N'-(3,4-dichlorophenyl)-N-(2-hydroxyethyl)- and N'-(3,4-dichlorophenyl)-N,N-bis(2-hydroxyethyl)-urea sharply reduced the activity of these inhibitors. On the other hand the dehydrated version of the latter

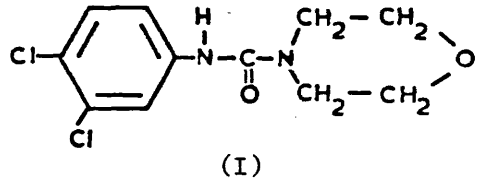


N'-(3,4-dichlorophenyl)-N-(2-hydroxyethyl)-urea



N'-(3,4-dichlorophenyl)N,N-bis(2-hydroxyethyl)-urea

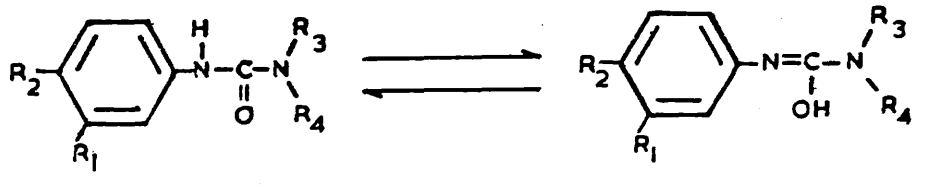
compound(I) inwhich the two hydroxyl groups were replaced by a relatively non-polar ether bridge, was a 100 fold more active than its polar counterpart. Finally, substitution of the carbonyl



(I)

oxygen of the 3,4-dichlorophenylureas with sulphur to give the corresponding 3,4-dichlorophenyl-thioureas gave rise to some derivatives with considerable activity, though in general these were from 300 to 2,000 times less effective than the oxygen analogues.

In a later study, Moreland and Hill (1963) examined the effect of fully saturated bicyclic and partially-saturated tricyclic dimethyl-ureas and dimethylthioureas in addition to substituted phenylureas on the photolytic activity (Hill reaction) of isolated chloroplasts. These workers reported that substituted phenylureas possessing the amide moiety (-N - C-) could undergo enol-keto tautomerization, represented by the following equilibrium:-



Resonance interaction between the ring system and the unshared electrons of the imino-nitrogen atom, and tautomerization, should both contribute to the strength of hydrogen bonds suggested to be involved in the binding of the herbicide to an as yet unidentified site in a chloroplast membrane (Good, 1961). The net effect of resonance interaction would be to decrease the electron density on the amide nitrogen with a resultant lowering of its electronegative charge. This consequently reduces the attraction of the amide nitrogen for the hydrogen atom enabling stronger hydrogen bonds to be formed between the herbicide and the binding site in the chloroplast. The fully or partially-saturated polycyclic dimethylureas were found to be weaker inhibitors, this being attributed to increased lipophilic properties and a lack of resonance interaction between the ring system and the imino nitrogen. Replacement of the carbonyl oxygen with a sulphur atom in the phenylthiourea series appeared to be accompanied by a degree of intermolecular association and polymer formation by virtue of the establishment of S ..... H-N bonds, the net result, however, being a dramatic reduction in inhibitory activity manifested against the Hill reaction attributed, at least in part, to the lower electronegativity of sulphur relative to oxygen (Moreland & Hill, 1963).

Good and Izawa (1964) attempted to generalise the structure/activity relationships of substituted phenylureas. They pointed out that an imino hydrogen (=N-H) was required if the substance was to be significantly inhibitory, and that furthermore, this imino hydrogen should be sterically accessible. This concept readily explained, for example, the ineffectiveness of derivatives of o-chlorophenylurea in

which the imino hydrogen is masked by the larger chlorine atom in such a manner that it could not participate in hydrogen bond formation.

Wessels & Van der Veen (1956) made the suggestion that the component of the chloroplast involved in the bonding of this group of herbicides was the chlorophyll molecule, the carbonyl oxygen of the cyclopentanone ring forming a hydrogen bond with the imino-hydrogen of the substituted phenylurea resulting in disruption of the photochemical apparatus. This view is by no means a generally accepted one and in the following discussion it will become apparent that the precise target site for phenylureas is still a matter for conjecture, largely resulting from an incomplete knowledge of the details of photosynthetic electron transport.

Mode of action

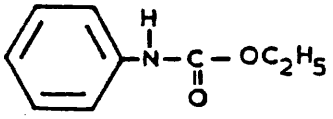
The phytotoxic symptoms of the phenylurea herbicides are primarily observed in the leaves, though in some cases changes in non-photosynthetic tissues are also induced (Minshall, 1957). Chlorosis is observed as one of the first visible effects of phenylurea treatment this being followed by more extensive leaf injury (Muzik, Cruzado & Loustalot, 1954; Minshall, 1957). Foliar application of a high concentration of herbicide results in the appearance within a few days of light green areas which eventually take on a water-soaked appearance and finally become necrotic. Symptoms take several days to develop when a low concentration dose is applied. In this case an initial wilting of the leaves is followed by the appearance of silver or grey blotches and rapid yellowing (Minshall, 1957). In wheat plants these

symptoms begin at the tip of the leaf and proceed downward toward the stem (Van Hiele, Hommes & Vervelde, 1970).

The movement of substituted phenylurea herbicides in plants has been examined by a number of authors, e.g., Haun & Peterson (1954), Muzik et al (1954), Fang, Freed, Johnson & Coffee (1955), Crafts (1959, 1962), Crafts & Yamaguchi (1960) and Geissbühler et al (1963a). There is general agreement that phenylurea herbicides are easily and rapidly taken up from both nutrient solution and soil by root systems and are subsequently translocated into stems and leaves by the transpiration stream. When applied to the leaf surface the herbicides appear able to penetrate epidermal layers to varying degrees, but apparently there is little or no entry into phloem tissue and therefore practically no translocation into stem or neighbouring leaves via the assimilate stream (Crafts & Yamaguchi, 1958). In a detailed study of absorption and distribution of (carbonyl-^{14}C)-diuron in red kidney bean, soybean and barley, Bayer & Yamaguchi (1965) found that following foliar application movement of the phenylurea in red kidney bean and soybean was restricted, whereas in barley the radioautographs indicated considerable movement of (carbonyl-^{14}C) diuron from the point of application towards leaf margins and tips and into adjacent leaves but not into the roots.

Soon after the introduction of the substituted phenylureas, Cooke (1955) observed that the sugar content of plants (sweet clover, Alfalfa and Lespedeza) treated with monuron decreased sharply and suggested that the mechanism of action was through an interference with photosynthesis. In another experiment leaf discs were incubated with monuron in the dark in the presence of sucrose. The leaf discs continued to accumulate starch, leading to the conclusion that monuron acted somewhere

prior to the formation of sugar (Cooke, 1956). In an attempt to determine whether monuron was actually affecting the photosynthetic mechanism, Cooke (1956) went on to make a study of the effect of the herbicide on the Hill reaction of isolated chloroplasts by a method proposed by Horwitz (1955) involving the use of Janus Green B as electron acceptor in the photolysis of H_2O reaction. He found that the Hill reaction of isolated spinach chloroplasts was completely inhibited by a very low concentration (10^{-7}M) of monuron. This report along with the known fact that phenylurethane, whose structure is



Phenylurethane

somewhat like that of phenylurea, had been shown to inhibit the Hill reaction (MacDowall, 1949) stimulated further and more detailed investigations on the effect of monuron and related compounds on the Hill reaction. It soon became well documented that monuron interfered with the light-dependent phases of photosynthesis (Wessels & Van der Veen, 1956), substituted phenylureas in general emerging as efficient and potent inhibitors of the Hill reaction (Spikes, 1956; Moreland, 1957). Moreland and Hill (1962) reported that although species

differences in susceptibility to phenylurea herbicides exist, chloroplasts from all plants examined proved to be equally susceptible to Hill reaction inhibition when treated with herbicide after isolation. According to Hill (1965), the inhibitory effect of phenylureas on the Hill reaction is related to their capacity to form hydrogen bonds with appropriate receptors at or near the active centre(s) of the chloroplast. Sweetser (1963), on the other hand, showed that flavin mononucleotides caused photochemical inactivation of phenylureas and drew attention to the close similarity between molecular models of substituted phenylureas and FMN.

In a study of the effect of monuron on carbon assimilation, Ashton, Uribe and Zweig (1961) found that when the herbicide was applied to the roots of plants carbon dioxide fixation by leaves in the light was greatly reduced. The subsequent distribution of radioactivity amongst fixation products following exposure of phenylurea-treated red kidney bean (Phaseolus vulgaris L.) to $^{14}CO_2$ both in the light and in the dark served to demonstrate that these herbicides almost completely blocked the pathway of photosynthetic carbon dioxide fixation, but that this did not involve an interference with any of the reactions of the photosynthetic carbon reduction cycle (Ashton et al, 1961). Jagendorf & Avron (1959) studied the inhibition of photosynthesis by monuron in considerable detail on the basis of the assumption that during the light phase of photosynthesis water is cleaved to give both an oxidised (Y.O) and a reduced (X.H) product. These workers postulated two steps in the evolution of O_2 from Y.O and that the effect of monuron was to inhibit the first of these reactions. Jagendorf & Avron (1959) also

found that in addition to inhibiting the Hill reaction, measured as the photo-induced reduction of ferricyanide or an indophenol dye, monuron also impaired ATP synthesis (cyclic photophosphorylation) in isolated chloroplasts though only when applied at higher concentrations. A similar result was obtained by St. John (1971) with the related herbicide, diuron, which was shown to inhibit the photochemical, but not the respiratory, production of ATP. Though diuron also impaired growth at the dosage required to inhibit ATP synthesis, the cell still maintained ATP levels in balance with growth. The effect of diuron upon photosynthesis and photoreduction in a hydrogenase-containing alga, Scenedesmus, which can use hydrogen in the absence of oxygen, was studied by Bishop (1958). Photosynthesis was reduced to about half by 5×10^{-7}M diuron and became completely inhibited in the presence of 3×10^{-6}M diuron, whereas after adaptation to hydrogen the effect of diuron disappeared. The effects caused by this substituted phenylurea were similar to other photosynthesis poisons, such as o-phenanthroline and naphthoquinone except that the potency of diuron as an inhibitor of oxygen evolution was substantially greater. Bishop (1958) concluded from these studies that diuron reacts specifically with some intermediate chemical or enzyme concerned with the production of oxygen in photosynthesis. Further evidence was obtained by Hoffman, Hersh, Sweetser & Todd (1960) for the site of inhibition of phenylureas proposed by Jagendorf & Avron (1959) who found that growth of the photosynthetic bacterium, Rhodospirillum rubrum, which lacks an O_2-evolving photosystem, was insensitive to monuron. In addition, photophosphorylation in cell-free extracts of this organism was equally insensitive to monuron (Hoffman et al, 1960). However, in green plants, possessing both

photosystems of photosynthesis, blockage of oxygen evolution interferes with the subsequent electron transport system, photophosphorylation and NADP (Nicotinamide adenine dinucleotide phosphate) photoreduction, and consequently CO_2 fixation is impaired. The introduction of a reducing agent such as ascorbate together with a suitable catalyst, such as 2,6-dichlorophenolindophenol (DCPIP), largely restores photophosphorylation and reduction of NADP, indicating that these latter processes are not directly inhibited by phenylureas (Monaco & Moreland, 1964; Van Overbeek, 1964).

The evidence presented so far thus suggested that compounds such as monuron and diuron inhibited the light-phase of photosynthesis through interference with the oxygen evolution process. The number of chlorophyll molecules which function together as a photosynthetic unit is of interest in relation to the concentration of urea herbicides required to produce inhibition. Thus Izawa and Good (1965) studied the adsorption and distribution of phenylurea herbicides in chloroplasts and found that one herbicide molecule was sufficient to inhibit a single O_2-evolving unit comprising approximately 250 chlorophyll molecules. Holly (1968) has pointed out that all these herbicides are very stable and are unlikely to react chemically though most have the potential for hydrogen bonding between their positive imino hydrogen and a negative carbonyl oxygen possibly located on the cyclopentanone ring of the chlorophyll molecule. The phenylureas also possess a carbonyl oxygen which could conceivably form a hydrogen bond with an imino hydrogen of chlorophyll.

Hoffman et al (1960) suggested that the toxicity of monuron to Euglena sp in the presence of succinate in the light, but not in the dark, was due to the formation in the light of a phytotoxic substance from monuron and a second component, possibly a flavoprotein complex. Along similar lines is the proposal (Sweetser & Todd, 1961) that the toxicity of monuron in the light to a culture of Chlorella pyrenoidosa supplemented with carbohydrate was due to accumulation of a toxic photosynthetic intermediate. Davis (1966) similarly suggested that the leaf injury in Shrub Live Oak (Quercus turbinella Greene) seedlings following fenuron treatment was due to the accumulation of toxic components in the blocked photosynthetic unit. Sweetser (1963) has obtained data to indicate that flavin mononucleotide (FMN) causes photochemical inactivation of monuron and other substituted phenylureas. This suggestion was based on the isolation from reaction mixtures containing FMN and monuron of a high molecular weight compound which was no longer inhibitory to photosynthesis. A close relationship apparently existed between the ability of a particular phenylurea compound to react photochemically with FMN and their potency in inhibiting photosynthesis. The interpretation of the above data was that the inhibition of photosynthesis by monuron may well result from an interaction of the herbicide with FMN or a flavoprotein moiety in the pathway of photosynthetic electron transport.

As part of a study on the effects of phenylureas on non-photosynthetic processes Sumida and Ueda (1973) investigated the effect of diuron on the biosynthesis of complex lipids in Chlorella ellipsoidea.

Their results indicated that incorporation of acetate into fatty acid residues was substantially impaired by treatment with diuron. It was argued, however, that since biosynthesis of fatty acids from acetate units requires the cofactors ATP, coenzyme A and NADPH, it was reasonable to assume that shortage of ATP and NADPH resulting from inhibition of photosynthesis was probably responsible for the observed inhibition of acetate assimilation into lipids. Another explanation however would be that under conditions where the ATP supply was reduced by the presence of a phenylurea herbicide lipids, particularly galactolipids, might be mobilized as an energy source. The final possibility of a direct effect of the herbicide on an enzyme(s) involved in lipid biosynthesis still remains.

Moreland, Malhotra, Gruenhagen and Shokraii (1969) studied the effect of substituted phenylurea herbicides on RNA and protein synthesis in excised soybean hypocotyls. Diuron, one of the most potent inhibitors of the Hill reaction of isolated chloroplasts was found to be moderately active as an inhibitor of RNA and protein synthesis as measured by incorporation of radioactively labelled ATP or orotate and leucine respectively. Since RNA and protein synthesis are vital processes for continued plant growth and development, any interference with these biosynthetic processes resulting from treatment with a phenylurea herbicide would be expected to exert a most adverse effect on the plant. The physiological and biochemical processes required for plant growth and development, including RNA and protein synthesis, are ultimately driven by energy derived from ATP. The observed inhibition of RNA and protein synthesis by diuron could thus be explained as the result of an

inhibition of cellular energy production though the possibility of a direct effect on one or both of these processes can not be excluded. In opposition to this view is the observation (Gruenhagen & Moreland, 1971) that treatment of excised soybean hypocotyls with diuron did not significantly reduce tissue ATP levels.

In a comparative study of the action of several herbicides on electron transport and associated phosphorylation processes in both chloroplasts and mitochondria (Moreland & Blackmon, 1968) it was found that whereas the noncyclic photosynthetic electron flow was inhibited by diuron, the respiratory electron transport chain was not affected. McDaniel & Frans (1969) studied the effect of the related phenylurea, fluometuron (N'-(m-trifluoromethylphenyl)-N,N-dimethylurea), on mitochondria from soybean. They observed a 50% uncoupling of oxidative phosphorylation from electron transport activity at fluometuron concentrations of 5.6×10^{-5}M with malate as substrate, and 4.6×10^{-5}M when succinate was the substrate employed. For comparison with these results, Moreland & Hill (1962) showed that a 50% inhibition of the Hill reaction could be achieved at a concentration of herbicide, in this case neburon(N'-(3,4-dichlorophenyl)-N-butyl-N-methylurea), of 2.2×10^{-7}M. The effect of monuron on oxidative phosphorylation was investigated by Lotlikar, Remmert & Freed (1968). Using mitochondria prepared from cabbage they found that appreciable inhibition of oxidative phosphorylation occurred only when the monuron concentration was as high as 3×10^{-3}M, this being some 10^{4} fold higher than the concentration required to inhibit the Hill reaction of isolated chloroplasts.

The above observations suggest that the Hill reaction of photosynthesis is the more likely primary site of action of the phenylurea herbicides, a conclusion supported by the numerous reports indicating good correlation between phytotoxicity and potency as Hill reaction inhibitors (Wessels & Van der Veen, 1956; Cooke, 1956; Moreland, 1957).

Brief reference was made earlier to a suggestion (Wessels & Van der Veen, 1956) that the observed inhibition of the Hill reaction might result through an association, stabilised by hydrogen bonds, of the phenylurea with the cyclopentanone ring of chlorophyll. A different view has been presented by Izawa and Good (1965) who considered that the substituted phenylureas inhibit the Hill reaction by reacting with the unidentified catalytic centre responsible for oxygen evolution. Good and Izawa (1964) initially disagreed with the view of Wessels & Van der Veen (1956) on the reasoning that since the herbicides are effective inhibitors at concentrations which are low relative to that of total chlorophyll, it becomes necessary to invoke the supplementary hypothesis that only one chlorophyll in hundreds possessed a carbonyl group accessible for hydrogen bonding, and that this one chlorophyll was also exceptional in being essential for photosynthesis and the Hill reaction. An additional observation which cannot be explained in terms of hydrogen bonding to chlorophyll is that several inhibitors of photosynthesis with little or no tendency to form hydrogen bonds were nevertheless among the most effective inhibitors of the Hill reaction. From their detailed study of the inhibitory action of phenylureas Izawa & Good (1965) concluded that interaction of diuron and monuron with chloroplasts involved an initial partitioning of the inhibitors between the aqueous solvent and the chloroplast phase followed by an

irreversible binding, saturated at low concentrations and not associated with inhibition, of about one inhibitor molecule per 1000 chlorophyll molecules and, finally, a reversible binding closely related to the degree of inhibition of electron transport. That the effectiveness of diuron could be attributed to its ability to inhibit the flow of electrons from water to NADP has been confirmed by many workers (e.g., Vernon & Zaugg, 1960; Schwartz, 1966; Levine, 1969). The same workers independently reported however that photoreduction of NADP could be restored in diuron-treated chloroplasts if an artificial electron-donor system such as ascorbate plus DCPIP or TMPD (N,N,N',N'-tetramethyl-p-phenylene diamine) was introduced into the reaction mixture. Since the ascorbate / DCPIP couple is known to donate electrons to a point in the photosynthetic electron transport chain linking the two photosystems (Vernon & Zaugg, 1960) the inference from these observations was that the phenylureas act at a site closely associated with photosystem 2 of photosynthesis. It was further noticed that in the presence of diuron both the c-type (cytochrome *f*) and b-type (cytochrome b_6, b_{559}) cytochromes of the photosynthetic electron transport chain could still be oxidised by photosystem 1, but they no longer became reduced in light absorbed by photosystem 2 (Levine, 1969). The assumption from these latter observations was that inhibition by diuron was at a site between photosystem 2 and the cytochrome components of the intermediate electron transport chain linking the two photosystems (see Fig. 1).

A somewhat different explanation for the mechanism of action of phenylurea herbicides was presented by Stanger & Appleby (1972) based on their observations that in diuron-treated chloroplasts degradation

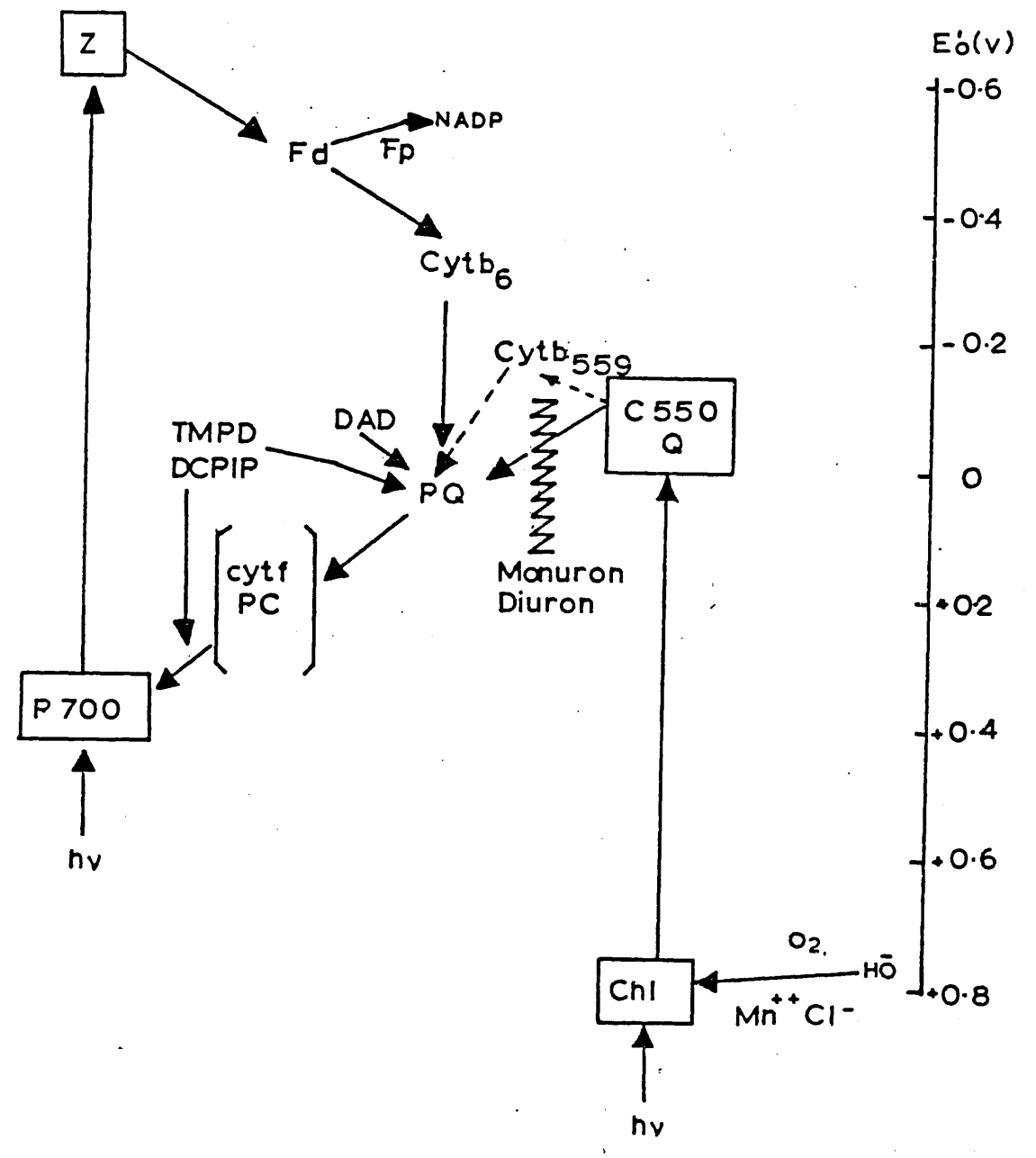


Fig. 1: Scheme showing the probable site of inhibition of photosynthetic electron flow by the substituted phenylureas, monuron and diuron

Fd = Ferredoxin; Fp = Ferredoxin:NADP reductase;

PQ = Plastoquinone; PC = Plastocyanin;

DAD = 2,3,5,6-tetramethyl-p-phenylenediamine;

TMPD = N,N,N',N'-tetramethyl-p-phenylenediamine

of carotenoid pigments preceded the breakdown of chlorophyll. These results were interpreted as lending support to a hypothesis that diuron induced phytotoxicity by catalyzing a lethal photosensitized oxidation in the chloroplast, occurring as a result of either a greater concentration of oxidized chlorophyll caused by an interruption of electron flow or an inhibition of NADPH formation necessary to maintain a functional carotenoid protective mechanism.

In a study of the effect of tris buffer (tris (hydroxymethyl)-aminomethane) on the Hill reaction of isolated chloroplasts Yamashita & Butler (1968) observed an ability of certain compounds, notably phenylene diamines, to donate electrons to photosystem 2 of photosynthesis. Thus although washing chloroplasts with tris buffer (0.8M) severely depleted the rate of electron donation from H_2O, a low concentration of the artificial electron donor p-phenylene diamine (PD) effectively restored the photoreducing capacity of the chloroplasts. The restored electron flow from p-phenylene diamine to an electron acceptor such as NADP was, however, found to be sensitive to substituted phenylureas such as diuron. At higher concentrations of the electron donors other sites beyond the diuron block seemed available for receipt of electrons from both p-phenylene diamine and TMPD so that the inhibition by diuron was no longer apparent. Yamashita & Butler (1968) concluded from these studies that diuron (10^{-6}M) inhibits photosynthetic electron transport at the site of the primary electron acceptor of photosystem 2, denoted by Q in Figs. 1 and 2, so that any diuron-sensitive electron flow implies donation of electrons prior to photosystem 2. Fig. 2 indicates a total of three sites for electron donation to the photosynthetic electron transport system. Measurements of fluorescence yield and of

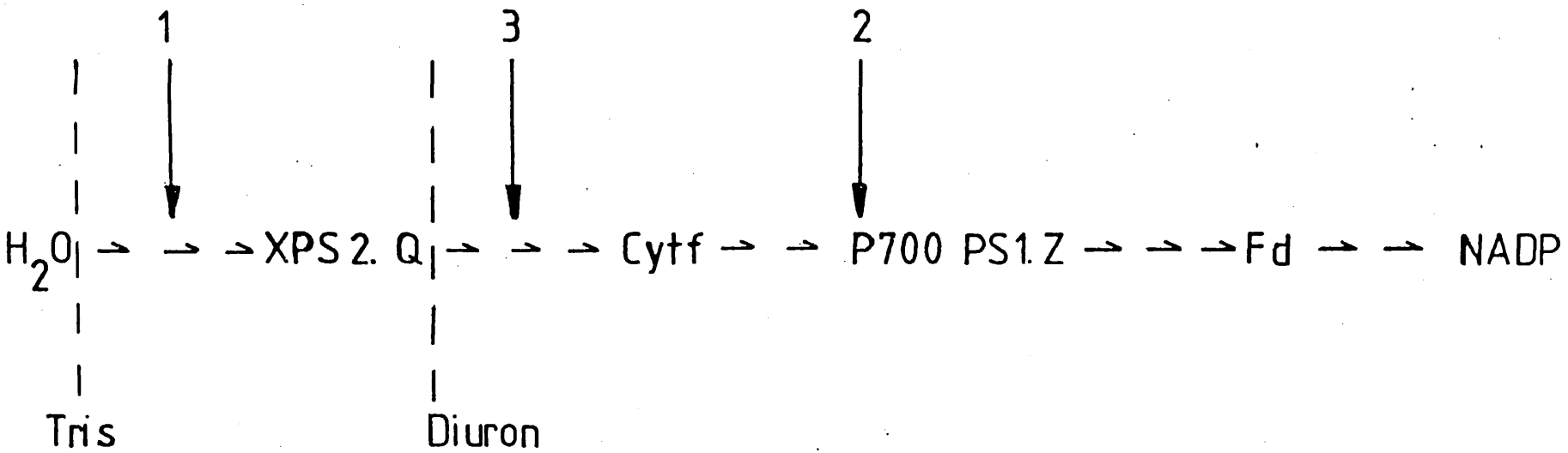


Fig. 2: Sites of electron donation to the photosynthetic electron transport system (after Yamashita & Butler, 1968)

PS1 = Photosystem 1; PS2 = Photosystem 2;

X = primary electron donor to photosystem 2.

photophosphorylation made by Yamashita & Butler (1968) indicated that all donor systems tested were capable of electron donation at site 1 to photosystem 2 in tris-washed chloroplasts in the absence of phenylurea herbicide. In the presence of diuron (10^{-6}M) which blocks electron flow through photosystem 2, donor site 2 is suggested to be available for receipt of electrons from low concentrations of both DCPIP and TMPD, but electron flow from this site to NADP was not associated with a phosphorylation site. At higher concentration both reduced DCPIP and TMPD were envisaged to supply electrons to NADP through site 3, an electron flow which encompasses the coupling site for photophosphorylation in the intermediate electron transport chain. According to Gromet-Elhanan & Redlich (1970) the related compound 2,3,5,6-tetramethyl-p-phenylenediamine (diaminodurene, DAD) is capable of functioning as an autoxidizable electron acceptor from photosystem 1 inducing a pseudocyclic electron flow from water to oxygen which is tightly coupled to phosphorylation with a P/2e ratio of approx. 1. This electron transport, as is the case for other pseudocyclic systems, was shown to be sensitive to substituted phenylurea herbicides. In the presence of diuron, however, DAD coupled with ascorbate appeared to mediate an open-chain electron flow to oxygen which was some 20 times more rapid than the pseudocyclic electron transport probably as a result of by-passing a rate limitation imposed by photosystem 2 (Fig. 3).

Izawa, Connelly, Winget and Good (1966) studied the effect of diuron on photosynthetic electron transport in the presence of an autoxidizable electron acceptor such as methyl viologen (M.V). They noticed also that diuron inhibited electron flow from water to an artificial electron

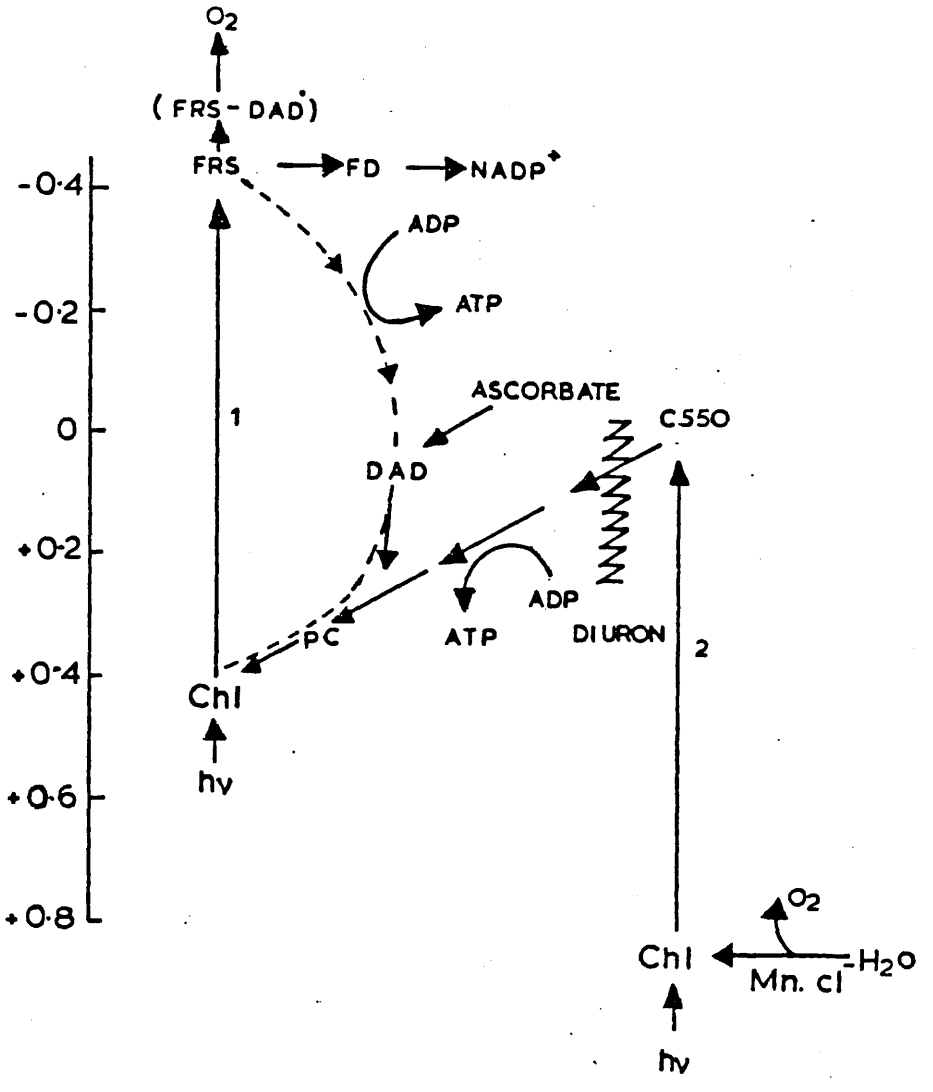


Fig. 3: Open-chain electron flow to oxygen mediated by DAD in the presence of diuron (after Gromet-Elhanan & Redlich, 1970)

Broken lines represent the route for cyclic electron flow.

PC = plastocyanin; FRS = Ferredoxin reducing substance;

DAD = 2,3,5,6-tetramethyl-p-phenylenediamine;

DAD· = free radical of DAD.

acceptor but that the electron flow could be restored by addition of an artificial electron donor such as the ascorbate/DCPIP couple. From their results Izawa et al (1966) placed the diuron-inhibition site near photosystem 2 but close to the component C550 (Q?, see pages 60-62) since inhibition by diuron was more influenced by light intensity than that caused by other photosystem 2 inhibitors. Trebst and Pistorius (1965) and Schwartz (1966) were also able to demonstrate that the inhibition of NADP photoreduction and oxygen evolution in diuron-treated chloroplasts could be reversed by electron donation from DCPIP- TMPD- and DAD-ascorbate couples. Whereas electron flow from DAD/ascorbate and DCPIP/ascorbate couples was accompanied by a stoichiometric formation of ATP that from the TMPD/ascorbate system was not. In these experiments DAD and DCPIP thus behaved similarly except that the former donor catalysed significantly higher rates of electron flow and concomitant ATP formation. A degree of caution is however necessary in arriving at any firm conclusions concerning the site of action of substituted phenylureas from studies with such artificial electron donors since there is difficulty in defining from the literature the precise sites of electron donation from DCPIP, DAD and TMPD, the site of donation from all three donors apparently depending on concentration (Trebst, 1964; Wessels, 1964; Trebst & Pistorius, 1965; Schwartz, 1966; Yamashita & Butler, 1968). Probably at best it may be said that these studies seem to indicate that photosystem 1 of photosynthesis does not contain a site sensitive to low concentrations (10^{-7}-10^{-6}M) of substituted phenylureas such as diuron. In contrast to the above interpretations which limits electron donation from DAD or TMPD to the intermediate electron transport chain

is the more recent view (Hauska, McCarty & Racker, 1970; Gromet-Elhanan & Redlich, 1970) that electron donation from DAD may be to a cyclic chain of electron carriers distinct from the intermediate electron transport chain, which is associated with cyclic photophosphorylation.

The most conclusive evidence for the location of the site of diuron inhibition on the reducing side of photosystem 2 has accumulated from numerous studies of chlorophyll fluorescence. Strehler and Arnold (1951) were the first to discover the phenomenon of light re-emission by higher plants and algae following a brief illumination period. All photosynthetic organisms studied, including the photosynthetic bacteria, were observed to emit a low intensity light after illumination, the spectrum of the re-emission closely resembling the fluorescence spectrum of chlorophyll a or bacteriochlorophyll in the case of green plants and photosynthetic bacteria respectively. The decay curves of the re-emitted light have been shown to result from several components with half lives ranging from 3 mS to 15 S or more (Arthur & Strehler, 1957). A study of the temperature-dependence of the decay of the various components (Tollin, Fujimori & Calvin, 1958) indicated that at temperatures as low as -170°C the rapid decay of re-emitted light was still apparent. On the basis of these findings Tollin et al (1958) suggested that the decaying components involved solid state processes such as the recombination of electrons and 'holes' or the emptying of shallow, electron-trapping levels.

In an attempt to obtain more information on the early reactions of photosynthesis, Sweetser, Todd and Hersh (1961) made a study of the effects of various inhibitors of photosynthesis upon the decay of the re-emitted light. It was found that the substituted phenylurea, monuron one of the most potent inhibitors of photosynthesis, showed a striking

specificity in its effects on components of the re-emitted light of both green algae (Chlorella sp & Euglena) and higher plants. From the first order treatment of the decay curve three decay components were obtained with half lives of 5-10 mS, 100-160 mS, and 0.75-3.0 S respectively. The results obtained indicated that the decay components with half lives of 5-10 and 100-160 mS were both affected by monuron, this being interpreted to mean that the chloroplast constituents responsible for the observed emissions probably function in a common reaction sequence. Complete inhibition of the fastest decaying component by monuron suggested that a very close correlation existed between the inhibitor and an early reaction in the O_2-liberating pathway of photosynthesis. The ratio of chlorophyll to monuron required for inhibition (over 280:1) led Sweetser et al (1961) to further suggest that the re-emitted light may originate from a special chlorophyll-enzyme complex. The absence of an inhibitory effect of monuron on the component with a half life of 0.75-3.0 S indicated that this component would not be expected to function beyond the monuron block with respect to chlorophyll. These results and conclusions are supported by the more recent studies of Bennoun (1970) and Mohanty, Mar & Govindjee (1971). who independently discovered that in the presence of diuron the light-induced increment of the variable chlorophyll a fluorescence remains undiminishingly high in the dark.

At physiological temperatures the observed fluorescence emanates mostly from the chlorophyll a of photosystem 2, or more precisely from the chlorophyll a_{678} component which has a main fluorescence band maximum at 685 nm. Normally only some 15% of the excitation energy of the photosynthetic pigments is thought to be lost as fluorescence or heat,

the remaining 85% being dissipated largely by driving electron transport across the reaction centre of photosystem 2 (Papageorgiou, 1975). The portion of the chlorophyll a excitation which is re-emitted as fluorescence (i.e., fluorescence yield) does vary, however, in response to the rate of photosynthesis. In general a weak chlorophyll a fluorescence typifies a vigorous photosynthesis whereas a strong fluorescence is indicative of a weak or inhibited photosystem 2 activity (Lavorel, 1959; Clayton, 1969). In order to perform an oxidoreduction against a redox potential gradient, the reaction centre complex of photosystem 2 is endowed with a photoreceptor chromophore (Chl a) and with a redox donor-acceptor couple (Z,Q), whose chemical nature remains unknown. A mechanism to account for the function of the reaction centre complex of photosystem 2 has been advanced by Kautsky, Appel & Amann (1960), Duysens & Sweers (1963), and Butler (1972), a direct electron exchange between Z and Q being considered to involve oxidation of the chlorophyll a photoreceptor itself as a result of the primary photoconversion.

$$Z\ Chl\underline{a}\ Q \xrightarrow{h_\nu} Z\ Chl\underline{a}^*\ Q \longrightarrow Z\ Chla^+\ Q^- \longrightarrow Z^+ Chl\underline{a}\ Q^-$$

The intimate association of the components of this reaction centre is evidenced by the occurrence of a photoinduced electron transport from Z to Q at -196°C (Erixon & Butler, 1971; Okayama & Butler, 1972) and by the fact that all known inhibitors, or inhibitory treatments, that block photosynthetic electron transport, act either before Z or after Q (Katoh, 1972). The primary electron acceptor of photosystem 2, Q, thus accepts electrons in its oxidised form from the photosensitized chlorophyll

thereby effecting a quenching of fluorescence. Any impairment imposed on the subsequent reoxidation of Q consequently results in an increase in fluorescence yield since the singlet excitation energy can not now be dissipated by supporting photosynthetic electron transport. Thus in the presence of diuron the fluorescence yield from photosystem 2 increases to a maximum suggesting that the herbicide prevents the re-oxidation of Q by the intersystem pool oxidants (Yamashita & Butler, 1968).

Mohanty, Braun & Govindjee (1972) showed that diuron only exerted such an effect when added during the dark period that precedes the excitation of fluorescence, since only then does it cause the reaction centres to accumulate in the state Z^+ Chl Q^- :

$$Z\ \text{Chl}\ Q\ A \xrightarrow{\text{Diuron}} Z\ \text{Chl}\ Q \,\|\, A \xrightarrow{h\nu} Z^+\ \text{Chl}\ Q^- \,\|\, A$$

(here, A denotes the intersystem oxidants; $\|$, the diuron-introduced block)

Added in the light, however, diuron generates no increase in fluorescence because the reaction centres would be already oxidised by the intersystem oxidants to the state Z^+ Chl Q :

$$Z\ \text{Chl}\ Q\ A \xrightarrow{h\nu} Z^+\ \text{Chl}\ Q^-A \qquad Z^+\ \text{Chl}\ Q\ A^- \xrightarrow{\text{Diuron}} Z^+\ \text{Chl}\ Q \,\|\, A$$

The fluorescence studies of Duysens & Sweers (1963), Duysens (1964) and Lavorel (1964) had led to the earlier conclusion that monuron prevents the oxidation by photosystem 1 of the primary electron acceptor, Q, of photosystem 2. Duysens (1964) located the site of diuron inhibition somewhat more precisely between Q and the secondary acceptor pool, A, of photosystem 2, considered to consist predominantly of plastoquinone (Amesz, 1973). This interpretation was based on the observation that low concentrations of diuron inhibited photoreduction of plastoquinone by

photosystem 2 but not its oxidation by photosystem 1, and now appears to be generally accepted by the majority of workers in photosynthesis. Velthuys & Amesz (1974) have reported an additional, as yet hypothetical component, designated R, situated between Q and plastoquinone. This conclusion was based on the results of a study of the reactions between the primary and secondary electron acceptors of photosystem 2 using measurements of the increase of chlorophyll fluorescence induced in darkness by diuron. On the basis of this interpretation, Velthuys & Amesz (1974) explained the mechanism of action of diuron in terms of a lowering of the mid point potential of R. Evidence for the existence of an additional acceptor between Q and plastoquinone possessing the same properties as R has also been obtained by Bouges-Bocquet (1973a) on the basis of measurements of methyl viologen reduction. This site of action is also consistent with the observed diuron inhibition (1 diuron : 375 Chl) of the dark oxidation, but not the photoreduction, at room temperature of the spectral component C_{550} associated with the reduction of the primary electron acceptor of photosystem 2 (Knaff & Arnon, 1969b). A recently discovered diuron-resistant electron transport to silicomolybdate mediated by photosystem 2 is also consistent with this site of action (Giaquinta, Dilley, Crane & Barr, 1974). The inhibition of the dark bleaching (1 diuron : 90 Chl) of another spectral band at 390 nm ("X-320") associated with the primary acceptor (Witt, 1973), and of oxygen evolution in Chlorella (50 μM diuron) after a single light flash (Duysens, 1971) provides additional support for a specific site of action of the herbicide on the acceptor side of photosystem 2, though some evidence does exist for a second site of inhibition on the donor side (Renger, 1973; Etienne, 1974).

Jagendorf & Margulies (1960) and Asahi & Jagendorf (1963) have reported that at high concentrations (10^{-4}M) monuron can be shown to inhibit photosystem 1-mediated activities such as PMS-catalysed cyclic photophosphorylation, this being interpreted as an indication of the existence of a possible third site of inhibition by monuron.

In spite of their specificity the actual component with which monuron and diuron interact on the reducing side of photosystem 2 remains unknown. The list of candidates for a component with which the herbicides might react would include those components which have been suggested to function in this part of the electron transport chain, including a quinone protein, non-haem iron proteins, component "B" (Bouges-Bocquet, 1973a), component "R" (Velthuys & Amesz, 1974) and cytochrome $\underline{b}_{559}$ (Cramer, Fan & Böhme, 1971). Cramer & Whitmarsh (1977) have presented evidence for cytochrome $\underline{b}_{559}$ being the site of action of diuron, though the light-induced absorbance changes of the cytochrome in the presence of the herbicide are complicated and not interpretable in terms of a well-defined inhibition site on the photosystem 2 side of cytochrome $\underline{b}_{559}$. However, an effect of low concentrations of diuron on cytochrome $\underline{b}_{559}$ can be seen in the ability of the inhibitor (~1 diuron : 400 Chl) to block on acid-induced reversible decrease in mid-point potential of the cytochrome, which can be ascribed to protonation of its oxidised form. The simplest interpretation of these data is that diuron interacts with cytochrome $\underline{b}_{559}$ and prevents its protonation. This interpretation has gained some support from the isolation of two independent highly fluorescent mutants of Chlamydomonas reinhardi which behave as though they are diuron-poisoned and which are deficient in ascorbate-reducible cytochrome $\underline{b}_{559}$ (Epel & Butler, 1972) and total cytochrome $\underline{b}_{559}$ (Horton, Donnell, Cramer, Lien, Togasaki &

San Pietro, 1977) respectively. An interesting observation in this context was that trypsin, which has been shown to cause conversion of cytochrome $\underline{b}_{559}$ from a "high potential" to a "low potential" form (Cox & Bendall, 1972), removed the diuron inhibition site of Chlamydomonas reinhardi when acting from the outside of the chloroplast membranes (Regitz & Ohad, 1976). If it is assumed that cytochrome $\underline{b}_{559}$ functions in the intermediate electron transport chain near photosystem 2 one might infer from the above considerations that diuron and monuron also act in this position. However, because photosystem 2 is a tightly integrated complex one should not rule out the alternative possibility that the measured effect of diuron on cytochrome $\underline{b}_{559}$ might arise from the herbicide affecting a component distinct from, but interacting strongly with cytochrome $\underline{b}_{559}$.

It will be apparent from the foregoing discussion that substituted phenylureas such as monuron and diuron have in their turn contributed greatly to photosynthesis research particularly with respect to photosystem 2 function. However, our knowledge of this system remains fragmentary and consequently final conclusions concerning the precise site of inhibition by monuron and diuron must be held in abeyance until such time as our understanding of the details of the mechanism of photosystem 2 activity is more complete.

Throughout this section constant reference has been made to results obtained from studies with monuron and diuron, this being a natural result of the widespread use of these two compounds as valuable tools in advanced photosynthesis research. Although the author has not encountered any reference in the above studies of chlorophyll fluorescence and redox properties of cytochrome $\underline{b}_{559}$ to phenylurea herbicides other than monuron

and diuron it is considered most likely, on the basis of their similar behaviour in studies of inhibition of the Hill reaction, that the above discussion can be extended to include all substituted phenylureas discussed in the present thesis.

SELECTIVE ACTION OF SUBSTITUTED PHENYLUREA HERBICIDES ON PLANTS

The earliest substituted phenylurea herbicides to be used commercially for weed control such as monuron, fenuron and diuron were relatively non-selective and were usually applied to the soil as general soil sterilants (Minshall, 1954; Fang et al, 1955). These compounds were primarily regarded as physiologically non-selective herbicides which inhibited the Hill reaction (see previous section). Later reports claimed that monuron for example exhibited a degree of selectivity, this however being attributed to some physical phenomenon such as placement of the herbicide (Rogers & Funderburk, 1967). Such an explanation, however, does not adequately explain the difference in susceptibility of tolerant and susceptible plants to the more recent substituted phenylureas.

Van Oorschot (1965) studied the effect of monuron on resistant (plantain) and susceptible (maize) species by following changes in photosynthetic CO_2 uptake with time. He found that CO_2 uptake by leaves of both plantain and maize plants decreased to a low value but that the activity gradually recovered in the case of the former plant. These results indicated that plantain appeared to have a marked ability to inactivate the herbicide whereas maize did not. Similar results were obtained for the action of cycluron on sugar beet (resistant) and maize (susceptible) and for N'-(4-butoxyphenyl)-N,N-dimethylurea on the leaves

of onion (resistant) and French bean (susceptible). Geissbühler et al (1963b) have investigated the metabolism of chloroxuron by plants showing different degrees of tolerance. The results showed that all plants studied were capable of metabolising the phenylurea to several derivatives but that there were differences in rate of metabolism between susceptible and tolerant species. These workers concluded that differences in the rate of metabolism represented a possible basis for the selective action of chloroxuron. However, Kuratle, Rahn & Woodmansee (1968) reported greater absorption of linuron by susceptible (common ragweed) compared to tolerant (carrot) species. Though metabolites of linuron were detected in both species certain of these showed retained phytotoxicity towards ragweed but caused no significant damage to carrot. From these results Kuratle et al (1968) suggested that differences in absorption, metabolism, and phytotoxicity of certain metabolic derivatives of linuron may all contribute to the observed selectivity of the herbicide. A similar conclusion was made by Nashed, Katz & Ilnicki (1970) from a study of the selective action of chlorbromuron (N'-(3,chloro-4-bromophenyl)-N-methoxy-N-methylurea on maize (resistant) and cucumber (susceptible). A degree of binding of the herbicide was detected in both species but cucumber was reported to absorb a two-fold greater quantity of chlorbromuron than maize. No metabolites of the parent herbicide were detected in cucumber leaves whereas in maize three metabolites were found to be present, none of which showed phytotoxicity.

In an investigation of the effect of fluometuron and its derivatives on the rate of photosynthesis in cotton (resistant) and redroot pigweed and foxtail (susceptible), Rubin & Eshel (1971) found that cotton was not affected by fluometuron concentrations sufficient to cause death of foxtail

and to severely reduce growth of the pigweed. The N-monodesmethyl derivative of fluometuron though generally less active was similarly relatively more phytotoxic to the weed species whereas the N-bis-desmethylated analogue was essentially nonphytotoxic to both weed and crop. These results are in agreement with the earlier observations of Kuratle et al (1968) and Nashed et al (1970) in that they provide additional evidence that the selective action of a phenylurea herbicide may result from differential susceptibility to metabolites of the herbicide in addition to differential metabolism of the herbicide itself and differing degrees of uptake by the roots as a result of depth protection. More recently Feeny, Parochetti & Colby (1974) studied the mode of selective action of chloroxuron on soybean (resistant) and tall morning glory (susceptible). In spite of a greater uptake of herbicide by the tolerant plant compared to the susceptible species only a very small amount of herbicide in soybean was located in the leaves, the amount of chloroxuron present in the leaves of morning glory being some 10-fold greater. From a study of the intracellular distribution of the absorbed chloroxuron, Feeny et al (1974) also found that the chloroplast fraction of tall morning glory contained between two to five times the amount of the phenylurea herbicide associated with the corresponding fraction from soybean. Since chloroxuron is a potent inhibitor of photosynthesis, Feeny et al (1974) logically concluded that the different amount of herbicide associated with chloroplasts of tolerant and susceptible species must be considered important to selectivity. In an attempt to elucidate the possible physiological reasons for the differences between parsnip (resistant) and tomato (susceptible) with respect to their

reaction to linuron, Hogue & Warren (1968) found that whereas tomato absorbed the herbicide readily and subsequently translocated it to the leaves, parsnip retained the majority of the absorbed linuron in the root fraction and translocated only a small percentage to the leaves. Further investigation again demonstrated that in the resistant parsnip the herbicide was metabolised to a large extent whereas very little metabolism was detected in the tomato. These observations explained the results of parallel studies of the effect of linuron on photosynthetic processes in the two species (Hogue & Warren, 1968). Whereas a rapid and complete inhibition of photosynthesis resulted when tomato plants were treated with the phenylurea, in parsnip impairment of photosynthesis was only temporary and the treated plants subsequently recovered from the effects of the herbicide. Hogue & Warren (1968) therefore concluded that the selective action of linuron on the two species examined was attributable to differences with respect to accumulation of the phenylurea in leaves and its subsequent metabolism.

From an earlier study of the effects of diuron on cotton (resistant) soybean, oat and corn (susceptible), Smith & Sheets (1967) suggested that the rate of herbicide metabolism was largely responsible for the tolerance of cotton to diuron and that differences in the inherent abilities of the species examined to metabolise the phenylurea was a major factor contributing to the selective action of diuron. Where differences in susceptibility to diuron were observed among oat, soybean and corn these were explained by Smith & Sheets (1967) in terms of differences in absorption and growth rate.

Rogers & Funderburk (1968) investigated the differential susceptibility of cotton (resistant) and cucumber (susceptible) to the related fluometuron. The results revealed that fluometuron had no appreciable effect on photosynthetic CO_2 fixation in cotton but in cucumber the same rate of application caused significant decrease in the relative concentrations of sucrose, glyceric acid and hexose phosphates. It was noticed also that although cucumber absorbed significantly more herbicide than cotton over a short exposure interval this difference was no longer apparent when uptake was monitored over a longer time period. The conclusion from this study was that differences in herbicide absorption did not appear to be an important factor in the selectivity of fluometuron towards cucumber and cotton, this being in agreement with Smith & Sheets (1967) but in contrast to the conclusions of Kuratle et al (1968) and Nashed et al (1970) arising from studies with related phenylureas. From a study of the uptake and subsequent distribution of fluometuron in cotton and cucumber, Rogers & Funderburk (1968) found that cotton retained the greatest percentage of the absorbed herbicide in the root whereas in cucumber there was invariably more fluometuron associated with the shoots than with the roots. Both species were reported to be capable of metabolising the herbicide, the N-monodesmethyl derivative of fluometuron showing approximately 50% of the phytotoxicity of the parent phenylurea whereas the N-bisdesmethylated product was essentially non-phytotoxic. Of the total ^{14}C detected as metabolites in cucumber some 90% represented phytotoxic components, whilst in cotton shoots only some 30% of the total metabolite fraction was phytotoxic. Consequently, the inference from these studies was that the major factor involved in the

differential susceptibility of cotton and cucumber to fluometuron is the ability of the former species to rapidly degrade the herbicide to less or non-phytotoxic derivatives (Rogers & Funderburk, 1968).

Osgood, Romanowski & Hilton (1972) were apparently the first workers to report varietal differences in response to a phenylurea herbicide. In a study of the effects of diuron on sugar cane these workers found that cultivars differed considerably in their tolerance to diuron. There was a greater concentration of diuron in the younger leaves of a sensitive cultivar, 'H53-263', compared with the tolerant cultivar, 'H50-7209'. Since the primary site of phenylurea activity is in green leaves, it was envisaged that the higher concentration of diuron in the leaves of 'H53-263' was at least partially responsible for its sensitivity to diuron. The results of uptake and distribution studies were essentially similar to those of Rogers & Funderburk (1968) and Smith & Sheets (1967) using resistant and susceptible plants of different species in that the sensitive strain of sugar cane accumulated a greater concentration of diuron in the younger leaves than the resistant strain. Investigation of the metabolic fate of diuron in the sugar cane cultivars studied revealed that the herbicide was degraded by successive demethylation to N-monodesmethyl and N-bisdesmethylated products in both tolerant and susceptible cultivars, though the metabolism was more complete in the case of the former strains. Evidence to suggest that varieties of crop plants can differ in their degree of tolerance to particular herbicides is increasing (Van Hiele et al, 1970). Of considerable importance have been the reports that varieties of winter wheat (Triticum aestivum L.) differ markedly with respect to their tolerance

to metoxuron and chlortoluron both in the field (Holroyd & Bailey, 1970) and in glasshouse trials (Van Hiele et al, 1970; Maas, 1971). These observations have given rise to some concern in recent years because both chlortoluron and metoxuron were generally non-selective when applied on certain of the currently commercially important wheat varieties. Thus many of the modern high-yielding wheat varieties suffer an unacceptable degree of damage at the herbicide dosage required for adequate control of competitive weeds.

The desirability of varieties of crop plants showing exceptional tolerance towards particular herbicide treatments, especially in the case of weed species difficult to control, was referred to earlier. Unfortunately, however, decisions with respect to which varieties are grown are of necessity made on the basis of such factors as high yield and resistance to disease rather than on the degree to which the strain will tolerate a potentially selective herbicide. In recent years such a dilemma has arisen in the case of winter wheat production. The major weed problems in winter-sown cereals are caused by the wild oat (Avena fatua, Av. Ludoviciana, Av. sterilis L. and Av. barbata Pott.) and black grass (Alopecurus myosuroides), species which constitute a most serious threat to the successful cultivation of winter-sown wheat in particular. Although herbicides have been available for the control of these troublesome weed species for many years, a recent survey has indicated that herbicides have been used on only a relatively small proportion of the infested areas (Phillipson, 1974). Even when herbicides are used annually on the same land the problem can remain for many years. Control of severe infestations was reported to often result in large increases in crop

yield though inevitably some Avena fatua and A. myosuroides plants survived to carry the problem into the following growing season. A. myosuroides constitutes a particularly difficult control problem in that germination can occur at any time between sowing and harvesting of the crop. Some 80% of the A. myosuroides seeds scattered in the course of harvesting apparently germinate during the following season whereas the remainder may remain dormant until the second or third year.. Experiments reported by Wormell (1972) have indicated that A. myosuroides co-existing with a cereal crop does result in a percentage loss in yield. This effect is apparent even when the weed is allowed to stand with the crop for periods as short as one week, which highlights the seriousness of unwanted competition for available sunlight and nutrients. By the very nature of its habit, Avena fatua might almost be said to have been designed to survive all man's efforts to exterminate it. The various species can be categorised into winter and spring germinating types. A particular infestation might either consist of a single species or of a combination of all species recognised as being capable of themselves causing a serious infestation. A factor of importance for the survival of Av. fatua is that its inflorescence ripens and scatters before the cereal ears. In addition, hairs at the base of the seed result in them becoming attached to other objects which assists transportation to neighbouring fields.

A. myosuroides and Av. fatua thus represent serious weed problems where winter wheat is grown in England and Europe. In view of this and because of the economic importance of the crop a large number of chemicals

have been tested for their ability to selectively control these weeds (Banting, Richardson & Holroyd, 1976). It is necessary that for any chemical showing promise in this area there should be a considerable discrepancy between the concentration necessary for acceptable weed control and that which results in the first signs of injury to the crop. Cereal cultivars showing exceptional tolerance to a particular herbicide are obviously attractive since they enable a more effective weed control programme based on herbicide applications at higher rates. Among the chemicals emerging as herbicides specifically recommended for the control of these two weed species in winter sown wheat are the more recently introduced substituted phenylureas, metoxuron (Berg, 1968) and chlortoluron (Smith & Tyson, 1970) and these are now being widely used for this purpose.

The efficacy of chlortoluron for weed control in winter wheat varieties was investigated in considerable detail by Van Hiele, Hommes & Vervelde (1970). In addition to controlling a range of broad leaved weeds the herbicide was also found to be effective against A. myosuroides, Av. Fatua and Poa annua. Selectivity in winter cereals was retained up to a chlortoluron concentration of some 4 Kg/ha. These observations have been confirmed by Tysoe (1974) who also reported that whereas pre- and post-emergence applications of chlortoluron appeared equally effective in controlling A. myosuroides, consistently better control of Avena fatua resulted from treatment at the pre-emergence stage. Ummel, Eder, Lichtblau & Stockl (1974) have also reported good control of Avena fatua and Alopecurus myosuroides in winter wheat by metoxuron both in the field and in greenhouse trials. In addition to studies with metoxuron and chlortoluron, Hewson (1974) also reported results obtained for a new

phenylurea, isoproturon. In agreement with the earlier reports, all three herbicides selectively controlled Alopecurus myosuroides and Avena fatua in winter wheat.

In view of the levels of control of wild oat and black grass that could be achieved in stands of winter wheat by use of chlortoluron or metoxuron and more recently, by isoproturon, the observations (Holroyd & Bailey, 1970; Maas, 1971) that a number of commercially important and high yielding wheat varieties, such as Maris Beacon, Maris Huntsman and Maris Nimrod, were themselves susceptible stimulated a thorough investigation of the differences in varietal response of winter wheat cultivars to these herbicides. Van Hiele et al (1970) compared the responses of selected cultivars of winter wheat to chlortoluron applied both pre- and post-emergence under glasshouse conditions. Whereas the cultivars Manella, Tadorna and Eno were all killed at a dosage equivalent to 8 Kg/ha one cultivar, namely Caribo, showed considerable tolerance to the herbicide applied at this high rate. Similar results have been obtained by Hewson (1974) and Hubbard and Livingston (1974) following treatment of wheat varieties with metoxuron and chlortoluron in field trials. Varieties susceptible to the phenylureas were severely scorched and thinned by herbicide application at various stages of growth, though these effects were not evident in the resistant varieties. Interestingly there was no evidence of varietal susceptibility to the related isoproturon. Similar results were obtained by Tottman, Holroyd, Lupton, Oliver, Barnes & Tysoe (1975) in a more recent study of the tolerance of chlortoluron and isoproturon by winter wheat varieties. Responses to isoproturon showed no clear-cut grouping of varieties, any differences being attributed

to the expression of several genetic characters interacting with the environment. In the case of chlortoluron, however, the varieties studied segregated into two distinct groups, one tolerant and the other susceptible. Inheritance studies showed a distinct segregation in the progeny of crosses between resistant and susceptible varieties, implying that herbicide tolerance was simply inherited and that it should be possible to include selection for tolerance of metoxuron and chlortoluron in a wheat breeding programme. On the basis of these results Tottman et al (1975) suggested that resistance to chlortoluron might be explained in terms of a simply inherited detoxifying mechanism in addition to genetic and environmental factors similar to those affecting isoproturon tolerance. Evidence for a genetic basis for the differences in susceptibility of winter wheat cultivars to chlortoluron and metoxuron has also been obtained by Stryckers, Van Himm, Persijn & Bulcke (1974). These workers concluded that the property of susceptibility to p-methyl- or p-methoxy-substituted N'-(phenyl)-N,N-dimethylurea compounds was derived from the parent line 'Pansar III', which is itself very susceptible. Tolerance, on the other hand, was considered to be derived from two parent cultivars 'Hybride du Joncquois' and 'Vilmorin 27'. Where intermediate responses were observed in some cultivars these were attributed to genetically determined differences in physiology and/or morphology rather than inability to detoxify the applied herbicide.

The investigations reported in the succeeding chapters concern studies made in this department by the present author on the biochemistry of the varietal susceptibility of winter wheat cultivars to metoxuron.

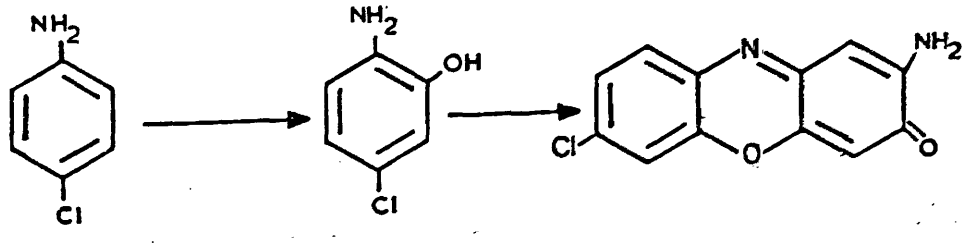
MOLECULAR FATE OF THE SUBSTITUTED PHENYLUREA HERBICIDES

Several published reports clearly indicate that plants and soil microorganisms are fully capable of metabolic attack on substituted phenylurea herbicides to which they are exposed in their environment (Freed, Montgomery & Kief, 1961; Geissbühler, 1969; Ashton & Crafts, 1973). In parallel with the situation in microorganisms, plants may modify phenylureas in several ways. In some species the metabolism may take the form of conjugation of the externally applied herbicide with proteins or carbohydrates or with simpler compounds such as amino acids and flavin mononucleotide (Freed et al, 1961). In addition the phenylureas may undergo demethylation, hydroxylation, hydrolysis and other chemical modifications (Ashton & Crafts, 1973). Results of several investigations have indicated that soil microorganisms play an important role in the degradation of soil-applied substituted phenylurea herbicides. Early studies made by Muzik et al (1954) showed that conditions which are well known to increase microbial activity in soils such as high temperatures, high moisture content and high organic matter also hastened the inactivation of phenylurea herbicides applied to the soil. Hill, McGahen, Baker, Finnerty & Bingeman (1955), Sheets & Crafts (1957) and Geissbühler et al (1963b) have independently compared rates of inactivation of phenylureas in sterilized and non-sterilized soils. Diuron for example was found to be consistently more persistent in sterilized than in non-sterilized soils, thus providing further evidence in support of the involvement of microorganisms in phenylurea degradation (Sheets & Crafts, 1957).

The first systematic search for organisms capable of decomposing phenylurea herbicides was carried out by Hill et al (1955). They succeeded in isolating a pseudomonad capable of utilising monuron as sole carbon source, the rate of oxygen uptake by the organism being shown to increase in parallel with herbicide concentration. A subsequent report (Hill & McGahen, 1955) showed that other species of common soil bacteria, belonging to the geni Xanthomonas, Sarcina and Bacillus were also able to utilize monuron as sole carbon source for growth on agar plates.

In a study of the decomposition of seven phenylurea herbicides by Bacillus sphaericus isolated from soil, Wallnofer (1969) found that the organism was able to metabolise the methoxy compounds monolinuron, linuron and metobromuron by the removal of CO_2 from the ureido portion of the molecule, leaving the corresponding chloroaniline moieties. Metabolism of linuron to 3,4-dichloroaniline was also achieved by disrupted cell preparations of B. sphaericus. In contrast, however, the organism was reported to be unable to decompose herbicides such as monuron, diuron and fluometuron. From a more detailed investigation of the degradation of substituted phenylurea herbicides by cell-free extracts of B. sphaericus Wallnofer & Bader (1970) reported that the N-methoxy-phenylureas were inactivated in the same manner as that reported for whole cells. In support of the earlier data obtained using whole cells they also found that the N,N-dimethylphenylurea herbicides tested were not significantly attacked by the enzyme system involved.

The ability of various soil bacteria to further metabolise products of hydrolysis of phenylureas has been investigated by a number of workers. Walker & Harris (1969) have isolated from soil a pseudomonad able to utilize aniline, a cleavage product of fenuron, as its sole carbon source. Washed cells of the organism oxidised aniline, by means of an induced enzyme system, with the concomitant liberation of ammonia leading Walker & Harris (1969) to postulate that the early stages of aniline metabolism involve oxidation to catechol which may subsequently be cleaved by the action of dioxygenase enzymes. More recently Briggs & Walker (1973) demonstrated that the growth of another soil isolate, Alcaligenes faecalis, on 4-chloroaniline as sole carbon source was accompanied by the production of a yellow pigment. The pigment, identified as a chlorinated phenoxazinone, was considered to result from a condensation reaction between two molecules of 5-chloro-2-aminophenol, hence establishing the first step in the microbial metabolism of 4-halogenoaniline as an hydroxylation ortho to the amino group.



Briggs & Walker (1973) speculated that the chlorophenoxazinone probably represented a by-product, the major role of the 5-chloro-2-aminophenol

being as a precursor to 4-chloroaniline formed prior to ring fission. In another report Briggs & Ogilvie (1971) observed a pink product in the aqueous phase of soil slurries containing 3-chloro-4-methoxyaniline, the parent amine of the phenylurea herbicide metoxuron and its N-mono-desmethylated metabolites. The pink product was identified as a mixture of 3,3'-dichloro-4,4'-dimethoxyazobenzene, 3-chlorobenzoquinone-4-(3-chloro-4-methoxy)anil and 2-3'-dichloro-4-hydroxy-4'-methoxy-diphenylamine, a reduced product of 3-chloro-4-methoxy aniline.

The first study concerning the metabolism of substituted phenyl-urea herbicides in plants was carried out by Fang et al (1955). Following foliar application of monuron to French bean (Phaseolus vulgaris var. Black Valentine) they observed a time-dependent formation of a monuron complex which, upon acid hydrolysis, yielded the unchanged herbicide. The component which complexed with the herbicide was later suggested by Freed et al (1961) to be of a low molecular weight protein or peptide nature.

Nashed & Ilnicki (1970) have more recently reported that a certain proportion of a phenylurea herbicide taken up by plants may become bound to protein components. In this study the roots of maize seedlings were treated with linuron and the protein-bound herbicide was subsequently extracted and separated according to the methods developed by Katz (1967), After the initial extraction of the plant material with acetone, appreciable amounts of additional herbicide were released from both root and shoot residues by submitting them to alkaline digestion or treatment with a proteolytic enzyme such as ficin. Further evidence for the binding of phenylureas to proteinaceous components within the

plant was obtained by Voss & Geissbühler (1966) in a study of the metabolic fate of fluometuron and metobromuron in cotton, wheat and potato. The influence of such factors as temperature, pH, ionic strength and chemical modification on the extent of sorption of phenylureas by protein has been investigated by Camper & Moreland (1971) in an in vitro system using diuron and bovine serum albumin. Studies with diuron derivatives suggested that the amide nitrogen and carbonyl oxygen function of the phenylamide were involved. The results also indicated that the molecular conformation of the protein also appeared to be important in herbicide binding. Conformational changes such as an unfolding in response to temperature tended to increase sorption, whereas a degree of contraction produced by changes in ionic strength resulted in decreased sorption of the diuron, suggesting that the herbicide may become bound to sites within the interior of the protein structure. Increased chlorination of the phenyl ring which would be expected to increase lipophilicity and relative acidity also resulted in a greater degree of binding of dimethylphenylureas. The light-dependent formation of a monuron-flavin mononucleotide complex observed by Sweetser (1963) in Chlorella also represents a potential complexing reaction which may result in inactivation of the herbicide. The binding of phenylurea herbicides to various constituents of plant tissues may therefore prevent phytotoxic material from reaching the site of biological activity and result in a modification of the dosage required to produce a given response.

Following their studies of the metabolic fate of chloroxuron in cultures of various soil bacteria Geissbühler et al (1963b) investigated the metabolism of the phenylurea in several weed and crop plants.

In common with other phenylureas studied and with the situation in bacteria, demethylation appeared to be the primary detoxification mechanism (Fig. 4), removal of one and both methyl groups from the parent dimethylated urea resulting in partial and complete reduction in phytotoxicity respectively. The very slow rate of release of $^{14}CO_2$ in plants treated with (carbonyl-^{14}C)-chloroxuron suggested that hydrolysis of the resulting N-bisdesmethylated phenylurea to give 4-chloro-4'-amino-diphenylether proceeds only at a very low rate. Very similar results were obtained in a study of the metabolic fate of monuron and diuron in cotton, soybean, oat and maize (Smith & Sheets, 1967) and of linuron in maize, soybean and crabgrass (Nashed & Ilnicki, 1970).

Onley, Yip & Aldridge (1968) studied the detoxication of diuron in maize seedlings and found that the mechanism involved was consistent with the route proposed by Geissbühler et al (1963b) and Smith & Sheets (1967). In general it was found that the relative concentrations of each metabolite found in the total methanol extracts decreased according to the sequence diuron>N-monodesmethyl diuron>N-bisdesmethylated diuron>3,4-dichloroaniline>3,4-dichloronitrobenzene. The amount of each compound present increased with time of exposure, indicating continued adsorption and metabolism. The identification of 3,4-dichloronitrobenzene in the plant extracts suggested that the small amount of 3,4-dichloroaniline formed may undergo oxidation. Very similar results were obtained in a study of the metabolic fate of fluometuron in cotton and wheat and of metobromuron in potato (Voss & Geissbühler, 1966). Further degradation to the corresponding free

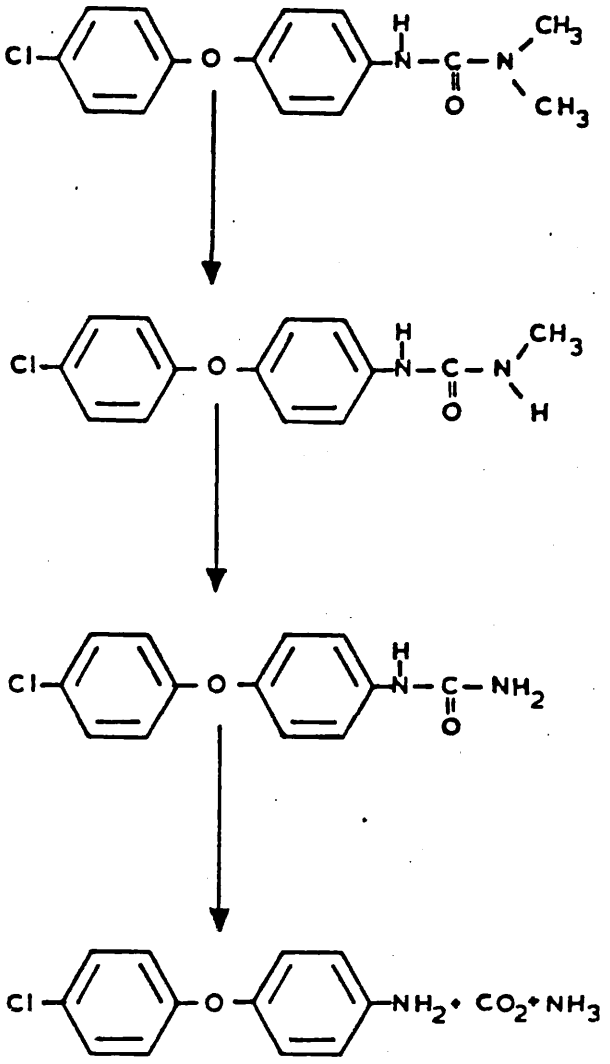


Fig. 4: Scheme for the metabolism of chloroxuron via N-demethylation and deamination/decarboxylation in various plant species (from Geissbühler et al, 1963b)

aromatic amines following an initial stepwise demethylation was either non-existent or very limited, and no ring-splitting or dehalogenation of either herbicide was observed.

Swanson & Swanson (1968) studied the metabolic fate of monuron and diuron in leaf discs prepared from cotton, soybean, maize and plantain. Though these studies confirmed the ability of certain species such as cotton and plantain to degrade phenylurea herbicides by progressive demethylation and hydrolysis, differences in the relative proportions of the various metabolites were observed between species. Thus whereas cotton and plantain appeared equally effective in demethylating the applied phenylurea, the ability to hydrolyse the amide bond was apparently greater in cotton which contained significantly larger quantities of the corresponding anilines. No demethylation was detectable in leaf discs of maize thus supporting data obtained using whole plants (Smith & Sheets, 1967; Onley et al, 1968), but in soybean discs demethylation occurred but apparently ceased at the level of the N-monodesmethyl-derivatives.

Rogers & Funderburk (1968) studied the detoxication of fluometuron in cotton and found that the mechanism involved was consistent with the route proposed by Geissbühler et al (1963b) and Smith & Sheets (1967). Similar results were obtained by Menashe & Goren (1973) in a study of the metabolic fate of fluometuron in citrus seedlings. Nashed et al (1970) conducted a time-course study of the metabolic fate of chlorbromuron (N'-(3-chloro-4-bromophenyl)-N-methoxy-N-methylurea) in cucumber and maize. In general it was found that a small portion of the parent compound was demethylated to give the N-desmethyl derivative

and that this metabolite increased with time whereas the N-desmethoxy and 3-chloro-4-bromophenylurea derivatives were detectable only at longer times after treatment. The proportion of the N-desmethoxy metabolite remained fairly constant relative to the amount of the parent herbicide present whilst the 3-chloro-4-bromophenylurea showed a marked increase in the week-old sample. There was no indication of the corresponding aniline derivative until the first week of treatment and even then this represented only a very small percentage of the total herbicide absorbed by the tissue.

In an investigation of the metabolism of monuron in excised leaves of French bean (Phaseolus vulgaris L. var. Black Valentine) and maize (Zea mays var. Batam Cross) Lee & Fang (1973) found that the leaves of both species were able to convert monuron to N'-(4-chlorophenyl)-N-methylurea, p-chlorophenylurea and unknown conjugates. Three metabolite conjugates were tentatively identified as the β-D-glucosides of N'-(2-hydroxy-4-chlorophenyl)-N-methylurea, N'-(2-hydroxy-4-chlorophenyl)-N,N-dimethylurea and 2-hydroxy-4-chlorophenylurea respectively (see Fig. 5), the identity of the latter two compounds being later confirmed by means of mass spectrometry (Lee, Griffin & Fang, 1973). Though at a herbicide concentration of 16 ppm aromatic hydroxylation represented the dominant pathway of monuron metabolism in French bean, sequential demethylation was invariably the major pathway of monuron transformation in maize in which the products of aromatic ring hydroxylation constituted minor metabolites only. Resulting from these studies Lee & Fang (1973) produced a scheme (Fig. 5) showing the various metabolic conversions of monuron in plants. Two additional major polar metabolites of monuron

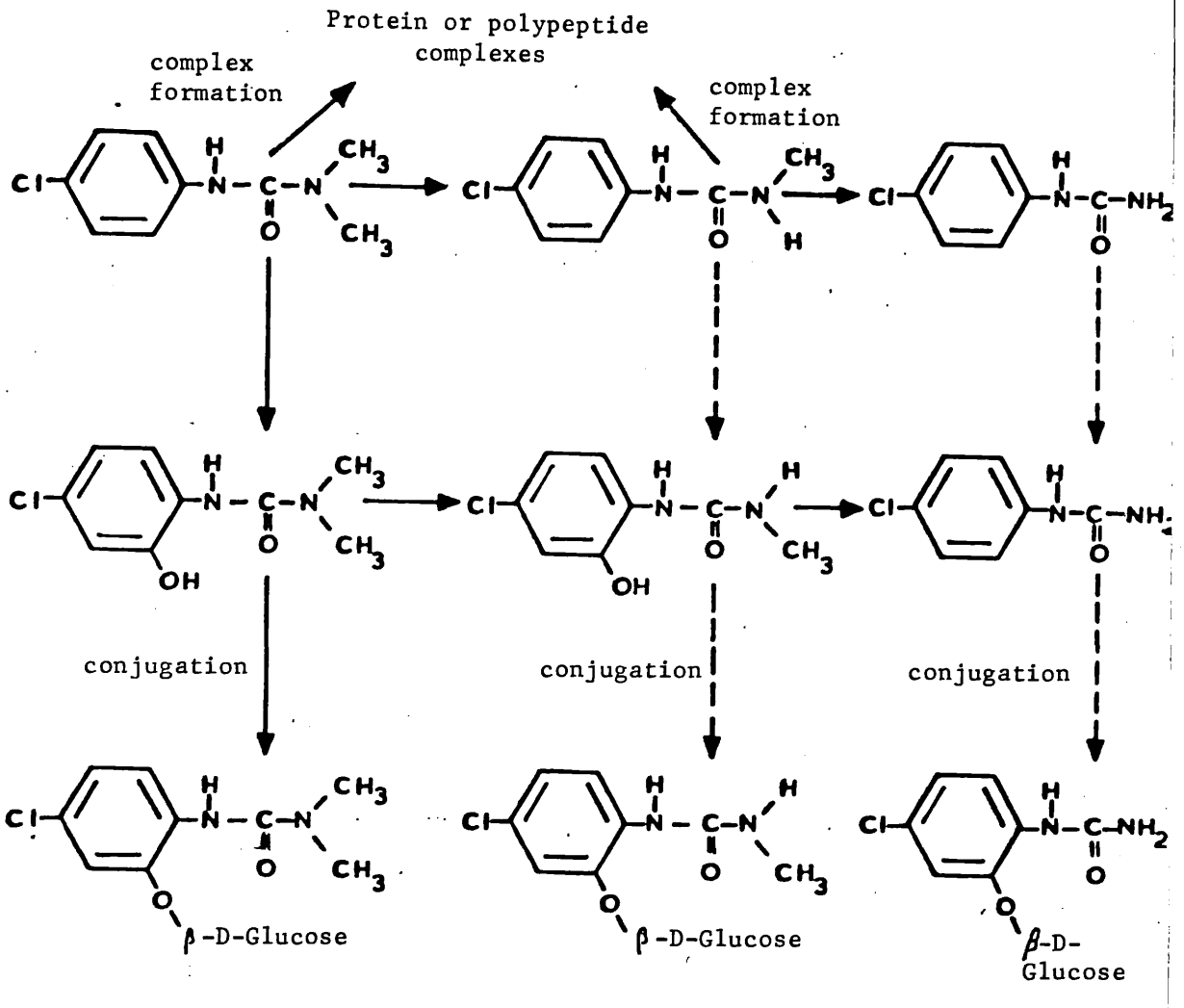
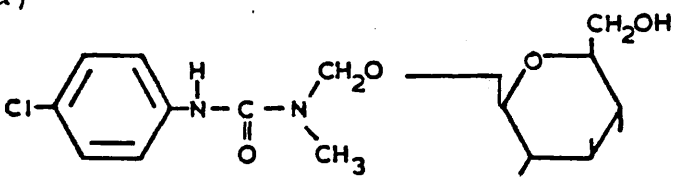


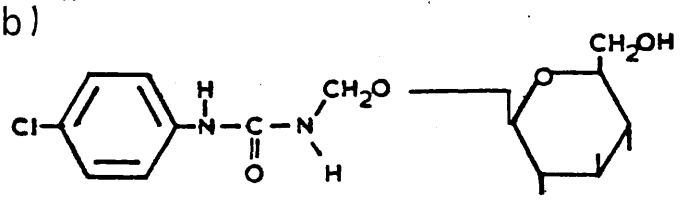
Fig. 5: Proposed routes of monuron metabolism in maize and french bean (After Lee & Fang, 1973)

have been reported in excised leaves of cotton (Frear & Swanson, 1972). These metabolites, which constituted some 20-25% of the total methanol soluble monuron metabolites present 24 h after treatment, were identified as β-D glucosides of N'-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea (a) and N'-(4-chlorophenyl)-N-hydroxymethylurea (b).

(a)



(b)



Isolation and identification of these polar monuron metabolites provided direct evidence for the formation of N-hydroxymethyl intermediates in the oxidative N-demethylation of substituted phenylurea herbicides by higher plants. In addition, some 20% of the ^{14}C-label applied to the leaves remained associated with the methanol-insoluble residue. A small fraction of this ^{14}C remained insoluble after hydrolysis with

1N hydrochloric acid for 16 h, the major portion of the ^{14}C recovered by this treatment being p-chlorophenylurea. Further treatment with trypsin, ficin, pepsin, protease and cellulose enzymes or with 6NHCl at 110°C failed to release significant additional quantities of methanol soluble ^{14}C-metabolites, suggesting that the residual insoluble ^{14}C-label may be associated with the lignin fraction of the plant. Other evidence that insoluble phenylurea residues may be associated with the lignin fraction was obtained by Neptune (1970) in a study of the metabolic fate of fluometuron in wheat and maize. Based on the results of the present studies and on their earlier work (Frear, Swanson & Tanaka, 1969), Frear & Swanson (1972) proposed a scheme for the metabolism of monuron in excised cotton leaves. The pathway proposed (Fig. 6) suggests a key role for N-hydroxymethyl intermediates, particularly N'-(4-chlorophenyl)-N-hydroxymethylurea in the formation of β-D-glucosides, and other polar, as yet unidentified methanol soluble metabolites and insoluble residues of monuron in higher plants. More recently, Frear & Swanson (1974) were able to confirm the earlier report by Lee & Fang (1973) of the existence of polar ring hydroxylation metabolites of monuron in that they were also able to detect a minor polar metabolite of monuron in cotton, and identified this as being an o-glycoside of 2-hydroxy-4-chlorophenylurea. Previous studies with other substituted phenylureas such as metobromuron in tobacco (Geissbühler & Voss, 1971) and fluometuron in wheat and maize (Neptune, 1970) had suggested that both of these herbicides were ring-hydroxylated, but specific metabolites and aglycones were not isolated and identified.

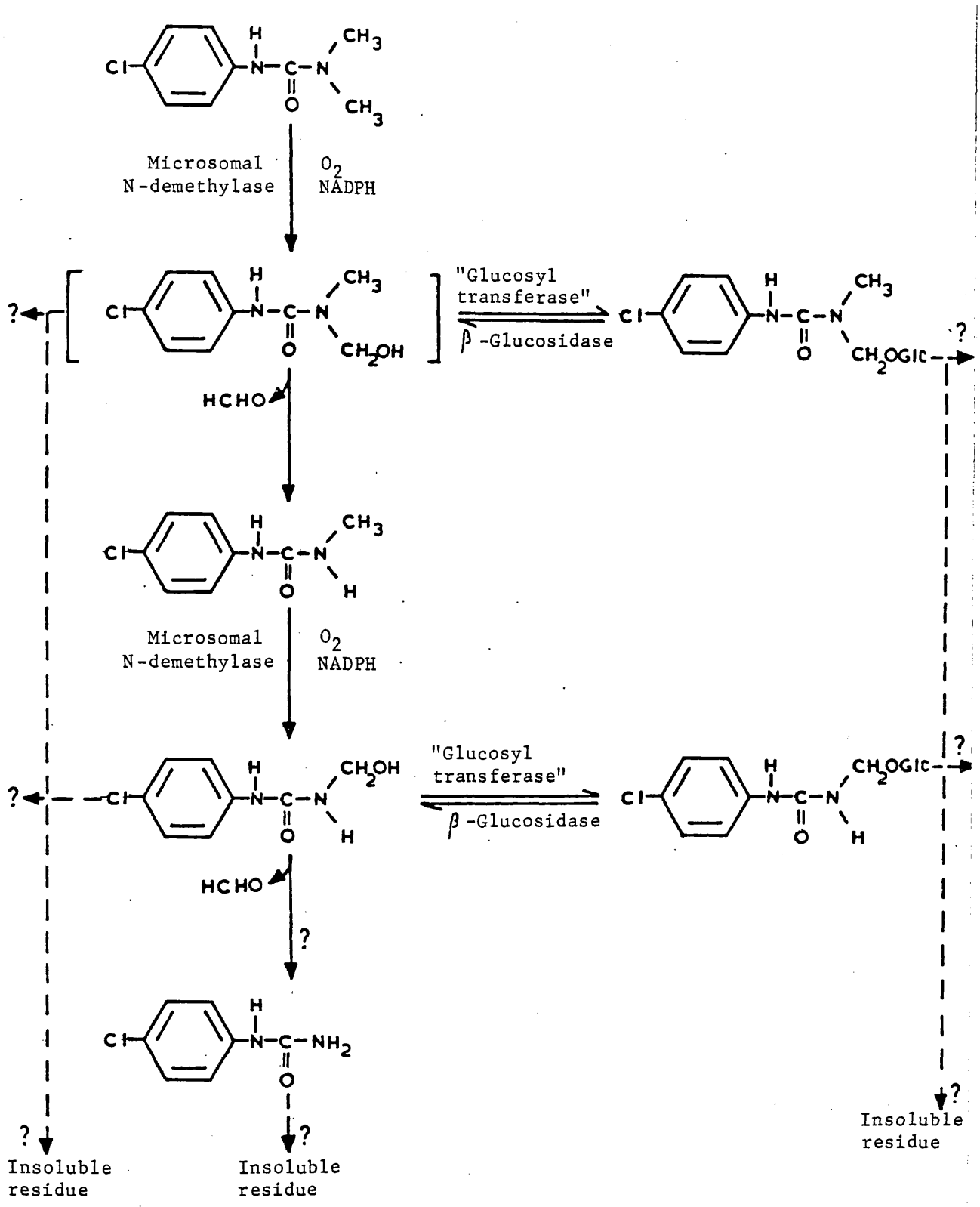


Fig. 6: Scheme for the metabolism of monuron in cotton leaves
(After Frear & Swanson, 1972)

The evidence for the metabolism of substituted phenylurea herbicides by various plant species is thus very considerable. The most common degradative route appears to proceed via N-demethylation and subsequent hydrolysis of the amide bond. Since these metabolic transformations constitute an effective detoxication mechanism an ability in a particular plant species to carry out these reactions confers a degree of immunity to substituted phenylureas. The more recent data on ring and methyl hydroxylation reactions as additional routes of phenylurea metabolism have been obtained using very few plant species only, in particular, cotton, and to the author's knowledge a study of the phytotoxicity of the products of hydroxylation reactions has not been carried out. It is consequently difficult to assess whether the hydroxylation pathways are of importance in the detoxication of the phenylureas in question.

N-dealkylation of various drug molecules is a common detoxification reaction of the microsomal fraction of the livers of mammalian species, this process usually requiring oxygen and NADPH as cofactor (Brodie, Gillete & La Du, 1958; Shuster, 1964). One of the first studies concerning the metabolism of substituted phenylurea herbicides in mammalian tissue was carried out by Ernst & Böh me(1965). Following oral administration of monuron and monolinuron (N'-(4-chlorophenyl)-N-methoxy-N-methylurea) to rats, the majority of the metabolites excreted in the urine retained the urea moiety. In common with the situation in bacteria and higher plants, hydroxylation and dealkylation appeared to be the primary detoxification mechanism in the rat. Very similar results were also obtained in a study of the metabolic fate of diuron and linuron in the rat (Böh me& Ernst, 1965). In an <u>in vivo</u> study of the metabolic fate of (trifluoromethyl-^{14}C)-fluometuron in the rat, Boyd & Fogleman

(1967) detected the N-monodesmethyl and N-bisdesmethylated compounds, these being excreted as conjugated products in the urine. A very small amount of the ^{14}C-labelled herbicide administered was excreted as the aniline derivative, though no $^{14}CO_2$ was detected during the first 48 h after administration.

In a study of the metabolism of chlortoluron, fluometuron and metobromuron in human embryonic lung cell cultures, Lin, Menzer & North (1976) detected metabolites resulting from oxidative reactions and identified these as N'-(3-chloro-4-methylphenyl)-N-formyl-N-methylurea, N'-(3-chloro-4-methylphenyl)-N-formylurea, N'-(3-chloro-4-methylphenyl)-N-methylurea and 3-chloro-4-methylphenylurea derived from chlortoluron, N'-(3-trifluoromethylphenyl)-N-formyl-N-methylurea, N'-(3-trifluoromethylphenyl)-N-methylurea and 3-trifluoromethylphenylurea from fluometuron and N'-(4-bromophenyl)-N-methylurea, p-bromophenylurea and N'-(4-bromophenyl)-N-hydroxy-N-methylurea in the case of metobromuron.

More recently, Hinderer & Menzer (1976a) made an *in vitro* study of chlortoluron metabolism using postmitochondrial, microsomal and supernatant fractions prepared from liver, testis, lung and kidney tissue of the rat. These experiments failed to reveal any evidence of degradation of chlortoluron by any of the postmitochondrial, microsomal or final supernatant fractions prepared from the various tissues. In addition, Mücke, Menzer, Esser & Geissbühler (1976) found that chlortoluron was not metabolised by rat liver microsomes even though it was extensively degraded by rats *in vivo*. Again, in a very similar experiment with Japanese quail, Hinderer & Menzer (1976b) found that chlortoluron was not metabolised by microsomal and supernatant preparations from any tissue nor by

postmitochondrial fractions from lung, kidney and testis. The post-mitochondrial fraction from quail liver homogenates was the only preparation found to degrade chlortoluron, the metabolites detected in significant quantities following 3-5 h incubations being N'-(3-chloro-4-methylphenyl)-N-methylurea, 3-chloro-4-methylphenylurea, N'-(3-chloro-4-carboxyphenyl)-N,N-dimethylurea, N'-(3-chloro-4-carboxyphenyl)-N-methylurea, N'-(3-chloro-4-hydroxymethylphenyl)-N-methylurea, N'-(3-chloro-4-hydroxymethylphenyl)-N,N-dimethylurea, 3-chloro-4-hydroxymethylphenylurea, N'-(3-chloro-4-methylphenyl)-N-formyl-N-methylurea and N'-(3-chloro-4-methylphenyl)-N-formylurea. This data led Hinderer & Menzer to propose a scheme (Fig. 7) for the metabolism of chlortoluron in postmitochondrial preparations of Japanese quail liver. Degradation of chlortoluron to N'-(3-chloro-4-hydroxymethylphenyl)-N-methylurea appeared to be the major metabolic pathway, the isolation of the intermediate N-formyl compounds suggesting that the metabolic pathway proceeds through conversion of the parent compound to N'-(3-chloro-4-methylphenyl)-N-formyl-N-methylurea and conversion of N'-(3-chloro-4-methylphenyl)-N-methylurea to N'-(3-chloro-4-methylphenyl)-N-formyl urea. The study revealed an additional metabolite which did not co-chromatograph with any of the unlabelled standards. However, upon acid hydrolysis this metabolite yielded N'-(3-chloro-4-carboxyphenyl)-N-methylurea. Along with the R_f values obtained from thin-layer chromatography, this indicated that the unknown metabolite was probably N'-(3-chloro-4-carboxyphenyl)-N-formyl-N-methylurea, which has also been isolated from the urine of rats treated with chlortoluron and characterised by mass spectrometry (Mucke, Menzer, Esser & Geissbühler, 1976).

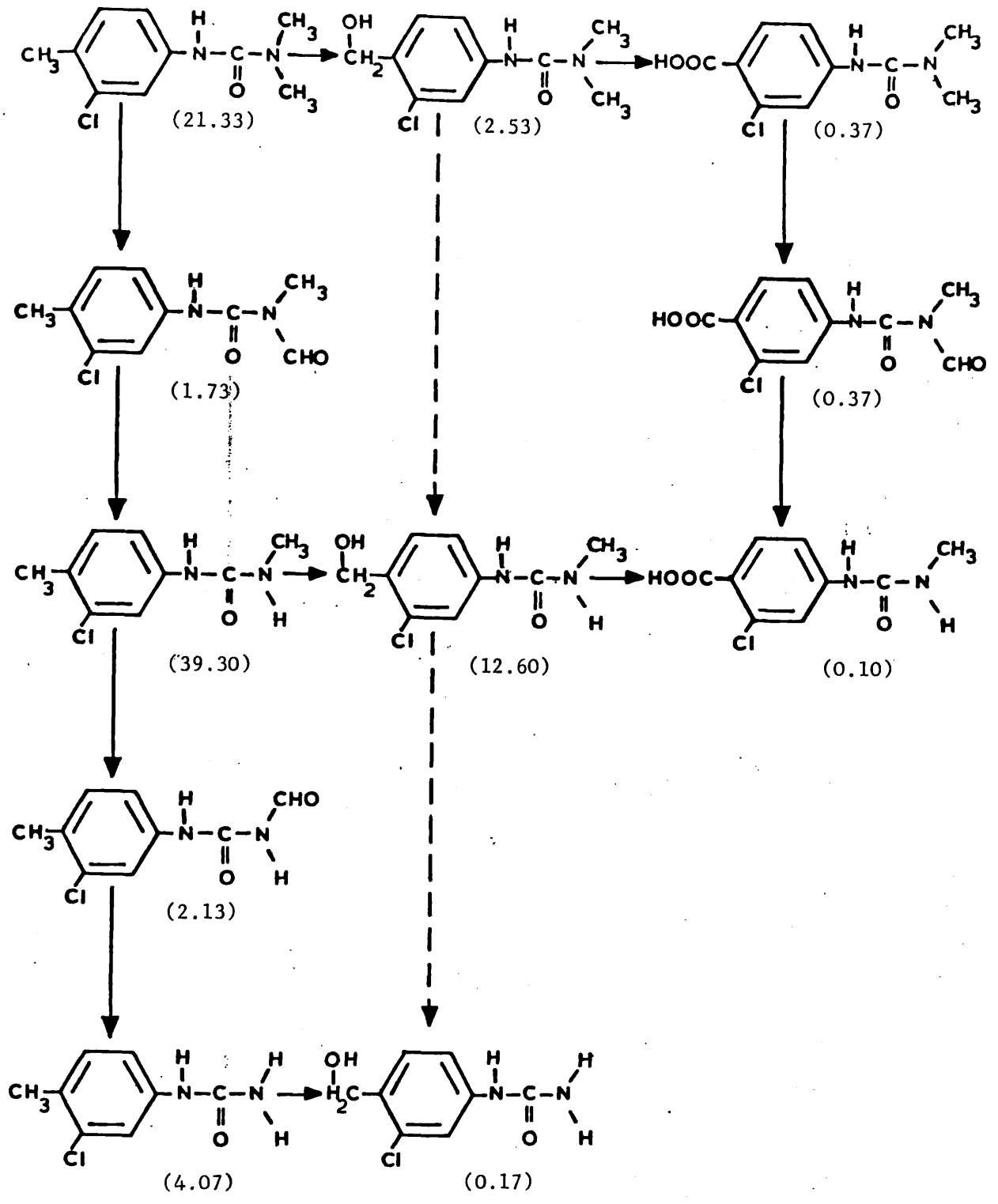
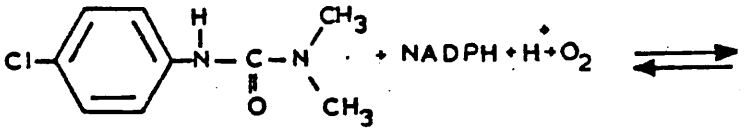
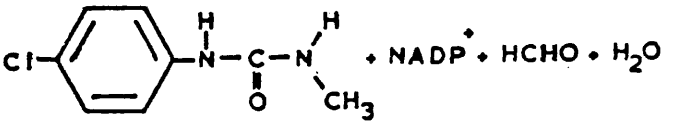


Fig. 7: Routes of chlortoluron metabolism in a liver post-mitochondrial preparation from Japanese quail (After Hinderer & Menzer, 1976b)

Figures in parenthesis represent the percentage of the total applied radioactivity recovered in the individual metabolites.

Earlier investigations had shown that diuron and certain other substituted phenylureas cause an increase in the activity of rat hepatic microsomal enzymes (Kinoshita & Du Bois, 1970). Hence, it would appear feasible that a similar microsomal enzyme system might effect N-dealkylation of phenylureas in plants. In an attempt to provide experimental evidence in support of this reasoning, Frear (1968) initiated an <u>in vitro</u> study of monuron metabolism in cotton, a species capable of rapid detoxication of the foliar applied herbicide. He was able to confirm the hypothesis by isolating an NADPH and oxygen dependent microsomal enzyme system capable of demethylating monuron to the N-mono-desmethyl derivative:-





An extension of the study to include plantain, buck wheat and broad bean revealed that the leaves of these species also contained an active microsomal monuron N-demethylase system, the enzyme being specific for substituted N'-(phenyl)-N-methylurea compounds (Frear, Swanson & Tanaka, 1969). The observed inhibition of the enzyme by carbon monoxide, ionic

detergents, sulphydryl reagents, chelating agents and electron acceptors and the demonstrated presence of a cytochrome b_5 and an active NADPH-cytochrome c reductase have been taken as confirmatory evidence for a microsomal electron transport system, similar to that reported for animals, operating in plants (Frear et al, 1969). Results of preliminary experiments on the metabolism of fluometuron by a microsomal preparation from citrus rootlets have led Menashe & Goren (1973) to suggest that this tissue contains an enzymatic demethylation system similar to that reported by Frear (1968) for cotton.

I N T R O D U C T I O N

Photosynthesis in Higher Plants

PHOTOSYNTHESIS IN HIGHER PLANTS

Photosynthesis is the process by which green plants reduce CO_2 to carbohydrates and oxidise water to O_2 at the expense of energy derived from sunlight. The process occurs in the chloroplasts of green plants and algae, the light energy being absorbed primarily by the pigment chlorophyll, and subsequently stored to the extent of some 5.1 eV/carbon atom (Arnold, 1976).

The reactions of photosynthesis can be conveniently divided into a light and dark phase. The light phase involves energy absorption which supports an electron transport pathway in chloroplasts which yields oxygen and NADPH (Hill reaction) and is coupled to ATP formation. The dark phase, on the other hand, does not require light and involves the reduction of carbon dioxide to carbohydrate at the expense of the ATP and reducing power generated in the light phase.

The Light Reaction of Photosynthesis

Since a knowledge of the mechanism of the light reaction of photosynthesis is a prerequisite for the understanding of the mode of action of the substituted phenylurea herbicides, and that data is presented in the present thesis concerning the effect of the phenylurea, metoxuron, on electron transport and some of the associated reactions of photosynthesis in wheat, a discussion of the light reaction of photosynthesis would seem particularly relevant. However, in view of the enormous literature which exists in this field it would appear almost futile to attempt an historical review of all of the information relevant

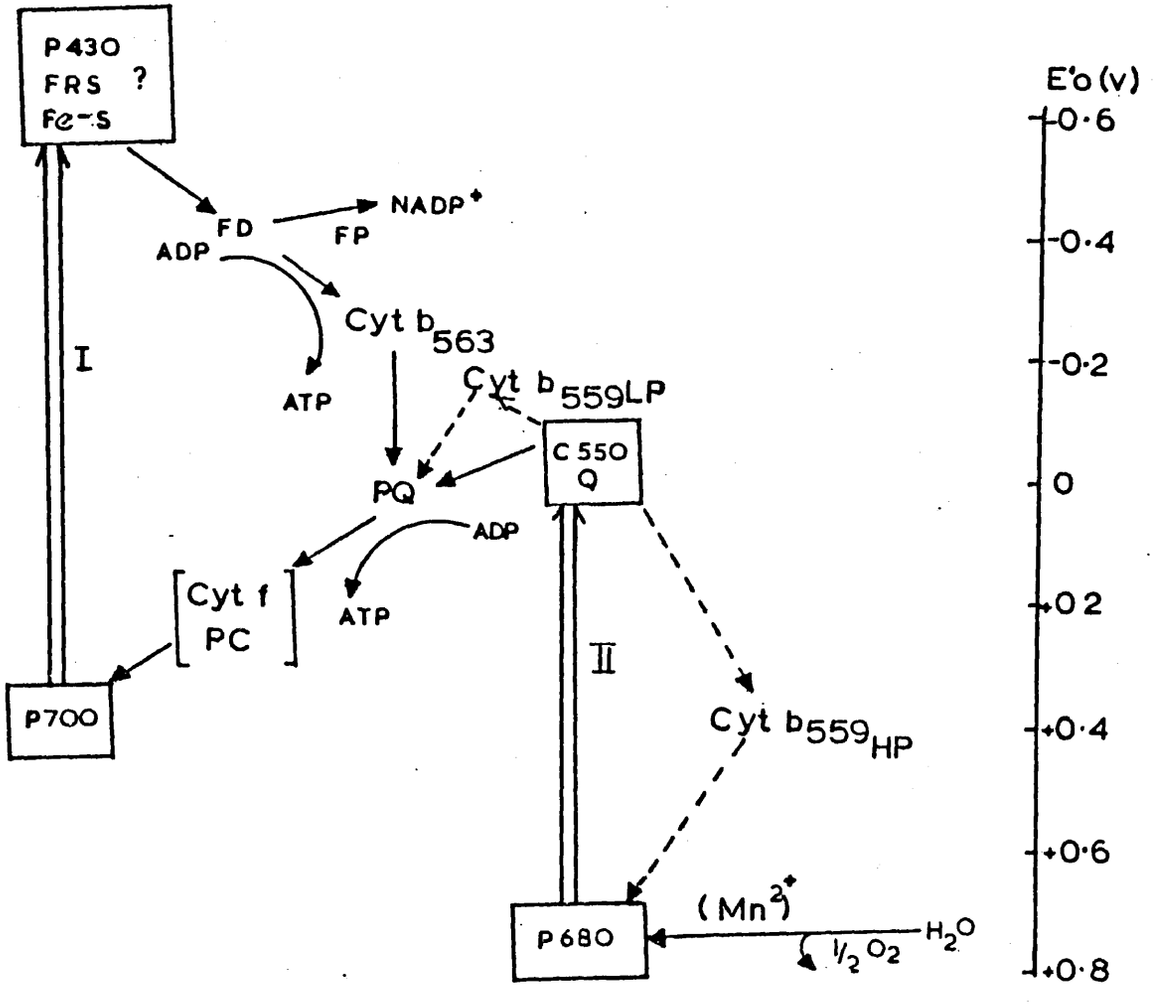
to the present study. Thus it seems reasonable to state, without detailed documentation, a number of generally accepted view points which form the basis of the experimental approach, and to reserve a more in-depth consideration of the literature to those aspects which bear directly on specific investigations and on the site of action of the substituted phenylurea herbicides. Many excellent articles dealing with the more general concepts of photosynthesis have appeared in the literature. Notable among these are the reviews of Boardman (1968, 1970), Walker & Crofts (1970), Owen (1971), Tollin (1974), Trebst (1974), Delaney (1975), Radmer & Kok (1975), Thornber (1975), Calvin (1976) and Cramer & Whitmarsh (1977).

The path of electron transport in photosynthesis used as a basis for the present investigation is shown in Fig. 8 which presents the arrangement of the popular Z-scheme for non-cyclic and cyclic electron flow generally accepted by the majority of workers in the field. Possible sites of photophosphorylation are shown although a current view is to think in terms of proton translocation across the membrane being coupled to ATP formation rather than to define sites of photophosphorylation (Hall & Evans, 1972). A different model for photosynthetic electronflow, involving three light reactions and separate non-cyclic and cyclic systems has been proposed (Knaff & Arnon, 1969a & b; Arnon, Knaff, McSwain, Chain & Tsujimoto, 1971) but will not be presented in detail in the present discussion since it is based on a very limited amount of evidence and has received little support in the literature.

Fig. 8: Current view of the conventional Z-scheme for photosynthetic electron transport

(Based on Hall & Evans, 1972 and Cramer & Whitmarsh, 1977)

PQ = plastoquinone; PC = plastocyanin;
FRS = ferredoxin-reducing substance; Fe-S = bound iron-sulphur protein;
Fd = ferredoxin; Fp = ferredoxin-NADP reductase (flavoprotein).
C550, Q, P_{430}, etc., have their conventional meaning.


P430
FRS ?
Fe-S
FD
FP
NADP+
ADP
ATP
Cyt b563
Cyt b559LP
C 550
Q
PQ
ADP
ATP
Cyt f
PC
P700
I
II
Cyt b559HP
P680
(Mn2+)
H2O
1/2 O2
E'o (v)
-0·6
-0·4
-0·2
0
+0 2
+0·4
+0·6
+0·8

In an overall sense, photosynthesis represents a photopotentiated transfer of electrons against a thermodynamic energy gradient from a donor (H_2O in the case of green plants) to an acceptor (generally pyridine nucleotide). A variety of electron carriers participate as intermediates in this process, e.g., plastoquinone, cytochromes, plastocyanin, non-haeme iron proteins and flavoproteins. Light energy enters the system at two points in green plants. One of these is located on the water oxidation side (photosystem 2) and the other on the pyridine nucleotide reduction side (photosystem 1). Specialised forms of chlorophyll participate in the energy conversion processes occurring at the so-called reaction centres in these two photosystems (Katz & Norris, 1973). These are distinguishable from the bulk of the chlorophyll, which serves a light harvesting function and feeds energy into reaction centres by non-radiative transfer, mainly on the basis of absorption spectral properties although recent developments have permitted fractionation of chloroplasts so as to provide a structural as well as functional separation (Tollin, 1974). The reaction centre chlorophyll of photosystem 2 (PS2) is usually designated P680 (Floyd, Chance & Devault, 1971) and that of photosystem 1 (PS1) as P700, based on the wavelengths at which spectral changes can be observed which correlate with the functioning of these species. In the most commonly accepted scheme of photosynthesis (Fig. 8) these two light reactions operate in series and are connected by a chain of electron carriers.

The reaction centre is one of the fundamental concepts in photosynthesis and is usually envisaged as a special chlorophyll complex associated with a primary electron donor (D) and acceptor (A). Upon excitation the following reaction sequence is presumed to occur

(Radmer & Kok, 1975) :

$$D\ Chl\ A \xrightarrow{h\nu} D\ Chl^{*}\ A \longrightarrow D\ Chl^{+}A^{-} \longrightarrow D^{+}\ Chl\ A^{-}$$

In photosystem 1 of green plants the reaction centre P700 has an absorption maximum at about 700 nm which is lost upon photo-oxidation. Plastocyanin or cytochrome f presumably functions as the donor and possibly ferredoxin as the acceptor (Muller & Witt, 1961; Tagawa & Arnon, 1962; Boardman, 1970), though the precise identity of the primary acceptor for this photosystem remains unresolved at the present time (see, e.g., Yocum & San Pietro, 1969, 1970; Hiyama & Ke, 1972). Although there has been some disagreement (Govindjee, Doring & Govindjee, 1970) with respect to the situation in photosystem 2, it is now generally accepted by most investigators that this reaction centre functions analogously. It had long been suspected that the photosystem 2 reaction centre would have a bleachable absorption band some 10-20 nm shorter than that of P700 (Radmer & Kok, 1975). However, measurements in this spectral region proved difficult because of high chlorophyll absorption and possible interference by changes in chlorophyll fluorescence yield (Butler, 1972). In addition the photosystem 2 reaction centre appeared to turn over much faster than P700 under most conditions thus adding to the experimental difficulties, the result being that the details of photosystem 2 function are less well understood.

The Electron Acceptor of Photosystem 2

The primary electron acceptor of photosystem 2 has generally been equated with Q, the notation employed by Duysens & Sweers (1963) for the quencher of the variable fluorescence emerging from photosystem 2,

which is followed by a much larger pool of a secondary quencher, A (Amesz, 1973), and which is generally considered to be plastoquinone. No comparable variable fluorescence component has been observed that correlates with the redox state of photosystem 1 (Duysens, 1963). The large plastoquinone pool A, whose concentration in the photosynthetic membrane is some 10-20 times larger than that of Q, functions as an electron buffer between the two light reactions (Witt, 1967, 1971). The chemical nature of Q remains unclear. Stiehl & Witt (1969) suggested that Q represented a separate small plastoquinone pool which becomes photoreduced to the semiquinone. This concept is supported by the electron spin resonance (ESR) studies of Kohl & Wood (1969) who concluded that the primary acceptor for photosystem 2 was a plastoquinone chromanoxyl-ion. In contrast, the experiments of Böhme & Cramer (1971, 1972a) suggested that the low potential form of the b-type cytochrome, b_{559} (cytochrome b_{559LP}) is located before the main pool of plastoquinone on the basis of an observed inhibition of its photosystem 1-dependent oxidation by the plastoquinone antagonist, dibromothymoquinone (DBMIB). This latter view of a possible role of cytochrome b_{559} has received further support from the observation that it acts as a second quencher of fluorescence in the presence of the substituted phenylurea herbicide, diuron (Ke, Vernon & Chaney, 1972) which has been shown to prevent electron flow between the primary and secondary acceptor pools (Duysens & Sweers, 1963).

Knaff & Arnon (1969a) discovered a light-induced absorbance change near 550 nm which they attributed to an electron transport component "C550", related to the primary acceptor of photosystem 2. Butler (1973) has suggested that C550 possibly represents an absorbance change in a

sensing pigment for the redox state of the quencher. The evidence for such a suggestion came from extraction and reconstitution experiments with plastoquinone and β -carotene which indicated that the presence of β -carotene was required to observe the C550 absorbance change (Okayama & Butler, 1972).

Artificial Electron Acceptors

Certain artificial electron acceptors may be reduced during photosystem 2 activity. Photoreduction of added electron acceptors by chloroplast preparations is widely referred to as a Hill reaction (after its discoverer R. Hill), potassium ferricyanide and the dye 2,6-dichlorophenol-indophenol (DCPIP) being among the most common of the Hill oxidants used (Nishimura, Sakurai & Takameya, 1964; Witt, 1967; Avron & Ben-Hayyim, 1969; Lien & Bannister; 1971). Results obtained from enhancement studies led some workers to conclude that both photosystems, and preferentially photosystem 1, operated in the photoreduction of these acceptors (Kok, Malkin, Owens & Forbush, 1966; Govindjee & Bazzaz, 1967; Lien & Bannister, 1971). Rumberg, Schmidt-Mende, Skerra, Vater, Weikard & Witt (1965) suggested that at high concentrations ferricyanide was capable of accepting electrons from photosystem 2 but only in experiments when illumination was by means of flashing light of moderate frequency. These problems concerning the specificity of photoreduction by photosystem 2 have recently been resolved (Trebst, 1974). The reduction of ferricyanide and DCPIP by photosystem 2 apparently depends on the accessibility of these compounds to the acceptor site for photosystem 2. In freshly prepared membranes the site is probably inaccessible to ferricyanide and DCPIP

and consequently both acceptors are reduced preferentially by photosystem 1. The rate of reduction by photosystem 2 is only some 40% of that by photosystem 1 (Bohme, Reimer & Trebst, 1971; Kimimura & Katoh, 1973) though a figure as low as 10% is given by Ouitrakul & Izawa (1973). However, in fragmented chloroplasts and isolated photosystem 2 particles the rate of ferricyanide and DCPIP photoreductions is increased because of exposure of the photosystem 2 acceptor site (Katoh & San Pietro, 1966, 1967; Vernon & Shaw, 1969). Consequently in equating rates of photoreduction of such polar acceptors to photosystem 2 activity the state of the chloroplast preparation is of great importance. Saha, Ouitrakul, Izawa & Good (1971) have recently grouped Hill acceptors into classes according to lipophilicity and polarity. It transpires that hydrophilic acceptors are photoreduced by photosystem 1 whilst lipophilic acceptors receive electrons from photosystem 2. On the basis of $P/2e^-$ ratios obtained during their photoreduction, oxidised phenylenediamines and benzoquinones thus proved to be excellent artificial electron acceptors for photosystem 2. This classification has received support from data obtained using inhibitors which block electron flow through the intermediate electron transport chain linking photosystem 2 to photosystem 1, such as DBMIB, KCN or $HgCl_2$ (Trebst, 1971; Ouitrakul & Izawa, 1973; Kimimura & Katoh, 1973). Photoreduction reactions mediated by photosystem 2 alone can therefore be measured even in intact lamellar systems by using a lipophilic acceptor and at the same time inhibiting electron flow to photosystem 1 using $HgCl_2$ (Katoh, 1972), KCN (Ouitrakul & Izawa, 1973) or polylysine (Ort, Izawa, Good & Krogmann, 1973).

The electron donor to photosystem 2

The ultimate electron donor in the photosynthesis of green plants is water. The nature of photosystem 2, incorporating the oxygen-evolving system of photosynthesis, continues to be poorly understood. This is partly due to the extreme lability of this tightly lamellar-bound photosystem but also to the fact that most of its components have not been identified. The biochemistry and biophysics of photosystem 2 and H_2O oxidation (oxygen evolution) have been reviewed by Cheniae (1970). From measurements of oxygen yield upon illumination of Chlorella or chloroplasts with single short flashes of light it has been deduced that oxygen evolution involves a four quantum process (Cheniae, 1970; Trebst, 1974). The absolute requirement of manganese (Mn) ions for a functional photosystem 2 and its involvement in the process of water oxidation is well documented. Cheniae & Martin (1970) have shown that treatment of isolated chloroplasts with the photosystem 2 inhibitor, hydroxylamine, results in a release of a substantial portion of the Mn in chloroplasts, with concomitant loss of Hill activities and photo-oxidation of artificial electron donors. A later study indicated that there are some 5 - 8 Mn atoms per photosystem 2 trapping centre in the green alga Scenedesmus, and some 4 - 6 in spinach chloroplasts (Cheniae & Martin, 1971). Heat treatment (Cheniae & Martin, 1966) and treatment of chloroplasts with tris (Itoh, Yamashita, Nishi, Konishi & Shibata, 1969) also results in the removal of manganese from the lamellar system, with accompanying decrease in electron donation from water. Although these studies have shown that manganese is a necessary requirement for photosystem 2 function, it is not certain whether Mn is involved directly

as an electron carrier, or whether it exerts some secondary effect on the structure and/or function of the photosystem. Anderson & Pyliotis (1969) reported, following electron microscope studies of manganese deficient and control chloroplasts, that the lamellar structure of chloroplasts with only 25% of the normal Mn content was indistinguishable from those of normal chloroplasts. However, Russell, Lyman & Heath (1969) have shown that under conditions of Mn deficiency the structural organisation of higher plant chloroplasts is altered, there being disappearance of intergranal lamellae or swelling and disruption of the thylakoids.

Early studies showed a stimulatory effect of Cl^- on the Hill reaction (Warburg & Lüttgens, 1946). The site of Cl^- involvement was later shown (Bové, Bové, Whatley & Arnon, 1963) to be the segment of the electron transport chain near photosystem 2. Hind, Nakatani & Izawa (1969) demonstrated a marked Cl^- effect in chloroplasts uncoupled by various agents and confirmed that the Cl^- is closely related to photosystem 2. More studies (Izawa, Heath & Hind, 1969) with artificial donors such as hydroxylamine and ascorbate, which donate electrons to photosystem 2, have located the Cl^- requiring step as being close to the terminal water oxidation mechanism.

Other compounds, notably β -carotene and cytochrome $\underline{b}_{559}$ have also been implicated on the donor side of photosystem 2 (Yamashita, Konishi, Itoh & Shibata, 1969; Sofrova & Bendall, 1971). In their extraction experiments Sofrova & Bendall (1971) found that maximum restoration of the high potential (HP) character of cytochrome $\underline{b}_{559HP}$ was found to depend on the presence of both β -carotene and plastoquinone.

The discovery of specific inhibitors between water oxidation and photosystem 2, such as high concentrations of carbonylcyanide phenylhydrazones (Izawa et al, 1969), NH_2OH (Bennoun & Joliot, 1969) and NH_3 or methylamine (Yamashita et al, 1969) and of specific treatments inactivating water oxidation but not photosystem 2 activity, such as incubation with Tris buffer (Yamashita & Butler, 1968a), KCl (Asada & Takahashi, 1971), urea, perchlorate, nitrate, thiocyanate or guanidine (Yamashita, Tsuji & Tomita, 1971) and gentle heating (Katoh & San Pietro, 1967; Yamashita & Butler, 1968b) have helped clarify reactions on the donor side of photosystem 2. Ort & Izawa (1974) reported a gentle and effective procedure for inactivation of oxygen evolution that involved treatment of the chloroplast preparation with hydroxylamine and EDTA. However, Cheniae & Martin (1971) have shown that the degree of inactivation of oxygen evolution by hydroxylamine is dictated to a considerable extent by the prevailing experimental conditions.

Artificial electron donors

In chloroplasts in which the electron donating side of photosystem 2 has been inhibited by certain of the treatments described in the foregoing section, water may be replaced by artificial electron donors. The list of compounds demonstrated to have the ability of donating electrons to photosystem 2 is, in fact, quite considerable and includes cysteine (Katoh & San Pietro, 1967), hydroquinones, phenylenediamines, benzidine, semicarbazide (Yamashita & Butler, 1969), diketogulonic acid (Habermann, Handel & McKeller, 1968), diphenylcarbazide (Vernon & Shaw,

1969), ascorbate, Mn^{++} ions (Ben-Hayyim & Avron, 1970), hydrazobenzol, hydroxylamine (Haveman & Donze, 1971), H_2O_2 (Inoue & Nishimura, 1971), tetraphenylboron (Homann, 1971) and hydrazine (Haveman, Duysens, Van der Geest & Van Gorkom, 1972). On the basis of differential activity by donors with some of the inactivation treatments mentioned in the previous section the implication from these studies is that there are several donation sites between the O_2-evolution site and photosystem 2 (Asada, Takahashi, 1971; Katoh, Satoh, Ikegami, Kimimura, Takamiya, 1971; Kimimura & Katoh, 1973). However, Trebst (1974) has pointed out that the ability of chloroplasts to utilise electrons from such a variety of artificial donors might merely reflect the degree of membrane damage (or of manganese extraction) in the water oxidation complex.

Some of the results obtained from the use of artificial electron donor systems for photosystem 2 are difficult to evaluate because of a number of side reactions that have not been taken into account sufficiently. For example, some of the artificial electron donor systems for photosystem 2 such as ascorbate, hydroquinone and phenylenediamines are also able to donate electrons to photosystem 1 (Yamashita & Butler, 1969) whilst carbonylcyanidephenylhydrazone (CCP) and tetraphenylboron also rapidly inactivate photosystem 2 (Homann, 1971) and consequently cannot be used for steady state studies. After initial oxidation by photosystem 2 hydrazine can be shown to be further oxidised chemically by molecular oxygen via chain reactions (Mantai & Hind, 1971). Of particular interest has been the oxidation of certain electron donors by the superoxide anion radical formed by autooxidation of the acceptor by molecular oxygen at the reducing side of photosystem 1. It has been

shown, for example, that the superoxide radical will oxidise ascorbate (Allen & Hall, 1973; Elstner & Kramer, 1973) and sulphite (Asada & Kiso, 1973). The discovery of the superoxide anion radical led Elstner, Heupel & Vaklinova (1970) to point out that the increased oxygen uptake observed when ascorbate is added to chloroplast preparations probably results from a superoxide radical oxidation rather than from electron donation. Electron donation to photosystem 2 by ascorbate does however occur in Tris-washed chloroplasts (Yamashita & Butler, 1969). Occasionally, the oxidation product of the electron donor may itself interact with the photosystems, as is the case for diphenylcarbazone derived from DPC (Vernon & Shaw, 1972) and azobenzene, the oxidation product of hydrazobenzene (Haveman & Donze, 1971). Finally some of the reported electron donors are active in fragmented chloroplasts or purified photosystem 2 particles but not in intact thylakoid membrane systems, an example being 1,5-diphenylcarbazide (DPC, Vernon & Shaw, 1969).

Function of cytochrome $\underline{b}_{559}$ in photosystem 2

The first studies of cytochrome $\underline{b}_{559}$ (then known as cytochrome $\underline{b}_3$) were made by Lundegardh in 1965. Since that time several hypotheses have been put forward concerning the function of the cytochrome. Bendall & Sofrova (1971) maintain that the cytochrome functions as an electron acceptor in the water-splitting reaction on the high potential (electron donor) side of photosystem 2. An alternative view which has received much support has been that of Cramer, Fan & Böhme (1971) who envisage cytochrome $\underline{b}_{559}$ as being situated near photosystem 2 in the main electron transport chain linking the two photosystems, as

depicted in Fig. 8. A more recent suggestion is that the cytochrome operates in a bypass or cyclic chain around photosystem 2 (Radmer & Kok, 1975).

Lundegardh (1965) concluded from his studies that cytochrome $\underline{b}_{559}$ might have a redox function in water splitting on the basis of an alteration in the photoresponse of the cytochrome in the presence of the phenylurea herbicide, diuron. The general concept that cytochrome $\underline{b}_{559}$ may have a role in water splitting was developed from the further observations that treatment of chloroplasts with agents that caused a large negatively directed shift in the midpoint potential of the cytochrome also tended to inhibit oxygen evolution (Wada & Arnon, 1971), and that algal mutants in which all or part of the cytochrome $\underline{b}_{559}$ complement was missing or shifted to a lower (more negative) potential were defective in photosystem 2 activity (Epel & Butler, 1972; Horton, Donnell, Cramer, Lien, Togasaki, San Pietro, 1977). Cytochrome $\underline{b}_{559}$ has been reported to be photooxidised by photosystem 2 at 77°K (Knaff & Arnon, 1969b), an observation which lends further support to the siting of the cytochrome prior to photosystem 2. Finally it can be added that purified triton-treated photosystem 2 particles still contain cytochrome $\underline{b}_{559}$ although always in a "low potential" form (Boardman, 1972; Ke, Sahu, Shaw, Beinert, 1974).

Bendall & Sofrova (1971) have suggested that the high potential form of the cytochrome (cytochrome $\underline{b}_{559\ HP}$) observable in undamaged chloroplasts may be an integral part of the mechanism of photolysis of water by photosystem 2, and have shown how the measured redox potential of the cytochrome can be reconciled with such function.

Further support comes from experiments on the time dependence in greening bean leaves of the low temperature photooxidation of cytochrome $\underline{b}_{559}$ (Baker & Butler, 1976). These studies showed that in addition to C550 photoreduction, photooxidation of cytochrome $\underline{b}_{559}$ at 77°K was also an indicator of photosystem 2 activity. These latter findings give support to the earlier observation of Ben-Hayyim (1972) that photosystem 2 is at least as good as photosystem 1 in oxidising cytochrome $\underline{b}_{559\ HP}$.

Low temperature studies of cytochrome $\underline{b}_{559}$ have been very important in quantitating ideas concerning a redox function of the cytochrome in water splitting. This approach initially pointed to the possibility that cytochrome $\underline{b}_{559}$ might serve as an electron donor to the reaction centre of photosystem 2 or that it at least formed an integral part of the photosystem 2 reaction centre complex (Cramer & Whitmarsh, 1977). The association of reduced or fairly high potential cytochrome $\underline{b}_{559}$ with photosystem 2 was made more quantitative by the observation that approximately one molecule of cytochrome $\underline{b}_{559}$ could be oxidised per reaction centre in continuous light at 77°K (Vermeglio & Mathis, 1974). The figure was increased to two molecules per reaction centre when the sample temperature was raised to -30°C in order to reoxidise the acceptor after the first illumination regime. The finding that the rate of cytochrome $\underline{b}_{559}$ photooxidation in continuous light at 77°K was a half to one third that of C550 photoreduction (Vermeglio & Mathis, 1974; Mathis, Michel-Villaz, Vermeglio, 1974) pointed to the existence of an alternative, preferred donor to the photosystem 2 reaction centre.

The earlier observation by the same workers (Vermeglio & Mathis, 1973) that oscillations in the C550 absorbance change at -50°C occur out of phase with those of cytochrome $\underline{b}_{559}$ similarly does not lend support to the conclusion (Knaff & Malkin, 1974) that C550 photoreduction depends on the presence of reduced cytochrome $\underline{b}_{559}$.

The finding that cytochrome $\underline{b}_{559HP}$ is much more labile to treatment with trypsin than is O_2 evolution (Cox & Bendall, 1972) is in agreement with these results. The inference to be drawn from this latter observation and from the lack of correlation between C550 photoreduction and cytochrome $\underline{b}_{559}$ photooxidation and between the appearance of water-splitting activity and cytochrome $\underline{b}_{559HP}$ in greening experiments is that cytochrome $\underline{b}_{559}$ is not an obligatory oxidation-reduction intermediate on the electron donor side of photosystem 2.

Evidence for the possible functioning of cytochrome $\underline{b}_{559}$ in a cyclic pathway centred on photosystem 2 arose from an observed photo-oxidation of cytochrome $\underline{b}_{559}$ by photosystem 2 at low temperatures and in the presence of carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) (Boardman, Anderson & Hiller, 1971), and from the correlation between an increase in fluorescence yield and loss of hydroquinone-reducible cytochrome $\underline{b}_{559HP}$ on addition of hydroxylamine or antimycin A to diuron-inhibited chloroplasts (Cramer & Böhme, 1972).

Although these latter observations would seem to indicate involvement of a cytochrome $\underline{b}_{559}$ in photosystem 2 it appears that in chloroplasts with an active photosystem 2 there is little evidence for an efficient cycle around this photosystem (Cramer & Whitmarsh, 1977).

The intermediate electron transport chain -
Electron transport from photosystem 2 to photosystem 1

Much of the work done on the photosynthetic electron transport chain in the last decade or two proceeded on the assumption that there should be as much correspondence as possible between the main electron transfer chain joining the two pigment systems of chloroplasts and the respiratory chain of mitochondria, exclusive of dehydrogenases, which was believed to be well established. Hill and co-workers (Hill, 1951; Davenport & Hill, 1952; Hill, 1954) discovered two distinctive types of cytochromes in chloroplasts distinct from those known to participate in mitochondrial electron transport. The first to be discovered was cytochrome f, which is bound to the chloroplast structure though it can be released by alkaline non-polar solvents. The α-band of its reduced form is at 555 nm, very similar to that of cytochrome c. In fact, cytochrome f is a cytochrome of the c class and is alternatively known as cytochrome c_{555}. Cytochrome b_6, or b_{563}, is found in the photosystem 1-enriched digitonin fraction as well as in a complex with cytochrome f where the limiting stoichiometry appears to be two molecules b_6 to one of f (Nelson & Neumann, 1972). More recently, another b cytochrome, namely, cytochrome b_3 or b_{559} has also been identified in chloroplasts (see pages 68-71).
Chloroplasts also contain a blue copper-protein called plastocyanin, two quinones, namely vitamin K_1, and plastoquinone, and in all probability other electron carriers which remain to be identified.

Elucidation of the sequence of electron carriers in the photosynthetic electron transport chain between photosystem 2 and photosystem 1 has been attempted by many of the same methods used in analysis of mitochondrial

electron transport. By piecing together information on the standard redox potentials of the carriers, their behaviour as observed spectroscopically, and the action of inhibitors and of artificial electron carriers, it has been possible to construct the sequence shown in Fig. 8. The chain leading from photosystem 2 begins with the compound C550, discovered by absorption changes at 550 nm by Knaff & Arnon (1969a & c), which is related to the primary acceptor of photosystem 2 and is probably a sensing pigment for the redox state of the quencher, Q (see Butler, 1973). Extraction and reconstitution experiments suggest that C550 may be β -carotene (Okayama & Butler, 1972). Following the next electron transfer agent, plastoquinone, the only known participants in the electron transport chain are cytochrome $\underline{b}_{559}$, cytochrome $\underline{f}$ and plastocyanin. The proposed function of plastoquinone in photosynthetic electron transport is supported by the observations that reduced plastoquinone is oxidised by light absorbed by photosystem 1, oxidised plastoquinone is reduced by light absorbed by photosystem 2, the observed absorbancy change at 252 nm is lost following petroleum ether extraction of chloroplasts but is restored by authentic plastoquinone, and that plastoquinone photoreduction is inhibited by diuron (Bishop, 1971). The evidence obtained by Böhme, Reimer & Trebst (1971) and Böhme & Cramer (1971) using 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), which interacts with the structurallly similar plastoquinone in the electron transport chain, suggests that plastoquinone may be positioned between cytochrome $\underline{b}_{559}$ and cytochrome $\underline{f}$ in the electron transfer sequence.

One of the most powerful experimental approaches to the identification of the sequence of carriers in the intermediate electron transport chain is the use of mutants of algae, such as Chlamydomonas and Scenedesmus which are deficient in P700 or one of the other electron carriers. Such mutants (e.g., Bishop, 1964) have yielded information which strongly supports the sequence shown in Fig.8 particularly with respect to the relative positions of plastocyanin and cytochrome f (Boardman, 1968; Levine, 1969). Cytochrome f was proposed to be the immediate physiological electron donor to photosystem 1 because of a supposed temperature insensitivity of its photooxidation (Chance & Bonner, 1963; Witt, 1967), but later work replaced cytochrome f by plastocyanin as the primary photooxidant of photosystem 1, as indicated in reviews by Levine & Reimann (1969) and Boardman (1970). Later, several groups reported the stimulation of cytochrome f photo-oxidation by plastocyanin in sonicated chloroplasts (Hind, 1968; Nelson & Neumann, 1972; Plesničar & Bendall, 1973). However, it had been argued that under such conditions plastocyanin would only replace cytochrome f displaced from the reaction centre by the fractionation procedure (Fork & Murata, 1971). Finally, Haehnel (1973) reported, on the basis of experiments using light flashes, that cytochrome f may not even be located in the main pathway of electron flow from plastoquinone to P700, in contrast to plastocyanin through which more than 90% of the electrons proceed. This seems to be in contradiction to those experiments by Larkum & Bonner (1972, a, b & c) and Biggins (1973) on cytochrome f absorption changes in continuous light and their dependence on excitation conditions, inhibitors, and the type of chloroplasts. These results suggested a functional role for cytochrome f

in both cyclic and non-cyclic electron flow. The recent use of a number of plastocyanin function inhibitors such as low concentrations of Hg^{++} (Kimimura & Katoh, 1973), KCN (10 mM, Ouitrakul & Izawa, 1973), histone and polylysine (Brand, San Pietro & Mayne, 1972; Berg, Cipollo, Armstrong & Krogmann, 1973), has shown that in otherwise untreated chloroplasts electron flow between cytochrome f and photosystem 1 is inhibited. The present evidence therefore favours plastocyanin as the immediate electron donor to photosystem 1 (Trebst, 1974).

An electron transport role for cytochrome b_{559} in the intermediate electron transport chain was proposed from studies made using chloroplast fragments of wild type and mutant strains of Chlamydomonas reinhardi (Levine & Gorman, 1966), such a role for a b-type cytochrome in chloroplasts being consistent with what was thought to be a well established text book scheme for linear electron transport in oxidative phosphorylation. The Chlamydomonas studies showed that a cytochrome component with a reduced α-band at 559 nm was autoxidisable, reduced by red light and by ascorbate, and was oxidised by far-red light in the wild type alga but not in mutants deficient in cytochrome f or plastocyanin (Gorman & Levine, 1966; Levine, 1969).

The precise location of cytochrome b_{559} in the sequence of carriers linking the two photosystems is a matter for continuing debate though the experiments of Böhme & Cramer (1971, 1972) with the plastoquinone antagonist, DBMIB, would suggest that the cytochrome is situated on the reducing side of plastoquinone. Though evidence for an additional electron transport component between "Q" and plastoquinone does exist

(Bouges-Bocquet, 1973b; Velthuys & Amesz, 1974) the interpretation adopted by Cramer and Whitmarsh (1977) is that the component is more likely to be of a quinone nature rather than a cytochrome.

The electron acceptor of photosystem 1

Among the first artificial compounds shown to be reduced by chloroplast preparations in the light were ferricyanide (Hill, 1937) and p-benzoquinone (Warburg & Lüttgens, 1944), observations which signified the discovery of photosynthetic oxygen evolution in cell-free systems long before the physiological electron acceptor of the Hill reaction was known. Besides benzo-, naphtho-, and anthraquinones, numerous dyes such as DCPIP, tetrazolium salts and methyl red and dipyridylium salts are also photoreduced by chloroplast preparations (Trebst, 1972).

The in vivo electron acceptor for photosystem 1 in chloroplasts is generally accepted to be ferredoxin (Tagawa & Arnon, 1962), the ferredoxin-catalysed reduction of $NADP^+$ mediated by ferredoxin-$NADP^+$ oxidoreductase, being the terminal stage of the physiological Hill reaction. The flavoprotein enzyme, which has been crystallised by Shin, Tawaga & Arnon (1963), is thought to be bound to the chloroplast lamella system though it can be readily solubilised from these after aging or fragmenting the chloroplasts. Ferredoxin has been shown to form a stoichiometric (1:1) complex with ferredoxin-$NADP^+$ reductase (Foust, Mayhew & Massey, 1969; Böger, 1971) and consequently may be loosely bound to a chloroplast membrane via such a complex (Hiedemann-Van Wyk & Kannangara, 1971).

Chance & San Pietro (1963) proposed that although ferredoxin was involved in the photosynthetic electron transport system, certain aspects of the kinetics of its reduction indicated the participation of an additional cofactor(s) between ferredoxin and the photochemistry of photosystem 1. Kamen (1961) suggested much earlier that a semi-chlorinogen with an oxidation reduction potential of about -0.6 to -0.7 V might serve as the primary acceptor of energy from excited chlorophyll. On the basis of refined spectral measurements Rumberg & Witt (1964) suggested that an unknown compound, designated as substance Z, having an oxidation reduction potential more negative than -0.44 V was functional between the photochemical reaction centre of photosystem 1 and ferredoxin. Chance, San Pietro, Avron & Hildreth (1965) noted that thoroughly washed chloroplasts devoid of ferredoxin retained the ability to photooxidize reduced cytochrome f whilst other investigators demonstrated that isolated chloroplasts could photoreduce a series of viologen dyes with oxidation reduction potentials as low as -0.7 V with varying degrees of efficiency depending upon the potential of the electron acceptor (Kok, Rurainski & Owens, 1965; Zweig & Avron, 1965; Black, 1966). From these studies it has been estimated that the primary acceptor should have an oxidation reduction potential of approximately -0.5 to -0.55 V (Bishop, 1971).

Soluble ferredoxin is not therefore considered to be the primary acceptor of photosystem 1, though recent experiments have indicated that a "bound ferredoxin" may play such a role (Hiyama & Ke, 1971; Malkin & Bearden, 1971). The present status of "bound ferredoxin" has been reviewed by Ke (1973). The main evidence for this suggestion stems

from the observation of an ESR signal resembling that of ferredoxin on illuminating chloroplast preparations, this persisting at temperatures of 25°K and below (Malkin & Bearden, 1971). However, no chemical analysis exists.

Yocum & San Pietro (1969, 1970) have isolated from spinach a component designated ferredoxin reducing substance (FRS) which they claim might function as the primary electron acceptor for photosystem 1. FRS was found to be autooxidisable, to possess absorption maxima at 265 - 270 nm and 325 - 330 nm and to consist of heat-stable and heat-labile components. Yocum & San Pietro were unable to identify the redox component, however, though at the time it was thought likely that a pteridene might be involved. The main evidence for identifying FRS with the primary acceptor of photosystem 1 was that it protected chloroplast fragments from inhibition by specific antibodies to photosystem 1-associated activities (Yocum & San Pietro, 1969). While these results have not been questioned, belief in FRS has now subsided in favour of a more structural role for the preparation (Gregory, 1977).

A more recent contender for the role of primary acceptor has been a component responsible for a spectral change occurring at 430 nm on exposure of chloroplasts to light absorbed by photosystem 1 (Hiyama & Ke, 1971). The main evidence in favour of this latter component, designated P430, is that the quantum yield and action spectrum for P430 photoreduction mimic those for P700 photooxidation and that subsequent reduction of ferredoxin coincides with the rate of disappearance of the 430 nm signal.

A number of other factors have been implicated as being associated with the primary electron acceptor site of photosystem 1, for example the oxygen reducing substance, ORS, (Honeycutt & Krogmann, 1970, 1972), the cytochrome reducing substance, CRS, (Fugita & MÜrano, 1967) and $S_{L\text{-}eth}$(Regitz, Berzborn, Trebst, 1970) but the ferredoxin reducing substance remains the most well characterised substance preceding ferredoxin in the electron transport chain associated with photosystem 1.

Cyclic electron flow mediated by photosystem 1

The existence of a cyclic photophosphorylation pathway was discovered by Arnon, Whatley & Allen (1955) as a result of the addition of menadione, a synthetic vitamin K derivative, to isolated chloroplasts in the light. Avron & Jagendorf (1956) showed that the cyclic electron flow involved only photosystem 1 and consequently was not inhibited by the phenylurea herbicide, diuron. Compounds which function as cofactors of cyclic photophosphorylation serve both as electron donors and electron acceptors to the photosystem 1 reaction centre, P700.

Cytochrome $\underline{b}_{563}$ has been implicated as a participant in cyclic electron flow (Avron & Neumann, 1968; Boardman, 1970), and Böhme & Cramer (1972b) have produced direct evidence for an energy coupling site linked to cytochrome $\underline{b}_{563}$ oxidation. Biggins (1973) demonstrated an involvement of cytochrome $\underline{f}$ in cyclic electron flow, an observation supported by Nelson & Neumann (1972) who were able to isolate from chloroplasts a cytochrome $\underline{b}_{563}$ particle which also contained cytochrome $\underline{f}$. The likely path of electron flow associated with cyclic photophosphorylation is shown in Fig. 8 and is in agreement with the majority of available experimental data.

Carbon Dioxide Assimilation in Photosynthesis

For plants to grow, net fixation of CO_2 into organic matter is essential and the only known series of reactions capable of achieving this end is the photosynthetic carbon reduction cycle, or Calvin cycle (Bassham & Calvin, 1957; Walker, 1974). The path of carbon in the Calvin cycle (Fig. 9) remains virtually unchanged from that outlined in the 1950's (e.g., Bassham & Calvin, 1957).

Losada, Trebst & Arnon (1960), using chloroplast preparations, identified and isolated the individual steps and enzymes of CO_2 assimilation and defined the Calvin cycle as consisting of three stages, carboxylation, reduction and regeneration. The carboxylative phase includes those enzymes leading from pentose-5-phosphate to 3-phosphoglyceric acid; the reductive phase, those leading from 3-phosphoglyceric acid to triose phosphate; and the regenerative phase, those leading from triose phosphate to pentose phosphate (Stiller, 1962).

In this cycle ribulose diphosphate is carboxylated to yield two moles of phosphoglyceric acid which are then reduced to the level of cell carbohydrate. This reductive sequence requires ATP and NADPH, generated by the light reaction, in amounts proportional to the amount of carbon dioxide fixed. The reactions of the photosynthetic carbon cycle are also arranged so as to regenerate ribulose diphosphate for the next round of carbon dioxide fixation. Following CO_2 fixation by RuDP carboxylase and subsequent rearrangements the eight participating reactions of the regenerative cycle (Krogmann, 1973) may be summarised as follows:

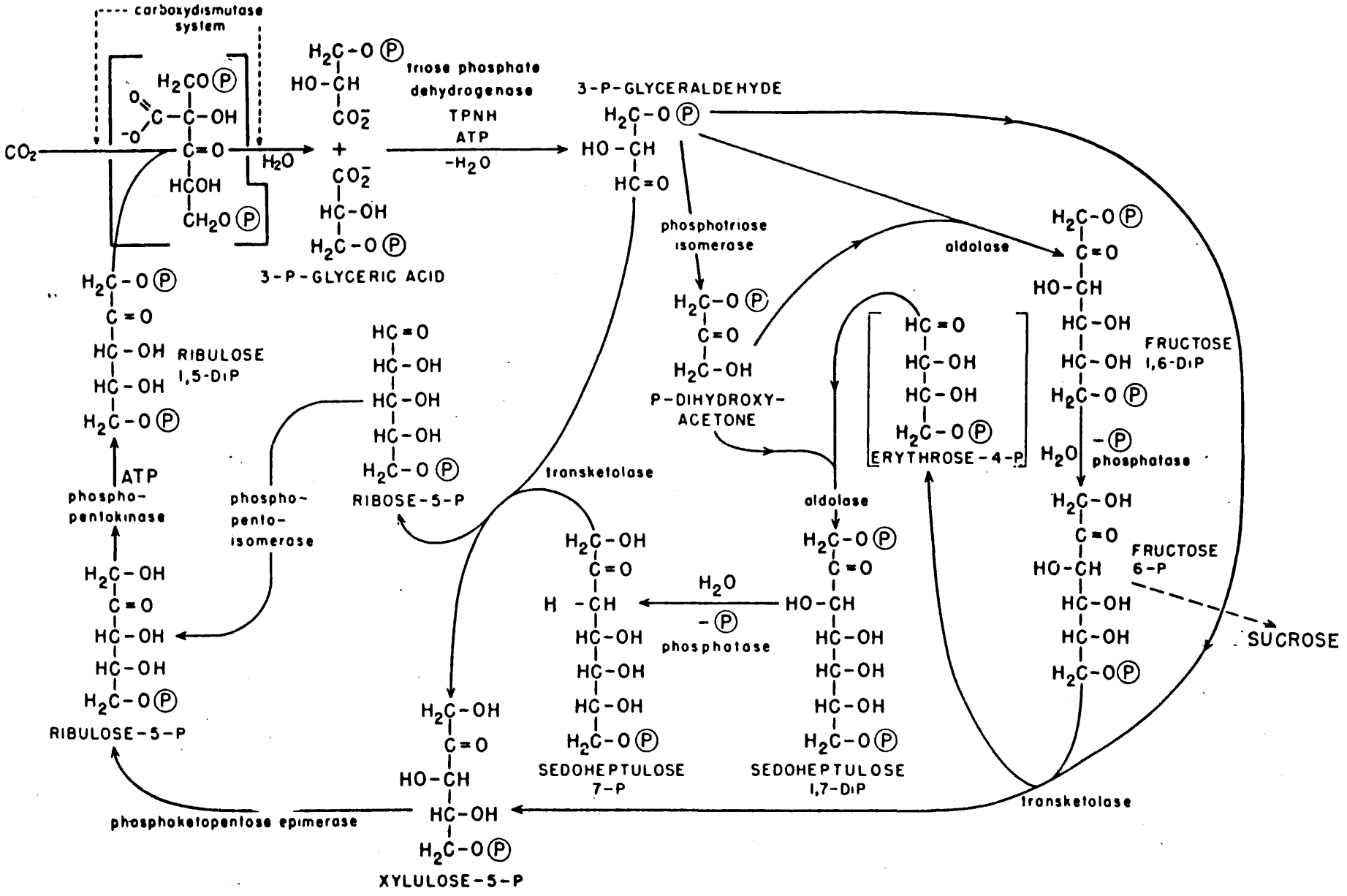


Fig. 9: The carbon reduction cycle (Calvin cycle) of photosynthesis.

(a) Conversion of fructose-1,6-diphosphate to fructose-6-phosphate by fructose-1,6-diphosphatase.

(b) Transfer of carbons 1 and 2 of fructose-6-phosphate to glyceraldehyde-3-phosphate by transketolase to give erythrose-4-phosphate and xylulose-5-phosphate.

(c) Condensation of erythrose-4-phosphate and dihydroxy acetone phosphate to give sedoheptulose-1,7-diphosphate, mediated by aldolase.

(d) The conversion of sedoheptulose-1,7-diphosphate to sedoheptulose-7-phosphate by sedoheptulose-1,7-diphosphatase.

(e) Transfer of carbons 1 and 2 of sedoheptulose-7-phosphate to glyceraldehyde-phosphate by transketolase to give ribose-5-phosphate and xylulose-5-phosphate.

(f) Isomerisation of ribose-5-phosphate to ribulose-5-phosphate by phosphopentoisomerase.

(g) Epimerisation of xylulose-5-phosphate to ribulose-5-phosphate by phosphoketopentose epimerase.

(h) Phosphorylation of ribulose-5-phosphate to ribulose-1,5-diphosphate catalysed by phospho-pentose kinase at the expense of the remaining ATP.

In one complete turn of the cycle, carboxylation of three ribulose diphosphate molecules gives six molecules of phosphoglyceric acid which are reduced to six molecules of triose phosphate by reactions requiring 6 ATP molecules and 6 NADPH molecules. Only 5 triose phosphate molecules are needed to regenerate 3 ribulose-5-phosphate molecules which are then converted to 3 molecules of ribulose diphosphate, requiring a total of 3 more ATP molecules. The triose phosphate gained from reduction of 3 CO_2 molecules may be condensed with more triose phosphate to give sugar

monophosphates and eventually carbohydrates, including starch, sugar and cellulose. Alternatively, the triose phosphate molecules can be converted to glycerophosphate for fat synthesis or transformed via phosphoglyceric acid and phosphoenolpyruvate to yield, for example, amino acid and fatty acids.

Continued studies, particularly of the individual enzymes, have resulted in the solution of some of the few lingering problems preventing the unqualified acceptance of the Calvin cycle. Gibbs & Kandler (1957) drew attention to an asymmetry in the distribution of radioactivity between the two halves of the hexose molecules produced during photosynthesis in $^{14}CO_2$ which was inconsistent with the proposed condensation of equally labelled triose phosphate. Stiller (1962), in a refreshingly sceptical article, challenged almost the entire scheme and Zelitch (1965) raised the possibility of an alternative carboxylation. However, this effect has since been attributed to the reversibility of the transketolase reaction and the influence of a triose phosphate pool (Bassham, 1964); the latter could possibly be enhanced by the easier transport of triose phosphate, in comparison to hexose phosphate, between chloroplast and cytoplasm (Heldt & Rapley, 1970). Low activities of the diphosphatases for fructose-1,6-diphosphate (FDP) and sedoheptulose-1,7-diphosphate (SDP) were also inconsistent with the operation of the cycle (Latzko & Gibbs, 1968), though the former enzyme has now been demonstrated to be sufficiently active to support CO_2 fixation (Carnier & Latzko, 1972). Both enzymes have been shown to be activated by the sulphydryl reducing agent dithiothreitol (Anderson, 1974; Schürmann & Buchanan, 1975) and reduced ferredoxin (Schürmann & Buchanan, 1975).

More recently two other protein factors in addition to reduced ferredoxin have been implicated in the activation mechanism (Schurmann, Wolosiuk, Buchanan, Breazeale & McKinney, 1977) the name thioredoxin being applied to one factor on the basis of the fact that it was replacable by thioredoxin extracted from E. coli. The second factor apparently possesses a thioredoxin dependent ferredoxin-thioredoxin reductase activity, the electrons for thioredoxin reduction being derived from reduced ferredoxin generated in the light (Schurmann et al, 1977). A disparity between the apparent low affinity of ribulose-1,5,diphosphate (RuDP) carboxylase in vitro for CO_2 and the over 20-fold higher affinity of whole leaves and isolated chloroplasts for CO_2 (see Preiss & Kosuge, 1970) no longer exists (Kelly, Latzko & Gibbs, 1976). An original report by Bahr & Jensen (1974) that RuDP carboxylase freshly released from broken chloroplasts had a K_m for CO_2 of 11 to 18 μM, similar to the level of CO_2 in air, has been confirmed in other laboratories (Badger & Andrews, 1974; Bowes, O gren & Hageman, 1975). Although a more recent estimate places the true K_m closer to 50 μM, the large amount of carboxylase activity detected was nevertheless sufficient to account for normal rates of photosynthesis by leaves in air (Lilley & Walker, 1975).

Although it has been reasonably assumed for many years that the metabolic processes of photosynthesis operate exclusively within chloroplasts, only comparatively recently have chloroplast preparations been obtained with capabilities for both CO_2-fixation and carbon cycling comparable to the rates in intact cells (Walker, 1965; Jensen & Bassham, 1966; Cockburn, Walker & Baldry, 1968). Such preparations have provided a useful adjunct to intact tissue and cells for studies on the Calvin

cycle, and kinetic and inhibitor studies with both chloroplasts and intact cells have served to confirm the earlier formulation of the pathway (Bassham & Jensen, 1967; Bassham & Kirk, 1968).

Although sucrose is generally thought to be synthesised in the chloroplast, only recently has evidence been presented establishing the net synthesis of the disaccharide by isolated chloroplasts (Gibbs, Latsko, Everson & Cockburn, 1967; Everson, Cockburn & Gibbs, 1967). In recent years the enzymes catalysing the conversion of hexose-phosphate to sucrose and starch have been detected and characterised. Evidence has been presented that chloroplasts isolated from sugar cane contain sucrose synthetases (Haq & Hassid, 1965). In addition to these enzymes, Bird, Porter & Stocking (1965) could also show the presence of sucrose-6-phosphate phosphatase, uridinediphosphate-glucose pyrophosphorylase, glucose-1-phosphate mutase and glucose-6-phosphate isomerase, but no fructose-6-phosphatase and invertase, in tobacco chloroplasts prepared by the non-aqueous technique. In these chloroplasts sucrose synthesis by way of sucrose-6-phosphate is the more active. The absence of invertase indicates that sucrose cleavage is presumably catalysed by sucrose synthetase (Avigad, 1964).

The intact leaf is capable of reducing CO_2 to carbohydrate at rates as high as 180-200 μmoles/mg chlorophyll/h. It will beapparent from the above discussion that present views indicate that three moles of ATP and two moles of reduced pyridine nucleotide are required for each mole of CO_2 assimilated via the Calvin cycle. Rates sufficient to satisfy each of the three phases of carbon dioxide assimilation have now

been achieved with isolated spinach chloroplasts. An $NADP^+$ photoreduction rate of approximately 300 μmoles/mg chlorophyll/h with unfractionated chloroplasts has been reported by Davenport (1960); photophosphorylation rates of up to 2500 μmoles/mg chlorophyll/h were recorded by Avron (1960), whilst Jensen & Bassham (1966) have observed CO_2 fixation rates in isolated chloroplasts of the same magnitude as intact material.

Research on the carbon metabolism of photosynthesis has proceeded with vigour during the last decade in the aftermath of the discoveries of the C_4-dicarboxylic acid pathway in tropical grasses such as maize and sugar cane as well as in certain dicotyledons (Karpilov, 1960; Hatch & Slack, 1966, 1970) and photorespiration (Forrester, Krotkov & Nelson, 1966). The primary importance of the ability of the photosynthetic carbon dioxide fixation pathway to support growth has, in recent years, received second place to the nature of the first compounds observed after feeding $^{14}CO_2$ in the light, these being the dicarboxylic acids (predominantly malate or aspartate) in the case of the C_4-dicarboxylic acid pathway (Hatch & Osmond, 1976) and glycollate in the case of photorespiration (Schnarrenberger & Fock, 1976). Special attention has been devoted to detailing the metabolic sequences of these two processes and their interplay with the Calvin cycle of CO_2 fixation. In addition, properties and distribution of the enzymes involved in photosynthetic carbon metabolism have been more closely examined with a view to identifying those with important regulatory functions in CO_2 fixation.

For a carbon dioxide fixation mechanism to permit continued growth it must entail an autocatalytic sequence able to generate more CO_2 acceptor than was present initially. Walker (1974) has clearly differentiated between the Calvin cycle and C_4-dicarboxylic acid metabolism in this respect, concluding that while the Calvin cycle is indeed autocatalytic the C_4 pathway is not. The uniqueness of the Calvin cycle as the only presently known pathway for the net incorporation of CO_2 for growth is a prerequisite for a realistic interpretation of much of the current literature on photosynthetic carbon metabolism such as Stiller (1962), Gibbs (1967), Walker & Crofts (1970), Bassham (1971), Black (1973) and Kelly et al (1976).

A discussion of the carbon metabolism associated with photorespiration does not seem particularly relevant to the present investigation and the reader is referred to the excellent review by Schnarrenberger & Fock (1976). The role of the C_4-dicarboxylic acid pathway in CO_2 fixation has been the subject of several reviews elsewhere (e.g., Hatch & Slack, 1970; Walker & Crofts, 1970; Hatch & Osmond, 1976) but since the pathway is apparently not present in wheat, the experimental material used in the investigations reported in this thesis, it will not be discussed further.

MATERIALS AND METHODS

Growth of Wheat Varieties

Seeds of the varieties used were supplied by the National Institute of Agricultural Botany, Cambridge. For experiments designed to locate any differences in biochemical functions between resistant and susceptible winter wheats, the varieties used were predominantly Cappelle and Cama (resistant) and Maris Nimrod, Maris Huntsman and Val (susceptible). No obvious morphological distinction exists between susceptible and resistant strains. For experiments requiring the routine isolation of chloroplast or microsomal fractions from unsprayed plants, or for studies on plants sprayed for various periods, the seeds of resistant and susceptible wheats were separately sown in John Innes No. 2 compost contained in 4" or 6" (11 - 16 cm) diameter plastic pots. Plants required in large quantity for the isolation of intact chloroplasts were also grown in John Innes No. 2 compost, but in well spaced rows in trays 22" x 15" (56 x 38 cm). These plants were grown in a Conviron environmental growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) maintaining day and night temperatures of 20 and 10°C respectively and a 16-hr day regime. The light intensity provided was 20,000 lx.

For experiments involving a study of herbicide uptake, seeds were germinated on moist filter paper in a petri dish for 3-4 days. Seedlings were subsequently transferred to a nutrient culture solution of the composition described by Hoagland & Arnon (1950). The culture medium employed therefore contained the following mixture, diluted to 1000 ml and adjusted to pH 6.5 with 0.1M H_2SO_4:

1M	ammonium acid phosphate	-	1 ml
1M	potassium nitrate	-	6 ml
1M	calcium nitrate	-	4 ml
1M	magnesium sulphate	-	2 ml
0.5%	ferric tartrate	-	1 ml
	trace element solution (see below)	-	1 ml

Trace element solution:

boric acid	-	2.86 g
manganese chloride	-	1.81 g
zinc sulphate	-	0.22 g
copper sulphate	-	0.08 g
water	-	to 1000 ml

These plants were grown in a greenhouse with facilities for regulated heating and lighting. During the winter months the plants were maintained under a 16 h day regime by means of an arrangement of 40 W fluorescent tubes. Vessels containing nutrient solution were covered with aluminium foil to exclude light from the root systems.

Plants were used for experimental studies approximately two weeks after germination (three-leaf stage) though plants at the four-leaf stage of development were used in some experiments, where indicated. When leaf material from sprayed plants was required only those leaves which had fully contacted the spray were taken for analysis, any emerging leaves being discarded since these were partly protected from the spray by the leaf sheath.

For investigations involving the isolation of coupled mitochondria from wheat, seeds were germinated on moist cotton wool in the dark at approximately 25°C and the hypocotyls harvested at the 4-5 day stage when they were some 1-2 cm in length.

Application of herbicides to intact plants

For routine spraying of plants approx. 0.05 g of formulated herbicide (80% phenylurea/20% wetting agent) was suspended in 100 ml of 0.1% Tween 60 in water. Emulsions were always prepared immediately before use and were applied as a spray, conveniently delivered from a Gallenkamp all-glass chromatography spray operated by a small compression pump. In all experiments the control plants were sprayed with a solution of 0.1% Tween 60 in water, not containing metoxuron.

For some translocation studies metoxuron was applied by immersing individual leaves for 5 s in an emulsion prepared by dissolving 0.1 g of formulated metoxuron in 100 ml of 0.1% Tween 60 in water. Plants dipped in this emulsion were subsequently kept suspended in an inverted position for a few minutes to allow drainage of excess emulsion from the surface of the leaves.

Isolation of Chloroplasts

The method of chloroplast isolation varied slightly according to the aim of the investigation. All manipulations were carried out in a cold room at 2-4°C or using pre-cooled equipment.

A. Small fragments (1 cm length) of leaves (6-10 g wet weight) were homogenised in an M.S.E. top drive homogeniser (M.S.E. Ltd., England) using approximately 40 ml of ice-cold buffered medium. The homogeniser was operated at maximum speed for two or three 10 s intervals, allowing a short interval between each homogenisation to reduce any rise in temperature. The leaf homogenate was then strained through four layers of cheesecloth, the leaf debris being discarded. In chloroplast isolation for individual experiments one of the following isolation media was employed :

Medium A — 0.5M sucrose; 0.05M $KHCO_3$, pH 7.5

Medium B — Isolation medium:
0.4M sucrose, 0.05M tetrasodium pyrophosphate, 0.001M $MgCl_2$, 0.001M EDTA, 0.01M NaCl, 0.6% carbowax 4000, 0.05% cysteine hydrochloride, 0.25% bovine serum albumin adjusted to pH 8.0 (Plesničar & Bendall, 1973).

Washing medium:
0.4M sucrose, 0.05M Tricine buffer (pH 8.0), 0.001M $MgCl_2$, 0.001M EDTA, 0.01M NaCl, 0.25% B.S.A. (Plesničar & Bendall, 1973).

The crude homogenate was then centrifuged at 200 x g for 90 s using the swing-out head of an M.S.E. bench centrifuge. The resulting pellet of residual cell wall debris, nuclei and some other particles

including some chloroplasts was discarded and the remaining supernatant centrifuged at 800 x g for 7 min. The chloroplast pellet from this latter centrifugation was washed once in 35 ml of the homogenising (for medium A) or washing medium (for medium B), and re-centrifuged at 800 x g for 5 min. Chloroplast preparations were suspended in the same medium as used for washing to give the appropriate chlorophyll concentration required for a particular assay. The suspensions were maintained at 0-4^{o}C in an ice-bath and used within 10 min of preparation. No further purification of the 800 x g fraction was carried out. Although this fraction is sometimes designated as "whole chloroplasts", microscopic examination has revealed the expected presence of variable quantities of broken chloroplasts as well as some contamination by other cell organelles, (Owen, 1971). In an electron microscope study of barley chloroplast preparations obtained using sucrose or NaCl-containing isolation media, Appelqvist, Stumpf & Von Wettstein (1968) found that the preparation made with 0.5M sucrose medium at pH 8.0 contained the highest percentage (16%) of whole chloroplasts (i.e. with chloroplast membrane and stroma intact). Only 10% whole chloroplasts were obtained after isolation with sucrose medium pH6.0 and still fewer after isolation with the 0.35M NaCl media. Although the chloroplast preparations obtained using the procedure described above were not subjected to electron microscopic examination, it is likely that they contained similar proportions of whole chloroplasts to those described by Appelqvist et al (1968) for sucrose media.

B. Isolation of Intact Chloroplasts

The isolation of chloroplasts with intact envelopes is facilitated by brief disruption, rapid separation and the use of a sugar or sugar alcohol rather than NaCl to maintain the osmotic pressure (Walker, 1964, 1965). Following invaluable work on new buffers for biological systems (Good, Winget, Winter, Conolly, Izawa & Singh, 1966) a further improvement to the technique was achieved by Jensen & Bassham (1966) when they substituted 2-(N-morpholino)-ethanesulphonic acid (M.E.S.) and inorganic pyrophosphate for orthophosphate in their isolation media. Together with a rapid isolation procedure such media permit the isolation of chloroplasts showing rates of CO_2 fixation in excess of 100 μmoles/mg chlorophyll/hr indicating a high degree of intactness of the chloroplast envelope. For experiments described in the present thesis requiring preparations enriched in the intact chloroplast fraction, the methods of chloroplast isolation used were those of Cockburn, Walker & Baldry (1968) and Miyachi & Hogetsu (1970).

Chloroplasts were isolated from seven day old wheat seedlings, in which only the first leaf was developed, by the methods described below:

Method A (Cockburn et al, 1968)

5 g of leaves were ground for 3-5 s in a chilled blender with 20 ml of ice-cold isolation medium consisting of sorbitol, 0.33M; $MgCl_2$, 5 mM; sodium isoascorbate, 2 mM and tetrasodium pyrophosphate, 10 mM adjusted to pH 6.5 at 0°C with HCl. Sodium isoascorbate was added after pH adjustment. The slurry was filtered through two layers of cheesecloth and subsequently filtered through a further four layers prior to centrifugation of the resulting filtrate at 0°C from rest to 4000 x g to

rest, in approx. 2 min using an M.S.E. High Speed 18 refrigerated centrifuge. The chloroplast pellet was then resuspended in a small volume (0.5 - 1.0 ml) of an ice-cold solution containing sorbitol, 0.33M; $MgCl_2$, 1.0mM; $MnCl_2$, 1.0mM; EDTA, 2mM and Hepes (N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid) buffer, 50 mM adjusted to pH 7.6 at 20°C with NaOH.

Method B (Miyachi & Hogetsu, 1970)

5 g of chilled leaves were homogenised for 3-5 s in a chilled blender with 50 ml of an ice-cold isolation medium containing sorbitol, 0.4M; $MgCl_2$, 5mM; sodium isoascorbate, 2mM; EDTA (tetrasodium salt) 2mM; bovine serum albumin, 0.1% and Hepes buffer, 12.5mM adjusted to pH 8.0 (the Na isoascorbate was added after pH adjustment). The resulting homogenate was immediately squeezed through two layers of cheesecloth and subsequently filtered through a further four layers prior to centrifugation of the filtrate at 2500 x g for 50 s, the supernatant being discarded. The resulting pellets were immediately resuspended in an ice-cold medium containing sorbitol, 0.4M; $MgCl_2$, 1.0mM; $MnCl_2$, 1.0mM; EDTA, 2mM; tetrasodium pyrophosphate, 10mM and Hepes buffer (pH 8.0), 50mM and used without delay.

For experiments requiring broken chloroplasts to facilitate interaction of herbicide with the binding site (s), the chloroplast preparations were subjected to osmotic shock by washing with 35 ml

of either deionised water or 5 mM tris buffer pH 7.5, followed by recentrifugation at 1000 x g for 7 min.

Application of substituted phenylurea herbicides and their metabolites to isolated chloroplasts

Chloroplasts isolated from the leaves of sprayed wheat plants contain herbicide which has arrived at these sites mainly by physical but possibly also by physiological mechanisms. It is the same former processes that transport herbicide to the active site (s) when chloroplasts are incubated with herbicide solution in vitro, though the mechanisms of transfer may be different. Numerous reports have been presented during the last two decades to indicate that the substituted phenylurea herbicides inhibit the Hill reaction of isolated chloroplasts (e.g. Cooke, 1956; Spikes, 1956; Wessels & Van Der Veen, 1956) and that the degree of inhibition observed generally correlated with phytotoxicity (Lee & Fang, 1972). Further studies (Bishop, 1958; Jagendorf & Avron, 1959; Vernon & Zaugg, 1960) indicated that the site of inhibition of photosynthesis by substituted phenylureas was probably closely associated with the O_2-evolving system of photosystem 2.

The solutions of substituted phenylureas referred to in studies where these herbicides were added to chloroplast preparations were derived from stock solutions (10 mM) of the technical phenylurea in methanol. The methanolic solutions were added directly to the final preparation of chloroplasts or 'broken' chloroplasts prior to the experimental investigations. Chloroplast preparations were generally left in contact with the herbicide for a period varying from 3-30 min prior to assay of photosynthetic activities.

In all assays the herbicide concentration employed was related to the chlorophyll content of the chloroplast preparations. In parallel control experiments chloroplasts were treated with the same volume of methanol not containing herbicide.

Determination of Chlorophyll

The chlorophyll content of chloroplast preparations was determined spectrophotometrically by the Arnon (1949) modification of an original method of MacKinney (1941). This method is based on the absorption of light by aqueous 80% acetone extracts of chlorophyll using the specific extinction coefficients for chlorophyll a and b given by MacKinney (1941).

In practice 0.5 ml of the aqueous suspension of chloroplasts was added to 4.5 ml of deionised water in a graduated 25 ml flask. Acetone was then added to the 25 ml mark and the flask stoppered and shaken vigorously. Precipitated protein was removed either by filtration through Whatman No. 1 filter paper or by centrifugation at maximum speed (2000 x g) on an M.S.E. bench centrifuge. The extinctions at 645 nm and 663 nm of the clear extract were determined using a Unicam SP 500 (Pye Unicam Ltd., Cambridge, England) spectrophotometer using 1 cm path length spectrophotometer cells and reading against an 80% acetone solution as blank.

The chlorophyll content of the chloroplast suspension was calculated by substitution of the extinction values in the following equation:-

$$C = \frac{(20.2 \times \underline{E}_{645} + 8.02 \times \underline{E}_{663})}{20} \text{ mg chlorophyll/ml}$$

Where C = concentration of chlorophyll (mg/ml)

$\underline{E}_{645}$ = optical density at 645 nm

$\underline{E}_{663}$ = optical density at 663 nm

The dilution (here 20-fold) can be varied depending on the chlorophyll content of the preparation.

Isolation of Mitochondria

Seeds of the winter wheat varieties used were germinated and grown at 25°C on moist absorbent cotton wool in the dark as previously described (p 89). 2-5 g batches of hypocotyls were harvested and homogenised for 10 to 20 s in a pre-cooled pestle and mortar with ice-cold grinding medium delivered at a uniform rate from a burette. When the first grinding was completed the mortar contained approx. 8 ml of grinding medium/g of tissue. The resulting homogenate was filtered through two layers of nylon cloth (pore size approx. 50) into centrifuge tubes. The residue in the nylon cloth was returned to the pestle and mortar, reground with grinding medium and refiltered through the nylon. Several grinding media were tested, the most satisfactory being that of Sarkissian & Srivastava (1968) composed of sucrose, 0.5M; EDTA, 1 mM; KH_2PO_4 (pH 7.2), 67 mM; and BSA, 0.75% (w/v).

Several centrifugation procedures were also tested including the rapid isolation procedure of Sarkissian & Srivastava (1968) and the more conventional procedures of Ikuma & Bonner (1967) and Raison & Lyons (1970). The procedure finally adopted, however, for the present study was a modification of the above methods based on the procedure of Pomeroy (1974).

After filtering through nylon, the homogenate was centrifuged at 2000 x g for 5 min, and the resulting supernatant fraction was centrifuged for a further 4 min at 20,000 x g. The mitrochondrial pellet resulting from the latter centrifugation was re-suspended in 10 ml of grinding medium, centrifuged at 1500 x g for 5 min, and the mitochondria re-sedimented from the supernatant by centrifuging for 15 min at 8000 x g. The mitochondrial pellet was re-suspended in a small volume (0.5 ml) of a resuspension medium composed of mannitol, 0.3M; EDTA, 1mM and BSA, 0.1% (w/v) adjusted to pH 7.2, prior to their use in experiments described in section 3, p.168.

Protein determination

Protein was determined in bovine serum albumin-free suspensions of organelle fractions by the method of Lowry, Rosebrough, Farr & Randall (1951).

The reagents used were:

A	2% Na_2CO_3 in 0.1M NaOH
B	0.5% $CuSO_4.5H_2O$, 1% sodium or potassium tartrate
C	50 ml A; 1 ml B
D	Folin & Ciocalteu reagent diluted 1:1 with deionised H_2O

To a sample of 5 - 100 µg of protein diluted to 1 ml with deionised water was added 5 ml of reagent C. After mixing the solutions were allowed to stand for 10 min at room temperature. 0.5 ml reagent D was then added with rapid mixing. After 30 min the blue colour was measured using a Unicam SP 500 or 600 (Pye Unicam Ltd., Cambridge)

spectrophotometer at 750 nm. A control estimation with solvent not containing protein was made on each occasion and this value was subtracted from those for the protein solution. Protein content was computed by reference to a standard curve prepared using crystalline bovine serum albumin.

Experimental methods for study of the light reactions in photosynthesis.

Spectrophotometric assays of electron transport in chloroplasts

Oxidation-reduction reactions in chloroplasts were first discovered independently of photophosphorylation by Hill (1937). The subsequent discovery of light driven phosphate esterification (Arnon, Allen & Whatley, 1954) could not be directly demonstrated to accompany oxidative or reductive steps. However a series of oxidative steps were postulated as the basis for ATP formation in the absence of oxygen uptake or evolution (Arnon, Allen & Whatley, 1956).

It was not until the discovery by Arnon and co-workers (quoted by Avron, Krogmann & Jagendorf, 1958) that phosphate esterification by chloroplasts could accompany ferricyanide reduction and the evolution of oxygen, that oxidative and phosphorylative reactions could be directly related. This finding was later extended with the postulate that $NADP^+$ was the natural electron acceptor (Arnon, 1959).

The Hill reaction can be considered as the coupling of electron transport from H_2O to the photoreduction of an acceptor which can be a dye such as 2,6-dichlorophenol indophenol (DCPIP) or potassium ferricyanide. The relationship of photosynthetic phosphorylation to the Hill reaction has been studied by Arnon, Whatley & Allen (1958).

Ferricyanide reduction and accompanying phosphorylation were shown to be increased by addition of Mg^{2+}, phosphate and ADP, the presence of all three components being necessary for maximum reductive and phosphorylative activity.

The Hill reaction studies described later were carried out using aqueously prepared chloroplasts or chloroplasts broken by osmotic shock, suspended in homogenising buffer. For measurement of potassium ferricyanide or 2,6-dichlorophenol indophenol photoreductions the 0.5M sucrose homogenising buffer (pH 7.5) was employed in chloroplast isolation.

All assays were carried out in 1 cm light path glass cuvettes illuminated by light of approx. 150,000 lx intensity. Illumination was provided by a 375 W photographic lamp (Atlas P1/6, E.S. E27/27) placed 15 cm from the cuvette in line with their light paths. A heat filter comprised of a chromatography tank filled with cold water was incorporated between the cuvettes and the light source to ensure that the temperature of the assay mixture did not rise above $20^{o}C$.

Assay of 2,6-dichlorophenol indophenol (DCPIP) reduction

The reaction medium was made up directly in 1 cm light path cuvettes from stock solutions of the following :

A	0.5M KH_2PO_4	-	1.7011 g/250 ml H_2O
	0.01M KCl	-	186.4 mg/250 ml H_2O
	pH adjusted to 6.5		
B	3.2×10^{-4}M DCPIP	-	2.32 mg/25 ml H_2O
	The pH of this solution is in the range 6.4 - 7.0.		

Each cuvette contained 2.5 ml solution A and 0.5 ml solution B before addition of the chloroplast preparations containing 30 - 40 γ chlorophyll. Each reaction cuvette thus contained KH_2PO_4, 125 μmoles; KCl, 25 μmoles; DCPIP, 0.16 μmoles and chloroplasts equivalent to 30-40 γ chlorophyll in a total volume of 3.0 ml. Each experiment incorporated the appropriate control containing 0.5 ml water in place of the DCPIP. The reaction cuvettes were kept covered with aluminium foil prior to illumination. Following inversion of the cuvettes to obtain thorough mixing of the contents, the initial extinction at 620 nm was measured with a Unicam SP 500 spectrophotometer against the minus DCPIP control as reference. The cuvettes were illuminated for a series of 30 or 60 s intervals, the reduction in extinction at 620 nm being determined at the end of each illumination period.

The rate of reduction of DCPIP by the chloroplast preparation, expressed as μmoles dye reduced per mg chlorophyll per h, was determined from the reduction after the first 30 or 60 s period of illumination. Reduction proceeded linearly over this interval. The data given by Punnett (1959) for the molar extinction coefficient of DCPIP at 620 nm was used in calculations and the dye, as supplied by British Drug Houses Ltd., Poole, England, was not purified further before use. Because the absorption of the dye changes with pH (pK = 5.7) all changes in optical density at pH 6.5 were multiplied by the factor 1.23 (Punnett, 1959).

The initial optical density of each cuvette was approximately 0.7. From the above data each cuvette can therefore be calculated to contain in practice only 0.12 γ moles of dye. This discrepancy to the theoretical

0.16 μ moles was attributed to impurity of DCPIP used in the investigations.

Assay of potassium ferricyanide reduction

The reaction medium was made up in 1 cm light path cuvettes from stock solutions of the following :

A	0.05M KH_2PO_4	-	1.7011 g/250 ml H_2O
	0.01M KCl	-	186.4 mg/250 ml H_2O
	pH adjusted to 6.5		
B	3mM potassium ferricyanide ($K_3Fe(CN)_6$)	-	24.75 mg/25 ml H_2O

Each cuvette contained 2.5 ml solution A and 0.5 ml solution B before addition of the chloroplast preparation containing 30-40 γ chlorophyll. Each reaction cuvette thus contained KH_2PO_4, 125 μmoles; KCl, 25 μmoles; $K_3Fe(CN)_6$, 1.5 μmoles and chloroplasts equivalent to approximately 30-40 γ chlorophyll in a total volume of 3.0 ml. A reference cuvette was set up containing water in place of the ferricyanide solution. Measurements of extinction at 420 nm were made after successive 2 min illumination periods. The rate of ferricyanide reduction was calculated from the decrease in extinction over the first 2 min illumination period using a molar extinction coefficient of 980 $l.\ mol^{-1}\ cm^{-1}$.

Assays involving the use of the Oxygen Electrode

Activities involving O_2 uptake were determined with a Yellow Springs Oxygen Monitor (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) using a Clark electrode.

Principles involved in the Polarographic Measurement of O_2 concentration

This account will deal only briefly with the principles involved in polarography. Further details can be found in a review of the historical, theoretical and experimental aspects of O_2 measurement with various types of electrode (Lessler & Brierley, 1968); also in another more recent review (Beechy & Libbons, 1971) there is a summary of the theory behind polarographic measurements.

The Clark electrode consists of a probe carrying a platinum cathode and a silver/silver chloride anode immersed in KCl solution and enclosed by a thin tightly fitting teflon membrane, permeable only to oxygen, which serves to separate the electrodes and KCl solution from the incubation medium under study.

A potential of 0.8V is applied across the electrodes which are connected by a resistance. Oxygen diffusing to the cathode is reduced according to the equation :

$$2H_2O + O_2 + 4e^- \longrightarrow 4OH^-$$

Electron flow from the anode through the external resistance compensates the loss of electrons from the cathode and causes the Ag atoms of the anode to form Ag^+ ions which are neutralised by Cl^- ions taken up at the anode from the KCl solution. The overall reaction is shown by the equation :

$$4Ag + 2H_2O + O_2 + 4Cl^- \longrightarrow 4\,AgCl + 4OH^-$$

The electron flow (current) through the resistance is proportional to the O_2 consumption at the cathode which itself depends on the rate at which O_2 diffuses to it. The rapidity with which oxygen is consumed once inside the teflon membrane is of such magnitude that it can be assumed that the rate of diffusion of oxygen to the cathode is directly proportional to its concentration in the incubation medium external to the membrane. Thus the current flowing in the resistance is a direct indication of the O_2 concentration in the incubation medium enabling the reactions involving O_2 uptake to be monitored.

The voltage produced when the current from the probe passes through the external resistance is amplified and the signal produced can be read directly on a meter or indirectly on a chart recorder. The oxygen monitor arrangement used in the present studies utilised a chart recorder.

Calibration of the Instrument

Each incubation mixture was stirred for at least 3 min to saturate it with air before the start of an assay. The electrode probe was then introduced into the incubation chamber and the readings on the meter and recorder adjusted to 100%. The rate of change in % O_2 saturation during each assay was obtained from the chart recorder. Experiments were normally carried out at either 20°C or 25°C, accurate thermostatic control which is essential because the permeability of the membrane and therefore the current registered for a given oxygen concentration is dependent on temperature, being achieved by an Ultra-thermostat type K2 (Messgeräte-Werk Lauda, W. Germany).

Typical calculation of change in oxygen concentration

The volume of O_2 (reduced to standard temperature and pressure) that will dissolve in 1 ml of water at, for example, 25°C when the gas pressure is 760 mm Hg is 28.22 µl (Bunsen's coefficient); this value is obtained from a standard curve (Fig. 10). The partial pressure of O_2 in air at normal temperatures = 0.21, therefore the amount of O_2 (reduced to standard temperature and pressure) dissolved in 1 ml H_2O at 25°C saturated with air is given by :

28.22×0.21 µl

$= 5.926$ µl O_2/ml H_2O

Thus in 1 ml of water at 25°C saturated with air there are dissolved

$5.926 \div 22.4$ µmoles O_2

$= 0.265$ µmoles O_2

Therefore in 3 ml of water (the chosen incubation volume for some experiments) saturated with air 0.795 µmoles are dissolved. If one assumes that differences in the amount of O_2 dissolving in water and the various buffers used are negligible we have :

$$\text{Rate of } O_2 \text{ uptake} = \frac{0.795 \times \Delta\%}{100} \text{ µmoles/min,}$$

where Δ % is the % change in O_2 saturation per min.

If C (mg) represents the amount of protein or chlorophyll (depending on the type of experiment in question) present in an incubation we have :

$$\text{Rate of } O_2 \text{ uptake} = \frac{0.795 \times 60 \times \Delta\%}{100 \times C} \text{ µmoles/h/mg protein or chlorophyll}$$

However, after the calibration procedure carried out prior to each assay it was invariably found that the reaction mixture contained between 90 - 98% of the O_2 dissolved in the same volume of deionised water.

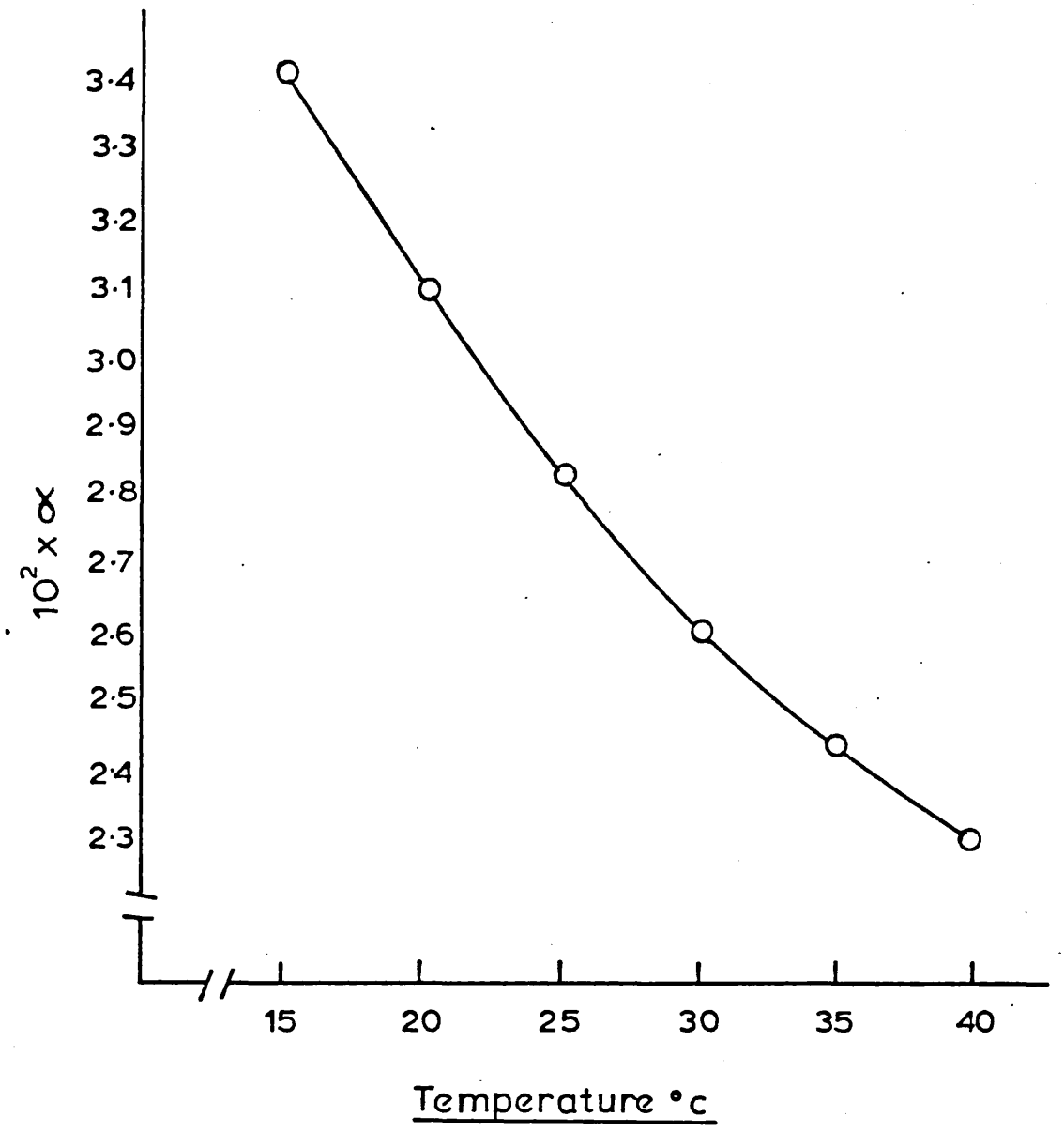


Fig. 10: Graph showing the relationship between Bunsen Coefficient and Temperature

(Based on data given in the Chemical Rubber Company Biochemistry Handbook)

In each case the final steady reading was noted and if, for example, the final reading noted was 95% then the amount of O_2 dissolved in the reaction medium at saturation was calculated as :

$$\frac{95}{100} \times 0.795 \text{ μmoles } O_2$$

Measurement of photosynthetic electron transport by oxygen evolution

Cockburn et al (1968) have shown that chloroplasts isolated from spinach leaves by their rapid isolation procedure involving pyrophosphate buffer are capable of catalysing photosynthetic O_2 evolution in the absence of an added Hill oxidant, indicating the retention of an intact chloroplast envelope.

Assays of photosynthetic oxygen evolution by wheat chloroplasts isolated by the procedures described on pages 92 - 93 were conducted at 20°C in an oxygen electrode vessel containing the following reaction medium :

0.33M sorbitol, 1.0 mM $MgCl_2$, 1.0mM $MnCl_2$, 2.0 mM EDTA, 10 mM $NaHCO_3$ and 50 mM Hepes buffer adjusted to pH 7.6. The chlorophyll concentration in a total volume of 3.0 ml was 60-100 γ.

After calibration of the instrument using 3.0 ml deionised H_2O, the subsequent reaction volume, the components indicated were added to the reaction vessel and the oxygen electrode probe was immediately carefully inserted so as to exclude all air bubbles. The solution was stirred for some 3 min to achieve equilibration, the reaction chamber being kept dark during this time by means of a light shield placed over the reaction assembly. While the reaction chamber was still enclosed by the light shield, the meter and recorder readings were noted and then

adjusted to 30%. The recorder was switched on and the reaction was initiated by removal of the light shield and illuminating the system at an intensity of approx. 55,000 lx using a 375 W photographic lamp (Atlas Pl/6, E.S. E27/27). A heat filter consisting of a 7 cm wide glass tank through which cold water was continually passing from tap to sink, was interposed between the lamp and the reaction chamber. Where indicated 10 µl of a methanol solution of metoxuron (final concentration in the reaction mixture = 3.3×10^{-7}M) was added to the reaction vessel, any effect being observed as a deflection on the recorder trace. Rates of oxygen evolution, measured over steady linear regions of the trace, were subsequently expressed as µmoles O_2 evolved/mg chlorophyll/h.

Assay of cyclic photophosphorylation in chloroplasts

In cyclic photophosphorylation, which results only in ATP formation (Arnon et al, 1954), photosystem 1 alone is involved. Photosystem 1 results in formation of reduced ferredoxin and an "oxidised" form of the chlorophyll a pigment system ($P700^+$). Electrons then flow in accordance with the thermochemical gradient from ferredoxin to a chain of electron carriers, probably including cytochrome components, via unknown intermediates, and then along the chain of carriers back to the chlorophyll a pigment system. The cyclic electron flow is accompanied by ATP formation thought to be at the primary phosphorylation site associated with non-cyclic electron flow (Losada, Whatley & Arnon, 1961; Arnon, 1967; Boardman, 1968; Knaff & Arnon, 1969c) though there may be an additional phosphorylation site(s) involved in cyclic electron transport.

Cyclic photophosphorylation can be catalysed by a number of electron carriers, of a physiological nature such as menadione (Arnon, 1955) or a non-physiological nature such as phenazine methosulphate (PMS) (Jagendorf & Avron, 1958). However, later evidence (Arnon, 1967) points to ferredoxin, an iron-containing protein found in chloroplasts, as being the endogenous catalyst for *in vivo* cyclic photophosphorylation. In the present studies phenazine methosulphate (PMS) has been used as an *in vitro* redox dye catalysing a cyclic electron flow which can be accompanied by photophosphorylation.

All assays were made using chloroplasts isolated by the procedure of Plesničar & Bendall (1973)(Medium B, page 90).

The reaction medium was made up in 1 cm light path cuvettes and was based on the method of Kleese (1966). Preliminary experiments indicated that maximum rates of photophosphorylation in wheat chloroplasts are observed at pH 8.0. The reaction medium was made up from stock solutions of the following :

A 2×10^{-4}M PMS (M. Wt. 306.3) - 6.12 mg/100 ml H_2O

This solution was made up immediately prior to use and kept in the dark.

B 68.06 mg tris buffer; 43.8 mg NaCl; 67.46 mg ADP; 30.50 mg $MgCl_2$, and 8.50 mg KH_2PO_4 in 25 ml H_2O, pH adjusted to 8.0.

Each cuvette was prepared by adding 0.5 ml of solution A and 2.0 ml of solution B before addition of the chloroplast preparation containing 50 - 80 γ chlorophyll. Each reaction cuvette thus contained 0.1 µmole PMS; 45 µmoles tris buffer (pH 8.0); 60 µmoles NaCl; 12 µmoles $MgCl_2$;

10.9 μmoles ADP; 5 μmoles KH_2PO_4 and chloroplasts equivalent to 50-80 γ chlorophyll in a total volume of 2.6 ml.

The cuvette contents were thoroughly mixed by inversion and a 0.5 ml sample was removed from each and added to 5.5 ml of 6.25% trichloroacetic acid. The cuvettes were placed in the illumination apparatus and illuminated for a fixed time (usually 3 min) after which a further 0.5 ml sample was removed and added to trichloroacetic acid to terminate the reaction.

The 0.5 ml aliquots represent the initial and final concentrations of inorganic phosphate in the reaction medium. Each acidified aliquot was submitted to an inorganic phosphorus determination by the method of Taussky & Shorr (1953). The rates of photophosphorylation were calculated using the difference in the initial and terminal inorganic phosphate concentrations, and were expressed as μmoles phosphate/ mg chlorophyll/h.

Determination of inorganic phosphate by the method of Taussky & Shorr (1953)

Reagents Required

5M H_2SO_4 - 278 ml of concentrated acid were slowly added to about 700 ml of deionised water and after cooling the volume was diluted to 1 litre.
6.25% TCA.

Ammonium molybdate stock solution: 50 g $(NH_4)_6Mo_7O_{24}$, $4H_2O$ were weighed into a litre beaker and about 400 ml 5M H_2SO_4 added with constant stirring to prevent "caking". When completely dissolved the solution was transferred to a 500 ml graduated flask and washed in quantitatively with 5M H_2SO_4 to the mark (final concentration 10%).

Ferrous sulphate-molybdate reagent: 10 ml of the ammonium molybdate solution were pipetted into a 100 ml volumetric flask and diluted to 70 ml with deionised water. 5 g $FeSO_4 . 7H_2O$ were added and the solution was made up to volume and shaken until the crystals dissolved. The solution was prepared immediately before use and kept in the dark.

0.5 ml Aliquots of the reaction mixture were added to 5.5 ml of 6.25% TCA to precipitate protein, and the acidified mixtures were centrifuged at 1000 x g for 5 min. To 1.5 ml of the supernatant was added 1 ml of the freshly prepared $FeSO_4$-molybdate reagent. The blue colour developed rapidly (maximum colour after 1 min) and is stable for at least 2 h. The calibration graph gave a linear plot passing through the origin from which the relationship $\underline{E}_{725}$ nm x 22.7 = γ phosphorus per 1.5 ml aliquot, was obtained (Fig. 11). The initial and final concentrations (γ) of phosphorus were thus determined by multiplying the observed optical densities by 22.7 and the actual concentrations of phosphorus in each cuvette were found by further multiplying by the dilution factor which in this case was 20.

Studies on the respiratory properties of isolated wheat mitochondria

Mitochondria were prepared from dark-grown hypocotyls as described on p. 96. Oxygen uptake was measured polarographically at 25°C in a 3 ml glass reaction vessel using a conventional Clark oxygen electrode (for details see p.102-6). After calibration of the instrument, reaction medium and mitochondria were added to the incubation chamber and the contents were stirred to saturate with O_2. The reaction mixture employed was composed of 0.3M mannitol, 10mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 10mM Tris-HCl buffer, and 0.75% (w/v) bovine serum albumin, adjusted to pH 7.2

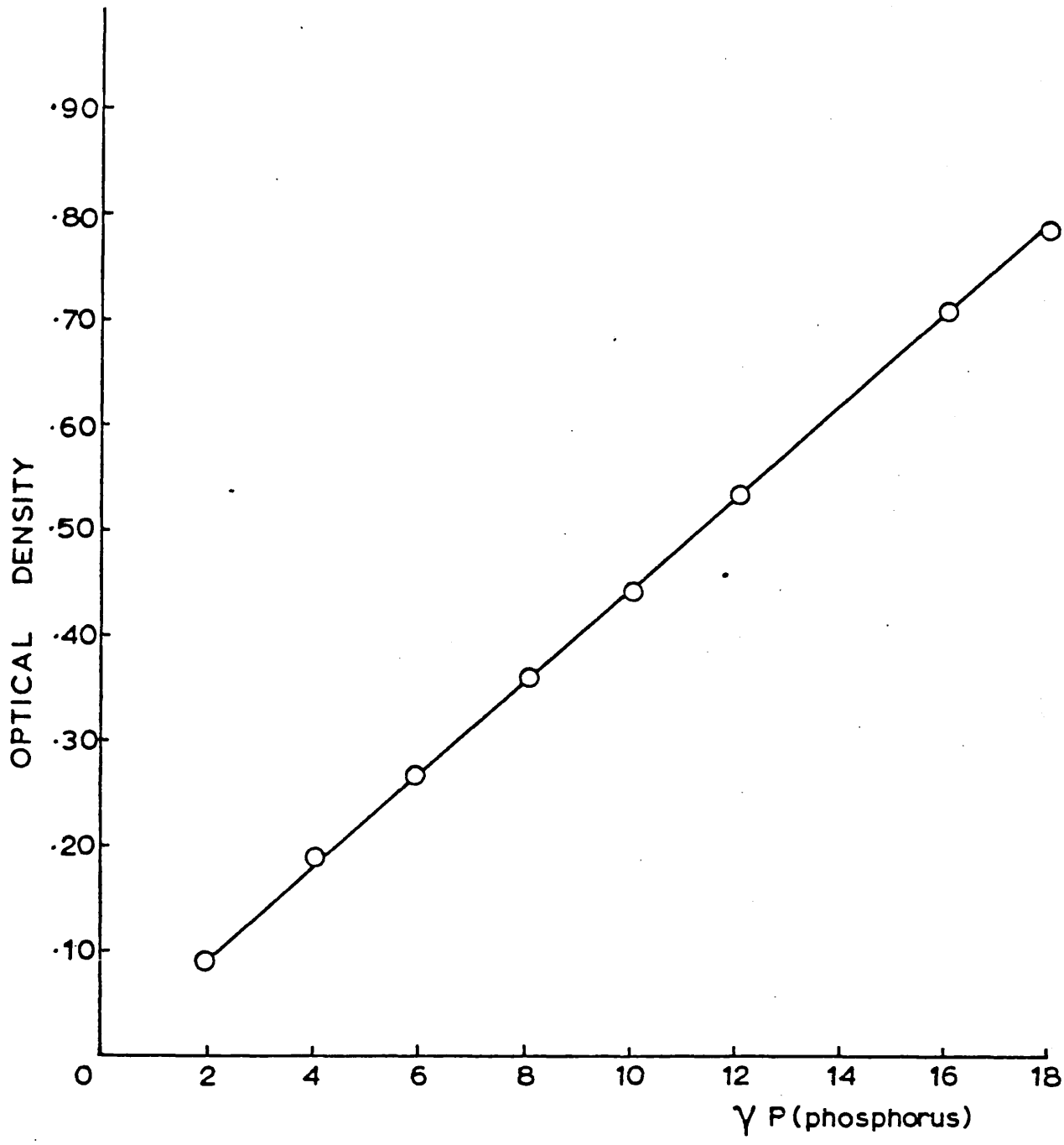


Fig. 11: Standard curve for the estimation of inorganic phosphate by the method of Taussky & Shorr (1953)

Oxygen uptake was initiated by the addition of the appropriate volume of stock solutions of substrates (0.75M, dissolved in 0.1M Tris, pH 7.2) by means of a long needled syringe, to give a final concentration of 10 mM for α-ketoglutarate, L-malate and succinate, and 1 mM for NADH. State 3 respiration was initiated by addition of 10 or 20 µl of a stock solution of ADP to give a final concentration of 50 or 100 µM. The effect of metoxuron on the rates of state 3 and state 4 respiration was examined following the addition of metoxuron during the course of the experiment to give final concentrations of metoxuron in the reaction mixture within the range of 3.3×10^{-6} to 3.3×10^{-4}M. Respiration rates were calculated from recorder tracings on the basis of a Bunsen's coefficient for O_2 at 25°C of 0.02822 ml/ml (Hodgman & Lange, 1930). All rates of O_2 consumption are expressed in µmoles/min/mg protein.

Carbon Dioxide assimilation studies.

$^{14}CO_2$ fixation by whole wheat plants

The reduction of $^{14}CO_2$ to form the intermediates used in the reductive and regenerative phase of the Calvin-Benson Cycle was studied by the distribution of radioactivity amongst the ethanol-soluble products of photosynthesis in leaves of sprayed and unsprayed resistant and susceptible plants.

The experiments were carried out with illuminated plants at various times after spraying, by exposing them to an atmosphere of $^{14}CO_2$ for 1 hour. Four plants (at the stage where the fourth leaf was just beginning to emerge) of both treated and untreated resistant and susceptible

varieties were uprooted, the roots washed, and the plants stood in 50 ml conical flasks containing a small amount of water. The flasks were then placed for exposure to $^{14}CO_2$ in the apparatus described in Fig. 12, being so arranged as to receive uniform illumination from a 375 W photographic lamp (Atlas P1/6 E.S. E27/27). The plants were illuminated in the reaction chamber for some 20 min prior to exposure to $^{14}CO_2$.

50 μCi of $Ba^{14}CO_3$ (specific activity 1.02 mCi/mmol) was placed in the weighing boat (Fig. 12) and $^{14}CO_2$ was generated by the addition of excess 10% lactic acid solution. The carbon dioxide concentration in the chamber after supply of $^{14}CO_2$ was approximately 0.08% ($^{14}CO_2$ concentration approx. 0.05%).

The leaves were allowed to photosynthesise for 1 h under a light intensity of approximately 16,000 lx. After the 1 h illumination period, the lamp was switched off and the air in the chamber was circulated for 10 min through Woulff bottles containing a saturated solution of barium hydroxide to absorb residual $^{14}CO_2$.

Following their removal from the photosynthetic chamber, the leaves of three of the plants (approx. 1 g wet weight) were immediately homogenised in hot 80% ethanol. In each case, the leaves of the fourth plant were used for wet weight-dry weight ratio determinations. The individual extracts were centrifuged to remove debris and the ethanolic solutions were carefully reduced to dryness by vacuum distillation at 36°C in a rotary vacuum evaporator (Büchi Glasapparatefabrik, Flawil, Switzerland). The residue was extracted with deionised water, filtered through a No. 3 sinta glass funnel and again reduced to dryness. The

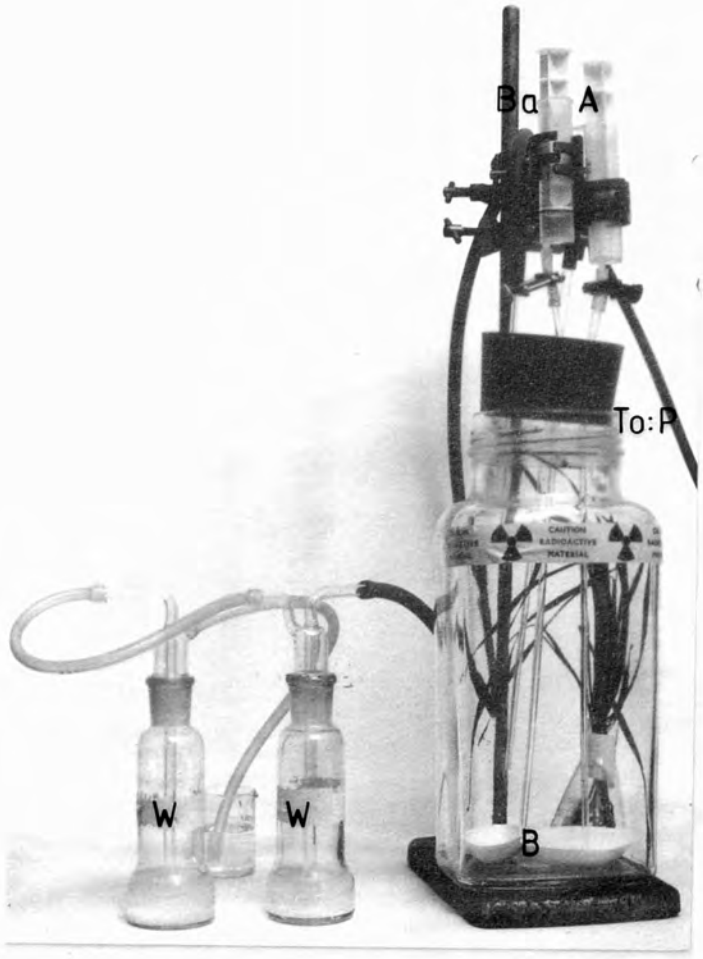


Fig. 12: Apparatus for exposure of whole wheat plants to $^{14}CO_2$

A = Acid reservoir

B = Weighing boats

P = Circulating pump

W = Woulff bottles containing saturated $Ba(OH)_2$

Ba = $Ba(OH)_2$ reservoir

Fig. 12: Apparatus for exposure of whole wheat plants to $^{14}CO_2$

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residue from the second evaporation was extracted into a small volume (5 ml) of 80% ethanol and transferred to a previously weighed 10 ml conical flask. The extract was concentrated to approximately 0.5 ml under N_2, and the remaining liquid was removed by freeze-drying in a commercial apparatus. Each flask was then re-weighed to determine the weight of the ethanol extracted material. The contents of each flask were then dissolved in 80% ethanol to a final concentration of 1 mg of ethanol soluble material per 0.1 ml solution. Suitable aliquots were removed from each extract for determination of total radioactivity using a Packard Tri-carb Liquid Scintillation Spectrometer, model 3390, equipped for external standardization. For the determination of radioactivity, the scintillation mixture recommended by Geissbühler et al (1963a) was employed. The scintillator consisted of a 0.5% solution of PPO (2,5-diphenyloxazole) in toluene and was rendered miscible with small aqueous aliquots by the addition of NCS solubilizer (Nuclear Chicago Solubilizer, Amersham/Searle Corporation, Illinois, U.S.A.) to the samples to be counted (for details see p. 132). Radioactivity was expressed as d.p.m. using the % efficiency calculated for each sample from a previously prepared quench curve (Fig. 15).

The soluble carbohydrates, amino and organic acids were separated by two-dimensional chromatography on Whatman No. 4 chromatography paper (52 cm x 44 cm), the solvent system recommended by Crowley, Moses & Ullrich (1963) being employed. The first dimension solvent, designated "semi-stench" consisted of the following mixture (prepared at least 24 h before use) :

17M Ammonia solution	100 ml
Water	950 ml
n-propanol	350 ml
Isopropanol	75 ml
n-Butanol	75 ml
Isobutyric acid	2500 ml
EDTA	1.2 g

The second dimension was developed with an n-butanol-propionic acid-water solvent (Benson, Bassham, Calvin, Goodale, Haas & Stepka, 1950) prepared from the following stock solutions :

A	n-butanol	1246 ml
	water	84 ml
B	Propionic acid (redistilled)	620 ml
	water	790 ml

In practice approximately equal volumes of A and B were adjusted to give a single phase solution. After the addition of a small amount (0.01 ml/ml) of 0.25M KH_2PO_4; K_2HPO_4 buffer (pH 6.6) to improve subsequent chromatography (Bassham & Calvin, 1957), a sample of the ethanol soluble products, equivalent to approximately 100,000 d.p.m., was applied as a spot 1 cm diameter near one corner of the chromatography paper, and the ethanol was evaporated in a stream of hot air. The paper was developed by descending chromatography in a sealed tank in an atmosphere previously equilibrated with the solvent. The first dimension was developed with the semi-stench solvent (approximately 8½ h) and the paper was then removed and dried in a fume cupboard. The second dimension was developed in a similar manner using the n-butanol-propionic acid-water solvent (7½ h). The paper was then dried in a fume cupboard and the radioactive materials were located by radioautography.

The identification of products of $^{14}CO_2$ fixation was tentatively made using the data supplied in Crowley et al (1963), Quayle (1972) and Delaney (1975).

$^{14}CO_2$ fixation by a single leaf

For experiments designed to investigate the effect of metoxuron, in resistant and susceptible wheat plants, on the translocation of photosynthetic carbon dioxide fixation products a procedure whereby individual leaves were exposed to $^{14}CO_2$ was followed.

These experiments were carried out with illuminated plants at various times after spraying, by exposing a single leaf to an atmosphere of $^{14}CO_2$ for 1 h. Plants were used at the four leaf stage and remained in their growing pots with their root systems undisturbed during the course of the experiment. Parallel experiments were carried out on control plants which had been sprayed with a solution of Tween 60 not containing metoxuron.

The photosynthetic chamber used in these studies consisted of a medium gauge polythene bag (25 x 36 cm) enclosing the single leaf to be exposed to $^{14}CO_2$ (Fig. 13). The aperture of the bag was sealed with sellotape, the leaf being subsequently inserted through a hole made in one of the bottom corners of the bag. The enclosure was made air-tight by clamping the polythene at the base of the leaf between two microscope slides, the leaf being protected from damage by the insertion of two pads of foam rubber impregnated with silicone grease. The adjacent corner of the bag was fitted with an inlet tube through which externally generated $^{14}CO_2$ was admitted by means of a small pump. Illumination was provided

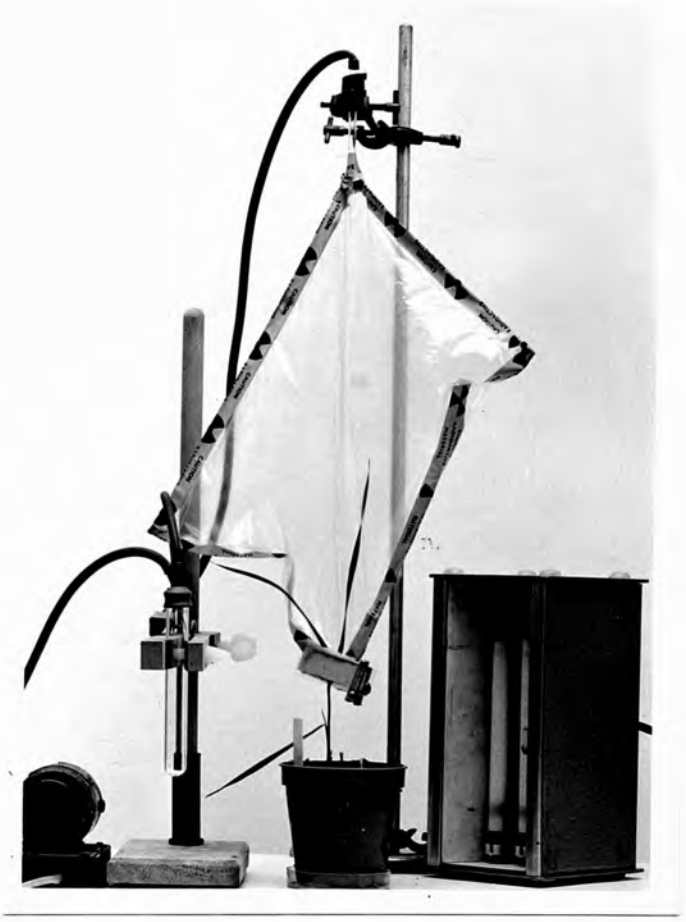


Fig. 13: Apparatus for exposure of a single leaf to $^{14}CO_2$

by means of four 6 W fluorescent strips arranged around the photosynthetic chamber to give a light intensity of approx. 16,000 lx at the leaf surface.

After rendering the photosynthetic chamber gas-tight 10 μCi of $^{14}CO_2$ was generated by the addition of excess 10% lactic acid solution to 2 mg of $Ba^{14}CO_3$ (specific activity 1.02 mCi/mmol) and was pumped into the sealed chamber. The pump was operated until the polythene bag was some 50% inflated before the inlet was closed, and $^{14}CO_2$ uptake was then initiated by turning on the fluorescent lamps.

Each leaf was allowed to photosynthesise for 1 h after which time the chamber was removed and the plants returned to the greenhouse. After some three days the plants were uprooted and prepared for autoradiography as described by Crafts & Yamaguchi (1964).

Isolation and estimation of ethanol-soluble carbohydrates from winter wheat

McDonald & Henderson (1964) pointed out that the cold water extract from Graminae consists mainly of glucose, fructose, sucrose, fructosan (fructose containing oligosaccharides) and some melibiose and raffinose. Although the fructosans of Graminae are extracted with cold water, they remain in the residue after ethanol extraction (Laidlow & Reid, 1952; McDonald & Henderson, 1964 and Adegbola & McKell, 1966).

The present studies were made using control and metoxuron sprayed plants of both susceptible and resistant winter wheats. The material to be analysed (2 g wet weight) was cut into pieces 1-2 cm long and immediately plunged into boiling ethanol. The supernatant was decanted and stored while the plant segments were transferred to a soxhlet thimble and were

extracted in a soxhlet extractor with 80% ethanol for 7 h. The ethanolic extract was recombined with the corresponding stored supernatant and the whole evaporated to dryness by vacuum distillation at 35°C on a rotary vacuum evaporator. The carbohydrates were extracted with deionised water, filtered through a No. 3 sinta glass funnel, and transferred (with water washings) to a 100 ml volumetric flask. After diluting to 100 ml the extracts, containing glucose, fructose and sucrose, were only very slightly coloured and were not further purified.

For the determination of carbohydrate, 1 ml aliquots of the above extracts were used. However, where the concentration of carbohydrate in the extract was very high suitable dilutions were assayed.

Carbohydrate was estimated by the phenol-sulphuric acid method, described by Dubois, Gilles, Hamilton, Rebers & Smith (1951, 1956). The method has the advantages of being simple, rapid, sensitive and specific for carbohydrates. Virtually all classes of sugars, including sugar derivatives, and oligo- and polysaccharides, can be determined. For this reason, as with similar methods (e.g., the anthrone determination of Dreywood, 1946), careful attention was given to the exclusion of cellulosic fibres from the final extracts.

1 ml of aqueous extract, containing between 10 and 70γ carbohydrate, was pipetted into a boiling tube and 1 ml of 5% (w/v) phenol (redistilled reagent grade) solution was added and mixed. Blanks for each determination were prepared with 1 ml of water in place of the sugar solution. 5 ml of 96% (w/w) H_2SO_4 (Reagent Grade) was rapidly pipetted into each tube so that the stream hit the liquid surface directly to produce rapid mixing and even heat distribution. In practice a 5 ml pipette with the orifice enlarged by grinding was used. Each tube was agitated during the acid addition and the same mixing procedure was used throughout. After 10 min

standing, the tubes were again shaken for a few seconds and placed in a water bath at 25-30°C for 20 min. The yellow-orange colour produced is stable for several hours. The extinction at 490 nm was measured using a Unicam SP 500 spectrophotometer against H_2O as reference. The average reading of the reference cell (H_2O instead of extract in assay mixture) was subtracted and the amount of carbohydrate then determined by reference to a standard graph previously prepared for a particular carbohydrate. For assays involving the determination of total ethanol soluble carbohydrate in the extracts (i.e., glucose, fructose and sucrose), determination was made through comparison with the standard graph prepared for glucose. Results are expressed on a percentage dry weight basis.

Determinations were made in duplicate in an attempt to identify and eliminate anomalies resulting from accidental contamination with cellulose support. This was particularly important for determination of carbohydrates eluted from paper chromatograms.

Paper chromatography of the ethanol-soluble carbohydrate fraction from winter wheat leaves

Chromatography was carried out on Whatman No. 1 chromatography paper (42 x 57 cm). By means of vertical lines the paper was delineated into 4 vertical channels of equal width. A 0.03 ml aliquot of the solution to be analysed was applied to each of the two centre channels along a line 10 cm from the edge of the paper. To another section was applied a mixture of reference carbohydrates consisting of D(-) fructose, sucrose, D(+) glucose, raffinose and D(-) ribose in 1% solution, while to the other section was applied 200 µg ribose in 0.03 ml. The

application of ribose enabled the percentage recovery of the individual sugars from the chromatography paper to be assessed. The chromatograms were allowed to equilibrate in the tank for about 30 min before addition of the developing solvent comprised of ethyl acetate, acetic acid and water in the ratio 3:3:1. The chromatograms were allowed to develop for 23 h and for this reason the chromatograms were previously serrated along the bottom edge to allow solvent to drip freely from the paper.

After irrigation, the paper was thoroughly air-dried, and the indicator strips cut off and sprayed with p-anisidine hydrochloride (a 3-4% solution of p-anisidine hydrochloride in 1-butanol-ethanol-H_2O (4:1:1, v/v) containing a trace of stannous chloride for stabilisation and sensitization). On heating to 100-120°C, pentoses are indicated by development of a cherry-red colour, aldohexoses a green-brown zone and ketohexoses a yellow zone. The method is capable of detecting less than 5 γ of a reducing sugar and, except for acid-labile oligosaccharides such as sucrose, non-reducing sugars are not reactive (Pridham, 1956).

The strips were carefully fitted to the papers from which they were cut, and sections corresponding to the indicator zones were marked and cut out for elution of sugars.

The zones containing sugars were cut into pieces 2-3 cm^2 and eluted by agitation in glass stoppered tubes for 10 min with 5 ml deionised water; the extracts contained 5-50 γ sugar/ml. After filtration through a No. 3 sinta glass funnel to remove cellulosic fibres, 1 ml aliquots of the filtrate were analysed for carbohydrate as previously described. Blank determinations were made on comparable areas of filter paper cut from sheets irrigated with the same solvent. Results are expressed as a percentage of the total carbohydrate eluted from the chromatograms.

Absorption, Distribution and Metabolism of Metoxuron by Resistant and Susceptible Winter Wheat

Substituted phenylurea herbicides have been shown to be rapidly absorbed by roots of plants and to be translocated acropetally in the transpiration stream (Haun & Peterson, 1954; Fang et al 1955; Crafts & Yamaguchi, 1960; Bayer & Yamaguchi, 1965; Smith & Sheets, 1967). Studies made using radioactively labelled monuron, diuron, linuron, chlortoluron and metoxuron have indicated absorption of these herbicides by leaves and stems as well as by the root systems of several plant species (Van Oorschot, 1965, 1968; Nashed & Ilnicki, 1970; Muller & Sanad, 1975). Accumulation of ^{14}C in aerial portions of plants demonstrated following root absorption of these labelled herbicides appeared to be dependent to a large extent on the rate of transpiration. Muzik et al (1954) and Minshall (1954) have shown conclusively that factors favouring reduced transpiration also delay or prevent the onset of phytotoxic symptoms in plants exposed to otherwise lethal concentrations of monuron. In studies reported by Bayer and Yamaguchi (1965) radioautographs of intact plants indicated no species differences in absorption, distribution or accumulation between barley and soybean plants exposed to (carbonyl-^{14}C)diuron via the culture medium. However other workers have demonstrated very substantial differences between different species, and in some cases varieties, with respect to absorption, distribution and accumulation of various substituted phenylurea herbicides. Smith and Sheets (1967) pointed out that the susceptibility differences observed among oat (Avena sativa L.), soybean (Glycine max L.) and corn (Zea mays L.) to (carbonyl-^{14}C)diuron were best explained by differences in absorption.

Hogue & Warren (1968) showed that tomato (Lycopersicum esculentum), a susceptible species, readily absorbed and translocated linuron to the leaves, only a small percentage of the herbicide being retained by the roots. This was in contrast to the resistant species, parsnip (Pastinaca sativa L.), in which the greatest part of the absorbed linuron was located in the root system with only a small percentage being translocated to the leaves. Osgood et al (1972) reported that sugarcane cultivars (Saccharum L. hybrids) differed considerably in their tolerance to diuron. Analysis of the plants following application of the herbicide to the roots showed that there was a greater concentration of diuron in the younger leaves of the susceptible compared to the resistant cultivars.

Many of the studies made using (^{14}C)monuron and (^{14}C)diuron have utilised gross radioautography of plants and could not therefore unequivocally demonstrate absorption, translocation or distribution of the intact herbicide molecule.

The metabolic fate of these widely used herbicides became of obvious concern in a society so concious of pesticide residues. Though much progress has now been made in this area our knowledge of the metabolism of substituted phenylureas is still incomplete. Fang et al (1955) have reported a monuron complex in the leaves of bean (Phaseolus vulgaris) after foliar application of the ^{14}C-labelled herbicide. The unidentified monuron complex increased with time concomitant with a parallel decrease in the concentration of free herbicide. That substituted phenylureas can be extensively degraded in biological systems was demonstrated by Hill et al (1955) who reported that certain soil

microorganisms were capable of utilising monuron as sole carbon source. Geissbühler et al (1963b) and Dalton, Evans & Rhodes (1966) independently identified decomposition products of phenylurea herbicides in soil and plant extracts treated with the herbicides. As a result of their more extensive study, Geissbühler et al (1963b) were able to present details of the relative phytotoxicities of the various metabolites and to suggest a degradation sequence. These studies indicated that metabolism of phenylurea herbicides appeared to involve successive N-demethylation reactions followed by hydrolysis of the resulting urea (see Fig. 4). The monomethyl product resulting from a single N-demethylation was reported to show some 50% of the phytotoxicity of the parent herbicide whereas the products formed by removing the second methyl group, and by the hydrolysis of the urea, were non-herbicidal (Geissbühler et al 1963b).

Smith and Sheets (1967) have presented schemes of degradation for monuron and diuron in cotton and soybean. The major ^{14}C-labelled constituents of leaf extracts of cotton (resistant) following treatment with (^{14}C)monuron or (^{14}C)diuron were non-phytotoxic, whereas those of soybean (susceptible) extracts retained considerable phytotoxicity. Rogers and Funderburk (1968) reported that the differential susceptibility of cotton (Gossypium hirsutum L.) (resistant) and cucumber (Cucumis sativus L.) (susceptible) to fluometuron could primarily be attributed to a difference in ability to degrade the applied phenylurea to less or non-phytotoxic compounds. Osgood et al (1972) reported that sugarcane cultivars degraded diuron to N-monodesmethyl and N-bisdesmethylated derivatives but degradation of diuron was more extensive in resistant

cultivars. Furthermore, a cotton leaf microsomal oxidase system which N-desmethylated monuron has been reported by Frear et al (1969). This enzyme was shown to require molecular oxygen and reduced pyridine nucleotides as cofactors.

The present studies were aimed at determining whether any differences could be detected between resistant and susceptible wheat plants with respect to (a) absorption, translocation and subsequent intracellular distribution of metoxuron; (b) nature and rate of metoxuron metabolism and (c) site of metabolism.

Preparation of ^{14}C-labelled Metoxuron

A sample of (methoxy-^{14}C)metoxuron was prepared from the unlabelled technical material by a procedure involving demethylation at the methoxy group followed by a remethylation reaction involving (^{14}C)methyl iodide (Fig. 14).

O-Demethylation of technical metoxuron

O-Demethylation was achieved by reacting technical metoxuron with boron trichloride in anhydrous methylene chloride by the procedure described below.

A solution of metoxuron (114 mg, 0.5 mmol) in 25 volumes of CH_2Cl_2 was added at -80°C to a solution of BCl_3 (117 mg, 1 mmol) in 7.5 volumes of CH_2Cl_2. The resulting mixture was allowed to warm to room temperature (22°C) over-night, being protected from ingress of water by fitting a calcium chloride tube to the reaction vessel. The methylene

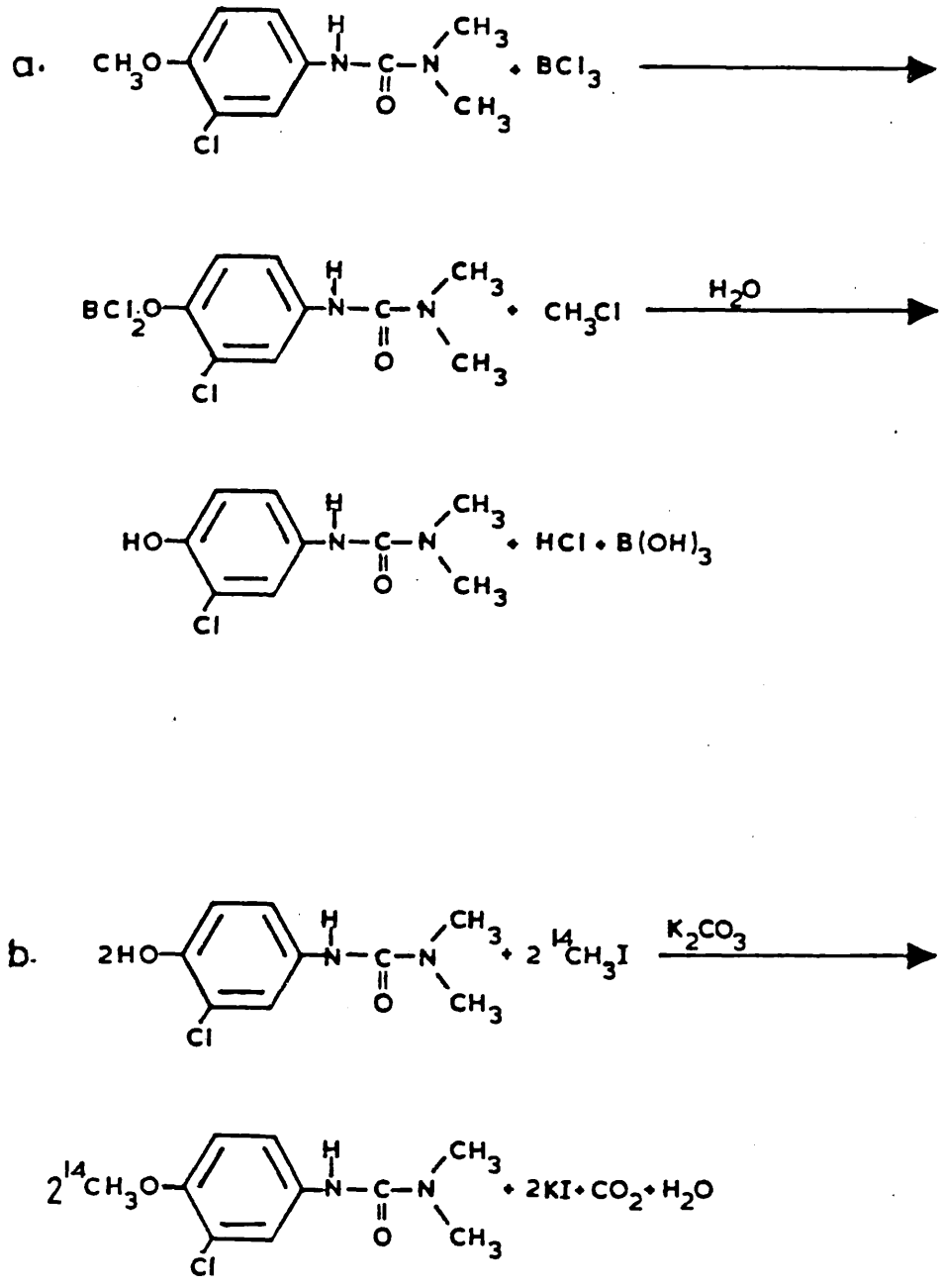


Fig. 14: Reactions involved in the preparation of (methoxy-^{14}C)metoxuron

a. O-demethylation of technical metoxuron using boron trichloride

b. Re-methylation of N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea with (^{14}C)methyl iodide

chloride and excess BCl_3 reagent were subsequently removed by rotary evaporation. The residue was dissolved in dry methanol and the methanol was removed by distillation. This procedure was repeated twice to hydrolyse boron complexes and to remove volatile boron products from solution. Following evaporation to dryness the phenolic product, N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea, was dissolved in diethyl ether and transferred to a separating funnel. The solution was shaken with an equal volume of 0.5M NaOH and the aqueous layer was collected and washed 2-3 times with ether. On neutralisation of the alkaline layer with IN HCl the phenol precipitated out of solution and was collected by vacuum filtration, washed and finally recrystallised from aqueous ethanol.

O-Methylation of N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea

Radioactivity was incorporated into the metoxuron molecule by the methylation of the phenolic derivative resulting from the procedure outlined above using (^{14}C)methyl iodide. The method adopted was based on a procedure reported by Vyas & Shah (1963). A solution of N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea (18 mg, 0.084 mmol) and (^{14}C)methyl iodide (6·9mg, 0.05 mmol, specific activity 10.0 μCi/μmol) in 1 ml dry acetone in a 5 ml round bottom flask was refluxed in the presence of potassium carbonate (23 mg, 0.166 mmol) for 4 h. A further amount of unlabelled methyl iodide (5.7 mg, 0.04 mmol) was then added to the reaction mixture and the refluxing was continued for a further 2 h. The acetone solution was decanted from the insoluble salts, reduced in volume and applied to a 1 mm preparative TLC plate coated with silica gel type H. The chromatogram was developed with a solvent

containing chloroform and methanol (9:1 v/v) and the radioactive herbicide was located by reference to a technical metoxuron standard applied to a marker zone. The zone containing the (methoxy-^{14}C)-metoxuron was scraped from the chromatographic plate and the material was recovered by exhaustive washing of the gel with methanol on a No. 3 sinta-glass funnel. This procedure yielded 14 mg of N'-(3-chloro-4-(^{14}C)methoxyphenyl)-N-N-dimethylurea (theoretical yield - 19 mg) of specific activity 4 mCi/mmol.

Absorption and Translocation of Metoxuron in Winter Wheat Plants

There is now general agreement that all substituted phenylureas so far examined are easily taken up from nutrient solution and soil by root systems and that most of them are subsequently rapidly translocated into stems and leaves by the transpiration stream (Geissbühler et al 1963a, Bayer & Yamaguchi, 1965). Movement of substituted phenylureas within plants has been investigated mainly with the aid of biological and gross autoradiographic techniques (Minshall, 1954; Bayer & Yamaguchi, 1965).

Uptake and distribution of metoxuron in the present study was investigated following exposure of plants at the three-leaf stage to 2 μCi of (methoxy-^{14}C)metoxuron (sp. act. 4 mCi/mmol) added to 40 ml of nutrient solution contained in 50 ml conical flasks. The roots were left exposed to the radioactive herbicide for three days, after which time the plants were removed and the roots rinsed with 50% aqueous methanol. The methanolic rinsings were returned to the culture flask and the combined contents were retained for radioactivity determination. After

blotting the roots dry, the intact plants were prepared for autoradiography by freezing at -70°C followed by freeze-drying in a commercial apparatus (Yamaguchi & Crafts, 1958). During this procedure plants were held flat by being placed in a folder of chromatography paper clamped between two sections of hardboard. The dried plants were placed in contact with Kodirex X-ray film, 35 x 43 cm (Kodak Ltd., Lond.) for some two weeks exposure prior to development.

Following the autoradiography procedure, the dried plants were exhaustively extracted with boiling methanol. A measure of the amount of herbicide within each plant was subsequently also achieved by subjecting the methanolic extracts to radioactivity determination in a liquid scintillation counter.

Intracellular distribution of absorbed metoxuron in wheat

Since it is now well documented that the mode of action of the phenylurea herbicides can be explained in terms of their potency in inhibiting photosynthetic electron transport, significant concentrations of herbicide would be expected to accumulate in the chloroplast fraction of the cell following initial uptake. In a study of the uptake of selected herbicides by isolated plant cells and protoplasts, Boulware & Camper (1973) observed a marked absorption of p-nitrophenyl-α,α,α-trifluoro-2-nitro-p-tolyl ether (fluorodifen) and α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine (trifluralin), whereas they were unable to detect any significant absorption of fluometuron and chlorbromuron by either isolated cells or protoplasts. When the intracellular distribution of the absorbed compounds was subsequently examined following separation of organelle fractions by density gradient centrifugation,

it was found that trifluralin was distributed evenly between the mitochondrial and the chloroplast-nuclei fraction, whereas fluorodifen (an inhibitor of photosynthetic electron transport) was concentrated in the chloroplast-nuclei fraction (Boulware & Camper, 1973). Since various herbicides thus show different patterns of distribution between subcellular organelles, it was of interest in the present study to see whether the same herbicide, in this case metoxuron, might behave differently in this respect in resistant and susceptible wheat varieties.

Nutrient grown wheat plants with intact roots were transferred to a second nutrient solution containing 2 μCi (methoxy-^{14}C)metoxuron (sp. act. 4 μCi/μmol) some four days prior to harvesting. 3 g fresh weight of leaf tissue from such plants were homogenised for 10 s with 50 ml of a buffer containing sorbitol, 0.3M; KCl, 0.01M; and Tricine buffer, 0.05M (pH 7.3). The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 200 x g for 5 min. The resulting supernatant fraction was subjected to differential centrifugation for 10 min at 1500 x g, 20 min at 9000 x g and finally 80 min at 100,000 x g. The 1,500 x g, 9000 x g and 100,000 x g pellets were resuspended in 1 ml of homogenising medium. After solubilisation with NCS the amount of radioactivity in each fraction and in the final supernatant was determined by liquid scintillation counting. Protein concentration was determined by the method of Lowry et al (1951). Results are expressed as d.p.m./mg protein for each fraction.

Organelle characterisation

The distribution of chloroplasts in the pellets obtained from differential centrifugation was ascertained on the basis of their chlorophyll content as determined by the method of Arnon (1949, see p. 95).

Mitochondria were determined by the presence of succinate-DCPIP reductase activity determined spectrophotometrically by the method of King (1967).

The reaction medium was made up directly in 1 cm light path cuvettes from stock solutions of the following.

A 0.2M phosphate buffer
pH adjusted to 7.4

B 3mM EDTA
pH adjusted to 7.4

C 0.6M succinic acid
pH adjusted to 7.4 with NaOH

D 5.3×10^{-4}M DCPIP

E 60 mM KCN

To each cuvette was added 1.5 ml solution A, 0.3 ml solution B, 0.1 ml solution C, 0.3 ml solution D, 0.05 ml solution E and 0.7 ml H_2O to a final volume of 3 ml. The reaction was initiated by the addition of 0.05 ml of resuspended pellet and the fall in extinction at 600 nm was recorded at 21°C on a Unicam SP 1800 spectrophotometer operated in the fixed wavelength mode using as reference a similarly prepared cuvette in which H_2O was substituted for the organelle fraction. Preliminary experiments showed that the reaction proceeded linearly over a 3 min period, the time employed for subsequent studies.

Microsomes can readily be characterized on the basis of their NADP-dependent electron transport system. The microsomal electron transport activity used in the present identification study was the NADPH-DCPIP reductase activity and was based on Dignam & Strobel (1975). The reaction mixture for acceptor reduction contained, in a final volume of 1.0 ml, 0.3 mmol KH_2PO_4, pH 7.7; 0.1 µmol NADPH, and 0.1 mmol DCPIP. After equilibration at 30°C the reactions were initiated by the addition of 0.01 ml of organelle fraction and the fall in extinction at 600 nm due to dye reduction was recorded on a Unicam SP 1800 spectrophotometer. In all cases the reference cuvette contained the same components as the reaction cuvette with the omission of NADPH.

Rates of marker enzyme activities were expressed as µmol/mg protein/h, protein concentrations being determined by the biuret method (Cornall, Bardawill & David, 1949).

Metabolism of metoxuron by winter wheat varieties

The importance of the ability of organisms to metabolise exogenous chemicals is gaining increasing recognition. It has now been demonstrated that such metabolism is of fundamental significance not only in the mode of action of herbicides and in the resistance of plants to the effects of applied herbicides but also in the determination of the amount of such chemicals that may remain in crops harvested for human utilisation (Freed & Montgomery, 1963).

The ability of biological systems to degrade substituted urea herbicides was initially established in studies concerned with their

dissipation from soils (Sheets, 1964). Recent interest in their metabolism by higher plants was apparently stimulated by a report by Welkner (1969) that two unidentified ^{14}C-labelled metabolites were formed from (^{14}C)monuron by Abutilon theophrasti. Subsequently Geissbühler et al (1963b) reported that Polygonum convolvulus and Golinsoga parviflora degraded (^{14}C)chloroxuron to the corresponding N-monodesmethylated,N-bisdesmethylated, and aniline derivatives. The same workers further suggested that differences observed in the rate of degradation by the two species might offer an explanation, at least in part, for their differential susceptibility. Smith & Sheets (1967) and Swanson & Swanson (1968) have reported similar degradative routes for monuron and diuron in cotton and soybean. The latter investigators demonstrated that cotton strongly metabolised monuron and diuron to the N-monodesmethylated, N-bisdesmethylated and aniline derivatives, whereas in soybean, metabolism did not progress beyond the first demethylation, and maize was unable to metabolise the herbicides in short term studies.

In a more detailed study, Frear, Swanson & Tanaka (1969) examined the extent to which certain phenylurea herbicides could be degraded by various plant species. Their results were in general agreement with those of Geissbühler et al (1963b), Smith & Sheets (1967) and Swanson & Swanson (1968) in that good correlation was observed between rate of metabolism of applied herbicide and the degree of resistance exhibited by the species studied. Of particular relevance to the present investigation is the report (Osgood et al, 1972) that certain sugarcane cultivars differ with respect to their rate of degradation of (^{14}C)-diuron.

In the present study a preliminary investigation was conducted to determine the metabolic fate of (^{14}C)metoxuron in resistant and susceptible wheat varieties. Further studies were made using leaf disc segments so that the inherent ability of photosynthetically-active tissue of resistant and susceptible varieties to degrade (^{14}C)metoxuron could be compared.

Metabolism of (^{14}C)metoxuron in susceptible and resistant wheat plants

For herbicide metabolism studies wheat plants were grown in nutrient culture as described previously (p 87). At the three-leaf stage of growth two plants of each variety were transferred to a fresh culture medium containing in addition 1 μCi (methoxy-^{14}C) metoxuron (sp. act. 4 mCi/mmol), the volume of the nutrient solution being approximately 80 ml per two plants. The roots of the plants were kept in darkness by covering the culture vessels with aluminium foil. Plants were harvested after 3-5 days treatment, care being taken to rinse the root systems free of adhering nutrient solution. The washings were returned to the culture vessel and the entire contents saved for determination of radioactivity not absorbed by plants. The roots were blotted lightly with tissue paper to remove excess water, and were then cut into small (1 cm length) segments prior to their exhaustive extraction with 40 ml boiling methanol. The individual extracts were centrifuged to remove debris and the methanolic solutions were carefully reduced to dryness by vacuum distillation at 40°C in a rotary vacuum evaporator (Büchi Glasapparatefabrik, Switzerland). The residue in each flask was dissolved

in 2 ml ethanol from which duplicate 0.2 ml and 0.1 ml aliquots were removed for analysis by thin-layer chromatography and for determination of total radioactivity extracted, respectively.

For a more detailed study in which the time-course of (^{14}C)-metoxuron metabolism in resistant and susceptible plants was being examined, the roots of the plants were exposed to 3 μCi (methoxy-^{14}C)-metoxuron dissolved in 10 ml of Hoagland nutrient solution contained in a small flask. Under the conditions used, (16 h day length at 21°C in the greenhouse) the nutrient solution containing the radioactive herbicide was almost completely absorbed via the transpiration stream within 8 h. The plants were subsequently removed and their root systems rinsed with distilled water, the washings being combined with any remaining contents in the treatment flasks and retained for determination of radioactivity not absorbed. The plants were then returned to their original culture flasks containing fresh nutrient solution without added herbicide, samples being harvested for analysis at 1, 2, 4, 7 and 14 days after treatment with (methoxy-^{14}C)metoxuron. Triplicate determinations were set up at each time interval following treatment. Plants were cut into small (1 cm length) pieces and were immediately extracted with boiling methanol. The extracts were then reduced to dryness under vacuum at 40°C, the resulting residue being taken up in 2 ml methanol. As previously, 0.1 ml samples were subjected to liquid scintillation counting for determination of total extracted radioactivity while duplicate 0.2 ml aliquots were analysed for metabolites by thin-layer chromatography and autoradiography (see p 133).

The residue remaining after the exhaustive extraction of the plants with methanol was weighed and ground to a fine powder in a mortar and pestle. Small samples (10 mg) were solubilised by treatment with 1 ml of NCS tissue solubiliser at 50°C (Hansen & Bush, 1967) for determination of total methanol-insoluble radioactivity by the liquid scintillation technique as described in the following section.

Sample preparation for liquid scintillation counting

For liquid scintillation spectrometry the toluene-based scintillation system alone or in combination with triton X-100 is widely used due to ease and low cost of preparation as compared to dioxane-based systems (Roberts, 1968). With certain qualifications the use of toluene/2,5-diphenyloxazole (PPO) scintillant is satisfactory for counting small aqueous samples containing ^{14}C (Turner, 1968, 1971). However studies have shown that the counting rate of several labelled compounds is not stabilised in the standard toluene/triton scintillation system. This may cause serious misrepresentation of data from a large number of replicated treatment samples counted over a period of several hours. The addition of NCS (3.3%, v/v) however, renders the system suitable for measuring a number of ^{14}C-labelled compounds (Chow, 1974).

Addition of NCS, a quaternary amine, to a scintillation system can, however, sometimes induce chemiluminescence and colour quenching due to reactions either with biological material (Herberg, 1958) or components of the scintillation system (Dunn, 1971). This does not represent a serious problem however since the reduction in counting efficiency for the colour-quenching effect can be corrected by means of a quench

correction curve prepared by the external standard channels ratio method, and chemiluminescence can be reduced to a minimum by neutralising the quaternary amine with glacial acetic acid after solubilisation.

In practice, 0.1 ml aliquots of the samples to be counted were pipetted into scintillation vials and 1 ml of NCS solubiliser was added. The vials were left at ambient temperature for 30 min after which time 0.03 ml of glacial acetic acid was added to each, followed by 19 ml toluene/PPO (5 g/litre) scintillation mixture. Each sample was counted for a 5 min period using a Packard Tri-carb liquid scintillation spectrometer, model 3390. Print outs were converted to disintegrations per minute (d.p.m.) by reference to a previously prepared quench correction curve (Fig. 15). Counting efficiency was generally in the region of 90%.

Thin-layer chromatographic analysis of the methanolic extracts of wheat plants treated with (methoxy-^{14}C)metoxuron

Thin-layer chromatography was carried out using ready-prepared glass plates coated with a 250 μm thickness of a mixture of silica gel and kieselguhr containing a fluorescence indicator (DC-Fertigplatten, kieselgel 60/kieselgur F_{254}, Merck, Darmstadt).

0.2 ml aliquots of the methanolic extracts to be analysed were further reduced in volume by gentle warming under a stream of nitrogen prior to being spotted on to the chromatography plates along the origin line. The solvent system employed for the development of the chromatograms was either benzene/acetone (2:1 v/v) or, more routinely, chloroform/ethanol (9:1 v/v), both of which gave adequate separation of the major

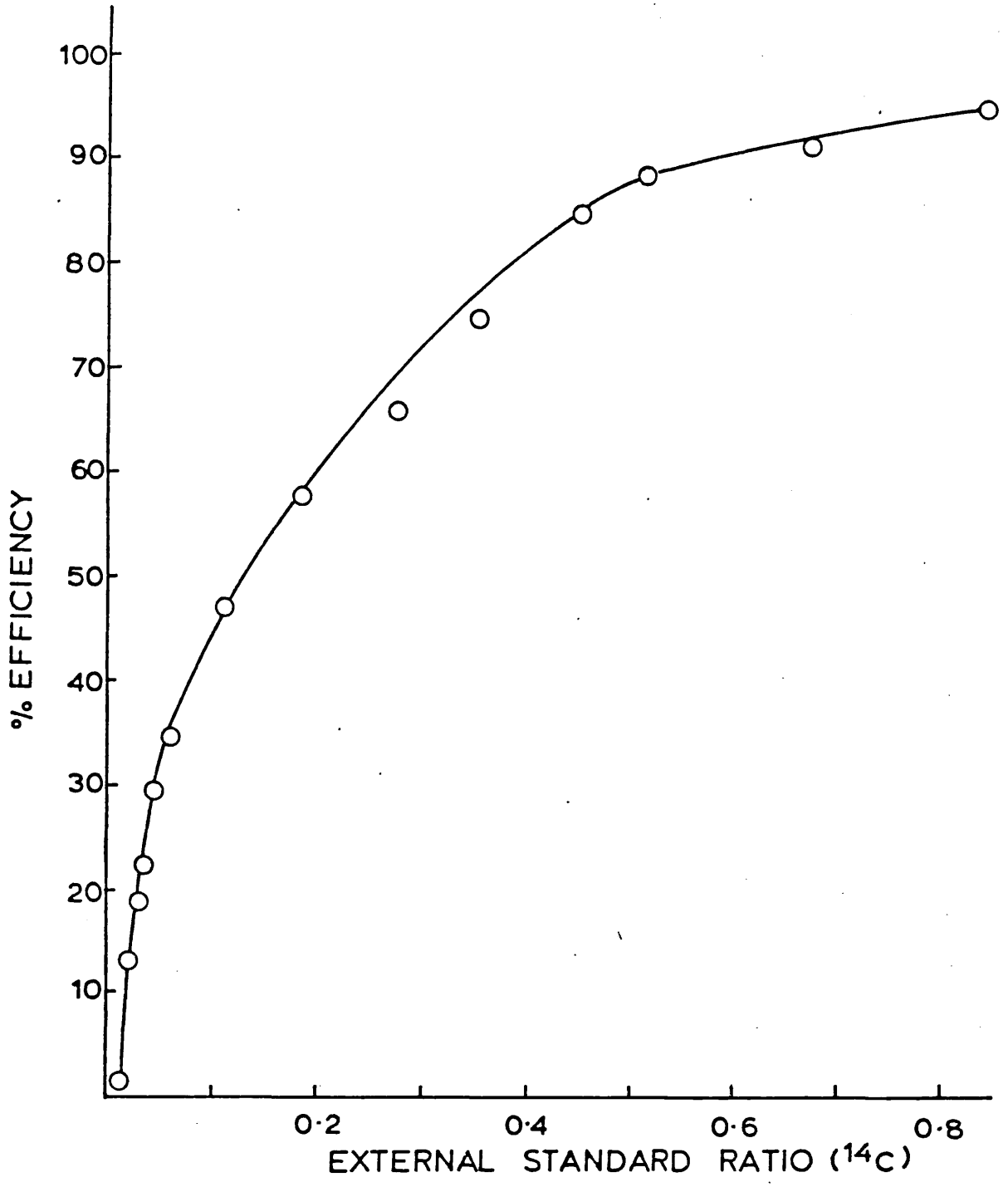


Fig. 15: Quench correction curve for the Packard Tri-carb liquid scintillation counter

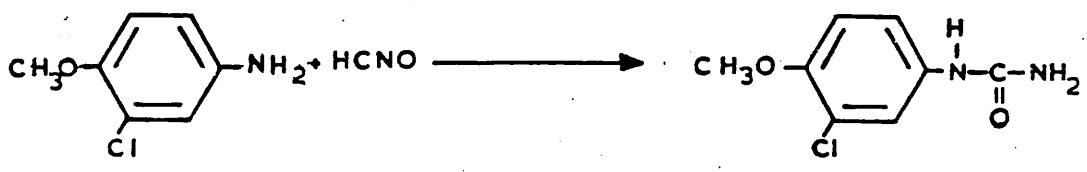
The curve was constructed by adding increasing amounts of a methanolic leaf extract to the standard scintillation cocktail described on p. 133.

metabolites. Non-labelled metabolites of metoxuron were chromatographed on the same TLC plates as the experimental samples to facilitate identification of the radioactive metabolites in the plant extracts. In the presence of the fluorescence indicator the non-labelled marker compounds were readily visualised under the UV lamp. Radioactive zones were located by autoradiography by exposing the developed chromatographic plates to Kodirex X-ray film of dimensions 18 x 24 cm.

Metabolites were tentatively identified by comparison of their R_f values in the two solvent systems used with those of the known authentic standards. In addition to the present herbicide the standards employed were N'-(3-chloro-4-methoxyphenyl)-N-methyl urea, N'-(3-chloro-4-methoxyphenyl) urea and 3-chloro-4-methoxyaniline, representing the products of partial and complete demethylation and hydrolysis respectively.

The N'-(3-chloro-4-methoxyphenyl)-N-methyl urea and 3-chloro-4-methoxyaniline standards were obtained as a generous gift from Dr. G.G. Briggs of the Rothamsted Experimental Station, Harpenden. The 3-chloro-4-methoxyphenylurea was synthesised by a procedure based on a reaction described by Kurzer (1963).

The reaction involved the addition of 5 ml of an aqueous solution of sodium cyanate (10 mmol) to 7.4 ml of a solution of 3-chloro-4-methoxyaniline (5 mmol) in 32.5% acetic acid at a temperature of 40°C:



The initial one ml of the sodium cyanate solution was added slowly with stirring until a white crystalline precipitate of the product appeared. The remaining 4 ml was then added quickly with vigorous agitation whereupon the separation of the product was very rapid and accompanied by a rise in temperature to 50-55°C. The thick, paste-like suspension was stirred for a further 10 min. After standing at room temperature for 2-3 h the suspension was diluted with 20 ml of H_2O and then cooled to 0°C. The product was harvested by vacuum filtration, washed with H_2O, and then thoroughly dried. The yield of crude N'-(3-chloro-4-methoxyphenyl)urea, a white crystalline powder, was approx. 90%. Further purification was achieved by recrystallisation from ethanol to a constant melting point of 176-8°C.

Following location of the ^{14}C-labelled metabolites by auto-radiography, radioactive zones were scraped from the chromatographic plates for elution with methanol. The amount of radioactivity present in each metabolite was then determined using the Packard Tri-carb scintillation spectrometer as outlined on p. 132.

Metoxuron metabolism studies using leaf disc segments

There are many reports in the literature which demonstrate that substituted phenylurea herbicides are generally readily absorbed by roots with subsequent translocation to the shoots of treated plants

(e.g. Fang et al, 1955; Geissbühler et al, 1963a; Smith & Sheets, 1967). Thus there is a potential for accumulation of the herbicides or their metabolites in leaves. Whether the accumulation in leaf tissue will result in phytotoxicity depends upon such factors as the rate of uptake and translocation, the inherent ability of a particular species or plant organ to degrade or otherwise detoxify the herbicide, and the rate at which detoxication occurs.

Experiments described in the foregoing section employed whole wheat plants that had contacted (^{14}C)metoxuron via their roots. In such studies it cannot be determined directly whether the metabolic conversion products found in the leaves are formed in the roots and subsequently translocated to the leaves. Furthermore, the ability of the leaf tissue to metabolise intact phenylurea herbicides translocated from the roots cannot be adequately assessed.

The present investigation was therefore conducted to compare the photosynthetically active tissue of resistant and susceptible varieties of wheat with respect to their inherent abilities to degrade metoxuron.

The plants used for these studies were grown in a greenhouse. The natural illumination being supplemented by means of an arrangement of fluorescent strips (p 87) for a minimum of 1 h prior to using the leaf tissue in an experiment. 8 mm diameter discs were subsequently cut using a sharp cork borer from washed expanded 2nd and 3rd leaves of plants at the four-leaf stage. After preparation the leaf discs were immediately transferred to 1 ml of a 0.05M phosphate buffer pH7 containing (methoxy-^{14}C)metoxuron (2 μCi) for incubation at room temperature in diffuse light for 1 h. At the end of the incubation period the discs were removed, rinsed exhaustively with distilled water and then prepared for subsequent experiments after removal of excess H_2O.

Experiments were conducted in a Warburg apparatus fitted with a bank of incandescent lamps. Leaf discs were arranged in a single layer around the main compartment of the Warburg flasks which contained 0.75 ml of 0.05M phosphate buffer, pH 7 to maintain turgidity of the tissue. Into the centre well of each flask was placed a piece of convoluted Whatman No. 42 filter paper and 0.6 ml of a CO_2 buffer consisting of a diethanolamine/potassium bicarbonate mixture (Pardee, 1949) to maintain a CO_2 atmosphere of 0.3% CO_2 at 25°C, the experimental temperature. For experiments designed to establish whether (^{14}C)-metoxuron metabolism resulted in any evolution of $^{14}CO_2$, the CO_2 buffer in the centre well was replaced by a CO_2 trap consisting of 0.2 ml of 6M potassium hydroxide adsorbed to filter paper to increase the effective surface area. These latter experiments were conducted in the dark. When illumination was required the adaxial surface of the leaf discs was placed in contact with the bottom of the flasks, illumination (approx. 16000 lx) being provided from below. All experiments were conducted over a 9 or 12 h period, duplicate samples of discs being removed for analysis after 2, 6, 9 and 12 h after transfer to the Warburg flasks.

Following their removal from the Warburg flasks after the appropriate time intervals, the discs were rinsed and transferred to test tubes which were immediately placed in dry ice. In each case the rinsings were combined with the ambient buffer, the amount of radioactivity in this combined solution being termed "leakage" to differentiate it from that retained in the leaf tissue at the time of sampling. The discs were then lyophilised over-night and were subsequently ground to a powder prior to extraction with methanol for 2 h. The methanol extract was

centrifuged at 30,000 x g for 20 min, and the plant residue was washed by shaking with a further 10 ml of methanol followed by a second centrifugation at 30,000 x g. The combined supernatants were reduced to dryness under vacuum at 40°C, the resulting residue being dissolved in 1 ml of methanol. 0.1 ml aliquots of this solution were removed to determine total radioactivity extracted using the liquid scintillation technique (p 132), whilst further 0.2 ml samples were analysed for radioactive metabolites by thin-layer chromatography and autoradiography (p133). In experiments employing a CO_2 trap the filter paper wicks were removed from the centre walls at the prescribed times and air dried. They were then transferred to scintillation vials and 10 ml of the toluene/PPO scintillation mixture was added. The amount of radioactivity in each sample was then determined using the Packard Tri-carb liquid scintillation spectrometer. In this case percentage counting efficiency was calculated using an internal standard.

Studies on the sub-cellular location of the metoxuron-metabolising enzyme system(s) in wheat

Recent in vivo studies, (Geissbühler et al, 1963b; Swanson & Swanson, 1968; Neptune, 1970) have unequivocally demonstrated that substituted N'-(phenyl)-N,N-dimethylurea compounds are progressively N-demethylated by higher plants to less phytotoxic N-monodesmethyl and N-bisdesmethylated derivatives. The only reports of such metabolism by an in vitro system have been those of Frear (1968, 1969) who found that a microsomal oxidase system from leaves of cotton and several other species was capable of N-demethylating monuron to its N-monodesmethyl derivative. The demethylation reaction was dependent on the presence

of NADPH and molecular oxygen and appeared to be confined to the microsomal fraction, other sub-cellular organelle fractions being ineffective (Frear, 1968).

The present investigation was therefore conducted to determine the site(s) of intracellular N-demethylation of metoxuron in winter wheat and to attempt to characterise the enzyme system(s) involved. Plants of resistant and susceptible wheat varieties were grown under identical conditions in a plant growth cabinet and were used at the three-leaf stage. The method employed for the isolation of sub-cellular organelle fractions in the present study was an adaptation of the procedure of Frear (1968).

Routinely, 50 g of excised leaf tissue were washed with distilled water and then ground to a fine powder in a pestle and mortar with liquid nitrogen and 10 g of acid-washed sand. The frozen leaf powder was then slurried with 200 ml of 0.3M potassium phosphate buffer, pH 7.5 containing sodium isoascorbate, 0.1M, NaCN, 1 mM, and 10 g polyclar AT (insoluble polyvinyl polypyrrolidone). After standing for 15 min with occasional stirring, the slurry was squeezed through cheesecloth and subjected to differential centrifugation. The cell-free extract (supernatant after centrifugation at 300 x g for 5 min) was centrifuged at 1500 x g for 10 min, 9000 x g for 20 min and 100,000 x g for 80 min. The 1500 x g, 9000 x g and 100,000 x g pellets were resuspended in 0.1M potassium phosphate buffer, pH 7.5 containing 1 mM NaCN and were used in assays immediately. All enzyme extraction and centrifugation procedures were carried out at $4^{o}C$.

Demethylation activity was assayed by following the rate of N'-(3-chloro-4-methoxyphenyl)-N-methylurea formation. The standard reaction mixture, adjusted to pH 7.5, contained (methoxy-^{14}C)metoxuron, 9 m µmol (sp. act. 4 µCi/µmol); NaCN, 0.05 µmol; K_2HPO_4, 50 µmol; NADPH, 1 µmol, and 1-3 mg of organelle protein in a final volume of 1.0 ml. Reactions were initiated by addition of the NADPH, the reaction vessels being maintained at 25°C for 30 min. Each experiment incorporated appropriate control assays from which reduced pyridine nucleotide was omitted. The reactions were terminated by rapid freezing in a dry ice-acetone bath followed by lyophilisation. The lyophilised reaction mixtures were subsequently extracted with one ml methanol, and the reaction product, N'-(3-chloro-4-methoxyphenyl)-N-methylurea, was then separated from the substrate by thin-layer chromatography of the methanol extract. Radioactive zones were visualised by autoradiography and the amount of radioactivity in the separated reaction product was quantitatively determined by liquid scintillation counting after elution from the chromatographic plates. Results are presented in disintegrations/min (d.p.m.) after applying appropriate corrections for efficiency and quenching. Recovery of radioactivity from the lyophilised reaction mixtures and from thin-layer chromatograms was approx. 95% and 90% respectively. Protein concentrations was determined by the method of Lowry et al (1951, see p 97).

In order to trap $^{14}CO_2$ that may be liberated from reaction mixtures as a result of (methoxy-^{14}C)metoxuron metabolism, some experiments incorporated a CO_2-trap consisting of 3 ml of a mixture of ethanolamine and ethyleneglycol monomethylether, 1:2 by volume (Jeffay & Alvarez, 1961).

Ethanolamine is an efficient and relatively inexpensive trapping material and has the advantage of not interfering with the liquid scintillation process. These experiments were carried out in tightly sealed test-tubes fitted with narrow inlet and outlet tubes so that the atmosphere above each reaction mixture could be continuously flushed with CO_2-free oxygen. The outlet from each reaction tube passed to the atmosphere via the CO_2-trap. The temperature and duration of the experiments were as described above. At the end of the incubation period the CO_2-trap solution was transferred to a scintillation vial and the amount of radioactivity present determined by liquid scintillation counting after the addition of 6 ml ethylene glycol monomethylether and 10 ml toluene containing 5 g PPO per litre. Use of the ethanolamine-ethylene glycol monomethylether mixture in the scintillation cocktail is required to facilitate solubility of ethanolamine carbonate in toluene (Jeffay & Alvarez, 1961). Counting efficiency was approx. 60% as determined by the use of an internal standard.

RESULTS AND DISCUSSION

Effect of Metoxuron on the Light Reactions in Photosynthesis. The Hill activity of chloroplasts isolated from intact wheat plants and treated with herbicide in vitro

The Hill activity of isolated chloroplasts as measured by the photoreductions of dichlorophenolindophenol (DCPIP) and potassium ferricyanide was assessed by the procedures described in the appropriate sections of the preceeding chapter. Chloroplasts were isolated from leaves of wheat plants at the three to four leaf stage and their Hill activity was subsequently assayed in the presence of varying concentrations of metoxuron.

Numerous reports have been presented during the last 25 years to indicate that the substituted phenylureas inhibit the Hill reaction of isolated chloroplasts and that the degree to which they inhibit photosynthetic activity correlates with their herbicidal potency (Cooke, 1956; Spikes, 1956; Wessels & Van der Veen, 1956). In a review of the literature on the mode of action of substituted phenylureas, Woodford (1958) concluded that the primary toxic effect was due to their absorption and concentration on active sites in the chloroplast and the subsequent inhibition of photosynthesis. Inhibition of the Hill reaction of isolated chloroplasts by substituted phenylureas was first reported by Wessels and Van der Veen (1956) and has subsequently been confirmed by Spikes (1956), Cooke (1956), Moreland (1957) and many other workers.

Though the efficacy of metoxuron as an effective herbicide has been attributed to its potency as an Hill reaction inhibitor, as for other more well documented phenylureas (Büchel, 1972; Van Leeuwen &

TABLE 2: Photoreduction of DCPIP and potassium ferricyanide by chloroplasts treated with metoxuron and related substituted phenylurea herbicides

Variety	Hill Reaction Activity (μmol dye reduced/mg chlorophyll/h)								% Inhibition Photoreduction					
	DCPIP photoreduction				$K_3Fe(CN)_6$ photoreduction				DCPIP photoreduction			$K_3Fe(CN)_6$ photoreduction		
	UT	monuron	chlor-toluron	metox-uron	UT	monuron	chlor-toluron	metox-uron	monuron	chlor-toluron	metox-uron	monuron	chlor-toluron	metox-uron
Maris Nimrod (S)	109	59	19	18	225	119	43	42	46	82	83	47	81	81
Cama (R)	98	53	16	13	188	110	43	38	46	83	86	43	78	80

Reactions were carried out in glass spectrophotometer cuvettes, illuminated with approx. 150,000 lx. Each reaction cuvette contained in μmol; KH_2PO_4 (pH 6.5), 125; KCl, 25; DCPIP, 0.16; $K_3Fe(CN)_6$, 1.5; monuron, chlortoluron or metoxuron, when present, 0.1 and chloroplasts (isolated in medium A, p.90) equivalent to 30-40% chlorophyll in a total volume of 3 ml. Molar Extinction Coefficients of DCPIP and ferricyanide were taken to be 22,400 and 980 at 620 nm and 420 nm respectively.

UT - chloroplasts treated with 0.1 ml methanol not containing herbicide

S - indicates susceptibility to metoxuron treatment in field trial experiments

R - indicates plants were resistant to metoxuron treatment

Van Oorschot, 1976), the present investigation was initiated to ascertain whether wheat varieties differ with respect to the inherent sensitivity of their chloroplasts to metoxuron. For purposes of comparison the related substituted phenylureas chlortoluron and monuron were included in the study.

The respective rates of reduction of DCPIP and potassium ferricyanide by chloroplasts from susceptible (Maris Nimrod) and tolerant (Cama) wheat varieties incubated in the presence of the various substituted phenylureas are shown in Table 2. Since the herbicides were applied to the chloroplasts as methanolic solutions the control assays incorporated an equivalent volume of methanol not containing herbicide. These results show that chloroplasts isolated from both resistant and susceptible wheat plants are inhibited by some 80% by treatment with metoxuron (3.3×10^{-6}M) in the case of both DCPIP and potassium ferricyanide photoreductions, indicating that resistant and susceptible varieties are not distinguishable on the basis of any difference in the inherent sensitivity of isolated chloroplasts to the herbicide. Though no attempt was made to obtain I_{50} values in the present investigation it would appear that the degree of inhibition of Hill activity resulting from treatment of chloroplasts with metoxuron and the related phenylurea, chlortoluron, are very similar and that these two herbicides are considerably more potent inhibitors of the Hill reaction that the more well documented monuron.

The effect of different concentrations of metoxuron on the Hill activity of chloroplasts from susceptible and tolerant wheat varieties is shown in Table 3. These results indicate that DCPIP and potassium

TABLE 3: Photoreduction of DCPIP and potassium ferricyanide by chloroplasts from susceptible and resistant wheat plants treated with different concentrations of metoxuron

Variety	metoxuron concentration	Hill Reaction Activity (μmol dye reduced/mg Chl/h)				% Inhibition photoreduction	
		DCPIP photoreduction		$K_3Fe(CN)_6$ photoreduction		DCPIP photoreduction	$K_3Fe(CN)_6$ photoreduction
		un-treated	metoxuron treated	un-treated	metoxuron treated		
Maris Nimrod (S)	3.3×10^{-7}	163	76	345	151	54	57
Cama (R)		161	88	336	187	45	46
Maris Nimrod (S)	6.6×10^{-7}	148	55	320	136	63	60
Cama (R)		122	41	290	104	67	64
Maris Nimrod (S)	3.3×10^{-6}	148	25	320	62	83	82
Cama (R)		122	13	290	52	89	86

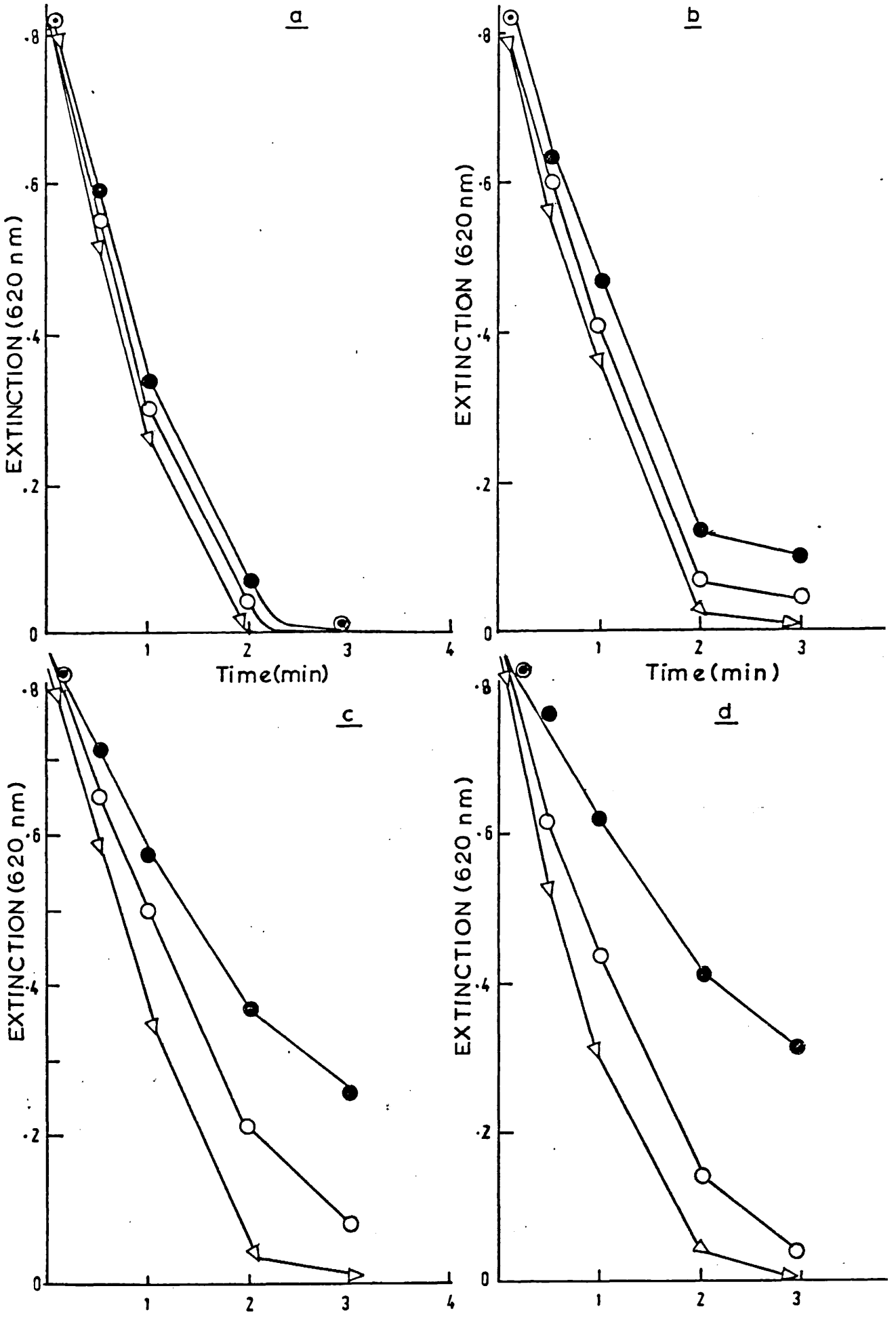
Reaction conditions as shown for Table 2 except that the metoxuron content of the assay mixtures was 0.01, 0.02 and 0.1 μmol respectively in the different experiments cited

ferricyanide photoreductions by chloroplasts isolated from both susceptible (Maris Nimrod) and resistant (Cama) plants are inhibited some 50% by a metoxuron concentration of 3.3×10^{-7}M. Increasing the metoxuron concentration further to 6.6×10^{-7}M and 3.3×10^{-6}M was accompanied by parallel increases in the percentage inhibition of Hill activity of chloroplasts from both varieties. Thus in these in vitro studies in which metoxuron has arrived at the site(s) of inhibition within the chloroplast by mainly physical processes, no distinction exists between susceptible and resistant wheat varieties. This result did not, however, rule out the possibility that chloroplasts isolated from metoxuron treated plants, in which physiological phenomena are involved in the subsequent distribution of the absorbed herbicide, might show different degrees of inhibition of photosynthetic activities.

The Hill activity of chloroplasts isolated from metoxuron-treated plants

Chloroplasts isolated from metoxuron-treated leaves some days after spraying have metoxuron associated with them. It was of obvious interest to see whether the metoxuron associated with the chloroplast fraction of the treated plants affected the photosynthetic activities of the chloroplasts of resistant and susceptible wheat varieties to the same extent.

In the present investigation a study was made of the time-course of inhibition of DCPIP and potassium ferricyanide photoreductions by chloroplasts isolated from metoxuron-treated resistant (Cama) and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Time(min)
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Fig. 16: Characteristics of DCPIP photoreduction by chloroplasts from susceptible and resistant wheat

Reaction conditions as previously described in Table 2.

Because of the close similarity between the results obtained for control plants of both varieties, only one set of data is presented.

Times after spraying :-

(a) 1 day (b) 2 days (c) 7 days (d) 14 days

-Δ-Δ- untreated control plants

-O-O- metoxuron-treated resistant wheat (Cappelle)

-●-● - metoxuron-treated susceptible wheat (Maris Nimrod)

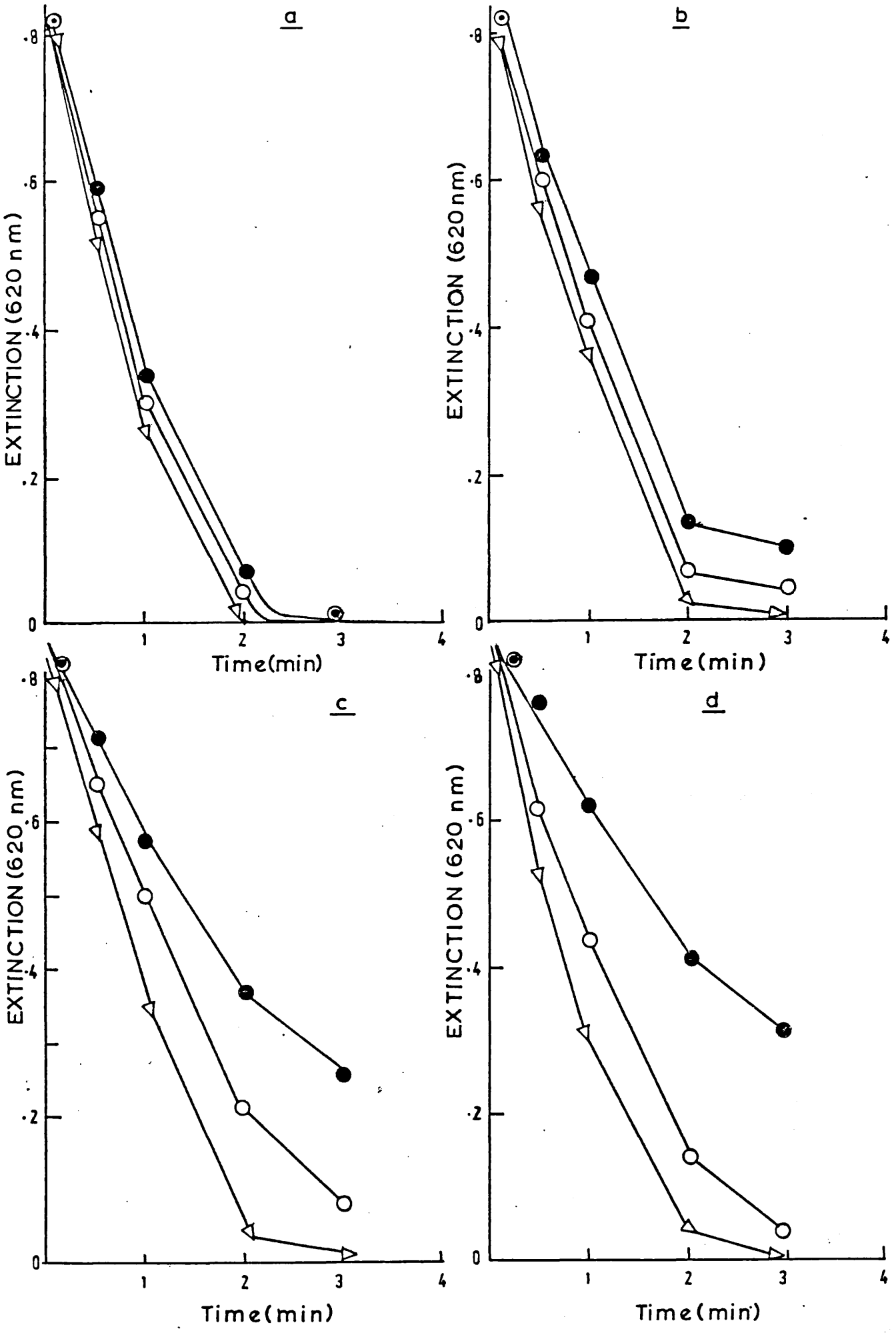
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Fig. 17: Characteristics of potassium ferricyanide photo-reduction by chloroplasts from susceptible and resistant wheat

Reaction conditions as previously described in Table 2.

Because of the close similarity between the results obtained for control plants of both varieties, only one set of data is presented.

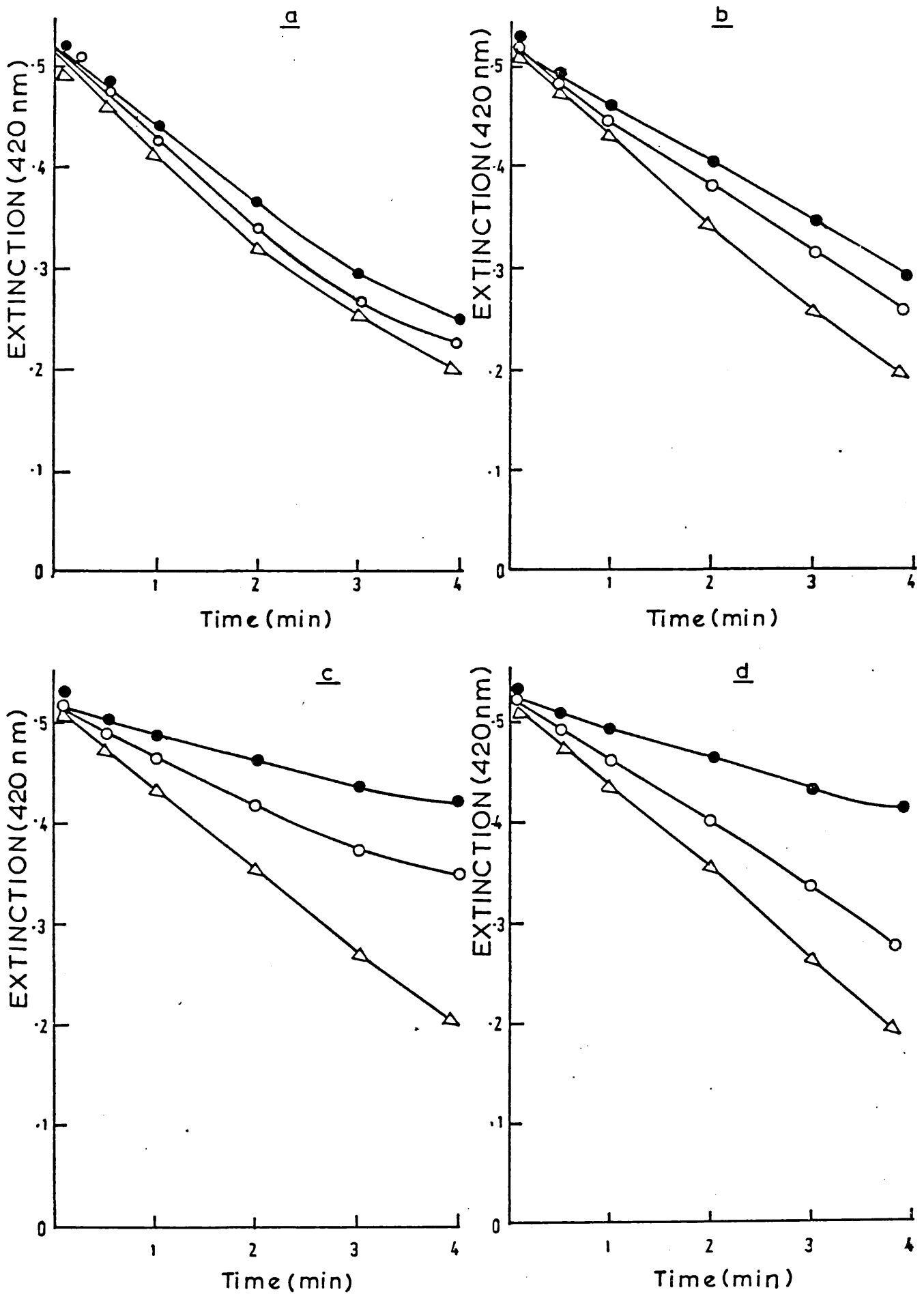
Time after spraying :-

(a) 1 day (b) 2 days (c) 7 days (d) 14 days

-Δ-Δ- untreated control plants

-O-O- metoxuron-treated resistant wheat (Cappelle)

-●-●- metoxuron-treated susceptible wheat (Maris Nimrod)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susceptible (Maris Nimrod) plants. Chloroplasts were extracted from plants initially at the three to four leaf stage at various times after spraying with a 0.05% metoxuron solution in 0.1% Tween 60. The characteristics of DCPIP photoreduction by such chloroplast preparations are shown in Fig. 16. The chloroplasts used in control experiments were isolated from plants of the same age which had been sprayed with a 0.1% Tween 60 solution not containing metoxuron. Similar characteristics for ferricyanide photoreduction are shown in Fig. 17. The respective rates of reduction of DCPIP and ferricyanide by chloroplasts from metoxuron-treated resistant and susceptible wheat are compared in Table 4. The results in Table 4, which includes data obtained for an additional resistant variety, Cama, indicate that 3 days after spraying the Hill activity of chloroplasts isolated from metoxuron-treated susceptible wheat measured by both DCPIP and ferricyanide photoreductions shows a two to three-fold greater inhibition than chloroplasts isolated from resistant plants. This distinction between the resistant and susceptible varieties is maintained at longer times after treatment, the Hill activity of chloroplasts of susceptible plants being reduced by some 55% after 7 days. Interestingly, when the corresponding figures for 14 days are compared it can be seen that a six-fold difference exists between the percentage inhibitions of both DCPIP and potassium ferricyanide photoreductions. The data indicate that though the percentage inhibition of the Hill activity of chloroplasts from susceptible wheat has continued to increase to some 60% at 14 days, chloroplasts from resistant wheat are now only some 10% inhibited, suggesting a considerable degree of recovery of photosynthetic activity in the resistant cultivars. The distinction between resistant and

TABLE 4: Photoreduction of DCPIP and potassium ferricyanide by chloroplasts from metoxuron-treated plants of both susceptible and resistant wheat

Time after spraying (days)	Variety	Reaction to metoxuron	Hill Reaction Activity (µmol dye reduced/mg Chl/h)				% Inhibition photoreduction	
			DCPIP photoreduction		$K_3Fe(CN)_6$ photoreduction		DCPIP photo-reduction	$K_3Fe(CN)_6$ photo-reduction
			un-treated	metoxuron treated	un-treated	metoxuron treated		
	Maris Nimrod	S	155	148	327	314	4	4
1	Cappelle	R	139	159	308	314	0	0
	Cama	R	153	161	285	288	0	0
	Maris Nimrod	S	157	147	303	280	6	8
2	Cappelle	R	152	159	342	339	0	1
	Cama	R	160	157	310	290	2	4
	Maris Nimrod	S	156	120	303	225	24	26
3	Cappelle	R	152	139	304	265	9	12
	Cama	R	-	-	-	-	-	-
	Maris Nimrod	S	156	95	281	167	38	41
4	Cappelle	R	152	128	285	225	16	20
	Cama	R	156	122	282	207	22	26
	Maris Nimrod	S	154	72	293	136	55	53
7	Cappelle	R	132	107	240	190	19	21
	Cama	R	150	100	281	200	34	30
	Maris Nimrod	S	155	65	293	120	58	60
14	Cappelle	R	144	130	300	260	10	13
	Cama	R	158	140	295	262	10	11

Reaction conditions were as given previously in Table 2

susceptible varieties observed in the present studies of the effect of foliar applied metoxuron on photosynthetic function in isolated chloroplasts reflects differences in visual symptoms of phytotoxicity noted in the course of the investigation. Treatment of susceptible wheat varieties with metoxuron is accompanied by yellowing of the leaves followed by scorching of the leaf tips, reduction of growth, wilting and ultimately death of the plant. In the case of the resistant cultivars, however, visible symptoms of damage,which are initially only very slight, disappear altogether some two-three weeks after treatment whereupon the plants continue to grow as normal.

In another series of experiments which incorporated an additional susceptible variety, Maris Huntsman, very similar results were obtained. The Hill activity of chloroplasts isolated from metoxuron-treated resistant and susceptible plants in this latter study are compared with those of untreated controls in Table 5. Since the data presented in both Table 4 and Table 5 was obtained using plants grown and treated with herbicide under greenhouse conditions, the temperature at which metoxuron-treated plants were maintained varied considerably with climatic conditions. The data contained in Table 5 was obtained during the summer months and although it does not contribute significant additional information concerning differences in response between resistant and susceptible varieties it is presented to illustrate that the onset of phytotoxicity and associated inhibition of Hill activity is much more rapid at elevated temperatures. Under these latter conditions it can be seen (Table 5) that the Hill activity of susceptible varieties is inhibited by some 30% 24 h after treatment with metoxuron and by some 80% after three days when photoreduction by chloroplasts from resistant cultivars is only some 20% inhibited compared to untreated controls.

TABLE 5: Photoreduction of DCPIP and potassium ferricyanide by chloroplasts from metoxuron-treated plants of both resistant and susceptible wheat varieties

Time after spraying (h)	Variety	Reaction to metoxuron	Hill Reaction Activity (µmol dye reduced/mg Chl/h)				% Inhibition photoreduction	
			DCPIP photoreduction		$K_3Fe(CN)_6$ photoreduction		DCPIP photo-reduction	$K_3Fe(CN)_6$ photo-reduction
			un-treated	metoxuron treated	un-treated	metoxuron treated		
12	Maris Nimrod	S	126	140	242	271	-	-
	Maris Huntsman	S	126	157	320	327	-	-
	Cama	R	128	153	249	305	-	-
	Cappelle	R	119	124	248	264	-	-
24	Maris Nimrod	S	138	94	255	167	32	35
	Maris Huntsman	S	150	112	270	219	28	24
	Cama	R	122	110	218	193	10	11
	Cappelle	R	133	121	257	229	9	10
48	Maris Nimrod	S	158	97	275	150	40	44
	Maris Huntsman	S	140	92	284	162	37	43
	Cama	R	130	105	230	202	19	21
	Cappelle	R	106	96	267	229	11	14
72	Maris Nimrod	S	158	24	377	75	85	86
	Maris Huntsman	S	-	-	-	-	-	-
	Cama	R	156	122	282	207	22	26
	Cappelle	R	152	128	285	235	16	18

Reaction conditions were as shown for Table 2

The results of the above studies of the Hill activity of chloroplasts isolated from metoxuron-treated plants thus show a fairly clear distinction between resistant and susceptible varieties, reflecting the different varietal responses observed in field trials (Van Hiele et al, 1970; Maas, 1971). Similar results have been obtained by Van Leeuwen and Van Oorschot (1976) in a study of the change in relative rate of net photosynthesis of resistant (Caribo) and susceptible (Manella) wheat varieties caused by short exposure of the roots to metoxuron and related substituted phenylureas in the nutrient solution. Though shortly after removal of the metoxuron the rates of net photosynthesis of both cultivars were reduced to almost zero, some 12 h after termination of treatment net photosynthesis in Caribo had recovered to some 40% of the pre-treatment rate whereas the corresponding value for Manella was 10%, indicating only very little recovery from metoxuron treatment. Additional support for these conclusions comes from a more recent study (Muller, Frahm & Sanad, 1977) of the influence of metoxuron, chlortoluron and isoproturon (see Table 1 for structure) on photosynthetic oxygen evolution (Hill activity) in two winter wheat cultivars. All three herbicides were found to depress photosynthesis of both the susceptible (Heines VII) and resistant (Jubilar) cultivars to some 6-10% of the rate measured in untreated control plants. Although oxygen evolution in herbicide-treated Heines VII remained at some 10% of the untreated control rates throughout the duration of the experiment, photosynthesis in Jubilar recovered to 63% and 86% of the control rates at three and six days respectively after cessation of treatment.

Effect of Metoxuron on Cyclic Photophosphorylation

The experiments described in the previous section indicated that in vitro treatment of chloroplasts isolated from resistant and susceptible varieties of winter wheat with 3.3×10^{-6}M metoxuron caused 80-90 per cent inhibition of the Hill activity in both varieties compared to chloroplasts from untreated controls. Avron (1964) and Wessels (1964) in their studies of the effects of substituted phenylurea herbicides on photosynthesis concluded that although electron transport from water to $NADP^+$ was very sensitive to inhibition by N'-(3,4-dichlorophenyl)-N,N-dimethylurea (diuron) NADP photoreduction in the presence of the DCPIP/ascorbate artificial donor system and cyclic photophosphorylation were relatively insensitive, suggesting that the sites of inhibition are confined to photosystem 2 of photosynthesis. In an earlier study, Jagendorf & Margulies (1960) showed that the Hill activity of chloroplasts with either potassium ferricyanide or an indophenol dye as electron acceptor was inhibited approximately 50% by a monuron concentration of 3×10^{-6}M whereas a concentration of 10^{-3}M was required to cause the same degree of inhibition of PMS-catalysed cyclic photophosphorylation, providing further evidence for photosystem 2 being a primary site of interference of photosynthesis by phenylurea herbicides. However, Asahi & Jagendorf (1963) later reported significant inhibition of photosystem 1-mediated activities such as PMS-catalysed cyclic photophosphorylation by somewhat lower concentrations of monuron, this being interpreted as an indication of the possible existence of a second site of inhibition by monuron which might contribute to the overall phytotoxicity of the herbicide.

Since it has clearly been demonstrated that substituted phenylurea herbicides can exert an inhibitory effect on photosystem 1-mediated activities, albeit at a greater concentration than that required to disrupt photosystem 2 function, it was considered relevant to examine the effect of metoxuron on these former activities in chloroplasts isolated from resistant and susceptible varieties of wheat. The photosystem 1 activity investigated in the present comparative study was PMS-catalysed cyclic photophosphorylation. In all experiments, chloroplasts were isolated in medium B (Page 90) developed by Plesničar and Bendall (1973) for the preparation of chloroplasts showing high rates of photophosphorylation. The optimum level of phenazine methosulphate used as a catalyst for cyclic photophosphorylation studies was 0.1 μmoles per 2.5 ml reaction volume (Table 6).

TABLE 6: Rates of PMS-catalysed cyclic photophosphorylation at varying concentrations of PMS

Phenazine methosulphate/ reaction cuvette (μmol)	μmol Pi esterified/ mg chl/h
0	83
0.05	692
0.1	776
0.2	672
0.4	650

Chloroplasts were isolated from Cama wheat by the method described by Plesničar & Bendall (1973). The reaction mixture maintained at 20°C contained in μmol; Tris buffer (pH 7.9), 45; NaCl, 60; $MgCl_2$, 12; ADP, 10.9; KH_2PO_4, 5; chloroplasts equivalent to 65% chlorophyll and PMS at the concentration specified, in a total volume of 2.6 ml. Reaction cuvettes were illuminated (150,000 lx) for 3 min. Phosphate estimation was by the method of Taussky & Shorr (1953).

This level of PMS was similar to that routinely used by Avron (1960) in studies of cyclic photophosphorylation in certain other species of higher plants and by Owen (1971) in barley.

Reaction mixtures were normally illuminated for 3 min, the relationship between uptake of inorganic phosphate and time being linear over this period. The effect of pH on the rate of cyclic photophosphorylation in wheat chloroplasts is shown in Fig. 18. The shape of the pH response curve is similar to that reported by Avron (1960), who observed maximum rates of activity in Swiss Chard chloroplasts at pH 7.6 to 7.9. Similar results were reported by Kleese (1966) in a study of cyclic photophosphorylation in barley chloroplasts in which the optimum pH was 8.2, with little or no activity being detectable at pH 7.5. Reference to Fig. 18 shows that the maximum rates of cyclic photophosphorylation obtained in the present study with wheat chloroplasts were observed at pH 7.9. Because of the sharp drop in activity which occurs on the acid side of the optimum pH, routine assays were performed at pH 8.0.

The results of a series of experiments in which chloroplasts were isolated from untreated plants and subsequently assayed in the presence of metoxuron are presented in Table 7. These data show that cyclic photophosphorylation in chloroplasts isolated from both resistant and susceptible varieties was inhibited some 20-25% by a metoxuron concentration of 6.6×10^{-4}M. Interestingly, a doubling of the herbicide concentration to 1.3×10^{-3}M did not appear to be accompanied by a corresponding increase in inhibition of cyclic photophosphorylation suggesting that inhibition of photosystem 1-mediated activities by metoxuron and possibly by substituted phenylureas in general may result from some non-specific

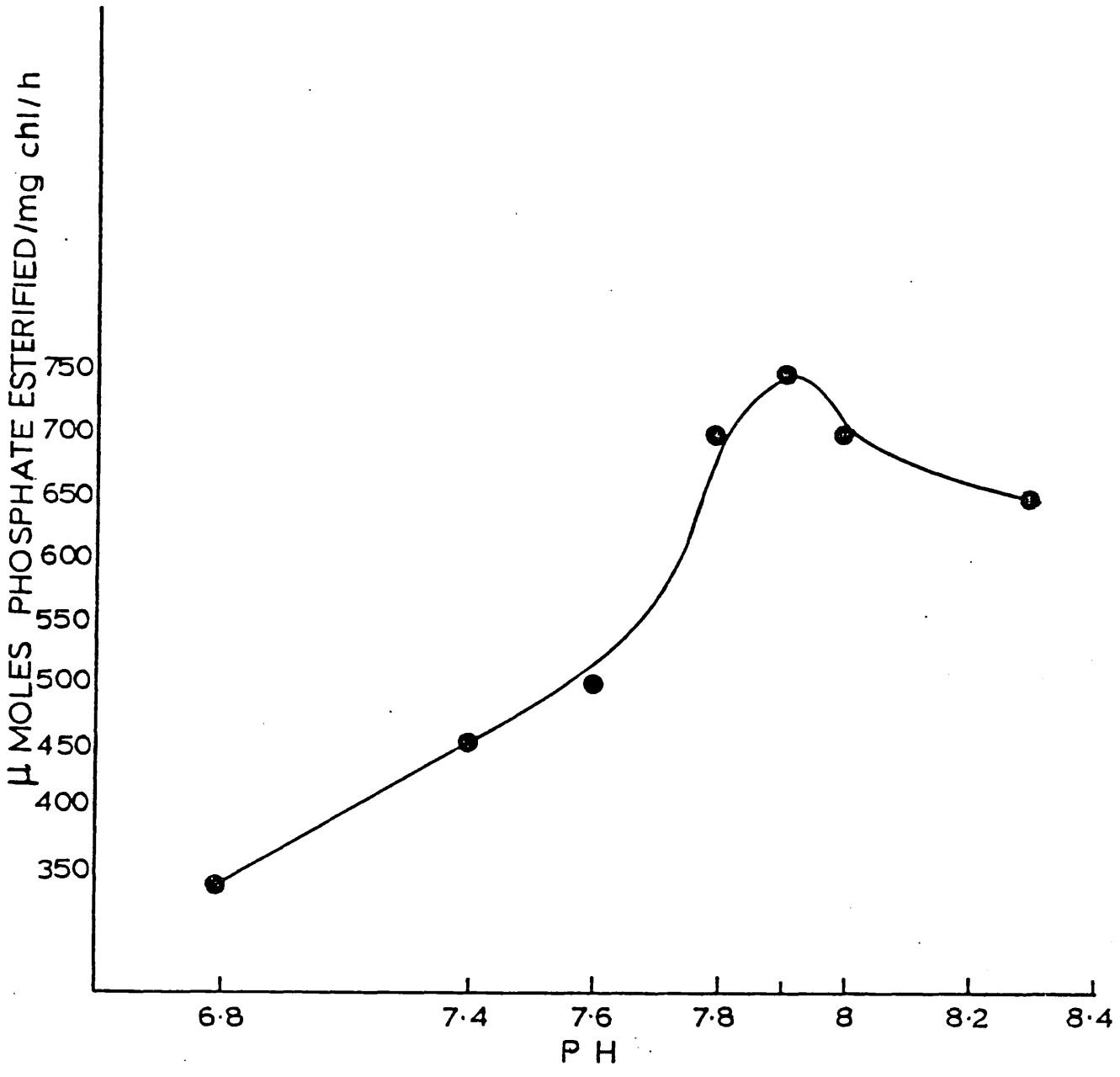


Fig. 18: Effect of pH on Cyclic Photophosphorylation in wheat chloroplasts

Reaction conditions were as shown for Table 6 employing the optimum PMS concentration of 0.1 μmol per total reaction volume of 2.6 ml.

TABLE 7: Effect of metoxuron and chlortoluron on cyclic photophosphorylation by chloroplasts from resistant and susceptible wheat varieties

Variety	Herbicide concentration	µmol Pi esterified/ mg chl/h			% Inhibition Photophos-phorylation
		un-treated	chlor-toluron treated	metoxuron treated	
Maris Nimrod (S)	6.6×10^{-4}M	904	670	670	25
Cappelle (R)		660	520	520	21
Maris Nimrod (S)	1.3×10^{-3}M	647	487	487	25
Cappelle (R)		530	410	410	23

Assays were carried out at pH 8.0. Other conditions were as previously described for Table 6 and Fig. 18. The herbicide concentrations shown represent final concentrations in the reaction cuvettes.

membrane damage rather than from binding to a component involved in cyclic electron flow. In support of this view are the results of parallel assays made using the related phenylurea, chlortoluron, which were identical to those obtained for metoxuron (Table 7).

The present results thus show that the effect of metoxuron on cyclic photophosphorylation cannot be correlated with the susceptibility or resistance of the wheat varieties to the herbicide in greenhouse trials. In addition, metoxuron concentrations some 100-fold greater than those required to inhibit Hill activity and associated O_2 evolution by 90-100%

only caused some 20% inhibition of cyclic photophosphorylation. The results of this *in vitro* study are thus in general agreement with the accepted view (Wessels & Van der Veen, 1956; Bishop, 1958; Asahi & Jagendorf, 1963; Cramer & Whitmarsh, 1977) that the substituted phenylurea herbicides act as potent inhibitors of photosystem 2 of photosynthesis and are virtually without effect on photosystem 1-catalysed activities such as cyclic electron flow and associated photophosphorylation.

It was reported earlier that the Hill activities of chloroplasts isolated from metoxuron-treated resistant and susceptible plants were inhibited to different extents even though such a distinction was not apparent in *in vitro* studies. Consequently, in a second series of experiments rates of cyclic photophosphorylation in chloroplasts isolated from plants sprayed with a 0.05% solution of metoxuron were compared with those for untreated controls some three days after treatment. The results of two separate experiments are compared in Table 8.

TABLE 8: Cyclic photophosphorylation by chloroplasts isolated from metoxuron-treated and untreated wheat varieties

Variety	µmol Pi esterified/ mg chl/h		% Inhibition Photophos-phorylation
	untreated	metoxuron treated	
Maris Nimrod	715	558	22
(S)	747	610	19
Cama	830	727	13
(R)	862	754	13

Reaction conditions as shown for Table 7.

Whereas the activity of chloroplasts isolated from metoxuron-susceptible plants was approximately 20% inhibited following contact with the spray, cyclic photophosphorylation in chloroplasts from resistant wheat was consistently some 13% lower than untreated controls. These data appear to reflect to a certain extent the results obtained for the DCPIP and potassium ferricyanide photoreduction assays reported earlier in that a measurable difference in rates of cyclic photophosphorylation between varieties does appear to exist when chloroplasts isolated from metoxuron-treated plants are examined. Since chloroplasts from both resistant and susceptible cultivars appear from in vitro studies to possess identical inherent sensitivities to metoxuron, the distinctions observable in the in vivo investigations presumably are a reflection of varietal differences in physiology and/or biochemistry which might influence uptake and subsequent metabolic fate of the herbicide within the plants.

Incorporation of $^{14}CO_2$ into Ethanol-soluble products of Photosynthesis

There have been many reports on the effect of substituted phenylurea herbicides on various aspects of photosynthesis, and these compounds have previously been shown to have an effect on CO_2 fixation and related processes. Soon after the introduction of substituted phenylureas Cooke (1955) observed that the sugar content of plants treated with monuron decreased sharply, and suggested that the mechanism of action was through an interference with photosynthesis.

In a study of the effect of monuron on carbon assimilation, Ashton et al (1961) found that when the herbicide was applied to the roots of red kidney bean (Phaseolus vulgaris L.) carbon dioxide fixation by leaves in the light was greatly reduced. The subsequent distribution of radioactivity amongst fixation products following exposure of monuron-treated red kidney bean plants to $^{14}CO_2$, both in the light and in the dark, served to demonstrate that these herbicides almost completely blocked the pathway of photosynthetic carbon dioxide fixation but that the observed inhibition was unlikely to result from an interference with any of the reactions of the photosynthetic carbon reduction cycle (Ashton et al, 1961). Of interest is the observation (Gimmler, Neimanis, Eilmann & Urbach, 1971) that whereas treatment of the alga Ankistrodesmus braunii with diuron caused an inhibition of $^{14}CO_2$-fixation when harvesting was carried out in the middle of a light period, the herbicide was a less effective inhibitor in the case of organisms harvested at the beginning of a light period or during a subsequent dark period. The majority of the available evidence suggests that the reduction in rates of CO_2 fixation in plants treated with phenylurea herbicides such as monuron and diuron is an indirect result of the inhibition of electron transport through photosystem 2, with the concomitant loss of generation of reducing power. Consequently, in the present studies the observed inhibition by metoxuron of the Hill activity, as determined by DCPIP and potassium ferricyanide photoreduction, of chloroplasts isolated from treated resistant and susceptible plants might be expected to be reflected in corresponding reductions in rates of CO_2 fixation, unless in the

susceptible variety one (or more) of the enzymes of the photosynthetic carbon reduction cycle constitutes an additional target site for the metoxuron molecule. It was therefore of interest to investigate the effect of spraying resistant and susceptible varieties of wheat plants with metoxuron on CO_2 fixation rates and the subsequent distribution of fixed carbon amongst the various photosynthetic products.

Plants of resistant and susceptible varieties were treated with metoxuron at approximately the three-leaf stage and were used in experiments some two-three days later when photosystem 2-mediated photoreduction in the susceptible variety was inhibited some 60-80%. Throughout this study control plants were sprayed with aqueous 0.1% Tween 60 not containing metoxuron. Rates of CO_2 fixation were determined by illuminating (16000 lx) the leaves of two plants for 1 h in an atmosphere containing 50 μCi of $^{14}CO_2$ and analysing the fixation products extracted with hot ethanol for radioactivity as described in Chapter 2.

The results of two separate experiments using plants which had contacted metoxuron for 48 and 72 h respectively are given in Table 9. These data show that 48 h after treatment with metoxuron $^{14}CO_2$ fixation by leaves of the susceptible variety Maris Nimrod, expressed as μCi incorporated per mg of total ethanol extract, was reduced some 10-fold compared to the rate determined for untreated controls. In contrast, inhibition in the resistant variety Cappelle was less severe, $^{14}CO_2$ fixation in metoxuron-treated leaves being reduced only four-fold compared to untreated controls. Similar results were obtained when $^{14}CO_2$ fixation was measured 72 h after treatment with metoxuron,

TABLE 9: Fixation of $^{14}CO_2$ into ethanol soluble material by metoxuron-treated and untreated wheat plants

Time after spraying (h)		48		72	
Variety		Maris Nimrod (S)	Cappelle (R)	Maris Nimrod (S)	Cappelle (R)
Wet weight of leaves (g)	UT	1.66	1.36	1.84	1.53
	T	1.70	1.75	1.55	1.36
Dry weight of leaves (mg)	UT	190	160	198	186
	T	188	196	187	176
Ethanol soluble extracts (mg)	UT	68	50	76	60
	T	68	74	66	58
Total Activity (μCi)	UT	1.39	0.86	1.01	0.71
	T	0.13	0.34	0.04	0.09
Sp. Activity X 10^3 (μCi/mg ethanol extract)	UT	20.5	18.0	14.00	12.00
	T	2.2	4.8	0.8	1.6

Leaves from two plants were taken for assay. Prior to exposure to $^{14}CO_2$ (50 μCi) for 60 min, the leaves were maintained in the light for the given length of time after spraying with metoxuron (T) or 0.1% Tween 60 (UT).

though rates of $^{14}CO_2$ fixation in both treated resistant and susceptible plants were now further reduced as a result of the extended contact with the herbicide.

The reduction in $^{14}CO_2$ fixation in metoxuron-treated leaves of susceptible and resistant wheat is made particularly apparent on examination of radioautograms of plants of which a single leaf was exposed to $^{14}CO_2$. The results shown in Figs. 19 & 20 were obtained using plants used at the four-leaf stage following illumination of the second leaf for 60 min in an atmosphere containing $^{14}CO_2$ some one and three days respectively after spraying the entire plants with metoxuron. Treatment with the herbicide has clearly resulted in less radioactivity being incorporated into both susceptible and resistant plants, this being particularly evident three days after metoxuron treatment. However, the distinction between resistant and suceptible varieties is not particularly evident in the presented photographs of the experimental radioautograms. The radioautograms recorded in Figs. 19 & 20 do seem to indicate, however, that translocation of photosynthate in plants of both susceptible and resistant wheat varieties is not impaired as a result of metoxuron treatment. This result is not unexpected in view of the belief that ATP for transport of photosynthetic products is provided by oxidative phosphorylation and cyclic photophosphorylation (Canney, 1963).

The results discussed above clearly show a quantitative difference in rates of $^{14}CO_2$ fixation between metoxuron-treated resistant and susceptible wheats. However, since the onset and extent of the observed inhibitions are very similar to those presented earlier in studies of

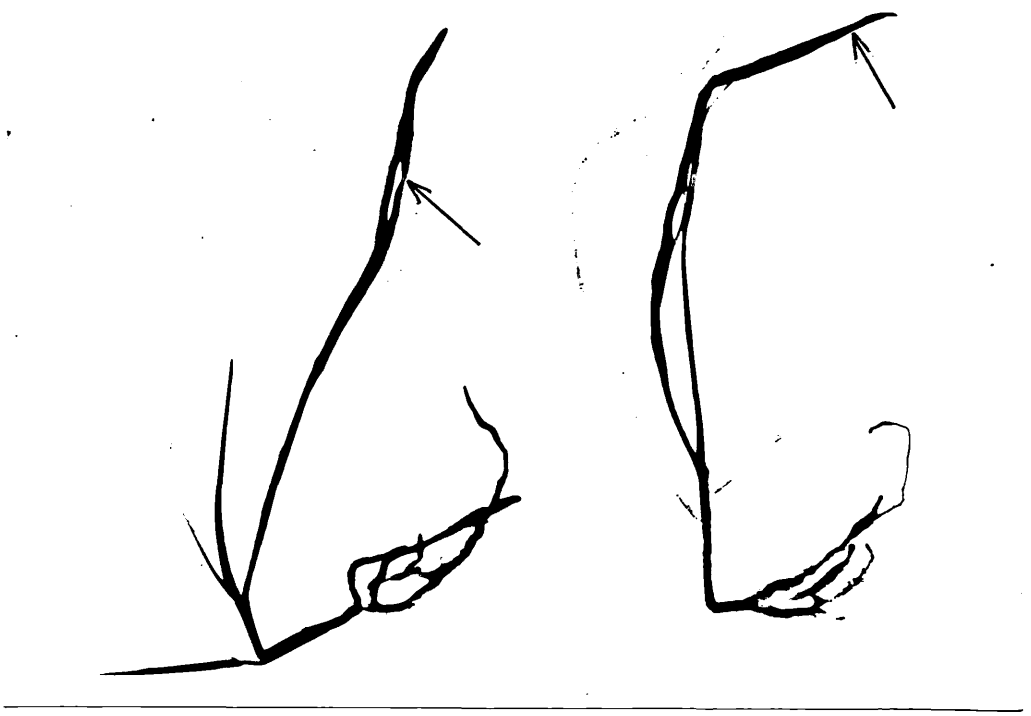
Fig. 19: Radioautographs of metoxuron-resistant and susceptible wheat plants 24 h after exposure of second leaf to 10 µCi $^{14}CO_2$

(a) metoxuron-treated and untreated Cappelle (R) wheat

(b) metoxuron-treated and untreated Maris Nimrod (S) wheat

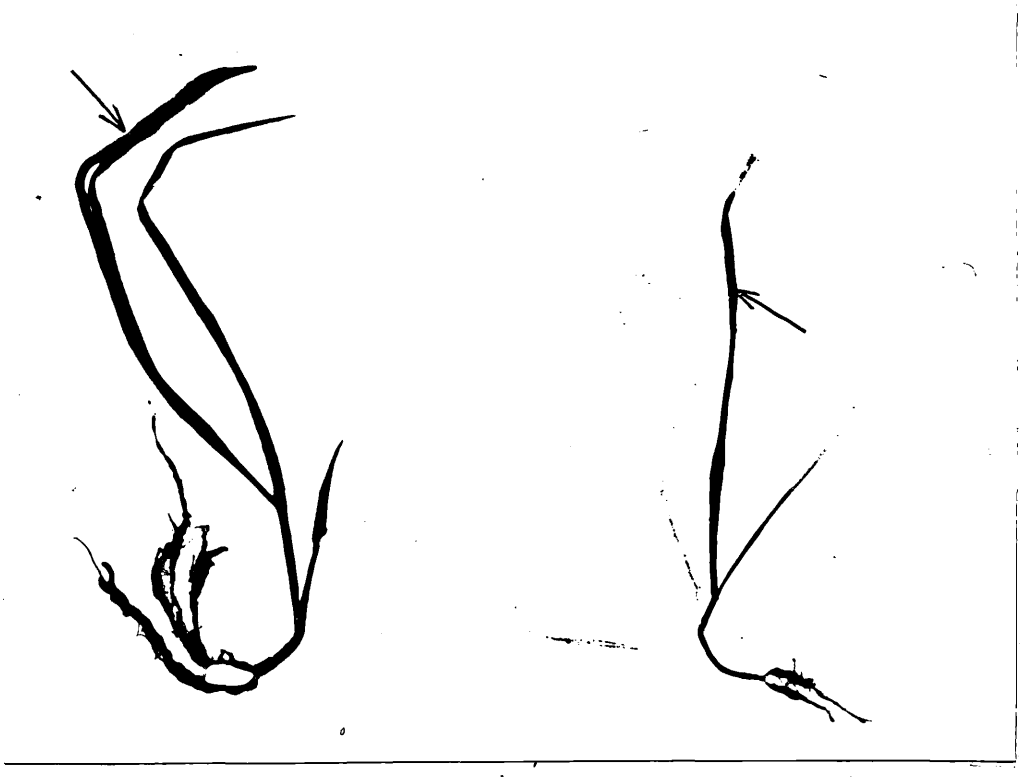
The arrows indicate the leaf exposed to $^{14}CO_2$ in each case.

(a)



untreated metoxuron treated

(b)



untreated metoxuron treated

Fig. 20: Radioautographs of metoxuron-resistant and susceptible wheat plants 72 h after exposure of the second leaf to 10 μCi $^{14}CO_2$

(a) metoxuron-treated and untreated Cappelle (R) wheat

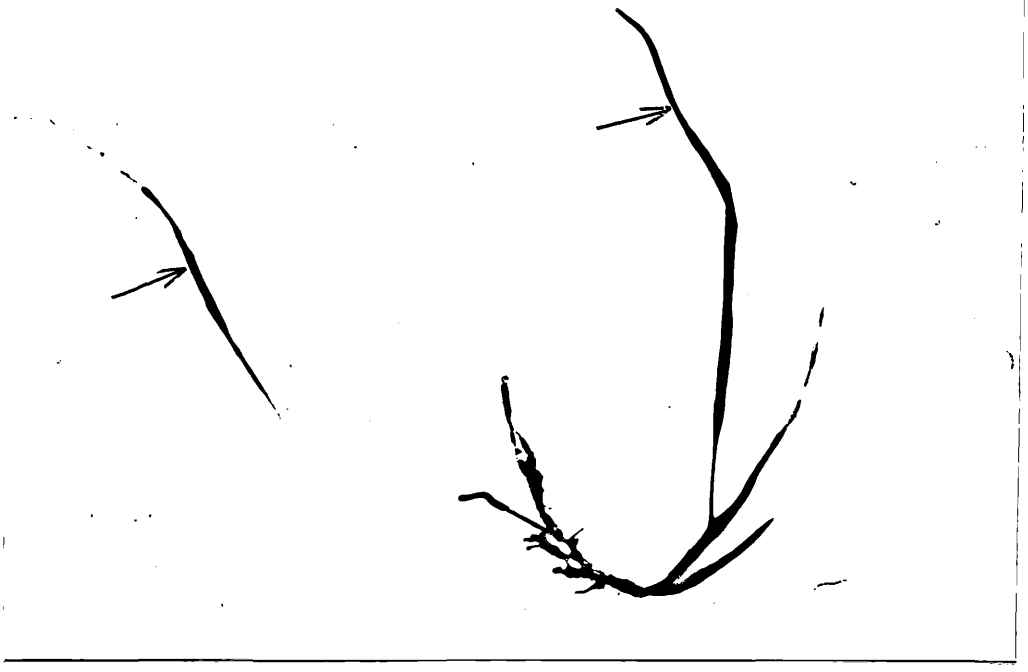
(b) metoxuron-treated and untreated Maris Nimrod (S) wheat

The arrows indicate the leaf exposed to $^{14}CO_2$ in each case.

(a)

metoxuron treated untreated

(b)

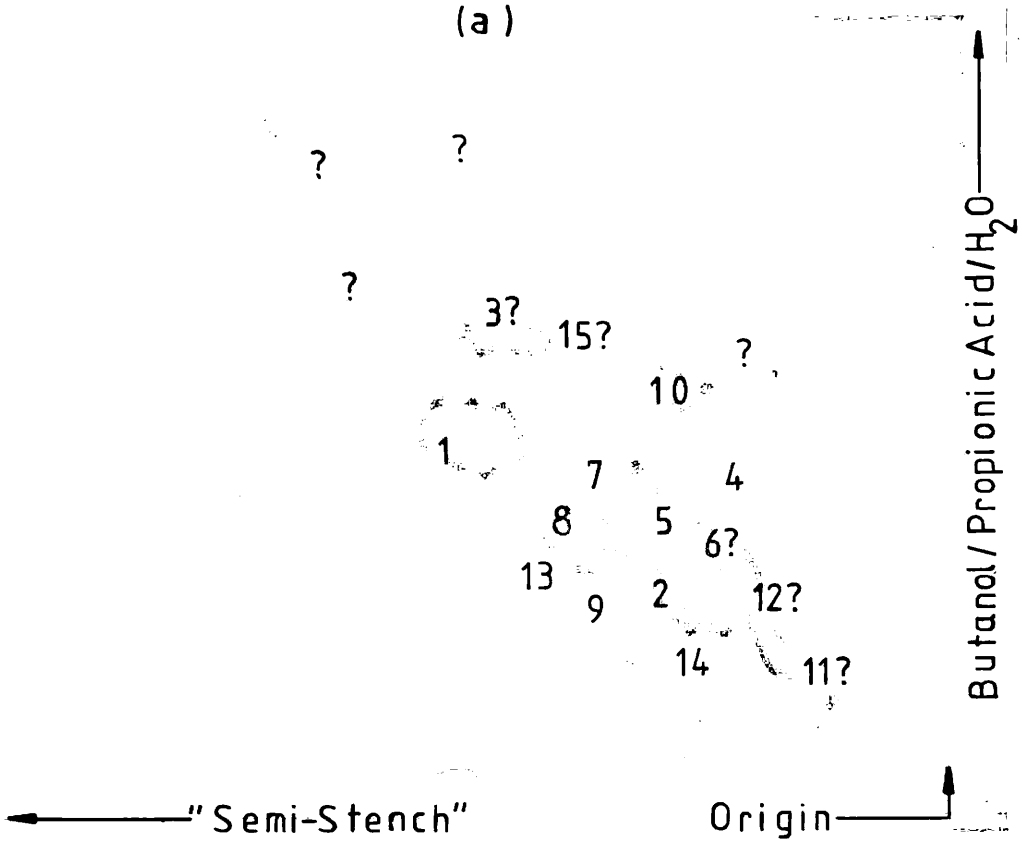


metoxuron treated untreated

Hill activity, the inference from the present results is that the observed inhibition of $^{14}CO_2$ fixation by metoxuron is a reflection of the reduction of ATP and NADPH levels accompanying the inhibition of photosynthetic electron transport at photosystem 2 rather than of a specific interference with the functioning of the Calvin cycle.

Examination of the data in Table 9 on the total radioactivity (μCi) fixed shows that in these studies the susceptible variety Maris Nimrod consistently incorporated significantly greater quantities of $^{14}CO_2$ into fixed carbon than did the resistant Cappelle. This higher rate of photosynthesis in Maris Nimrod possibly represents an important factor in the high yields obtained with this variety, which has been more extensively used than Cappelle in agriculture in recent years.

To investigate the effect of metoxuron on the subsequent metabolism of the initial products of $^{14}CO_2$ fixation in resistant and susceptible wheats the distribution of radioactivity amongst the components of the ethanolic extracts of plants exposed to $^{14}CO_2$ was examined. The ethanol soluble extracts used to compile the data presented in Table 9 were therefore subsequently subjected to two-dimensional paper chromatography using the solvent systems and procedures described in Chapter 2, and the separated radioactive products were located by autoradiography. Though no marker compounds were studied in the present system a tentative identification of the components corresponding to the major radioactive zones was attempted from a comparison with similar radioautograms and Rf values reported by Crowley et al (1963), Owen (1971) and Delaney (1975). The radioautograms for a selection of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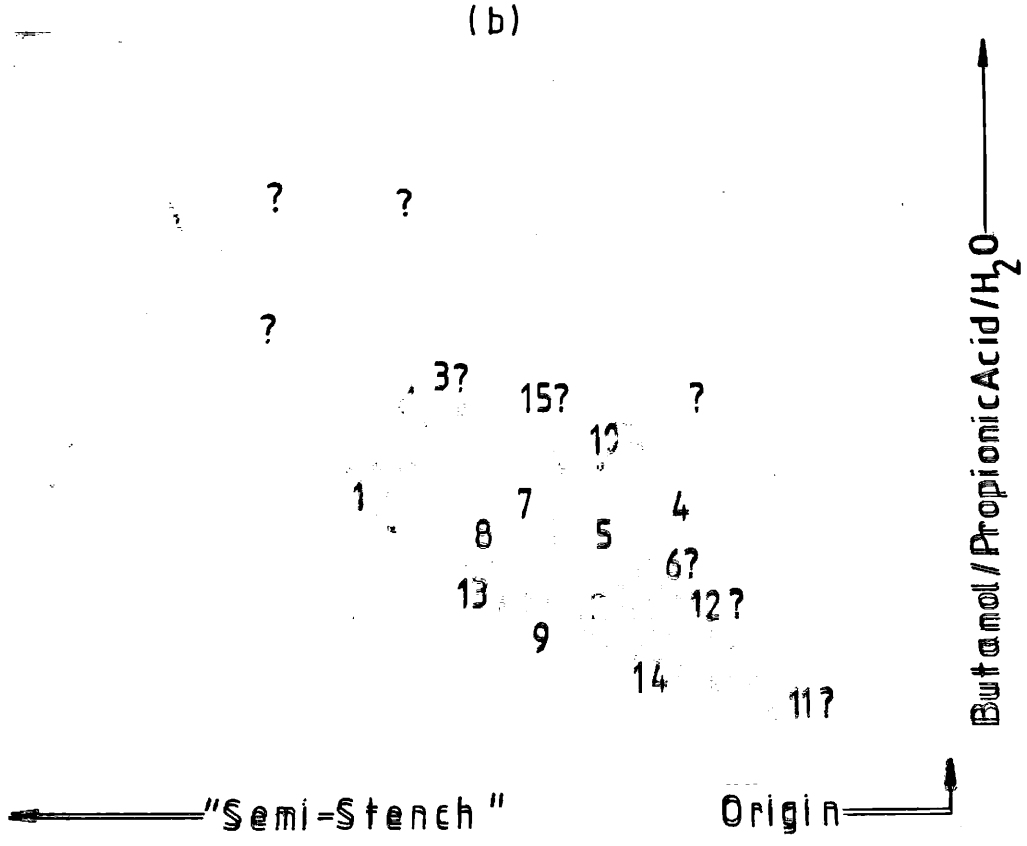
Butanol / Propionic Acid / H_2O
"Semi-Stench"
Origin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Butanol / Propionic Acid / H_2O
"Semi-Stench"
Origin

Fig. 21: Autoradiographs of chromatograms of extracts of wheat after exposure to $^{14}CO_2$

Leaves were extracted immediately after exposure to 50 μCi $^{14}CO_2$ for 1 h during illumination.

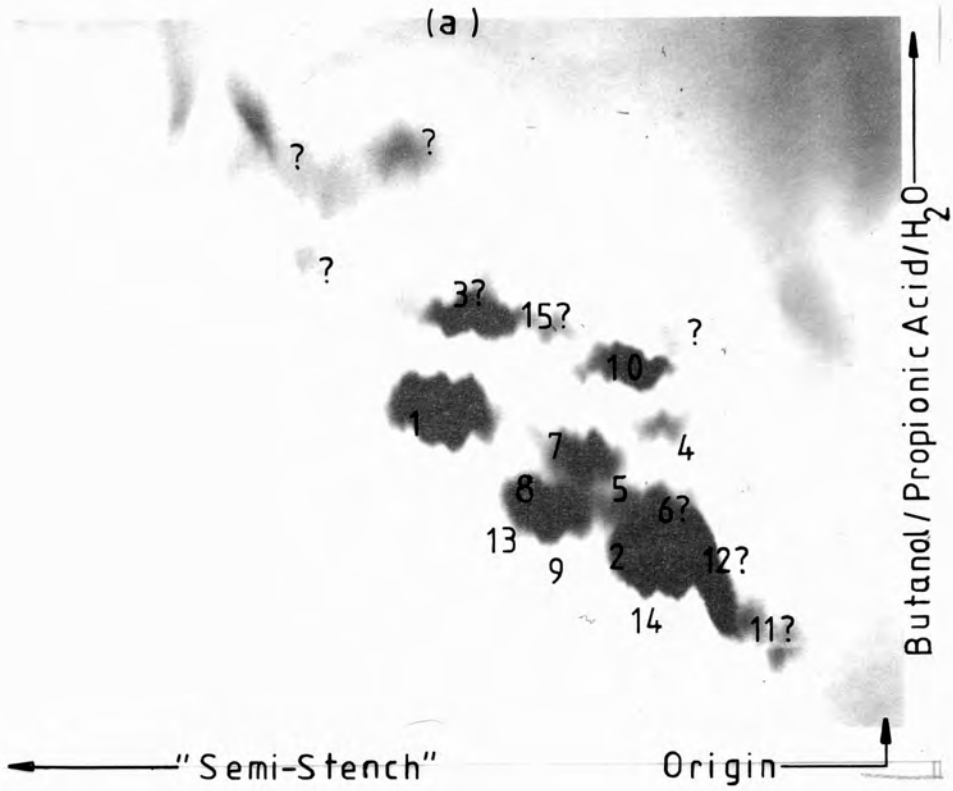
(a) Untreated Cappelle (R) wheat
(b) Untreated Maris Nimrod (S) wheat

Identification of the separated compounds has been attempted from a comparison with similar autoradiographs and Rf values reported by Crowley et al (1963), Owen (1971) and Delaney (1975),

1. Alanine
2. Aspartate
3. Glycollate
4. Citrate
5. Fructose
6. Glucose
7. Glutamate
8. Glutamine
9. Glycine
10. Malate
11. Monophosphates
12. Phosphoglycerate
13. Serine
14. Sucrose
15. Glycerate

Where numbers are accompanied by (?), identification remains tentative.

(?) alone indicates zones for which no identification has been attempted.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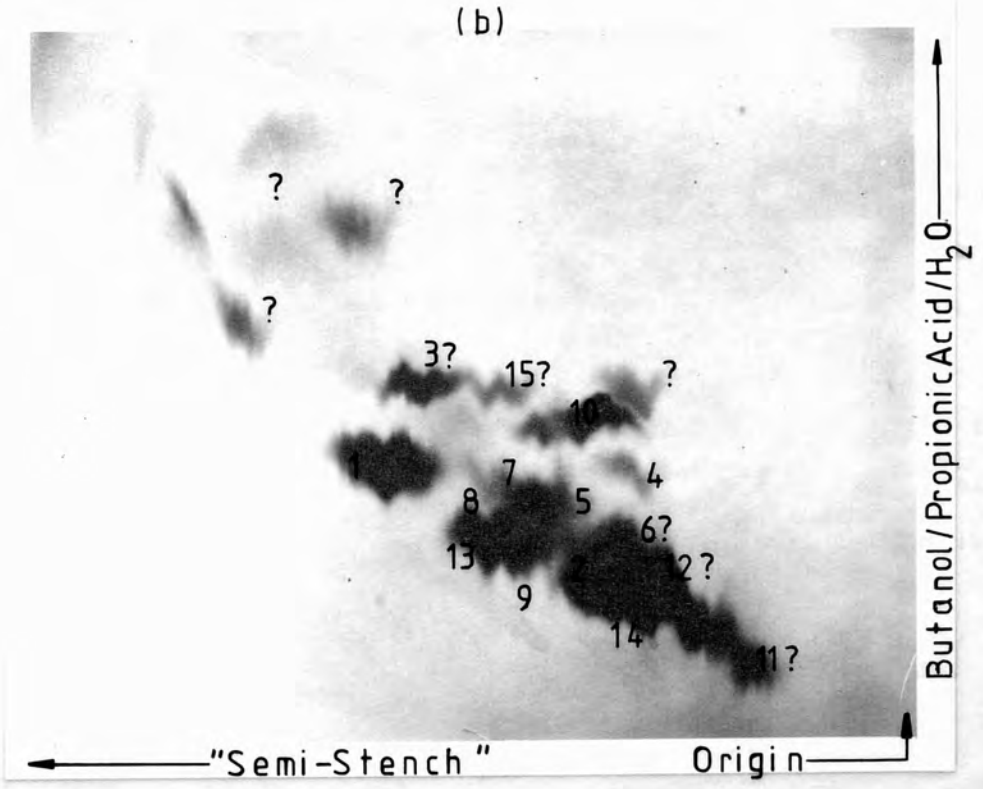
"Semi-Stench"
Origin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Butanol/Propionic Acid/H_2O
"Semi-Stench"
Origin

experiments are presented in Figs. 21-23. Volumes of ethanol extracts from resistant and susceptible plants were adjusted in each experiment so that the same amount of radioactivity (approx. 100,000 cpm) was applied to each chromatogram prior to development. The radioautograms presented in Fig. 21 are fairly typical of those obtained for equivalent amounts of ethanol extract from control plants of susceptible and resistant wheat varieties. Figs. 22 & 23 compare the radioautograms obtained for metoxuron-treated resistant and susceptible wheat respectively with those for corresponding untreated controls some 48 h after spraying. It can be seen that the separation patterns obtained for metoxuron-treated resistant and susceptible plants are qualitatively very similar, not only with respect to each other but also to untreated controls, thus lending additional support to the earlier conclusion that treatment with metoxuron does not result in any specific interference with the reactions of the photosynthetic carbon reduction cycle and associated metabolic pathways.

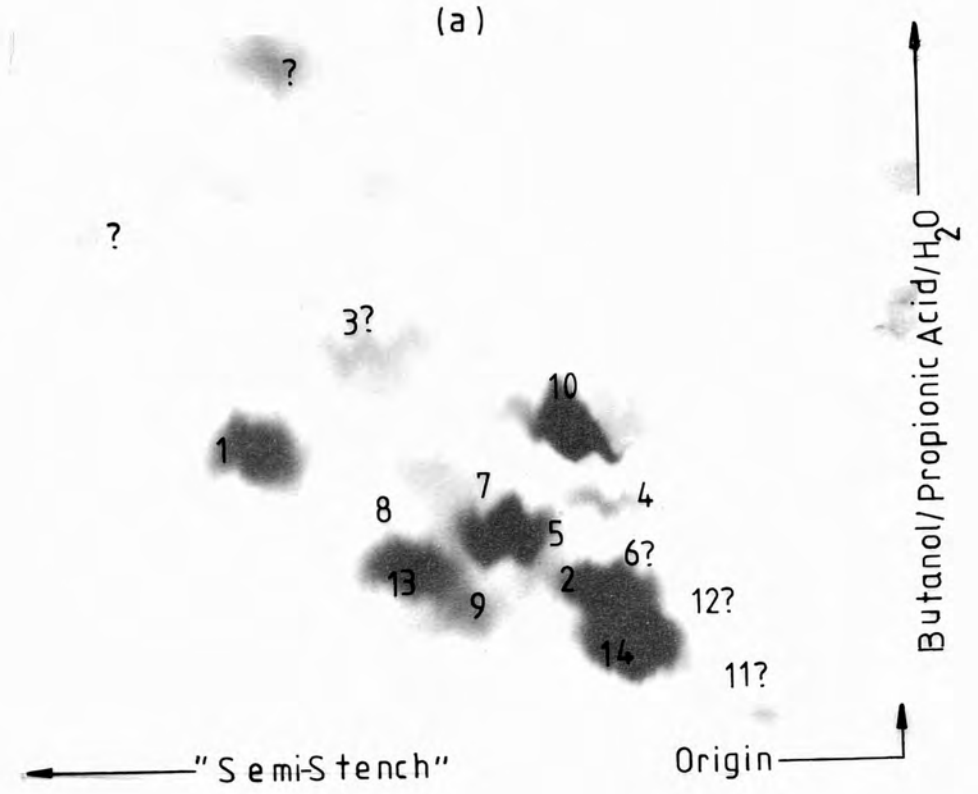
The results presented in Table 9 showed no significant reduction in the amount of ethanol soluble material extracted from the leaves of metoxuron-treated plants compared to untreated controls. These data contrast with the observation that $^{14}CO_2$ fixation in resistant and susceptible wheat treated with metoxuron can be diminished by some four and ten-fold respectively. A further investigation of the effect of metoxuron on the level of ethanol soluble carbohydrates in susceptible and resistant wheats was therefore made.

Fig. 22: Autoradiographs of chromatograms of extracts of metoxuron-resistant (Cappelle) wheat after exposure to $^{14}CO_2$

Experimental details and identification of radioactive zones as for Table 9 and Fig. 21.

(a) Untreated wheat

(b) Metoxuron-treated wheat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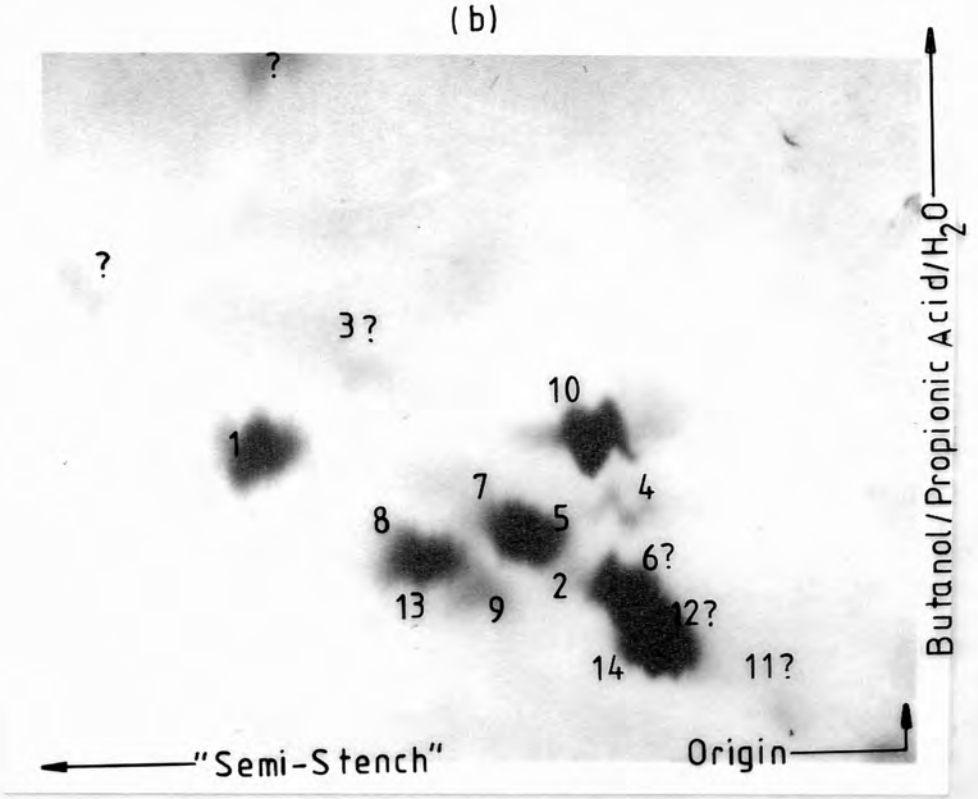
Butanol/Propionic Acid/H_2O
"Semi-Stench"
Origin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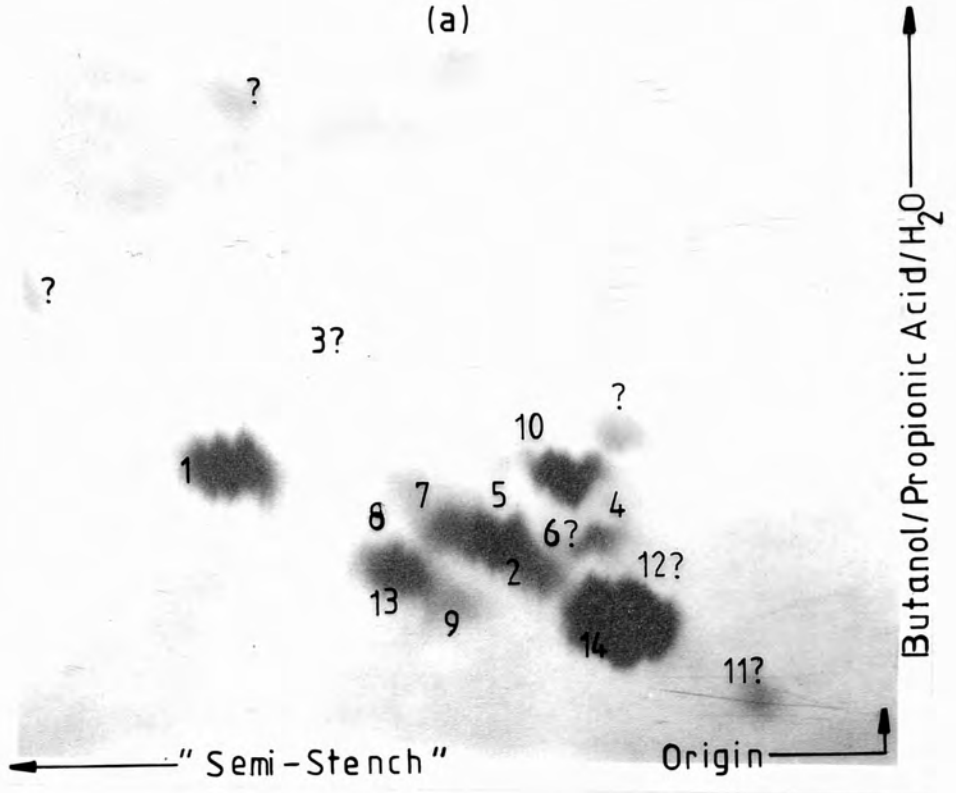
Butanol/Propionic Acid/H_2O
"Semi-Stench"
Origin

Fig. 23: Autoradiographs of chromatograms of extracts of metoxuron-susceptible (Maris Nimrod) wheat after exposure to $^{14}CO_2$

Experimental details and identification of radioactive zones as for Table 9 and Fig. 21.

(a) Untreated wheat

(b) Metoxuron-treated wheat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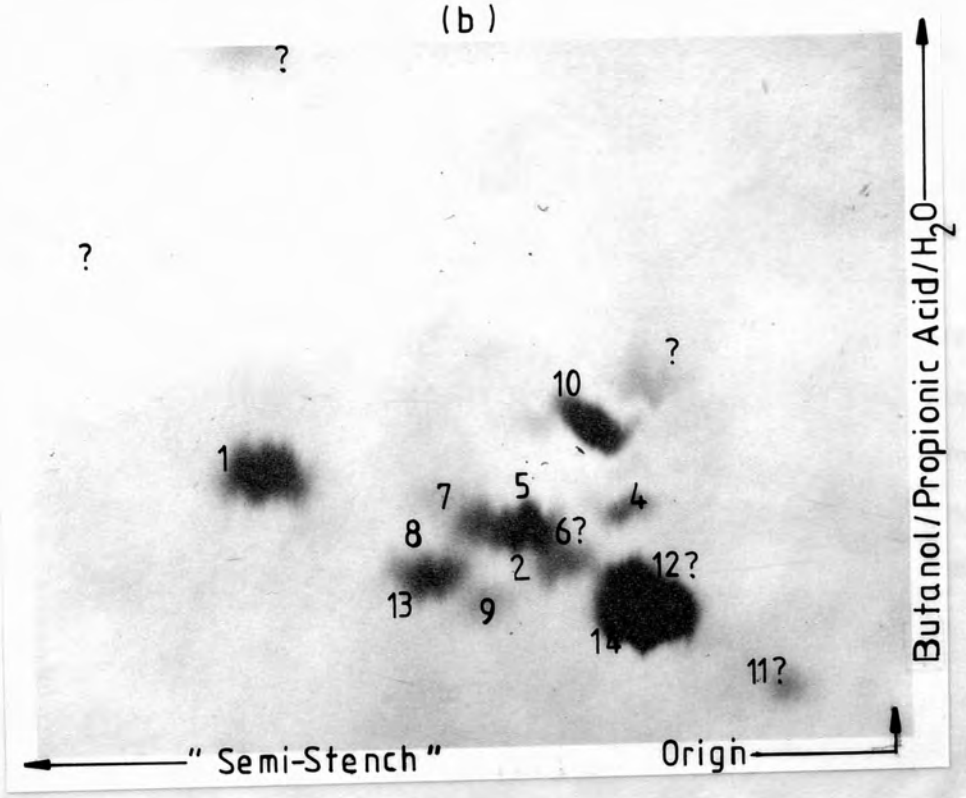
" Semi-Stench "
Origin
Butanol/Propionic Acid/H_2O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" Semi-Stench "
Origin
Butanol/Propionic Acid/H_2O

Effect of Metoxuron on Ethanol Soluble Carbohydrates

Cooke (1955) demonstrated that the level of carbohydrate in several leguminous species was decreased by treatment with monuron. This reduction in sugar content was attributed either to an interference of monuron with photosynthesis or to a monuron induced increase in sugar utilization. In an extension of this study (Cooke, 1956) it was found that leaf discs incubated with monuron in the presence of sucrose in the dark still accumulated starch, indicating that monuron acted at a stage prior to the formation of sugars.

The present studies were made using control and metoxuron-treated plants of both susceptible and resistant wheats at various times after treatment. Plants were grown in John Innes No. 2 compost under greenhouse conditions and were sprayed with metoxuron at the three-leaf stage. The material to be analysed (2 g wet weight) was obtained by exhaustive extraction with 80% ethanol in a soxhlet apparatus (p. 115). The final extracts (100 ml) containing principally glucose, fructose and sucrose were analysed for total carbohydrate by the phenol-sulphuric acid procedure of Dubois et al (1951, 1956). Carbohydrate content was computed by reference to a standard curve prepared for glucose.

Results obtained at various times after metoxuron treatment are given in Table 10 and are expressed as percentage dry weight of ethanol soluble carbohydrates. These data clearly show a decrease in the level of carbohydrate in the leaves of both susceptible and resistant varieties in response to metoxuron treatment. However, the reduction in carbohydrate content is more severe in the case of metoxuron-treated susceptible

TABLE 10: Effect of Metoxuron on Ethanol Soluble Carbohydrate in Wheat.

Time after spraying (days)	Ethanol Soluble Carbohydrate (% Dry Weight)			
	Maris Nimrod (S)		Cappelle (R)	
	Treated	Untreated	Treated	Untreated
0	2.40	2.30	2.27	2.20
1	1.90	3.77	2.36	3.75
2	1.78	3.70	1.95	3.50
4	1.61	3.78	2.20	3.78
6	1.80	3.90	2.83	3.85

Carbohydrate was estimated using the phenol-sulphuric acid procedure of Dubois et al (1951, 1956)

compared to resistant plants, amounting to some 55% and 25% respectively of untreated control levels six days after contacting the herbicide. These results are in agreement with those of Cooke (1955) obtained using monuron and also with the data given earlier in the present thesis on $^{14}CO_2$ fixation in metoxuron-treated resistant and susceptible wheats.

Walsh & Grow (1971) have studied the effects of substituted phenylurea herbicides on the level of carbohydrate in certain marine algae. These workers found that monuron, diuron, fenuron and neburon

all caused a decrease in the carbohydrate content of the algae. In agreement with the present observations, Walsh & Grow (1971) reported that the extent of the reduction in carbohydrate content following herbicide treatment was not uniform amongst the algae screened, but varied from some 66% in susceptible types to only 18% in the more resistant populations. The present results are also in agreement with the more recent report (Zemanek & Kovar, 1975) that treatment of a resistant wheat cultivar with the related phenylurea, chlortoluron, did not result in a large difference in reducing sugar content of the leaves compared to untreated controls. Unfortunately, however, this latter study did not include a chlortoluron-sensitive cultivar and thus a more direct comparison with the data presented in the present thesis is not possible.

Having demonstrated significant reductions in the amount of ethanol-soluble carbohydrate present in the leaves of wheat plants treated with metoxuron, particularly in the case of the susceptible variety Maris Nimrod, it was of interest to see whether the observed reduction could be accounted for by a decrease in an identifiable component(s). In another experiment therefore each individual extract was subjected to quantitative paper chromatography on Whatman No. 3 paper. After concentration of the extracts duplicate 0.05 ml aliquots containing 300 µg ethanol extracted material were chromatographed as previously described (p. 117). Following development of the chromatograms, one chromatographic zone was sprayed with p-anisidine hydrochloride to locate the sugars, this serving as a reference for subsequent elution of sugars from the duplicate zone for estimation by the phenol-

sulphuric acid method (p. 116). A mixture of reference carbohydrates and 200 µg of ribose as a standard to check percentage recovery from the chromatography paper, were also applied to each chromatogram. The results obtained are summarised in Table 11. These data again

TABLE 11: Effect of metoxuron on the principal components of the ethanol soluble carbohydrate fraction of susceptible and resistant wheat

Time after spraying (days)	Variety	Treatment	Sugar content (% Dry Weight)			
			Sucrose	Glucose	Fructose	Total
0.5	Maris Nimrod (S)	untreated	1.52	1.02	0.91	3.37
		treated	1.68	1.10	0.98	3.52
	Cappelle (R)	untreated	1.50	1.03	0.75	3.22
		treated	1.52	1.02	0.80	3.34
5	Maris Nimrod (S)	untreated	4.58	2.10	1.25	8.3
		treated	1.27	0.75	0.48	2.18
	Cappelle (R)	untreated	3.92	2.20	1.43	7.3
		treated	1.56	0.97	0.67	3.12

The sugars were separated by paper chromatography (p.117) and, following elution with deionised water, were estimated using the phenol-sulphuric acid procedure of Dubois et al (1951, 1956).

indicate a clear decrease in total ethanol soluble carbohydrate in metoxuron-treated resistant and susceptible varieties some 5 days after treatment, the greater reduction being observed in the case of the

susceptible Maris Nimrod. However, the reduction in total ethanol soluble sugars can be attributed to a significant decrease in all three sugars analysed in that 5 days after metoxuron treatment the reductions in fructose, glucose and sucrose are proportionally the same in both varieties.

Effect of Metoxuron on the Respiratory Properties of Wheat Mitochondria

The results presented in the previous section showed that the levels of ethanol-soluble carbohydrate in the leaves of metoxuron-treated susceptible wheat plants were substantially reduced compared to untreated controls. Though this result was not unexpected in view of the observed reduction in rates of $^{14}CO_2$ fixation in leaves of wheat plants treated with metoxuron (Table 9), the possibility remained that the data might also be explained in terms of a metoxuron-induced stimulation of respiratory processes with an accompanying increase in sugar utilisation.

In a comparative study of the action of diuron on electron transport and accompanying phosphorylation processes in mitochondria and chloroplasts (Moreland & Blackmon, 1968), it was found that whereas non-cyclic electron flow and the coupled phosphorylation reaction in spinach chloroplasts was strongly inhibited by the herbicide, oxidative phosphorylation in potato mitochondria was not. However, an effect of monuron on mitochondrial function has been observed by Lotlikar et al (1968) who reported that

at a concentration of 3×10^{-3}M monuron caused a reduction of some 50% in the P/O ratio of cabbage mitochondria. The concentration of monuron employed in this latter study, however, was far in excess of that required to give complete inhibition of Hill activity of isolated chloroplasts in in vitro assays (Bishop, 1958).

In the present investigation, mitochondria were isolated from the shoots of three-days old dark grown metoxuron-resistant and suceptible wheat seedlings, and their respiratory properties were subsequently examined using a Clark oxygen electrode as previously described (p. 102) with L - malate, succinate and NADH as substrates. In such a study it is essential to define both the substrate and ADP levels in order to identify the steady-state condition of the mitochondria during an experiment. The steady-state conditions of mitochondria relevant to the present discussion are commonly referred to as states 3 and 4 (Chance & Williams, 1956). State 4 ("resting state") is an aerobic state characterised by a low respiration rate, even though adequate substrate may be present. This state requires definite conditions for its establishment in that mitochondria must be very carefully prepared and supplied with substrate in the absence of phosphate acceptor. State 3, on the other hand, is the "active" state of rapid respiration and phosphorylation in the presence of adequate supplies of substrate and phosphate acceptor (ADP). The ratio of the rates of oxygen uptake during states 3 and 4 of respiration is referred to as the respiratory control (R.C.) index, the value of which for a particular batch of mitochondria provides a measure of the biochemical integrity of the preparation (Chance & Williams, 1956). Substances which uncouple

oxidative phosphorylation from electron transport not only result in a lowering of the measured P/O ratios but are also characterised by causing stimulation of state 4 respiration through releasing the limitation imposed by the absence of a supply of phosphate acceptor. Consequently, in the present study any ability of metoxuron to uncouple mitochondria prepared from resistant or susceptible wheat would be recognised as an increase in oxygen uptake when the herbicide is added during state 4 of respiration.

The data presented in Figs. 24-26 show that wheat mitochondria oxidise all three substrates examined with good respiratory control and P/O ratios. Succinate oxidation can be seen to proceed at a fairly constant rate with uniform ADP ratio. Under similar conditions, however, the rate of malate oxidation decreased with each additional increment of ADP added.. Addition of ADP to all reaction mixtures clearly stimulated oxidation of substrates (state 3) and concomitant with the subsequent exhaustion of the ADP supply substrate oxidation decreased (state 4). However, no very significant alteration in the rate of state 4 respiration was observed on the addition of metoxuron (final concentration in the reaction mixture = 3.3×10^{-4}M) to assay mixtures containing mitochondria from either resistant or susceptible wheats, irrespective of the substrate being oxidised (Figs. 24-26). The inference from these results is that the mitochondrial electron transport chain and associated oxidative phosphorylation does not represent an additional site of action of metoxuron in treated wheat plants. The possibility still remains, however, that the decrease in ethanol soluble carbohydrate accompanying treatment of wheat plants with metoxuron might result from an interference

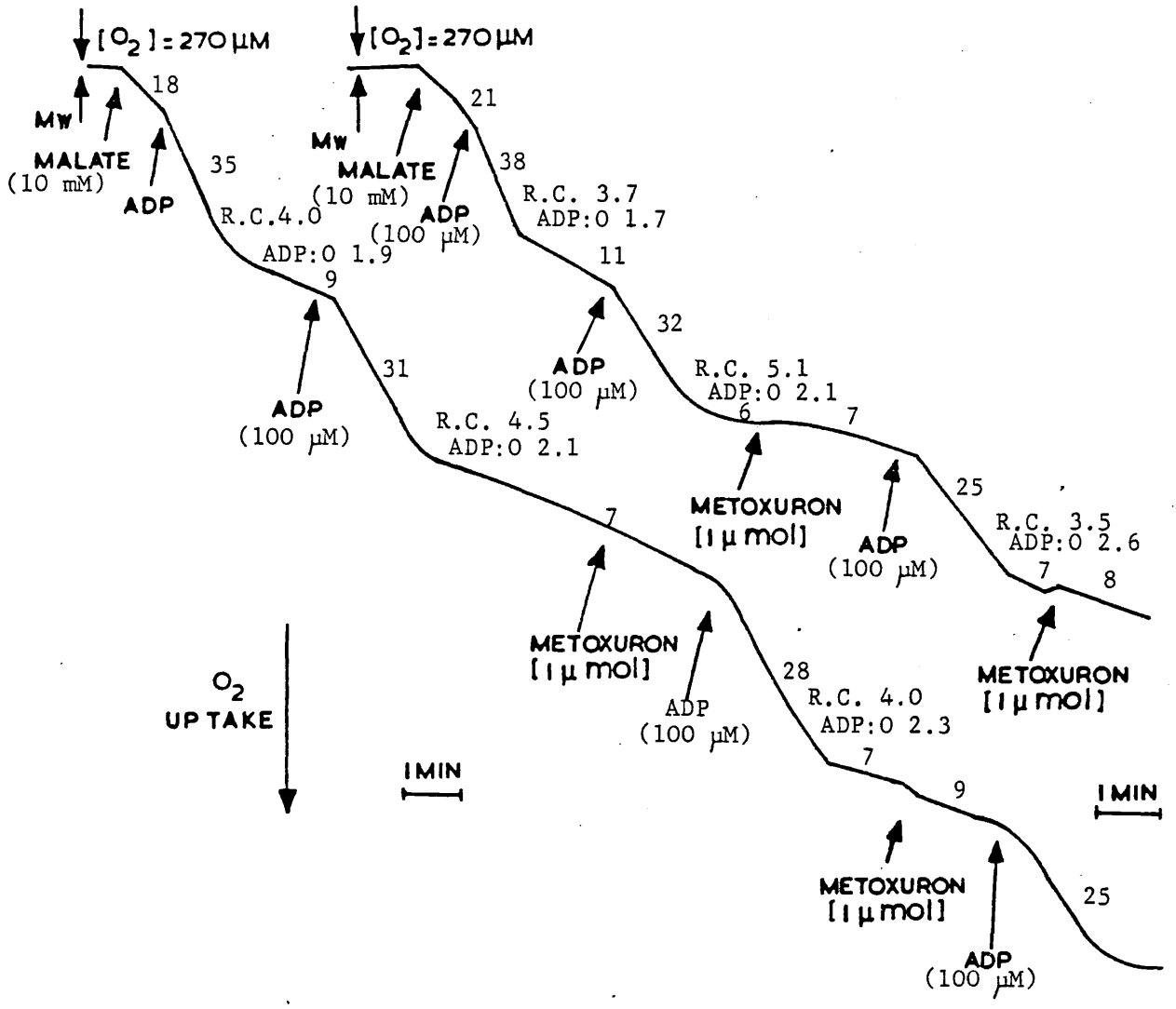


Fig. 24: Polarograph traces of O_2 uptake by wheat mitochondria utilising malate as substrate

Mitochondria were isolated from the shoots of 4 day old dark-grown wheat seedlings by the procedure described in the "Methods" section (p. 96). O_2 uptake was measured using a Clark Oxygen Electrode (p. 102).

Numbers along the traces are rates of O_2 uptake in µmol/min/mg protein.
Concentrations of reactants are final concentrations in the 3.0 ml reaction mixture.

Mw = addition of mitochondria containing approx. 1.4 mg mitochondrial protein.
RC = Respiratory control index.
Metoxuron was added as 10 µl of a stock solution of the technical herbicide in methanol.

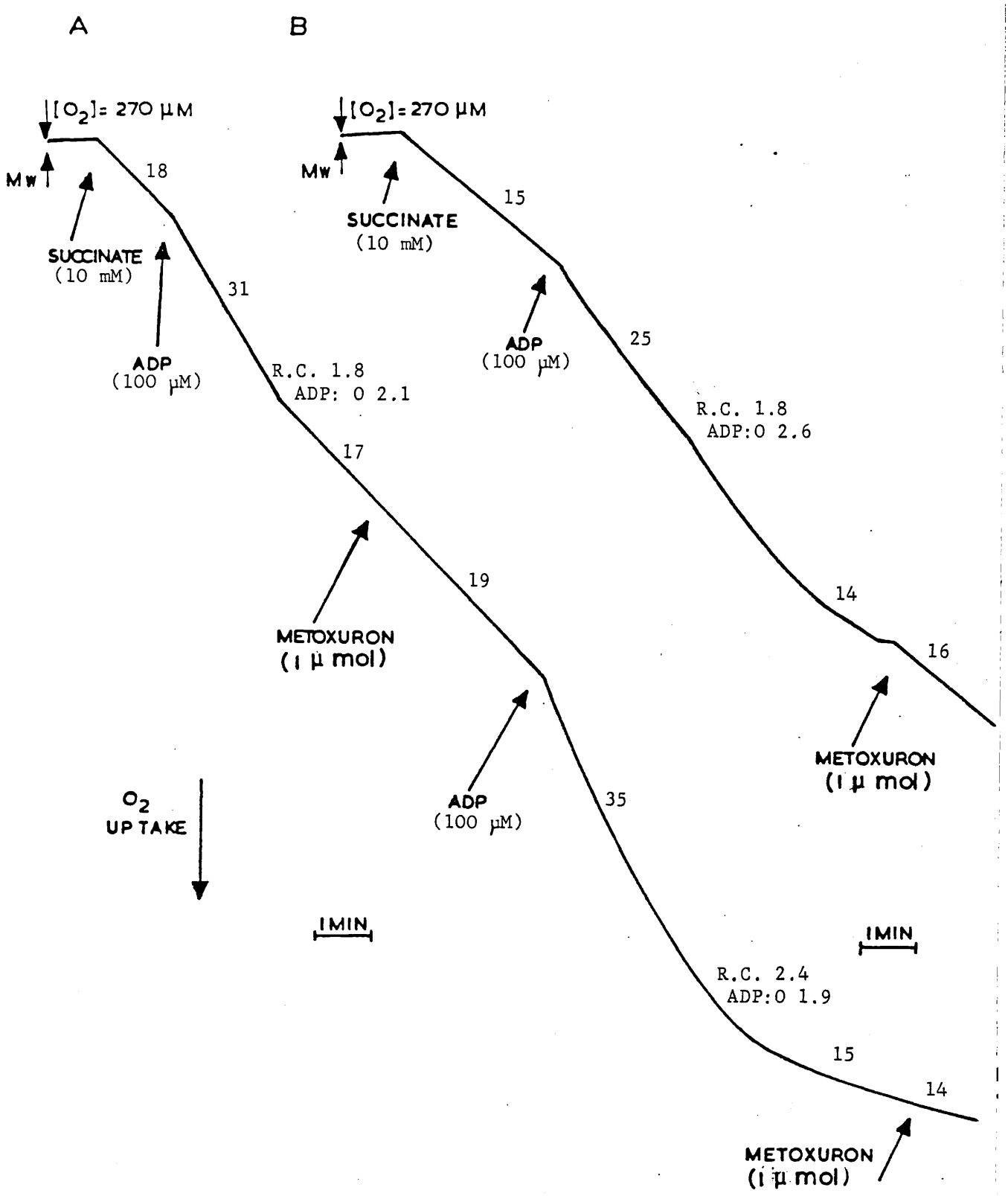


Fig. 25: Polarograph traces of O_2 uptake by wheat mitochondria in the presence of succinate as substrate

The procedures employed for the isolation and assay of the mitochondria were as previously described in the "Methods" section (pages 96 and 102).

The various abbreviations have the same meaning as for Fig. 24.

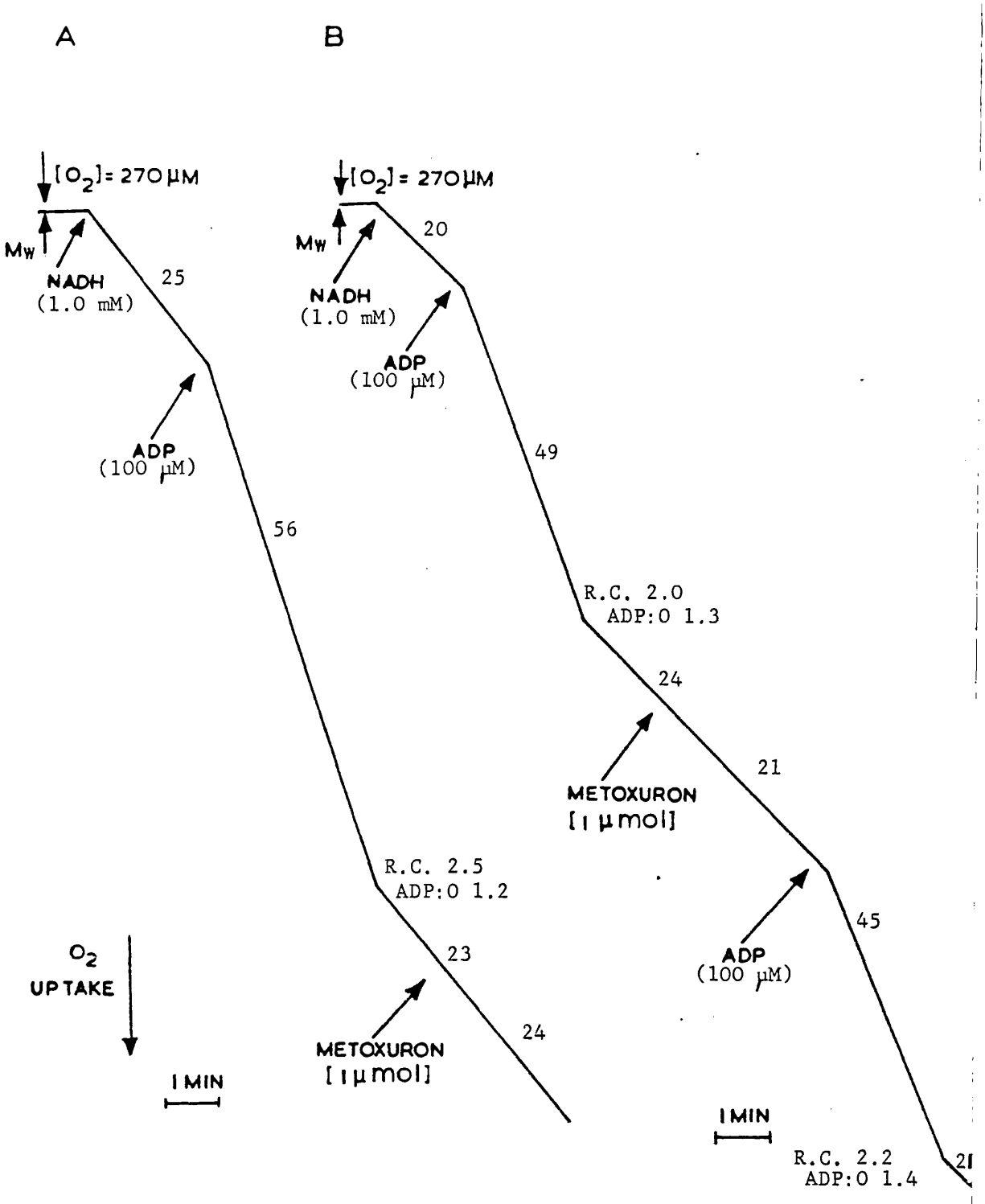


Fig. 26: Polarograph traces of O_2 uptake by wheat mitochondria in the presence of NADH as substrate

See Fig. 24 for experimental details and explanation of terms.

with mitochondrial function by some metabolite or complex of the parent herbicide formed within the plant. This latter possibility was not investigated in the present study.

The results presented in Figs. 24-26 are consistent with the findings of other workers (Mukasa, Itoh & Nosoh, 1966; Lotlikar *et al*, 1968; Moreland & Blackmon, 1968) for similar concentrations of related phenylurea herbicides. In an extensive study, Mukasa *et al* (1966) examined the effect of diuron on the respiratory properties of rat liver mitochondria and of some 18 microorganisms including *E. coli*, *Proteus vulgaris*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. The only respiratory system to be significantly affected by diuron was that of the yeasts for which a 50% inhibition was obtained at a diuron concentration of 1.6×10^{-4}M. In a recent extension to this investigation, however, Colson, The Van, Convent, Briquet & Goffeau (1977) demonstrated a resistance to diuron treatment in the mitochondrial respiratory chains of three mutants of *Saccharomyces cerevisiae*. This resistance to diuron was expressed *in vitro* as well as *in vivo* since the oxidation rates of succinate by sonicated sub-mitochondrial particles from the mutants were clearly less sensitive to diuron-treatment than that of the wild type organism.

The present data contrast, however, with the observation (McDaniel & Frans, 1969) that fluometuron caused a 50% uncoupling of oxidative phosphorylation in soybean mitochondria at a concentration of 5.6×10^{-5}M with malate as substrate, and at 4.6×10^{-5}M when succinate was the substrate employed. Though Lotlikar *et al* (1968) were also able to demonstrate an effect of monuron on oxidative phosphorylation in cabbage mitochondria, the concentration required (some 3×10^{-3}M) was some 10^4

times higher than that required to cause extensive inhibition of the Hill reaction of isolated chloroplasts.

Studies on the Uptake and Translocation of Metoxuron in Wheat

An ability of young wheat plants to translocate foliar applied metoxuron was noted in a preliminary study of the effect of the herbicide on photosynthetic electron transport. In this study the Hill activities of chloroplasts isolated from the second and third leaves only of resistant and susceptible wheat plants were measured over a period of time following application of metoxuron to the first leaf as a dip. In view of the severe potency of metoxuron as an inhibitor of photosynthetic electron transport demonstrated in the in vitro studies presented earlier, care was taken in the present investigation to remove deposits of metoxuron from leaf surfaces by wiping with cotton wool impregnated with 80% methanol prior to chloroplast isolation. Rates of DCPIP and potassium ferricyanide photoreductions obtained for chloroplasts isolated from leaves of metoxuron-treated resistant and susceptible wheat varieties are compared to those for untreated controls in Table 12. These results show that inhibition of Hill activity becomes evident in chloroplasts isolated from the second and third leaves of both metoxuron-resistant and susceptible varieties within 5 days of application of the herbicide to the first leaf. As was noted in an earlier study, however, involving treatment of the entire plant with metoxuron (Table 4), a distinction exists between the percentage inhibitions of photosynthetic activity measured in the two varieties. In the present study this distinction between varieties is particularly evident some 14 days after treatment with the herbicide, this being largely attributable to an apparent recovery from inhibition of photosynthesis in chloroplasts isolated from the resistant Cappelle.

TABLE 12: The Hill activity of chloroplasts isolated from the untreated leaves of metoxuron-resistant and susceptible wheat plants treated with the herbicide via the first leaf

Time after treatment (days)	Variety	Hill Reaction Activity (μmol dye reduced/mg Chl/h)				% Inhibition photoreduction	
		DCPIP photoreduction		$K_3Fe(CN)_6$ photoreduction			
		Untreated	Treated	Untreated	Treated	DCPIP photoreduction	$K_3Fe(CN)_6$ photoreduction
2	Maris Nimrod (S)	127	130	240	243	-	-
	Cappelle (R)	126	127	237	239	-	-
5	Maris Nimrod (S)	127	108	242	200	16	17
	Cappelle (R)	126	112	235	206	12	12
7	Maris Nimrod (S)	124	101	242	194	20	19
	Cappelle (R)	129	113	242	214	12	12
14	Maris Nimrod (S)	119	102	194	166	14	14
	Cappelle (R)	110	104	183	173	5	6

Assay conditions as shown for Table 2

The data presented in Table 12 clearly demonstrates that following application to a single leaf movement of metoxuron (or of a phytotoxic metabolite) into other leaves can take place, this presumably indicating that a degree of downward movement of metoxuron in the assimilation stream may be possible. This result is apparently in contrast to much of the data in the literature which have led to the widespread acceptance of the view (Hay, 1976) that substituted phenylurea herbicides, in particular monuron and diuron, represent classical examples of herbicides translocated only in the apoplast (transpiration stream). A herbicide translocated exclusively by this mechanism would consequently be unlikely to show any basipetal movement, as was the case in a study of the translocation of ^{14}C-chloroxuron in buckwheat (Polygonum convolvolus) following application to the leaf surface (Geissbühler, 1963a). Although the majority of experimental evidence thus suggests that movement of the phenylureas examined to date is largely apoplastic, entry into the symplast (protoplasm) must occur at some point if the applied herbicide is to exert its toxic effect in the chloroplast. Though the mechanism by which phenylureas actually cross the plasmalemma remains unknown (Hay, 1976), it is considered conceivable that this movement may occur passively by simple diffusion in view of the fact that the distance between cell wall and chloroplast in mesophyll tissue is generally quite short (Ashton & Crafts, 1973). The latter authors have also expressed the possibility that movement of monuron for example into the symplast may occur at concentrations so low as to have evaded detection by the standard technique of autoradiography following application of the ^{14}C-labelled herbicide. This suggestion is of particular interest to the present investigation and may well provide an explanation for the

data presented in Table 12 for which the assay for movement into the protoplasm of the leaf cells was both specific and very sensitive, i.e, the degree of inhibition of photosynthetic activity apparent in isolated chloroplasts.

Although it was realised that the distinction between metoxuron-resistant and susceptible varieties evident in the data presented in Table 12 might be explainable on the basis of an enhanced rate of metoxuron detoxification in the resistant cultivar, it was considered conceivable that the present results might also reflect differences between the two varieties with respect to permeability of the chloroplast envelope towards metoxuron. To test this latter possibility, however, chloroplasts with intact outer envelopes (whole chloroplasts) were required for use in in vitro assays.

There is ample evidence that the ability of chloroplasts to catalyse extra-cellular photosynthesis is related to the integrity of the bounding membrane (Walker, 1964; Jensen & Bassham, 1966; Kalberer, Buchanan & Arnon, 1967). Electron microscopy studies have shown that the most active chloroplasts retain an intact double envelope (Walker, 1965; Jensen & Bassham, 1966). The isolation of chloroplasts with intact envelopes is facilitated by brief disruption, rapid separation and the use of a sugar, such as sucrose, glucose or fructose (Walker, 1964, 1965) or sugar alcohol such as sorbitol (Jensen & Bassham, 1966) or mannitol (Kalberer et al, 1967), rather than NaCl to maintain the osmotic pressure. Chloroplasts isolated under such conditions show high rates of carbon dioxide fixation and associated evolution of oxygen (Walker 1964, 1965). The substitution of pyro-phosphate and/or Hepes for orthophosphate and other buffer systems

previously employed (Jensen & Bassham, 1966; Good et al, 1966) resulted in further increases in the rates obtained, this presumably reflecting additional improvements in the integrity of the chloroplasts isolated.

The method of chloroplast isolation initially adopted in the present study utilised a combination of pyrophosphate and Hepes buffers and was adapted from a procedure developed by Cockburn et al (1968) for the isolation of intact chloroplasts from spinach (see p. 92). An ability of a particular chloroplast preparation to mediate photosynthetic oxygen evolution (expressed as μmol O_2 evolved/mg chl/h) in the absence of an added Hill reagent, as measured using a Clark oxygen electrode (p. 102), was taken as an indication of the presence of a significant percentage of chloroplasts with intact envelopes. The data presented in Fig. 27 represent the results of a preliminary study made using chloroplasts isolated from spinach, grown under greenhouse conditions. These results indicate a high degree of intactness of the chloroplast envelope in the spinach preparation in that the rate of oxygen evolution obtained (82 μmol/mg chl/h) compares favourably with data presented by Cockburn et al (1968). During the first hour or so of storage on ice it was found that, in agreement with Cockburn et al (1968), the chloroplasts retained their capacity to evolve oxygen but subsequent to this progressive loss of activity was observed. The addition of metoxuron (final concentration in assay medium 3.3×10^{-7}M) to the assay medium, where indicated, caused an immediate and complete inhibition of oxygen evolution. Since the concentration of metoxuron employed in the present study was analogous to that found in earlier in vitro

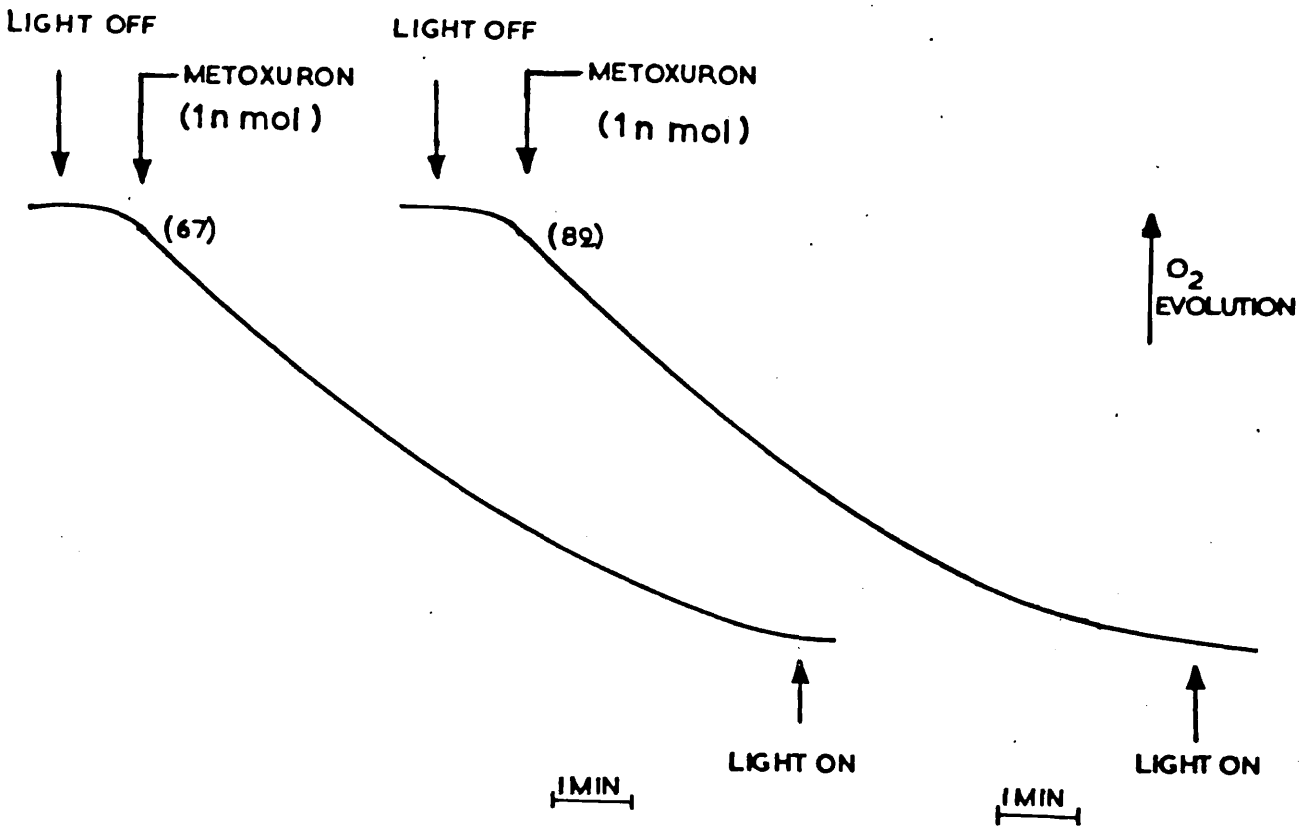


Fig. 27: Polarograph traces of O_2 evolution by spinach chloroplasts

Chloroplasts were isolated by Method A described on p. 92. Oxygen evolution was measured in the absence of an added Hill reagent using a Clark-type Oxygen electrode as previously described in the "Methods" section (p. 102).

Each reaction mixture contained: sorbitol, 100 µmol; $MgCl_2$, 3 µmol; $MnCl_2$, 3 µmol; EDTA, 6 µmol; $NaHCO_3$, 30 µmol and Hepes buffer, 150 µmol, adjusted to pH 7.6. The chlorophyll concentration in a total volume of 3.0 ml was 60-100 γ. Where indicated, metoxuron was added as 10 µl of a stock solution of the technical herbicide in methanol.

Numbers in parenthesis represent rates of O_2 evolution expressed as µmol/mg chl/h.

studies using broken chloroplasts to cause extensive inhibition of DCPIP and potassium ferricyanide photoreductions (Tables 2 and 3), one might infer from this result that the spinach chloroplast envelope poses no particular problem for the entry of the metoxuron molecule.

Subsequent attempts to isolate intact chloroplasts from metoxuron-resistant and susceptible wheat plants achieved very little success in that the rates of oxygen evolution obtained were very low. A degree of success was obtained, however, using an isolation procedure adapted from Miyachi & Hogetsu (1970) involving the use of Hepes in place of the tetrasodium pyrophosphate buffer system in the homogenising medium. Chloroplasts were isolated from seven day old wheat seedlings in which the second leaf was just beginning to emerge. Figs. 28 and 29 present data obtained for chloroplasts isolated from both metoxuron-resistant and susceptible varieties respectively. These results show that rates of oxygen evolution obtained for wheat chloroplasts were considerably lower than those presented in Fig. 27 for spinach. This presumably is a reflection of the greater difficulty involved in the preparation of intact chloroplasts from wheat as a result of the increased fibre content of the leaf tissue, though species differences in capacity for photosynthesis clearly do exist. As was the case with spinach chloroplasts, addition of metoxuron (final concentration 3.3×10^{-7}M) to the assay solutions at the points indicated caused an immediate and complete inhibition of oxygen evolution mediated by chloroplasts from both resistant and susceptible varieties (Figs. 28 and 29). These results would thus seem to indicate that the metoxuron molecule can readily penetrate the

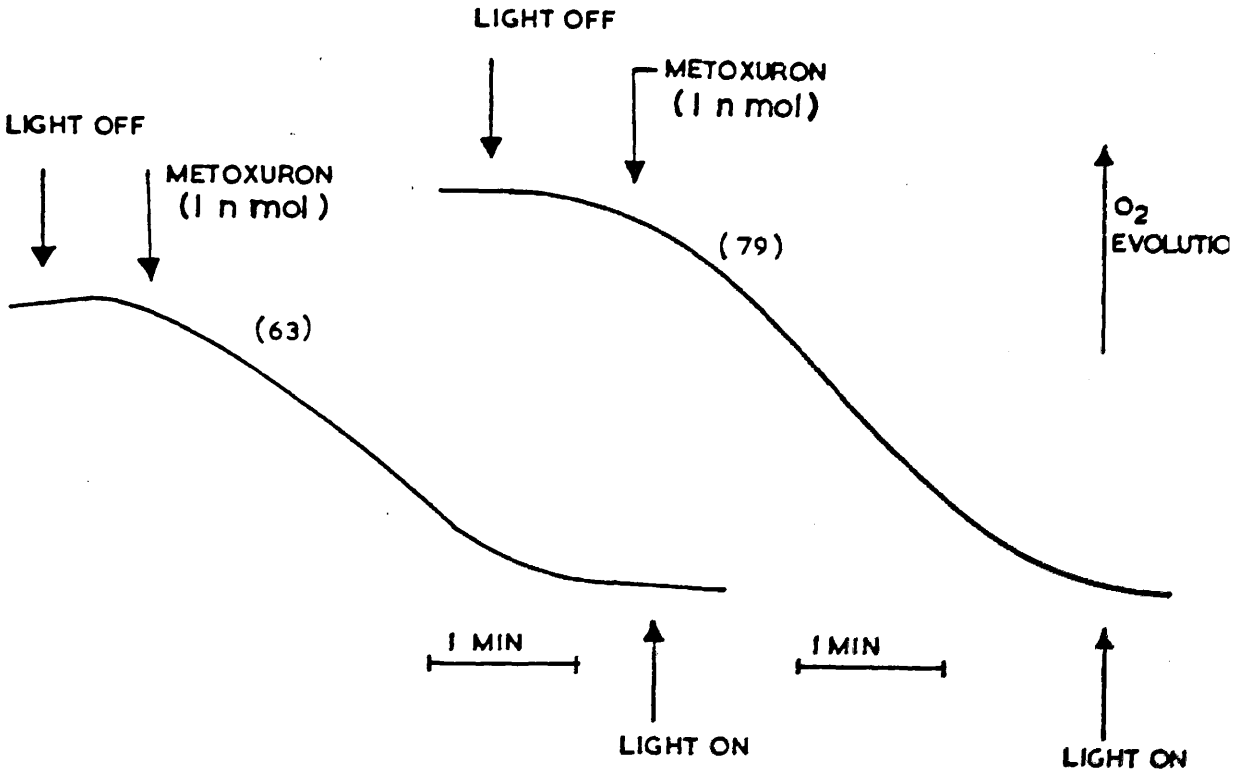


Fig. 28: Polarograph traces of O_2 evolution by chloroplasts isolated from metoxuron-resistant (Cappelle) wheat

Chloroplasts were isolated by Method B described on page 93.

Other experimental details were as for Fig. 27.

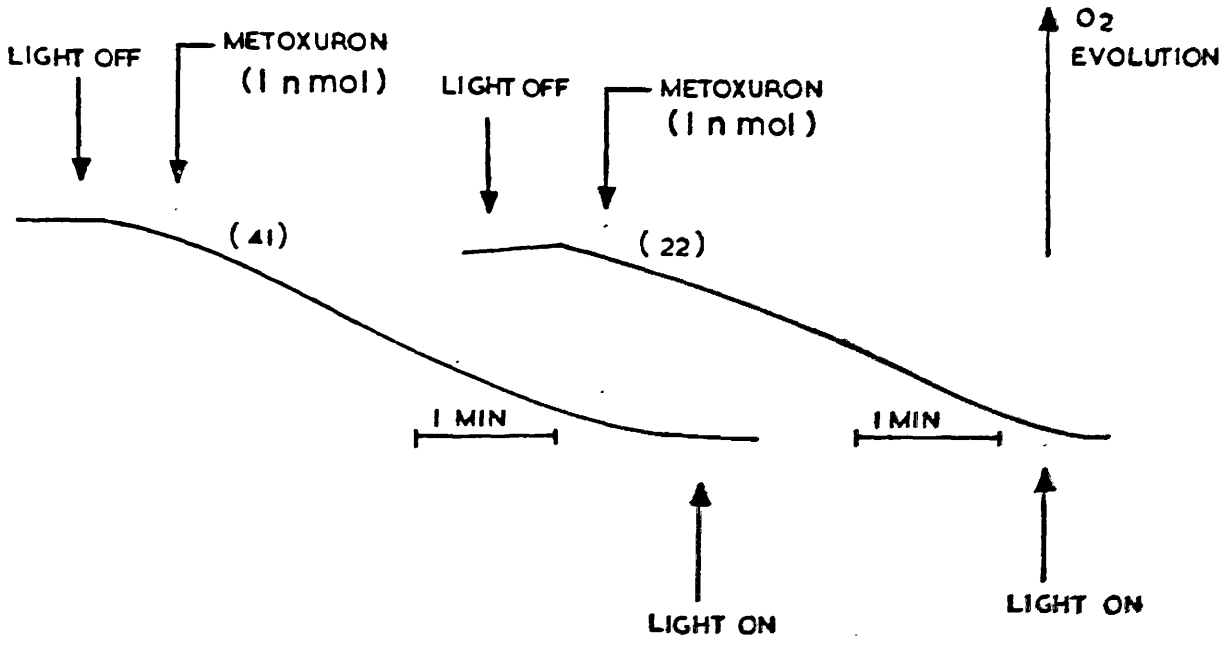


Fig. 29: Polarograph traces of O_2 evolution by chloroplasts isolated from metoxuron-susceptible (Maris Nimrod) wheat

Chloroplasts were isolated by Method B described on page 93.

Other experimental details were as for Fig. 27.

chloroplast membrane of chloroplasts of both varieties studied so that impeded access to the site(s) of inhibition in the chloroplast is therefore unlikely to represent a factor contributing to metoxuron resistance. During the present investigation it was noted that the rates of oxygen evolution mediated by chloroplasts isolated from the susceptible variety, Maris Nimrod, were consistently lower than those obtained using chloroplasts from resistant Cappelle, whereas in all previous studies rates of photosynthetic activities of chloroplasts isolated from both varieties were generally comparable. The reason for this discrepancy remains unknown.

A more extensive study of uptake and translocation of metoxuron by resistant and susceptible wheat varieties was facilitated by the synthesis of a sample of (methoxy-^{14}C) metoxuron (sp. activity 4 mCi/mmol) by the procedures outlined previously (p. 122). For the present study plants of resistant and susceptible varieties were grown in nutrient culture under greenhouse conditions (p. 87). At the three to four leaf stage the seedlings were transferred to a fresh culture medium supplemented with 2 μCi of (methoxy-^{14}C)metoxuron (concentration in nutrient medium 2.8 ppm), samples being removed and prepared for autoradiography at various time intervals as described previously (p. 124). Fig. 30 represents a photographic record of the radioautograms obtained for both resistant and susceptible plants some 12 h after transference to the culture medium containing ^{14}C-labelled herbicide. These radioautograms clearly indicate that considerable radioactivity is absorbed by the root systems of plants of both varieties during the course of the experiment, and that subsequent translocation to aerial plant parts

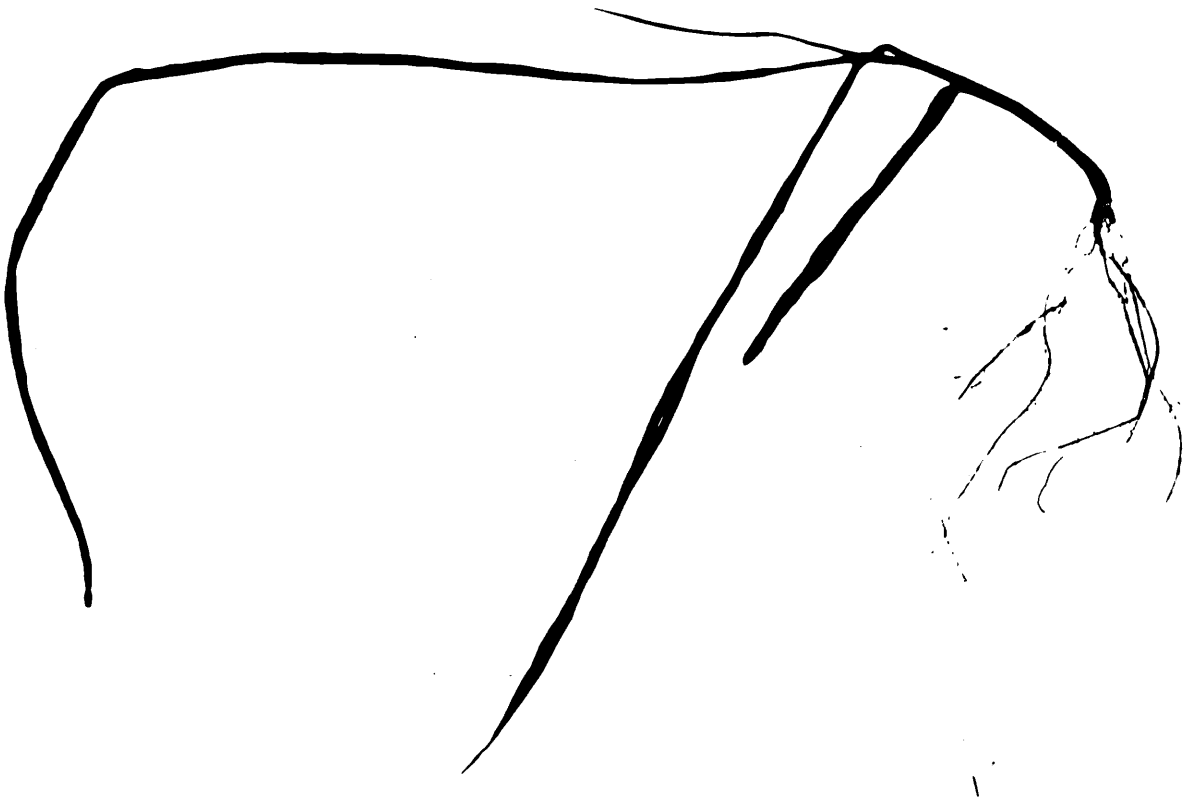


Fig. 30: Radioautographs of metoxuron-resistant and susceptible wheat plants 12 hours after transfer to nutrient culture containing 2 µCi (methoxy-^{14}C)metoxuron

The procedures employed in the preparation of the radioautographs were described previously in the "Methods" section (p. 132).

Left : susceptible (Maris Nimrod) wheat

Right : resistant (Cappelle) wheat

readily occurs. Fig. 31 presents the results of a second experiment made using plants some six weeks old, in the early stages of tillering, which had contacted the radioactive herbicide for 48 h. Radioautographs prepared for plants treated for longer than 48 h are not presented since these merely showed even greater darkening of the film adjacent to the plant material. Although under present conditions radioautography cannot be regarded as a particularly quantitative technique, visual assessment of the radioautograms recorded in Figs. 30 and 31 suggested that plants of the metoxuron-susceptible variety accumulated more radioactivity than similarly treated resistant plants. This is not apparent, however, in the photographic records which show little or no distinction between wheat varieties with respect to uptake of ^{14}C-labelled metoxuron. The uptake and distribution pattern for metoxuron obtained in the present study with wheat is very similar to data obtained in previous studies (Bayer & Yamaguchi, 1965; Kuratle et al, 1968; Neptune, 1970) made with other substituted phenylurea herbicides in a variety of plant species.

Though not presented in the present thesis, radioautographs prepared following the application of ^{14}C-labelled metoxuron to the leaf surface of wheat plants indicated a slight basipetal translocation of metoxuron (or its metabolites) in both resistant and susceptible varieties, thus lending support to the data presented earlier on the effect of treatment of a single leaf with metoxuron on the Hill activity of chloroplasts isolated from leaves that had not contacted the herbicide. The basipetally translocated activity, however, accounted for only a very small amount of the total radioactivity absorbed. Though it has been reported (Crafts, 1959) that the substituted phenylureas accumulate only in the apices of

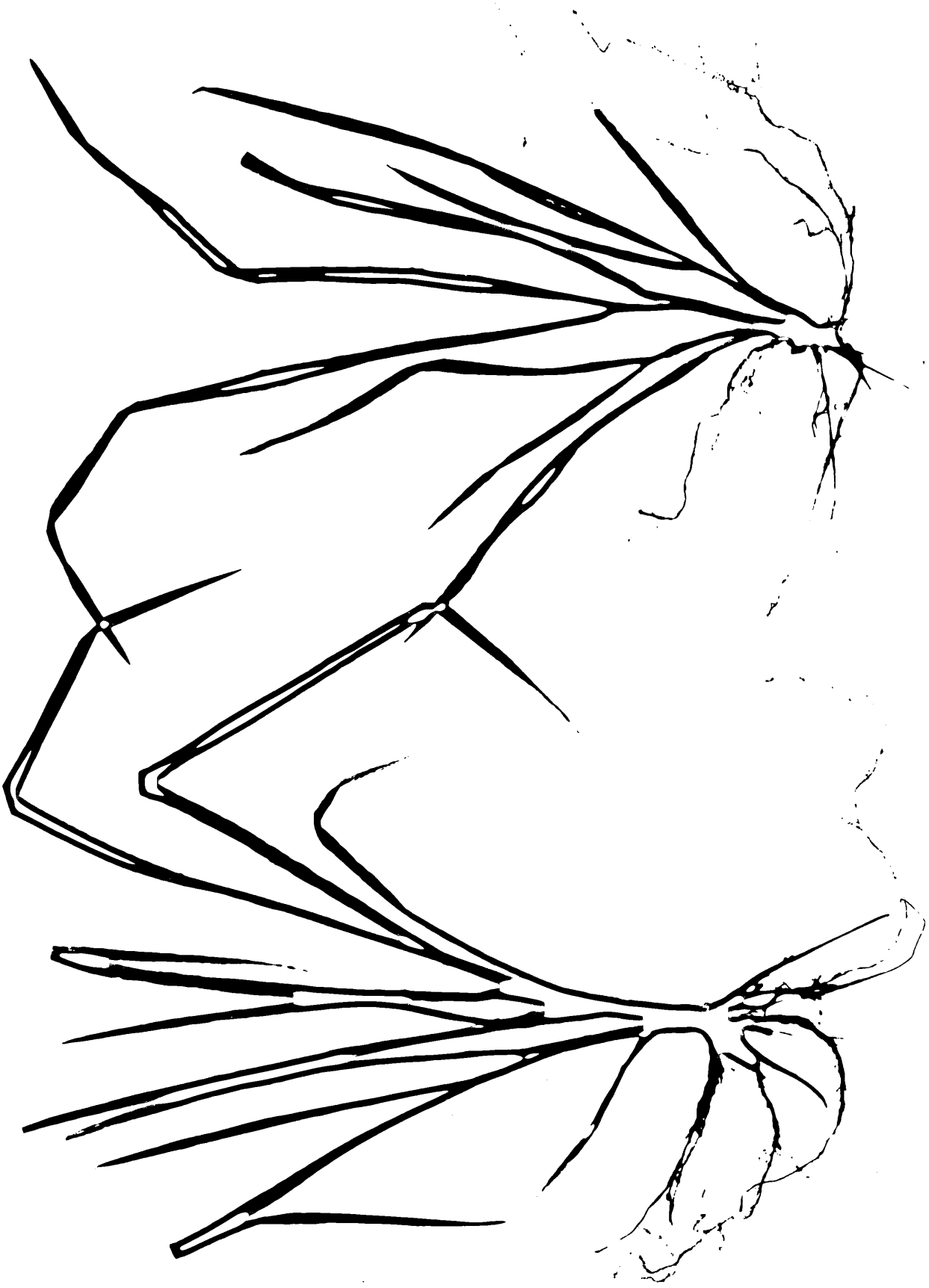


Fig. 31: Radioautographs of metoxuron-resistant and susceptible wheat plants 48 hours after transfer to nutrient culture containing 2 µCi (methoxy-^{14}C)metoxuron

The procedures employed in the preparation of the radioautographs were described previously in the "Methods" section (p. 132).

Left : resistant (Cappelle) wheat

Right : susceptible (Maris Nimrod) wheat

the leaves with no basipetal movement, Fang et al (1955), in agreement with the present study, recorded a slight basipetal translocation following treatment of bean leaves with ^{14}C-labelled monuron.

In order to obtain a quantitative assessment of the movement of (methoxy-^{14}C)metoxuron absorbed from the nutrient medium by plants of both varieties, samples were removed for analysis of total radioactivity after various times of contact with the herbicide. Following removal of the plants from the culture medium the roots were rinsed with 80% methanol to remove radioactive herbicide adhering to their surface prior to exhaustive extraction with methanol. The methanolic extracts were then concentrated and the amount of radioactivity present was determined by the liquid scintillation technique. Results obtained for resistant and susceptible wheats, expressed in d.p.m./g fresh weight, are compared in Fig. 32. These data indicate a progressive absorption of (methoxy-^{14}C)-metoxuron by both cultivars throughout the 5 days over which the experiment was conducted. However, uptake of radioactivity is considerably greater in the case of the susceptible variety, Maris Nimrod, amounting to some twice that achieved by the resistant strain after 5 days. A similar result was obtained in the course of a related study concerning the distribution of the absorbed radioactivity from ^{14}C-labelled metoxuron between various subcellular organelle fractions obtained from leaves at various times after contacting the herbicide. Though the presentation of these latter results is reserved for the next section, Fig. 33 is presented here since it compares the sums of the total radioactivity measured in all organelle fractions obtained for resistant and susceptible varieties. The distinction between resistant and susceptible varieties is even more apparent in these results in that after some 5 days of

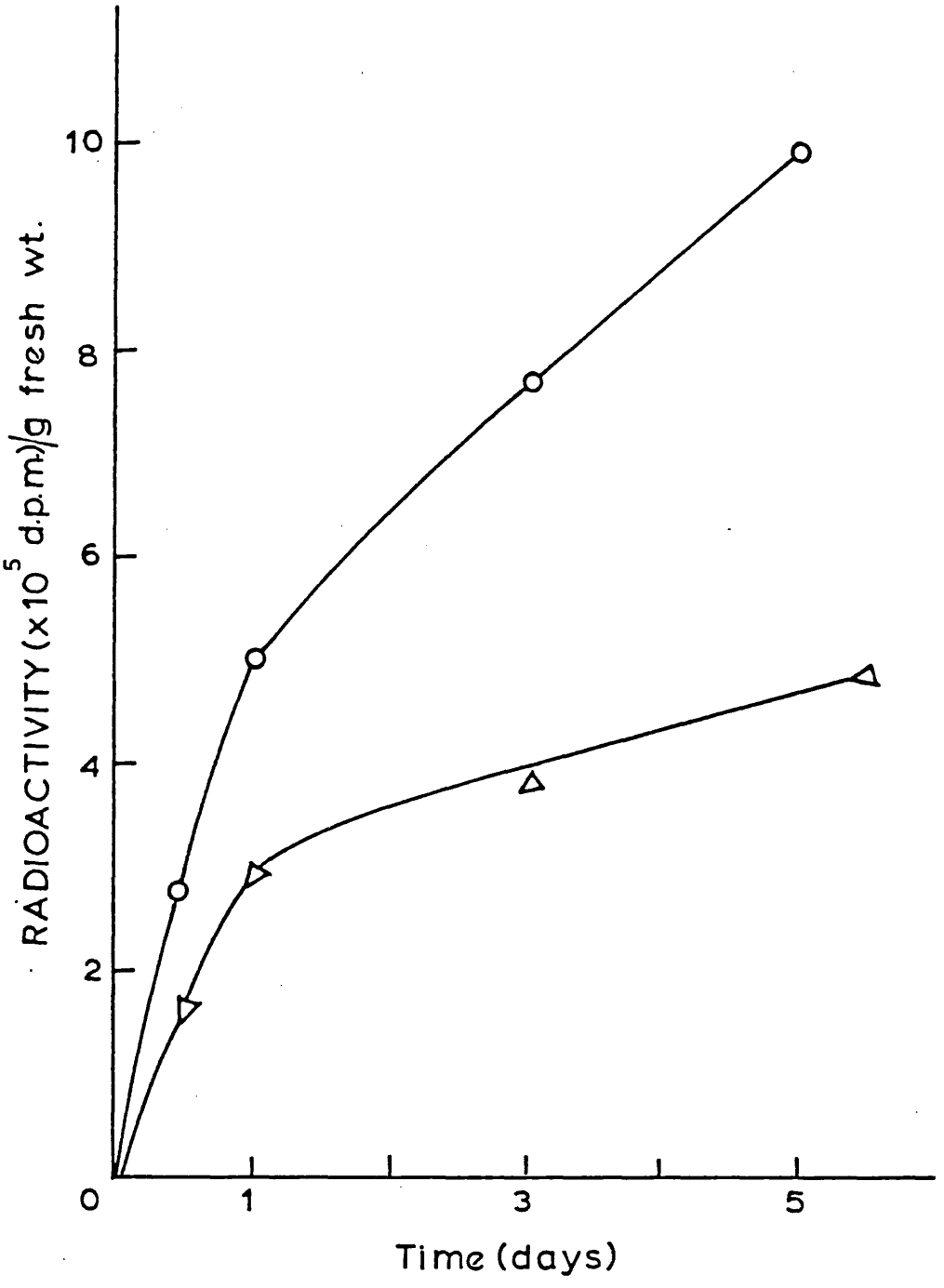


Fig. 32: Absorption of (methoxy-^{14}C)metoxuron by resistant and susceptible wheat plants

Plants were grown in nutrient culture and transferred to a fresh medium supplemented with radioactive metoxuron (2 µCi, sp. activity 4 µCi/µmol) at the three leaf stage.

The values plotted are for total radioactivity present in methanol extracts of whole plant tissue at various times after contacting the herbicide.

- O - O - metoxuron-susceptible (Maris Nimrod) wheat

- Δ - Δ - metoxuron-resistant (Cappelle) wheat

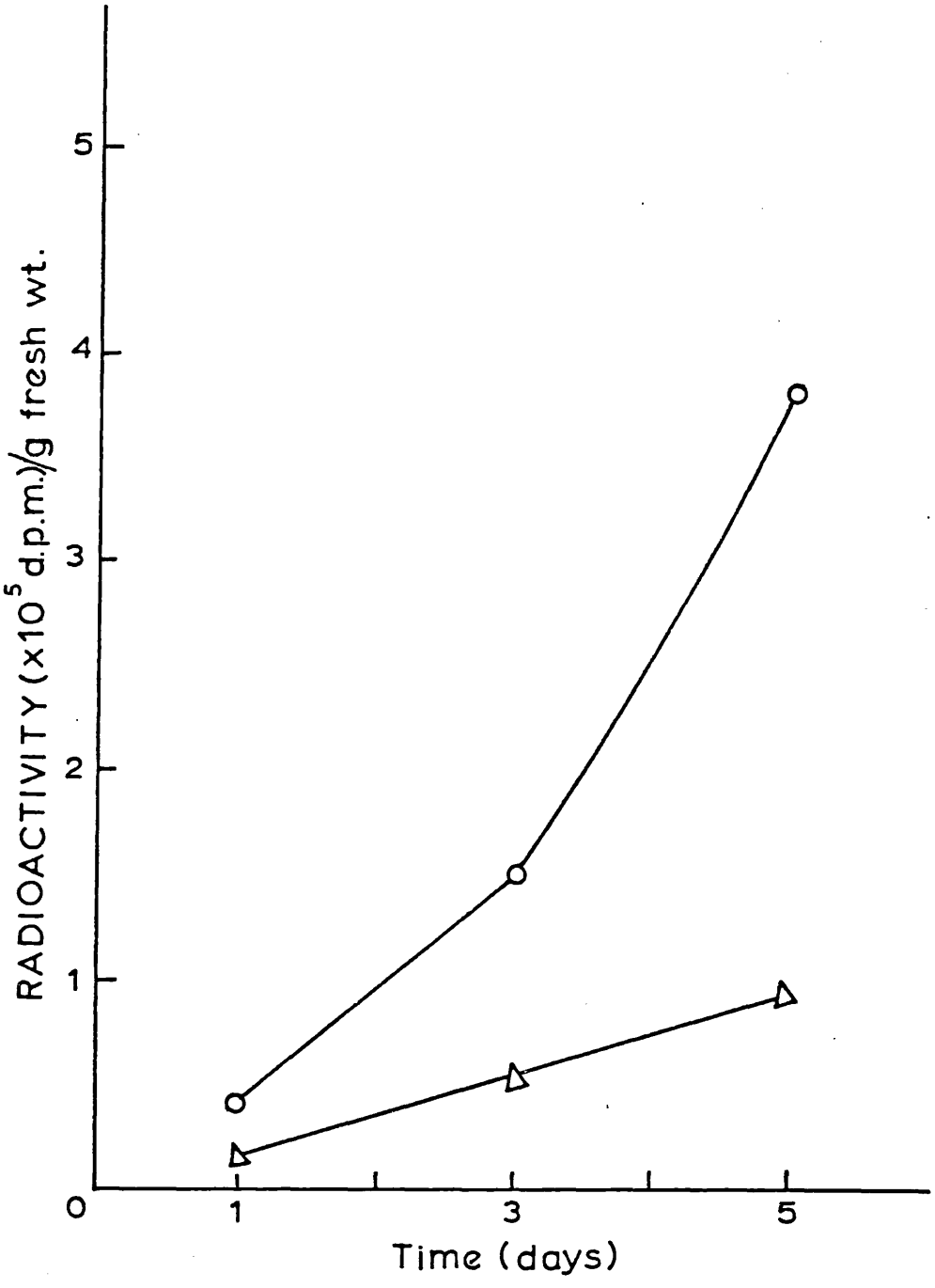


Fig. 33: Absorption of (methoxy-^{14}C)metoxuron into leaf tissue of resistant and susceptible wheat plants

The values plotted are of total radioactivity associated with all sub-cellular organelle and final supernatant fractions prepared from leaves at the times indicated.

Other experimental details as for Fig. 32.

- O - O - metoxuron-susceptible (Maris Nimrod) wheat

- Δ - Δ - metoxuron-resistant (Cappelle) wheat

treatment of the roots with (methoxy-^{14}C)metoxuron there is a 4-5 fold greater uptake of radioactivity into the leaves of susceptible compared to resistant wheat. Comparison of the data presented in Figs. 32 and 33 obtained under similar conditions for growth and treatment of the plants, would suggest that not only is the uptake of ^{14}C-labelled herbicide more extensive in the case of the susceptible cultivar but that the subsequent accumulation into the leaves may also be more rapid.

Previous studies have shown that differences in susceptibility of several crop plants to monuron and diuron appear to be related to differential absorption (Smith & Sheets, 1967). Similar conclusions were arrived at by Kuratle et al (1968) in a study of the selective action of linuron on ragweed (susceptible) and carrot (resistant) and by Neptune (1970) following a study of the selective action of fluometuron on maize (resistant) and wheat (susceptible). In the present study, for metoxuron to be effective it must arrive at the active site(s) within the plant. Since substituted phenylureas are potent inhibitors of the Hill reaction of photosynthesis, the herbicide must be absorbed by the roots and translocated to the leaves. The association of different concentrations of chloroxuron, for example, with chloroplasts has been previously shown to have a direct bearing on the selective action of the herbicide towards soybean and tall morning glory (Feeny et al, 1974). Thus the accumulation of an approximately 4-5 fold greater amount of metoxuron in the leaves of susceptible compared to resistant wheat demonstrated under the experimental conditions employed in the present studies would appear to be a very significant observation and may be responsible, at least in part, for the different reactions of the two wheat varieties to metoxuron.

Sub-cellular distribution of ^{14}C-labelled metoxuron

Having demonstrated that a distinction exists between resistant and susceptible wheat plants with respect to uptake of the radioactive herbicide, it was realised that such a result was only significant in the present context if it could be shown to be reflected in similar differences between the varieties with respect to the amounts of radioactive metoxuron associated with various sub-cellular organelle fractions, particularly the chloroplasts. In the present study therefore an attempt was made to obtain a quantitative measure of the amount of radioactivity associated with several organelle fractions following the uptake of ^{14}C-labelled metoxuron from the nutrient medium. For this purpose five plants of each wheat variety were treated with (methoxy-^{14}C)-metoxuron (2 μCi) at the four-leaf stage some four days prior to harvesting. In the isolation of organelle fractions, 3 g fresh weight of leaf tissue from such plants were homogenised and the resulting brei was passed through two layers of cheesecloth. The various organelle fractions were obtained by subjecting the filtrate to differential centrifugation at 200 X g, 1,500 X g, 9,000 X g and, finally, 100,000 X g to sediment nuclei and cell walls, chloroplasts, mitochondria and microsomes respectively, as previously described (p. 125). After harvesting each fraction was washed with buffer and re-sedimented in an attempt to remove any contaminating particles prior to determination of radioactivity and protein content. Radioactivity was measured by the liquid scintillation technique following solubilisation of the organelle fraction with NCS solubiliser (p. 132).

The results of a preliminary organelle characterisation experiment for wheat are shown in Tables 13 and 14, and are based on assays of the marker enzymes succinate:DCPIP reductase and NADPH:DCPIP reductase for mitochondria and microsomes respectively, (see p. 127). These data

TABLE 13: Succinate-DCPIP reductase activity of various sub-cellular fractions prepared from wheat leaves

Fractions	Succinate-DCPIP reductase activity	
	Total Activity (International units)	Specific Activity (μmol/min/mg protein)
200 X g supernatant	1210	0.11
9000 X g pellet.	780	0.65
100,000 X g pellet	120	0.095

Reactions were carried out in 1 cm light path cuvettes as previously described (p. 127). Each reaction mixture contained, in a final volume of 3.0 ml, 300 μmol potassium phosphate buffer, pH 7.4; 1 μmol EDTA; 180 μmol Na succinate; 0.16 μmol DCPIP; 1 μmol KCN and 0.05 ml of organelle fraction containing approximately 0.03 mg of protein.

would suggest that the pellets obtained by centrifugation at 9,000 X g and 100,000 X g consist predominantly of mitochondria and microsomes respectively, with little cross contamination. A high NADPH:DCPIP reductase activity is also associated with the 100,000 X g supernatant fraction, indicating the presence of "light" microsomal particles.

TABLE 14: NADPH-DCPIP reductase activity of various sub-cellular fractions prepared from wheat leaves

Fraction	NADPH-DCPIP reductase activity	
	Total Activity (International units)	Specific Activity (μmol/min/mg protein)
300 X g supernatant	3590	0.32
1,500 X g pellet	230	0.19
9,000 X g pellet	160	0.14
9,000 X g supernatant	3430	0.37
100,000 X g pellet	1410	1.16
100,000 X g supernatant	1760	0.23

The reaction mixture contained, in a final volume of 1.0 ml, 300 μmol potassium phosphate buffer pH 7.7; 0.1 μmol NADPH; 0.1 μmol DCPIP and a volume of a particular organelle fraction equivalent to approx. 0.03 mg protein.

The concentration of chloroplasts in the pellet obtained by centrifugation at 1,500 X g was apparent from the intense green colour of this fraction.

The distribution of radioactivity amongst organelle fractions prepared from metoxuron-resistant wheat is compared to that obtained for susceptible plants in Table 15. From these results also it is immediately apparent that the susceptible cultivar absorbs far greater

TABLE 15: Sub-cellular distribution of ^{14}C-labelled metoxuron in resistant and susceptible wheat plants

Fraction	Total radioactivty (d.p.m./fraction)		Specific activity (d.p.m./mg protein)	
	Cappelle (R)	Maris Nimrod (S)	Cappelle (R)	Maris Nimrod (S)
300 X g pellet	14320	50,060	1288	4908
1500 X g pellet	4450	17990	3685	10620
9000 X g pellet	3800	5450	4330	6284
100,000 X g pellet	1800	3080	2620	3888
100,000 X g supernatant	58500	432450	2321	12813

Radioactivity in each organelle fraction was determined using the scintillation technique described on p. 132. Protein content was assayed by the method of Folin & Lowry (p. 97).

amounts of radioactive metoxuron than does the resistant strain. When the data are expressed as d.p.m./mg protein for each fraction it can be seen that the largest differences between the two varieties occur in the chloroplast, nuclear and cell wall and 100,000 X g supernatant fractions, though smaller differences also exist between the respective mitochondrial and microsomal fractions. The two to three-fold greater concentration of metoxuron associated with the chloroplast fraction of susceptible compared to resistant wheat is, however, of particular significance in the present investigation in

view of the fact that the mode of action is through an inhibition of photosynthesis (Büchel, 1972; Leeuwen & Oorshot, 1976). Consequently, it is concluded from the present study that the observed differences in uptake and subsequent distribution of metoxuron would appear sufficiently significant so as to offer at least a partial explanation for the observed varietal susceptibility in winter wheat to the herbicide.

Considerable differences in uptake of metoxuron into roots and leaves of some six winter wheat cultivars were also observed in a recent study by Müller & Sanad (1975). In contrast to the results presented in the present study, however, no clear correlation was found to exist between reaction to metoxuron and degree of absorption of the herbicide when applied to the root system. Of particular interest is the result obtained for the variety, Hanno, designated resistant in field trial experiments, which was found to absorb the greatest quantity of metoxuron into its leaf system. Though the data presented by Müller & Sanad (1975) are not directly comparable with the results obtained in the present study in that the herbicide was applied to the soil rather than via nutrient culture so that factors of a non-physiological nature may also be involved, they do suggest that a degree of caution should be used in interpreting data from such a study involving only single examples of resistant and susceptible wheat cultivars.

Results presented earlier on the effect of metoxuron on the Hill activity of chloroplasts isolated from treated plants, in which chloroplasts from the resistant variety showed a marked recovery from

inhibition at longer times after treatment, suggested that the resistant variety in particular possessed an ability to detoxify the absorbed herbicide to form less or non toxic products. On the basis of these observations a sample of ^{14}C-labelled metoxuron was synthesised (p. 122) so that a more detailed investigation of the metabolic fate of metoxuron in wheat could be inhibited. The results of these studies are presented in the final section.

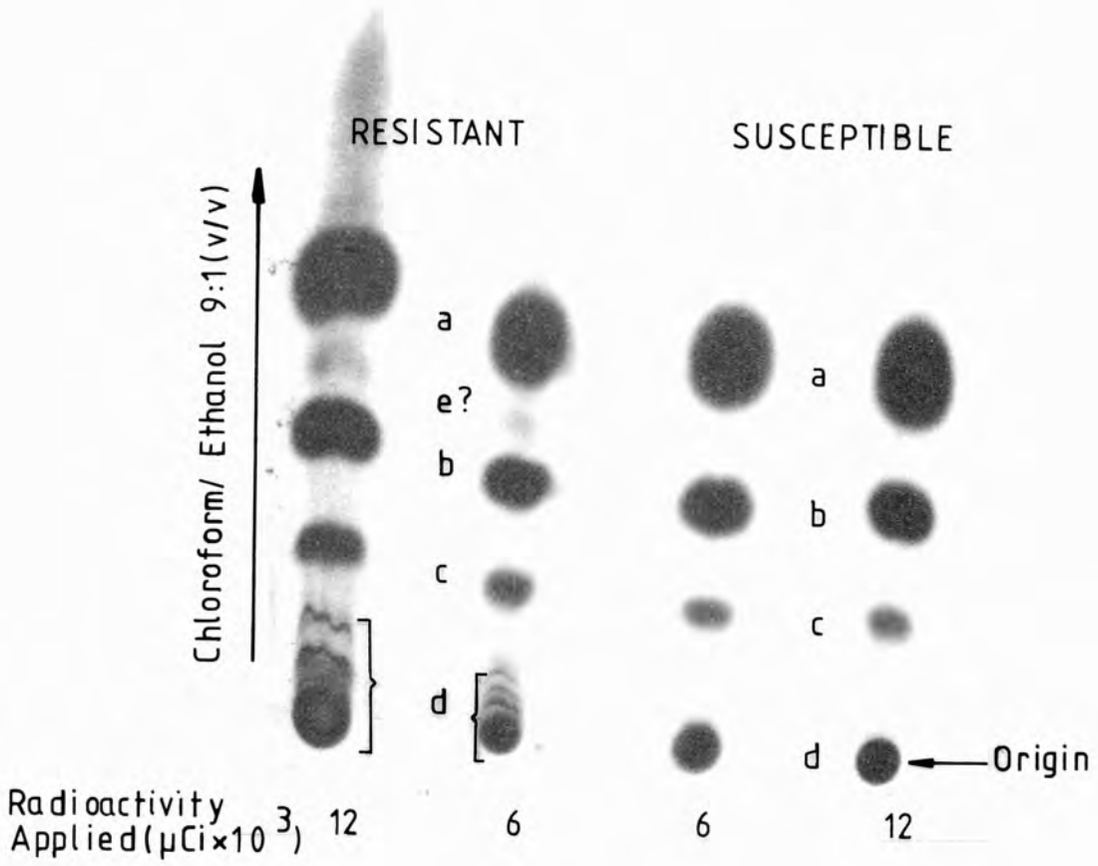
Metabolic fate of metoxuron in wheat cultivars

This study utilised plants of both resistant and susceptible wheat cultivars grown in nutrient culture in a growth cabinet under the environmental conditions previously described (p. 87). For metabolism studies plants at the three to four leaf stage were transferred to fresh nutrient solution to which was added 1 μCi of (methoxy-^{14}C)metoxuron (final concentration in the nutrient solution, 1.4 p.p.m.). In an initial experiment plants were subsequently left with their roots in contact with the ^{14}C-labelled herbicide for three days prior to the exhaustive extraction of metoxuron and its metabolites with 80% methanol. After concentration of the methanolic extracts the metabolites were separated from the parent herbicide by thin layer chromatography and were subsequently located by radioautography (p. 132 & 133). A photographic record of the radioautograms obtained for resistant and susceptible wheat plants is presented in Fig. 34, and represents the chromatographic patterns obtained for the same quantities of radioactivity applied to the chromatographic plates. The higher amounts of radioactivity were applied in an attempt to ensure that more minor metabolites did not escape detection. These results show that metoxuron is degraded to several metabolites by

Fig. 34: Radioautograph of Thin Layer Chromatograms of 80% methanol extracts of wheat plants treated with (methoxy-^{14}C)metoxuron for 3 days

Plants of resistant (Cappelle) and susceptible (Maris Nimrod) wheat were transferred to a culture medium supplemented with (methoxy-^{14}C)-metoxuron (1 µCi, sp. act. 4 µCi/µmol) at the three-leaf stage. The extraction and chromatographic/radioautographic techniques were as described previously in the "Methods" (pages 132-133).

The chromatograms were developed using the chloroform:ethanol (9:1 v/v) solvent system.


RESISTANT
SUSCEPTIBLE
Chloroform/ Ethanol 9:1(v/v)
a
e?
b
c
d
a
b
c
d
Origin
Radioactivity Applied(μCi×10 3) 12
6
6
12

plants of both resistant (Cappelle) and susceptible (Maris Nimrod) wheat. The radioactive zone designated (a) in Fig. 34 was identified as the unchanged parent herbicide by chromatographing ^{14}C-labelled metoxuron along an adjacent zone. The R_f values of the major unidentified metabolites, designated (b) and (c), were the same in both varieties and thus probably represent the same compounds. Identification of the major metabolites (b) and (c) was subsequently attempted using as a basis the N-demethylation pathway established by other workers for several substituted phenylureas in various species (e.g., Geissbühler et al, 1963b; Rogers & Funderburk, 1968; Lee & Fang, 1973; Frear & Swanson, 1974). If such a pathway similarly existed in wheat then the metabolites present in the methanolic extracts could be expected to include N'-(3-chloro-4-methoxyphenyl)-N-methylurea, 3-chloro-4-methoxyaniline and N'-(3-chloro-4-methoxyphenyl) urea. Samples of the former two compounds were obtained as a generous gift from Dr. G.G. Briggs of the Rothamsted Experimental Station, whereas the N'-(3-chloro-4-methoxyphenyl)urea was synthesised by the procedure described on p. 134. The standard compounds were dissolved in methanol and applied both as single spots and as a mixture to TLC plates to which were also applied samples of the radioactive extracts from both resistant and susceptible wheat plants. The chromatograms were developed by irrigation with the chloroform-ethanol (9:1 v/v) solvent system after which the authentic standards were visualised by irradiating the plates with ultra-violet light prior to the location of the unknown radioactive metabolites by autoradiography. For comparison, some chromatograms were developed with the benzene:acetone (2:1 v/v) solvent system of Geissbühler et al,

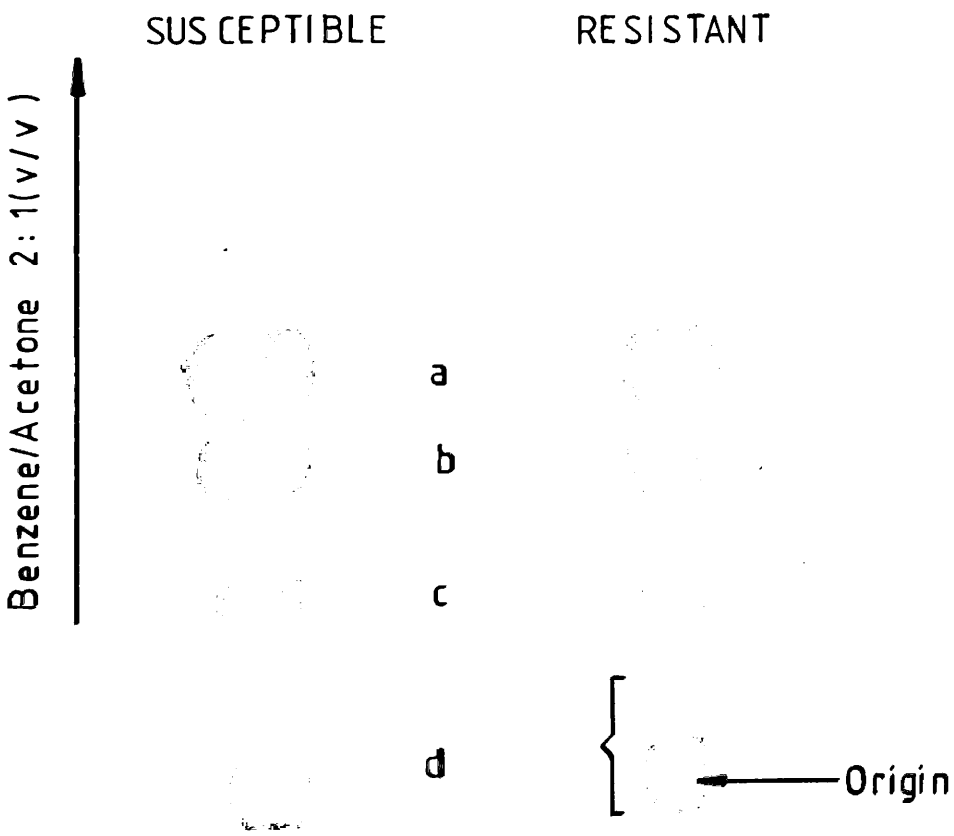

SUSCEPTIBLE
RESISTANT
Benzene/Acetone 2:1(v/v)
a
b
c
d
Origin

Fig. 35: Radioautograph of Thin Layer Chromatograms of 80% methanol extracts of wheat plants treated with (methoxy-^{14}C)metoxuron for 3 days

Experimental details as for Fig. 34 except that the benzene:acetone (2:1 v/v) solvent system was used for irrigation of the chromatograms.

A volume of 80% methanol extract equivalent to 15×10^{-3} µCi radioactivity was applied at the origin of each zone.

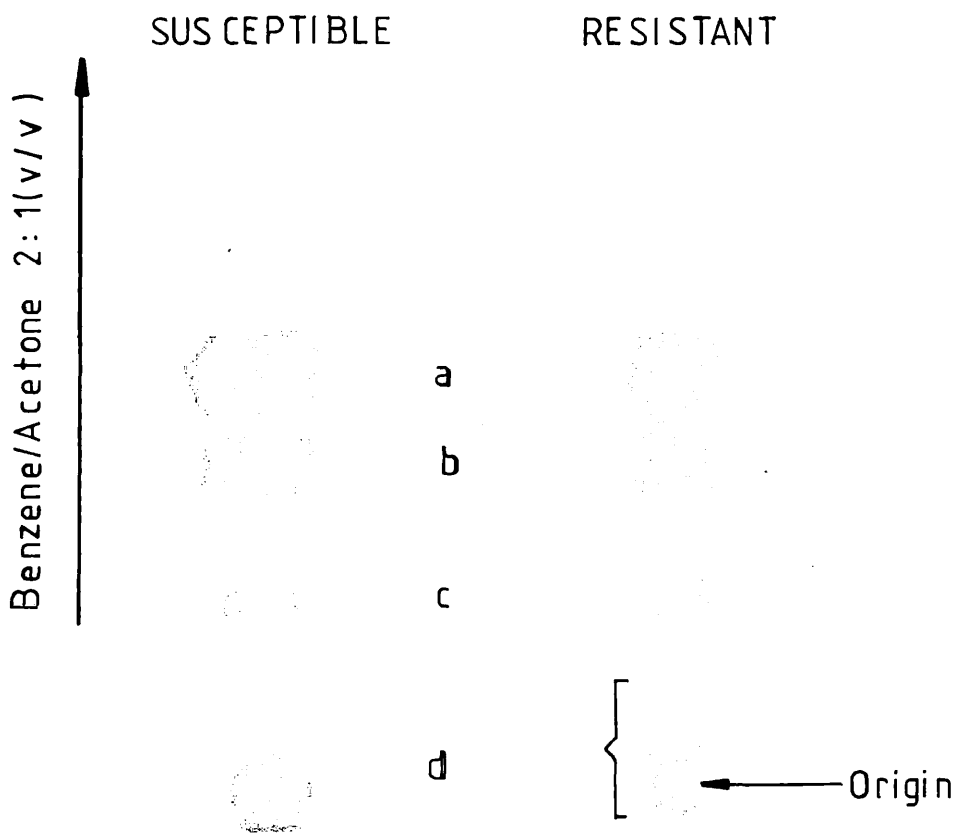
Key to radioactive metabolites :

a = metoxuron

b = N-monodesmethylated metoxuron

c = N-bisdesmethylated metoxuron

d = conjugates ?


SUSCEPTIBLE
RESISTANT
Benzene/Acetone 2:1(v/v)
a
b
c
d
Origin

(1963b) and Smith & Sheets (1967). Table 16 compares the Rf values obtained in the two solvent systems employed for the authentic standards with those for the major metabolites present in the radioactive zones, and represent average values obtained for some ten different experiments. These results show that very good agreement exists between the Rf values obtained for metoxuron and its metabolites and those for the various standards in both solvent systems, and that the metabolites designated (b) and (c) in Fig. 34 most probably correspond to N'-(3-chloro-4-methoxyphenyl)-N-methylurea and N'-(3-chloro-4-methoxyphenyl)urea respectively, confirming the existence of an N-demethylation pathway in wheat. Examination of the Rf values obtained in the benzene:acetone solvent system reveals that the values for the parent herbicide and the monodesmethylated metabolite are very close, these values being 0.4 and 0.3 respectively. The poorer separation obtained between these two compounds in this latter solvent system is apparent also in the radio-autogram presented in Fig. 35 and consequently throughout the remainder of the present investigation the chloroform:ethanol solvent system was routinely employed. Examination of the results presented in Fig. 34 reveals that although the separation patterns obtained for both metoxuron-resistant and susceptible varieties are very similar, a notable difference is the presence of significantly more polar material at or near the origin in the case of the former variety. Furthermore, the chromatograms prepared for extracts of resistant plants show the presence of an additional though apparently very minor metabolite, labelled (e), with an Rf value intermediate between that of metoxuron and its N-mono-desmethylated derivative. This latter material was not, however, present in all radioautograms examined in the present investigation and its

TABLE 16: Comparison of Rf values obtained for metoxuron and several of its metabolites

Compound	Authentic standards		^{14}C-labelled compounds present in plant extracts			
			Cappelle (R)		Maris Nimrod (S)	
	Benzene: Acetone 2:1 (v/v)	Chloroform: Ethanol 9:1 (v/v)	Benzene: Acetone 2:1 (v/v)	Chloroform: Ethanol 9:1 (v/v)	Benzene: Acetone 2:1 (v/v)	Chloroform: Ethanol 9:1 (v/v)
Metoxuron	0.40	0.70	0.39	0.70	0.39	0.70
N'-(3-chloro-4-methoxyphenyl)-N-methylurea	0.30	0.56	0.30	0.58	0.31	0.58
N'-(3-chloro-4-methoxyphenyl)-urea	0.17	0.38	0.18	0.40	0.18	0.40
3-chloro-4-methoxyaniline	0.54	0.77	0.55	0.77	0.55	0.77

The Rf values given for metabolites present in plant extracts represent average values obtained in ten different experiments. Metabolites were separated by TLC on ready-prepared glass plates coated with a fluorescence indicator (p.133). Authentic standards were visualised by irradiation with U.V. light while the ^{14}C-labelled compounds present in plant extracts were located by autoradiography.

identity remains unknown. Reference to Table 16 indicates that this substance is most likely to be 3-chloro-4-methoxyaniline, a product of hydrolysis of the ureido group of metoxuron, which has a significantly greater Rf value than the parent herbicide and is therefore seemingly absent from the chromatograms depicted in Fig. 34. However, very small amounts of the aniline derivative were detectable in another experiment on chromatograms of extracts prepared from wheat plants that had contacted ^{14}C-labelled metoxuron for 4 days prior to analysis of radioactive metabolites (see metabolite (f), Fig. 36). These results again show the good separation of metoxuron and its metabolites achieved with the TLC system employing chloroform:ethanol as developing solvent. The radioactive zones were sufficiently compact and well separated to facilitate the ready recovery of the various metabolites from the chromatographic plates by removal of the appropriate areas of gel and eluting with methanol. To enable a quantitative comparison to be made between the extent of metoxuron metabolism in resistant and susceptible plants, the radioactivity present in each methanol eluate was determined using the scintillation counting technique. The results obtained are given in Table 17 and are expressed in d.p.m. per metabolite in the total plant extracts. These data again show a greater amount of radioactivity associated with the extract prepared from susceptible wheat. This difference is mainly attributable to the some two-fold higher level of metoxuron in the latter extracts since, with the exception of the polar material at or near the origin (designated (d) in Fig. 36), the radioactivity associated with the various metabolite fractions obtained in this particular experiment is very similar for both resistant and susceptible varieties. However, differences

Fig. 36: Radioautograph of Thin Layer Chromatograms of 80% methanol extracts of wheat plants treated with (methoxy-^{14}C)metoxuron for 4 days

Details of experimental procedures and metabolite identification were given in Figs. 34 and 35 respectively. The additional radioactive zone, (f), visible as a minor metabolite was provisionally identified as 3-chloro-4-methoxyaniline.

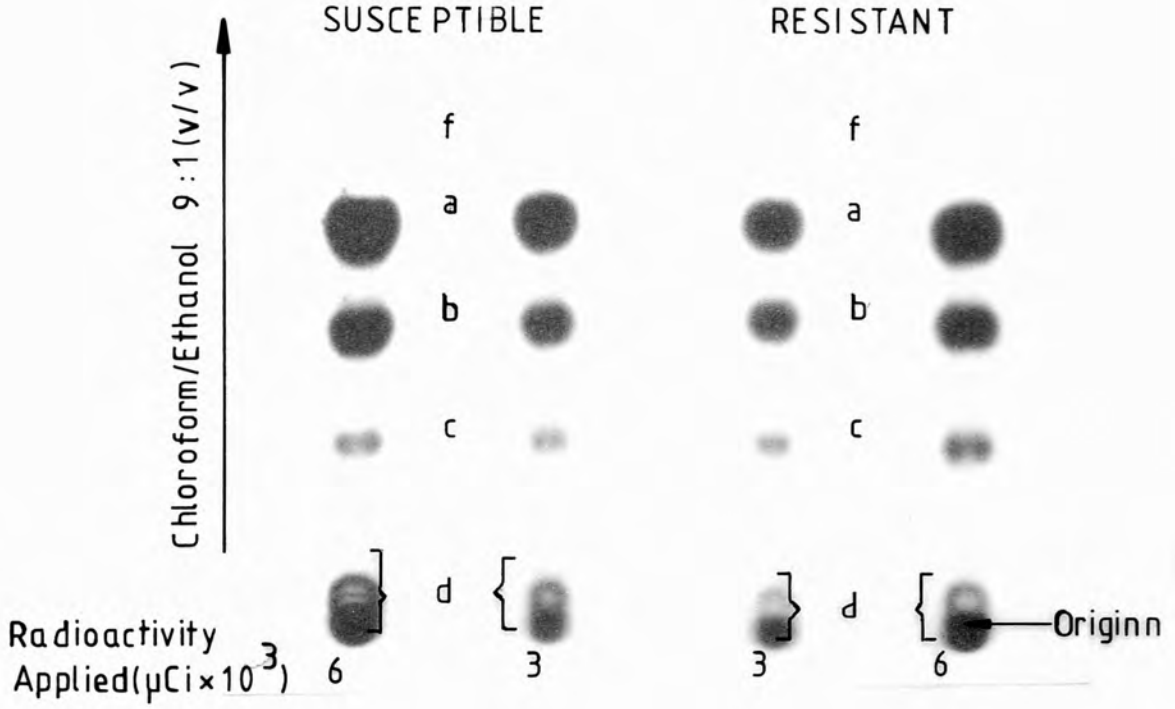

SUSCEPTIBLE
RESISTANT
Chloroform/Ethanol 9:1 (v/v)
f
a
b
c
d
Origin
Radioactivity Applied (μCi × 10⁻³)
6
3
3
6

TABLE 17: Distribution of radioactivity between metoxuron and its metabolites in methanolic extracts of resistant and susceptible wheat

Radioactive compound	Total radioactivity present (d.p.m.)		% distribution of radioactivity	
	Cappelle (R)	Maris Nimrod (S)	Cappelle (R)	Maris Nimrod (S)
Metoxuron	59,000	1 021 00	45.8	61.0
N'-(3-chloro-4-methoxyphenyl)-N-methylurea	41,000	44,000	31.8	26.5
N'-(3-chloro-4-methoxyphenyl)-urea	6,900	6,400	5.4	3.8
3-chloro-4-methoxyaniline	2,800	2,200	2.2	1.3
Origin*	19,000	12,400	14.8	7.4

After development of the chromatograms using the chloroform: ethanol (9:1 v/v) solvent system the radioactive compounds were eluted from the TLC plates with methanol and a quantitative assessment of the amount of radioactivity associated with each metabolite was obtained using the scintillation technique (p. 132).

*In the present context the term "origin" incorporates the polar metabolites of very low Rf adjacent to the origin.

do become apparent when the results are expressed as percentage distribution of radioactivity between metoxuron and its metabolites. Therefore some 4 days after treatment metoxuron constitutes some 46% of the total radioactivity in extracts of resistant wheat compared

to 61% in the case of the susceptible variety. The results presented in this form also show that the polar material of low Rf comprises a greater percentage of the total radioactivity extracted from resistant wheat than for the susceptible variety, these values being 15% and 7% respectively. Smaller differences also exist between the N-monodesmethylated (b) and N-bisdesmethylated (c) metabolites (Table 17 and Fig. 36).

Having demonstrated a significantly greater rate of metoxuron breakdown in resistant compared to susceptible wheat plants over a period of four days of contact with the ^{14}C-labelled herbicide, it was considered of particular interest to compare metoxuron metabolism in the two varieties both qualitatively and quantitatively over a considerably wider time-scale. In this second experiment plants of both resistant and susceptible varieties were treated with (methoxy-^{14}C)metoxuron in the nutrient medium for 24 h only so as to avoid any possible complication in interpretation resulting from the continued uptake of unchanged ^{14}C-labelled herbicide that might otherwise occur throughout the subsequent duration of the experiment. After treatment, the roots were rinsed and the plants were transferred to a second vessel containing fresh nutrient solution without added herbicide, samples being removed for extraction and analysis of radioactive metabolites after various time intervals. These results are presented in Fig. 37 and Table 18 which show respectively photographic records of the autoradiograms obtained and details of the radioactivity eluted from each radioactive zone expressed as a percentage of total radioactivity recovered, for each sampling time. Examination of the radioautograms reveals striking differences between varieties with

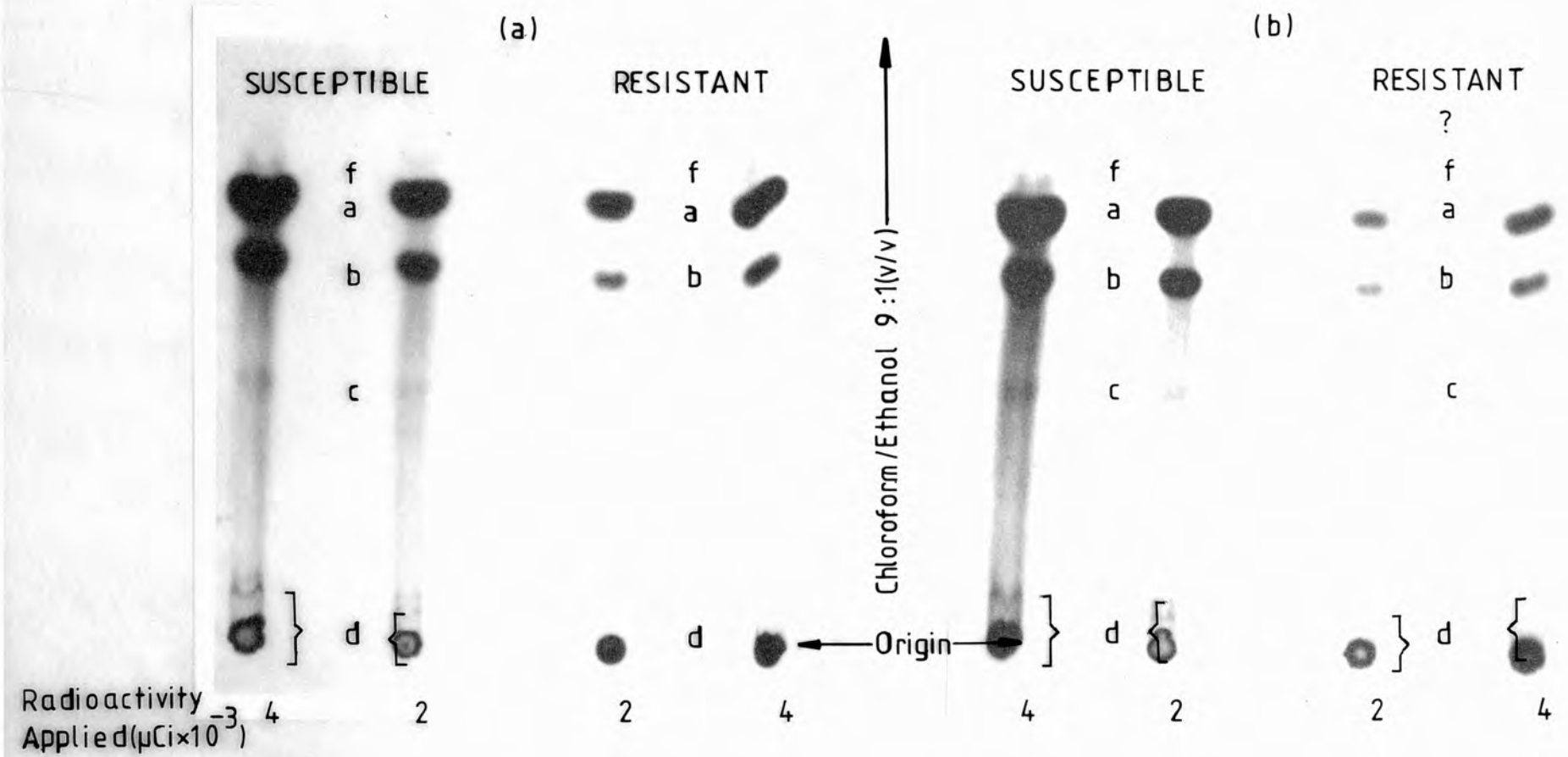
Fig. 37: Radioautographs of Thin Layer Chromatograms of 80% methanol extracts of wheat plants treated with (methoxy-^{14}C)metoxuron

Experimental details as for Fig. 34, except that the culture medium was supplemented with 3 µCi (methoxy-^{14}C)metoxuron (sp. act. 4 µCi/µmol). Plants were left in contact with the treatment solution for 24 h before being transferred to fresh nutrient solution.

Details of metabolite identification were given in Figs. 35 and 36.

Times after removal from treatment solution :

(a) 1 day (b) 2 days (c) 4 days (d) 14 days


(a)
SUSCEPTIBLE
RESISTANT
(b)
SUSCEPTIBLE
RESISTANT
?
f
a
b
c
d
Chloroform/Ethanol 9:1(v/v)
Origin
Radioactivity Applied(μCi×10⁻³)
4
2
2
4
4
2
2
4

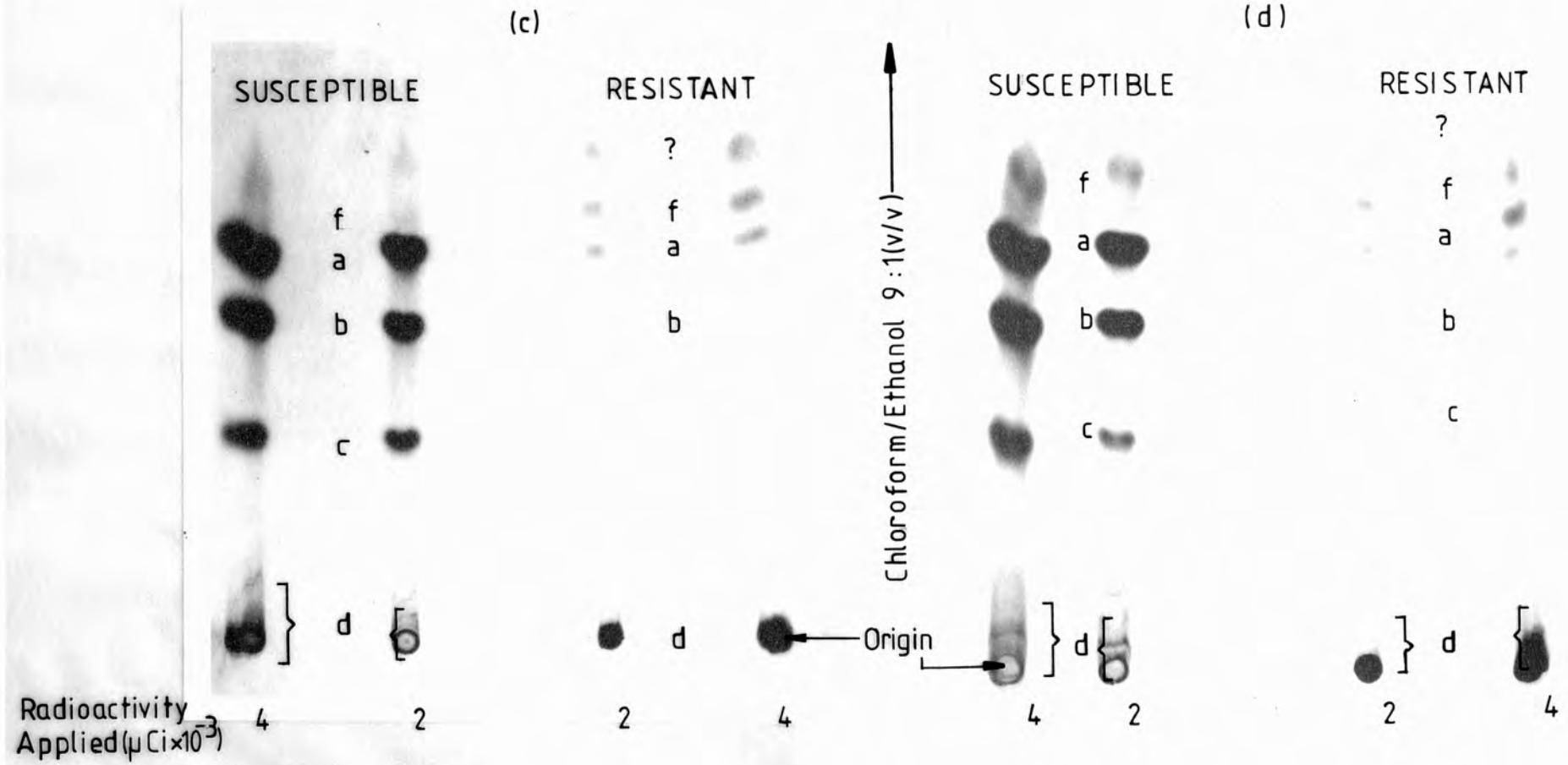

(c)
(d)
SUSCEPTIBLE
RESISTANT
SUSCEPTIBLE
RESISTANT
?
f
a
b
c
d
Origin
Chloroform/Ethanol 9:1(v/v)
Radioactivity Applied(μCi×10⁻³)
4
2
2
4
4
2
2
4

TABLE 18: Distribution of radioactivity between metoxuron and its metabolites in methanol extracts of resistant and susceptible wheat prepared at various times after treatment

Variety	Time after treatment (days)	% Total radioactivity recovered				
		Metoxuron	N'-(3-chloro-4-methoxyphenyl)-N-methylurea	N'-(3-chloro-4-methoxyphenyl)-urea	3-chloro-4-methoxyaniline	Origin
		(a)	(b)	(c)	(f)	(d)
Cappelle (R)	1	39.3	14.5	6.0	6.2	34.0
	2	22.0	15.5	8.7	6.9	41.1
	4	8.3	8.4	7.0	12.5	59.8
	14	6.5	3.9	7.1	8.1	60.4
Maris Nimrod (S)	1	61.1	16.1	4.5	2.6	15.7
	2	55.8	16.8	6.3	3.7	6.3
	4	42.1	23.9	11.8	4.5	17.7
	14	31.5	27.0	13.7	8.7	19.7

Experimental conditions as described for Table 17.
Letters in parenthesis refer to the various radioactive zones visible on the radioautograph presented in Fig. 37.

respect to the amount of radioactivity associated with identifiable metabolites at all times of sampling. Throughout the duration of the experiment the concentration of the parent herbicide in extracts from both resistant and susceptible plants can be seen to decrease, this being accompanied by a concomitant increase in the proportion of radioactive metabolites. Reference to Table 18 shows that in this experiment after one day following removal from the treatment solution the proportion of total radioactivity in the methanol extracts contributed by unchanged metoxuron amounted to some 60% and 40% for suceptible and resistant plants respectively. The corresponding values fourteen days after treatment are respectively 32% and 7%, this being a clear indication of the more rapid metabolism of metoxuron in the resistant cultivar. Whereas in the resistant variety, Cappelle, the radioactivity lost from the parent herbicide can largely be accounted for by a substantial increase in radioactivity associated with polar material remaining at the origin (60% of total after 14 days) and to a smaller increase provisionally attributed to the 3-chloro-4-methoxyaniline, in susceptible Maris Nimrod the corresponding increase occurs in the initial products of N-demethylation, the N'-(3-chloro-4-methoxyphenyl)-urea not being particularly apparent until four days after treatment. Reference to Fig. 37 shows the presence of an additional, as yet unidentified, metabolite (Rf = 0.81) particularly apparent and well defined in the case of extracts of resistant wheat in which it constituted some 14% of the total radioactivity present after fourteen days. This latter metabolite may represent a product of an additional metabolic pathway so far undiscovered, a suggestion also made by Geissbühler (1969) on the basis of minor metabolites present in extracts of metobromuron-

treated maize seedlings. Relatively little formation of the aniline derivative of metoxuron was noted in the present investigation as was previously the case in studies on the metabolism of related phenylureas such as monuron (Smith & Sheets, 1967; Swanson & Swanson, 1968), diuron (Onley et al, 1968; Osgood et al, 1972) and fluometuron (Rogers & Funderburk, 1968; Neptune, 1970) in both plants and soil. A possible explanation might be the loss of the aniline metabolite during the extraction procedure or the occurrence of a rapid transformation by conjugation and/or oxidation thus preventing its accumulation in significant amount (Neptune & Funderburk, 1968). An alternative possibility presented by Rogers & Funderburk (1968) is the existence of an enzyme system with so low an affinity for the N-bisdesmethylated substrate that the aniline derivative would be formed to only a very slight extent or not at all, as was the case in their study of the metabolism of fluometuron in cucumber.

The results presented in Table 18 and Fig. 37 thus show a clear distinction between resistant and susceptible wheat with respect to rate and extent of metabolism of ^{14}C-labelled metoxuron. However, to assess whether the N-demethylation reactions giving rise to the observed metabolites in resistant wheat constitute an effective detoxification mechanism it must necessarily be demonstrated that the derivatives are less phytotoxic than the parent herbicide. In order to compare differences in phytotoxicity between metoxuron and its major metabolites, the effects of N'-(3-chloro-4-methoxyphenyl)-N-methylurea and N'-(3-chloro-4-methoxyphenyl)urea on the Hill activity of chloroplasts isolated from both

resistant and susceptible wheat plants were investigated. The rates of DCPIP photoreduction obtained are compared with those for untreated control chloroplasts in Table 19 for two different concentrations of the phenylureas in the assay medium. These results show that though a considerable ability to inhibit photosynthetic electron transport is retained in the N-monodesmethylated metabolite, the N-bisdesmethylated derivative is only very slightly inhibitory and consequently would not be expected to display significant phytotoxicity. It would therefore appear that N-demethylation of metoxuron (see Fig. 38) does represent an effective detoxication mechanism and offers an explanation for the recovery of the resistant variety from metoxuron treatment observed in studies presented earlier.

In the course of the present investigation it was noted that not all of the ^{14}C-labelled metoxuron taken up from the treatment solutions could be accounted for in the total radioactivity present in the methanolic extracts and the remaining solid residues. Since the metoxuron used in the present studies was labelled with ^{14}C in the 4-methoxy group it was realised that an ability in wheat to O-demethylate the herbicide might result in some of the observed radioactivity being lost as $^{14}CO_2$. To test this latter possibility, metabolism of (methoxy-^{14}C)metoxuron was investigated in leaf disc segments of resistant and susceptible wheat plants in the presence of a CO_2 trap. In addition, it was hoped that a study made using leaf discs would clarify whether the photosynthetic tissue of wheat possessed an ability to detoxicate the herbicide. The previous studies reported in the present thesis on the metabolism of metoxuron employed whole plants treated with the herbicide in the nutrient

TABLE 19: Effect of metoxuron and its metabolites on the photoreduction of DCPIP by chloroplasts from resistant and susceptible wheat

Variety	Concentration of phenylurea	Hill Reaction Activity (μmol DCPIP reduced/mg chl/h)				% Inhibition photoreduction		
		Untreated	Metoxuron	N'-(3-chloro-4-methoxyphenyl)-N-methylurea	N'-(3-chloro-4-methoxyphenyl)-urea	Metoxuron	N'-(3-chloro-4-methoxyphenyl)-N-methylurea	N'-(3-chloro-4-methoxyphenyl)-urea
Maris Nimrod (S)	3.3×10^{-4} M	80	5	10	64	93	87	17
Cappelle (R)		79	6	11	67	93	87	14
Maris Nimrod (S)	3.3×10^{-5} M	114	5	54	104	95	52	9
Cappelle (R)		122	8	57	112	93	46	7

Reaction conditions as shown for Table 2.
The concentrations of the various substituted phenylureas employed represent final concentrations in the reaction mixtures.

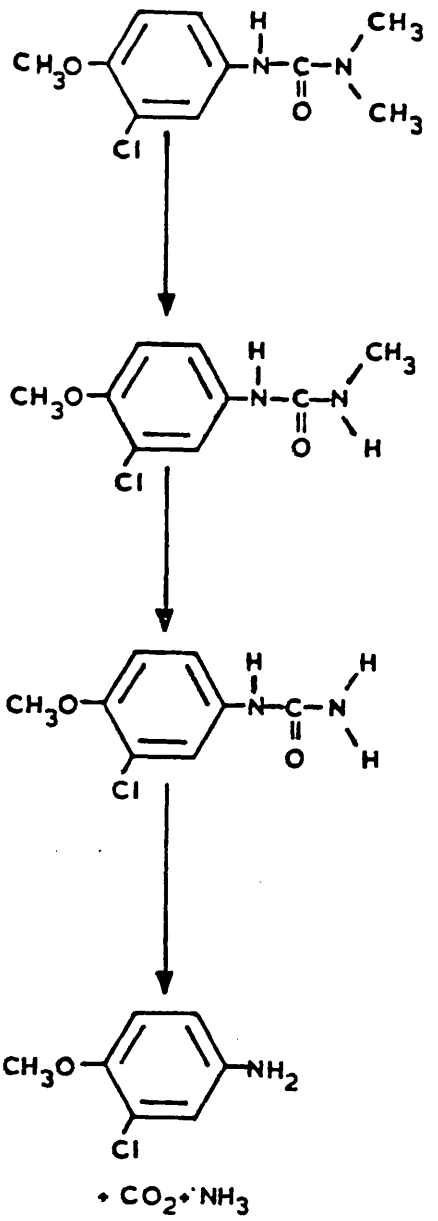


Fig. 38: Metabolism of metoxuron in wheat via successive N-demethylation

solution, with the result that it could not be determined directly whether the metabolites found in the shoots were in fact produced in the roots and subsequently translocated to the leaves.

The present studies utilized 8 mm diameter discs cut from the expanded second and third leaves of both resistant and susceptible wheat plants at the four-leaf stage of development. The prepared discs were treated with ^{14}C-labelled metoxuron by suspension for 1 h in diffuse light in 1 ml of 0.05M phosphate buffer containing 2 μCi (methoxy-^{14}C)metoxuron. All experiments were carried out in a Warburg apparatus fitted with facilities for illumination as described previously (p. 135-138).

A preliminary experiment was carried out to examine the inherent ability of the photosynthetic tissue of wheat to N-demethylate absorbed metoxuron. During the experiment an attempt was made to maintain the discs in an actively photosynthesising condition by illuminating and maintaining a constant concentration of CO_2 within the Warburg flasks by means of a diethanolamine/bicarbonate CO_2 buffer situated in the centre well. Following removal from the treatment solution, the discs were rinsed and placed in the Warburg flasks as previously described (p. 135-138) after removal of excess water. Samples were removed for extraction and analysis of radioactive metabolites at various intervals. The radioautograms prepared from chromatograms of the total methanol extracts are shown in Fig. 39 whilst the amount of radioactivity detected in each metabolite is expressed as a percentage of the total methanol soluble ^{14}C in Table 20. Examination of Fig. 39 shows that leaf tissue of both susceptible and resistant wheat is capable of N-demethylation of metoxuron though the extent of this metabolism can be seen to be

Fig. 39: Radioautograph of Thin Layer Chromatograms of 80% methanol extracts of leaf discs treated with (methoxy-^{14}C)metoxuron

Experimental details were given previously in the "Methods" section (pages 132 and 135). Leaf discs were prepared from the fully expanded leaves of resistant (Cappelle) and susceptible (Maris Nimrod) wheat plants at the three leaf stage of development, and were treated with (methoxy-^{14}C)metoxuron (1 µCi, sp. act. 4 µCi/µmol) for 1 h prior to incubation in Warburg flasks in the presence of a CO_2 buffer. Samples were removed for analysis of radioactive metabolites at the times indicated.

Identification of metabolites was as for Figs. 35 and 36.

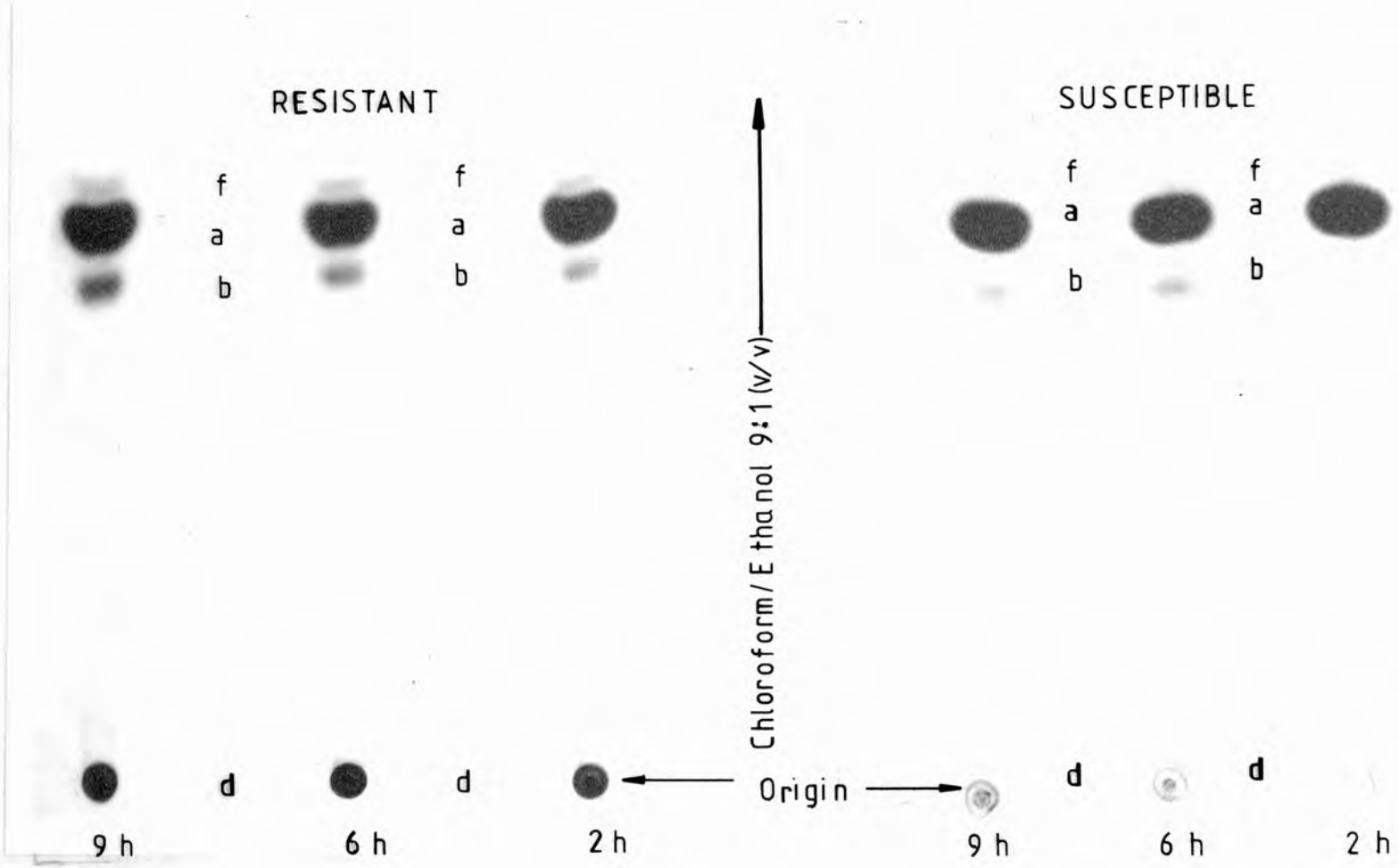

RESISTANT
SUSCEPTIBLE
f
a
b
d
Chloroform/Ethanol 9:1 (v/v)
Origin
9 h
6 h
2 h

TABLE 20: Metabolism of (methoxy-^{14}C)metoxuron in leaf discs

Variety	Time after treatment (h)	% Total Radioactivity Recovered			
		Metoxuron	N'-(3-chloro-4-methoxyphenyl)-N-methylurea	3-chloro-4-methoxyaniline	Origin
Maris Nimrod (S)	2	88.4	3.7	3.3	4.6
	6	83.6	8.6	3.7	4.1
	9	83.4	7.9	3.9	4.8
Cappelle (R)	2	75.5	9.7	4.3	10.6
	6	67.8	10.5	4.9	18.1
	9	60.0	14.6	7.3	18.8

Leaf discs were incubated for the times indicated in Warburg flasks in the presence of a CO_2 buffer as previously described in the "Methods" (p. 137). Other experimental conditions were as given in Table 17.

more pronounced in the latter variety. The distinction between the two varieties with respect to the amount of the aniline metabolite (f) formed and the amount of polar material (d) remaining at the origin is again apparent in the present study. Under the conditions of this particular experiment little or no bis-desmethylated metoxuron was detected in the methanol extracts prepared from either variety. Reference to Table 20 reveals that the concentration of metoxuron in the methanol extracts prepared from leaf discs of resistant wheat plants decreased progressively over the duration of the experiment, and after 9 h represented only some 60% of the total ^{14}C-label. In contrast, in the case of susceptible wheat unchanged metoxuron still constituted some 83% of the total methanol-extracted radioactivity after 9 h of incubation. The greater loss of ^{14}C-label from (methoxy-^{14}C)metoxuron in leaf discs of resistant compared to susceptible wheat can be seen to result in concomitant substantial increases in radioactivity associated in particular with monodesmethylated metoxuron and the unidentified metabolite(s) remaining at the origin.

Having demonstrated that the leaf tissue of resistant wheat possessed an ability to rapidly metabolise the metoxuron molecule, a second experiment was designed to detect any evolution of $^{14}CO_2$ which might be expected to be released if O-demethylation of metoxuron represented an additional metabolic route. For this particular purpose, with the exception of the replacement of the CO_2 buffer in the centre well with a CO_2 trap consisting of a roll of filter paper impregnated with 6M KOH, reaction conditions and the procedure employed were as described for the previous experiment. At 3 h intervals the filter paper wicks were removed from the Warburg flasks and, after drying, the amount of radioactivity

present was measured using the liquid scintillation technique as previously described (p. 132). The results obtained are presented in Table 21 and are expressed as d.p.m./g fresh wt. of discs.

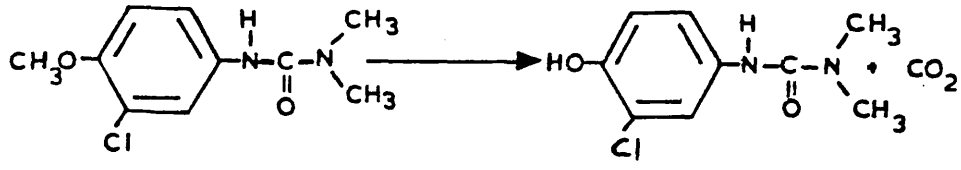
TABLE 21: Liberation of $^{14}CO_2$ from (methoxy-^{14}C)metoxuron-treated leaf discs of resistant and suceptible wheat plants

Time after treatment (h)	$^{14}CO_2$ trapped (d.p.m./g fresh weight)	
	Resistant Wheat (Cappelle)	Susceptible Wheat (Maris Nimrod)
2	12,800	5,100
6	33,100	6,000
9	41,500	8,000
12	51,500	7,200

Following treatment with (methoxy-^{14}C)metoxuron, the discs were incubated for the prescribed times in Warburg flasks, the released $^{14}CO_2$ being trapped by means of a piece of convoluted filter paper saturated with 6M KOH situated in the centre well. The amount of radioactivity trapped was subsequently determined by the scintillation technique.

These data clearly demonstrate an ability of leaf tissue of both resistant and susceptible wheat plants to liberate $^{14}CO_2$ from (methoxy-^{14}C)metoxuron. However, a clear distinction can again be seen to exist between the two varieties in that the leaf discs of the resistant variety evolved a five to six-fold greater quantity of $^{14}CO_2$ over the duration of the experiment. If, indeed, the observed evolution of $^{14}CO_2$ results from an initial O-demethylation of (methoxy-^{14}C)metoxuron, an initial product of this reaction pathway

might be expected to be N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea. This latter compound would, however, be non-radioactive and consequently would have escaped detection in the earlier experiments described in the present thesis had it represented an additional component of the methanolic extracts examined.



Though no attempt was made in the present study to detect the presence of O-desmethylated metoxuron in the methanol extracts, partly because it was considered that such a compound might become rapidly conjugated to form a glycoside derivative (s), a comparison was made between the phytotoxicities of this latter metabolite and metoxuron based on percentage inhibition of the Hill reaction of isolated chloroplasts in in vitro assays. Rates of DCPIP photoreduction obtained for chloroplasts separately treated with the two compounds are compared to those for untreated controls in Table 22.

TABLE 22: Effect of metoxuron and its O-desmethylated derivative on the Hill activity of chloroplasts from resistant and susceptible wheat.

Variety	Hill Reaction Activity (µmol DCPIP reduced/mg chl/h)			% Inhibition photoreduction	
	Untreated Control	Metoxuron	O-desmethylated derivative	Metoxuron	O-desmethylated derivative
Cappelle (R)	148	14	138	90	8
Maris Nimrod (S)	123	8	113	95	10

Reaction conditions as shown for Table 2.

These data show that N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea inhibited DCPIP photoreduction by chloroplasts from both resistant and susceptible wheat by only some 10% when applied at a final concentration of 3.3×10^{-5}M. In contrast, metoxuron applied at the same concentration caused some 90% inhibition of photoreduction activity indicating that in addition to removal of the N-methyl groups, cleavage of the 4-methoxy group also results in a marked reduction in efficacy as an inhibitor of photosynthesis. Phytotoxicity might be expected to be even further reduced in the event of a subsequent conjugation of the N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea.

Demethoxylation of urea herbicides containing a methoxy group (e.g., linuron, monolinuron, metabromuron, chlorbromuron) does not appear to have been studied as thoroughly as demethylation. In contrast to metoxuron, the methoxy group in the case of the above compounds is attached directly to an amide nitrogen rather than to the phenyl ring so that a direct comparison between the present results and earlier reports may not prove particularly meaningful. Previous reports have demonstrated, however, that both metabromuron (Voss & Geissbühler, 1966) and linuron (Nashed & Ilnichi, 1970) are readily demethoxylated to less phytotoxic products by potato and maize, and soybean and crabgrass (Digitaria sanguinalis) respectively.

In summary, the results presented in the foregoing section would suggest that the distinction between resistant and susceptible wheat with respect to reaction to metoxuron can seemingly be adequately accounted for by a combination of mainly two phenomena: (1) a greater rate of uptake of the herbicide by the root system of susceptible

compared to resistant wheat, and (2) a faster rate of N-demethylation and demethoxylation of the absorbed herbicide by the latter variety.

In a final series of experiments an attempt was made to locate the site(s) of metoxuron metabolism within the plant cell. The existence of an *in vitro* cotton leaf microsomal oxidase system, which N-demethylated monuron to form the N-monodesmethylated derivative, has been reported previously by Frear (1968). In a further report, Frear *et al* (1969) located an N-demethylase enzyme requiring oxygen and NADPH as co-factor in the microsomal fraction of cotton. This enzyme was reported to be specific for substituted N'-phenyl-N-methyl ureas and was shown to be responsible for the breakdown of both monuron and fluometuron in cotton. The present study utilised plants of both metoxuron-resistant and susceptible wheat grown under identical conditions in a growth cabinet. At the three-leaf stage of development plants were harvested for use in the preparation of sub-cellular organelle fractions by an adaptation of the procedure of Frear (1968). The original brei obtained by homogenising the leaf tissue was subsequently fractionated by subjecting to differential centrifugation at 300 x g, 1500 x g, 9000 x g and 100,000 x g as described previously (p. 139). The results of organelle characterisation experiments were presented earlier when the intracellular distribution of absorbed ^{14}C-labelled metoxuron was discussed (see p. 183). After resuspension in phosphate buffer the individual fractions were immediately assayed for N-demethylase activity by following the formation of N'-(3-chloro-4-methoxyphenyl)-N-methylurea. Following the termination of the reaction by rapid-freezing and lyophilisation, the metoxuron and reaction product(s) were extracted with methanol and were subsequently separated by thin layer chromatography.

Radioactive zones were visualised by autoradiography and the amount of radioactivity in the separated reaction product was quantitatively determined by liquid scintillation counting after elution from the chromatographic plates.

The results obtained, typical examples of which are presented in Fig. 40 and Table 23, clearly demonstrate the association of a metoxuron N-demethylase activity with the microsomal fraction of the leaves of both resistant and susceptible wheat.

TABLE 23: Metoxuron N-demethylase activity of various sub-cellular organelle fractions prepared from wheat leaves

Fraction	N-demethylase activity			
	Specific Activity*		Total Activity	
	Maris Nimrod (S)	Cappelle (R)	Maris Nimrod (S)	Cappelle (R)
Cell-free extract	175	236	96,250	119,400
1500 x g pellet	174	210	7,100	7,220
9000 x g supernatant	178	198	80,100	85,140
9000 x g pellet	180	190	11,020	10,350
100,000 x g supernatant	176	194	58,100	61,100
100,000 x g pellet	340	420	15,500	17,700

Each reaction mixture contained (in total volume of 1 ml), KH_2PO_4 buffer (pH 7.5) 50 µmol; NaCN, 0.5 µmol; NADPH, 1 µmol; (methoxy-^{14}C)metoxuron, 0.09 µmol (sp. act. 4 µCi/µmol) and an aliquot of a sub-cellular organelle fraction equivalent to approx. 2 mg protein. Reactions were carried out at 25°C for 30 min. In the case of control assays, the NADPH was omitted.

*Specific activity is expressed as d.p.m. N'-(3-chloro-4-methoxyphenyl)-N-methylurea formed/mg protein/30 min.

Fig. 40: Radioautographs of Thin Layer Chromatograms of 80% methanol extracts of various sub-cellular organelle fractions of wheat incubated with (methoxy-^{14}C)metoxuron

Assay conditions were as for Table 23. Details of the chromatographic and autoradiographic procedures were given in the "Methods" section (pages 132-133).

Identification of metabolites was as for Figs. 35 and 36.

In each case a volume of methanolic extract equivalent to 10×10^{-3} µCi of radioactivity was applied at the origin of each zone.

Key to sub-cellular fractions :-

1 = 100,000 x g pellet;

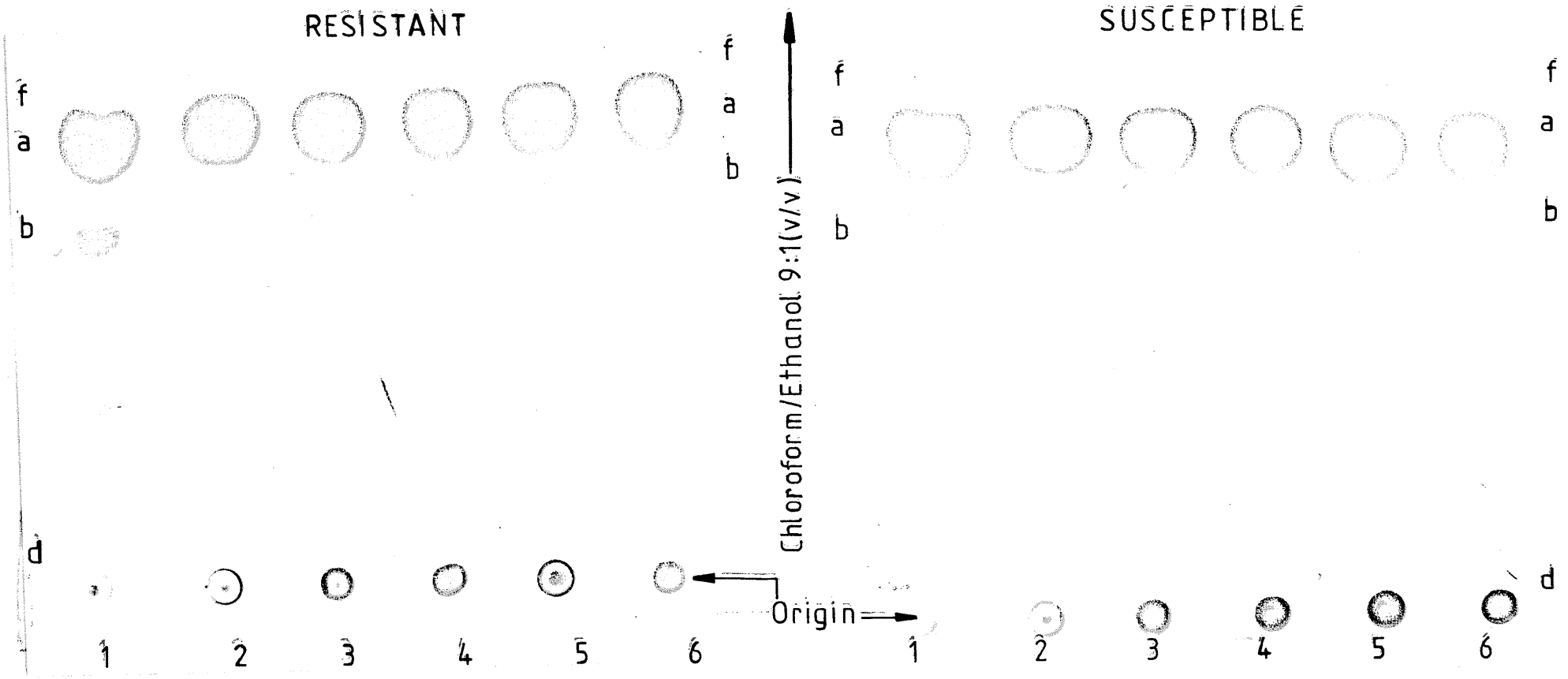
2 = 100,000 x g supernatant;

3 = 9,000 x g pellet;

4 = 9,000 x g supernatant;

5 = 1,500 x g pellet;

6 = Cell free extract


RESISTANT
SUSCEPTIBLE
f
a
b
d
Chloroform/Ethanol 9:1(v/v)
Origin
1
2
3
4
5
6

Reference to Table 23, however, which expresses the specific activity of the N-demethylation reaction in the various organelle fractions as d.p.m./30 min/mg protein, shows that appreciable activity is also detectable in the 1500 x g (chloroplasts) and 9000 x g (mitochondria) pellets. Though each pellet was washed and resedimented prior to being used in a subsequent assay, it is considered likely that the activity associated with these latter fractions will ultimately be shown to result from contamination with microsomal fragments. In addition, the specific activity of the N-demethylase can be seen to be somewhat higher in the microsomal fraction of resistant compared to susceptible wheat, though it would be difficult to argue that such an apparently small difference could alone account for the observed differences in varietal response to metoxuron treatment. Attempts to detect larger differences between the two varieties with respect to activity of the N-demethylase, through modifications to the extraction and assay procedures, met with very little success and usually resulted in a reduction in activity measured in both varieties. Results presented in the foregoing section, however, suggested that O-demethylation of metoxuron may also be effected by the leaf tissue of wheat. If this latter metabolic reaction can also be ascribed to the microsomal fraction of the leaf cells, then it would clearly interfere with the N-demethylase assay if it occurs simultaneously in the microsomal preparations used. The product of any O-demethylation reaction involving either metoxuron or N-monodesmethylated metoxuron would be non-radioactive. Therefore, since cleavage of the 4-(^{14}C)methoxy group was demonstrated previously in the present studies using leaf discs to occur at a five to six-fold greater rate in resistant wheat, the amount of N-monodesmethylated metoxuron

detected in the microsomal assays for this latter variety may have been reduced relative to that measured for the susceptible variety by a corresponding order of magnitude. Consequently, these studies on the sub-cellular location of metoxuron N-methylation activity should be repeated using a metoxuron sample labelled with ^{14}C in an alternative position (either universally in the phenyl ring, or in the carbonyl group) so that this particular point may be clarified. Though no reports of O-demethylation by plant microsomal preparations were encountered by the present author, such reactions are well documented in mammalian microsomes, particularly those obtained from liver tissue, which oxidatively cleave arylalkyl ethers to produce the corresponding phenols (see, e.g., Brodie, Gillette & La Du, 1958; Nilsson & Johnson, 1963; Watabe, Yoshimura & Tsukamoto, 1964; and Shigematsu, Yamano & Yoshimura, 1976). Though not reported in the present thesis, the results of a preliminary metabolism experiment incorporating a CO_2-trap made using microsomes prepared from plants of metoxuron-resistant and susceptible wheat showed that O-demethylation activity could not be detected in these latter preparations on the basis of release of radioactive carbon dioxide. Therefore, confirmatory evidence as to whether O-demethylation of metoxuron can be achieved by wheat microsomal preparations will seemingly have to await the availability of a sample of radioactive metoxuron labelled with ^{14}C in a position other than the 4-methoxy group.

CONCLUDING DISCUSSION

In the preceeding sections the results of each experiment or series of experiments have been directly discussed. In this section it is proposed to briefly outline suggestions for future research arising from conclusions made as a result of the present investigations.

Studies of the Hill activity of isolated chloroplasts incubated with metoxuron showed a similar inhibition of DCPIP and potassium ferricyanide photoreductions in both resistant and susceptible wheat plants. Thus in these studies in which metoxuron has arrived at the site(s) of inhibition within the chloroplast by mainly physical processes, no distinction exists between the two varieties. This did not, however, rule out the possibility that chloroplasts isolated from metoxuron-treated plants, in which physiological phenomena are also involved in the subsequent distribution of the absorbed herbicide, might show different degrees of inhibition of photosynthetic activities. Indeed, examination of the Hill activity of chloroplasts isolated from metoxuron-treated plants showed a greater inhibition of DCPIP and potassium ferricyanide photoreductions in susceptible compared to resistant varieties. This result correlated with the visual symptoms displayed by resistant and susceptible varieties following foliar application of metoxuron. Whereas treatment of the susceptible varieties with metoxuron was followed by scorching and eventual death of the plant, tolerant varieties recovered from an initial slight visible injury in parallel with a return of Hill activity to normal levels. This result is in agreement with data obtained by Van Leeuwen & Van Oorschot (1976) in a study of the change in relative rate of net photosynthesis of resistant and susceptible wheat varieties following short exposures of

the roots to metoxuron in the nutrient solution. Though net photosynthesis in both resistant and susceptible cultivars was reduced initially to almost zero, some 12 hours after removal from the treatment solution photosynthesis in the resistant variety had recovered to 40% of the control rate whereas the corresponding degree of recovery for susceptible wheat was only some 10%. Similar results have also been obtained more recently by Muller, Frahm & Sanad (1977) in a study of the influence of the substituted phenylureas, chlortoluron, isoproturon and metoxuron on photosynthetic oxygen evolution (Hill activity) in two winter wheat cultivars. All three herbicides were found to depress photosynthesis of both the susceptible and resistant cultivars to some 6-10% of the rate measured in untreated control plants. Although oxygen evolution in herbicide-treated susceptible plants remained at some 10% of the untreated control rates throughout the duration of the experiment, photosynthesis in the resistant cultivar recovered to 63% and 86% of the control rates at three and six days respectively after cessation of treatment.

The cyclic photophosphorylation studies described in the present thesis were carried out utilising phenazine methosulphate as catalyst. These studies showed that metoxuron concentrations some 100-fold greater than those required to inhibit Hill activity and associated oxygen evolution by 90-100% were found to cause only some 20% inhibition of cyclic photophosphorylation in chloroplasts of both resistant and susceptible varieties. The results of this <u>in vitro</u> study are thus in general agreement with the accepted view (Wessels & Van der Veen, 1956; Asahi & Jagendorf, 1963; Cramer & Whitmarsh, 1977) that the substituted phenylurea herbicides

act as potent inhibitors of photosystem 2 of photosynthesis and are virtually without effect on photosystem 1-catalysed activities such as cyclic electron flow and associated photophosphorylation. However, the results of a subsequent in vivo study, in which cyclic photophosphorylation activity of chloroplasts isolated from metoxuron-treated plants was measured, appear to reflect data obtained for the DCPIP and potassium ferricyanide photoreduction assays in that a measurable difference between rates of cyclic photophosphorylation between resistant and susceptible varieties is now seen to exist. The varietal distinctions observed in the present investigation of the effect of metoxuron on various photosynthetic activities presumably are a reflection of varietal differences in physiology and biochemistry influencing uptake and subsequent metabolic fate of the herbicide within the plants.

The studies reported in this thesis have also observed a reduction in the proportion of ethanol soluble material of leaves 24 hours following treatment of wheat plants with metoxuron, the susceptible varieties showing the greater decrease. This distinction between resistant and susceptible varieties was more apparent when plants that had contacted the herbicide for longer periods were investigated. These results were also reflected in a greater inhibition of $^{14}CO_2$ fixation by metoxuron-treated leaves of susceptible plants compared to similarly treated resistant strains. The radioautograms presented in Figs. 21-23 also seem to indicate that translocation of photosynthate in plants of both varieties is not drastically impaired as a result of metoxuron treatment. This result is not unexpected in view of the observed absence of a significant effect of metoxuron on ATP synthesis by cyclic photophosphorylation and mito-

chondrial oxidative phosphorylation when applied at concentrations adequate to cause total inhibition of non-cyclic photosynthetic electron flow.

Comparison of the separation patterns obtained on subjecting the $^{14}CO_2$-fixation products extracted from metoxuron-treated resistant and susceptible wheats to two-dimensional paper chromatography revealed very little qualitative or quantitative difference between cultivars. This result is interpreted as implying that the observed inhibition of photosynthetic carbon dioxide fixation by metoxuron occurs as a consequence of a diminished supply of reducing power and ATP resulting from inhibition of non-cyclic electron flow through photosystem 2, rather than through an interference with any specific reaction(s) of the photosynthetic carbon reduction cycle. Such reduced levels of $NADPH_2$ and ATP might be revealed by comparison of the chemical analysis of metoxuron-treated susceptible wheat and untreated controls for these cofactors. However, a recent study on the effect of metoxuron on the in vitro activity of wheat ribulose-1,5-diphosphate carboxylase revealed a considerable degree of inhibition, the enzyme from metoxuron-susceptible strains being consistently reduced by the greatest extent (G. Anderson, personal communication). This latter observation suggests the possibility that factors other than a lack of reducing power and ATP may be contributing to the observed reduction in CO_2 fixation. It would therefore be of considerable interest to extend this study to examine the effect of metoxuron on the activities of the individual enzymes of the Calvin cycle to see whether any additional effects on levels of activity can be detected, and whether the above result for ribulose 1,5-diphosphate carboxylase can be confirmed.

The studies reported in this thesis have also shown that differences in absorption and translocation of metoxuron in resistant and susceptible wheat varieties may represent additional factors contributing to the observed difference in varietal response. Considerable differences in uptake of metoxuron into roots and leaves of some six winter wheat cultivars were also observed in a recent study by Müller & Sanad (1975). In contrast to the results presented in the present thesis, however, no clear correlation was found to exist between reaction to metoxuron and degree of absorption of the herbicide when applied to the root system. Of particular interest is the result obtained for the variety, Hanno, designated resistant in field trial experiments, which was found to absorb the greatest quantity of metoxuron into its leaf system. Though the data presented by Müller & Sanad (1975) are not directly comparable with the results obtained in the present study in that the herbicide was applied to the soil rather than via nutrient culture so that factors of a non-physiological nature may also be involved, they do suggest that the difference in uptake of ^{14}C-labelled metoxuron observed in the present study may not be a major factor contributing to the difference in varietal reaction.

The results of a sub-cellular organelle localisation study of absorbed ^{14}C-labelled metoxuron indicate that on the basis of protein content the greatest amounts of radioactivity were associated with the chloroplast fraction. In addition, chloroplasts from susceptible varieties contain a several fold greater quantity of metoxuron than those of resistant plants, a very significant observation in view of the fact that phytotoxicity is mediated primarily through an inhibition of photosynthesis. A degree of caution, however, is necessary in interpreting

results obtained in such sub-cellular organelle distribution studies made using whole plants since it is difficult to assess the extent to which extracellular herbicide (present in the apoplast) becomes associated with the various organelle fractions during their isolation. This difficulty might be at least partially overcome in a study of the uptake of ^{14}C-labelled metoxuron by isolated protoplasts, prepared from leaves of both resistant and susceptible wheat plants. A procedure for preparing protoplasts from cereal leaves has been reported by Evans, Keates & Cocking (1972). The advantage of this technique stems from the fact that protoplast membranes are readily ruptured by gentle osmotic shock thereby facilitating the release of structurally intact organelles which may then be separated by density gradient centrifugation employing media which retain structural integrity of the bounding membranes (Honda, Hongladarom & Laties, 1966). Such a study would also enable a direct comparison of the rates of penetration of metoxuron across the cell membrane of resistant and susceptible wheats to be made.

Studies reported in the present thesis on the metabolism of metoxuron, made using (methoxy-^{14}C)metoxuron, again indicate a greater amount of radioactivity associated with methanolic extracts prepared from susceptible wheat. A major degradative pathway in wheat is shown to involve a two-step N-demethylation which seems to be followed by a slow hydrolysis of the ureido group to give the corresponding aniline derivative. Reference to Table 18 shows that after one day the proportion of total radioactivity in the methanol extracts contributed by unchanged metoxuron amounts to some 60% and 40% for susceptible and resistant plants respectively. The corresponding values fourteen days after treatment were respectively 32%

and 7%, this being a clear indication of the more rapid metabolism of metoxuron in the resistant cultivar. Whereas in the resistant variety, Cappelle, the radioactivity lost from the parent herbicide can largely be accounted for by a substantial increase in radioactivity associated with polar material remaining at the origin (60% of total after 14 days) and to a smaller increase provisionally attributed to the 3-chloro-4-methoxy aniline, in susceptible Maris Nimrod the corresponding increase occurs in the initial products of N-demethylation, particularly the N-monodesmethylated metabolite, which retained a considerable ability to inhibit photosynthetic electron transport. The metabolic route outlined is similar to that reported for the metabolism of other phenylurea herbicides in various plant species (Geissbühler et al, 1963b; Smith & Sheets, 1967; Neptune, 1970) and was shown to result in an effective detoxication of metoxuron in in vitro assays of Hill activity in which though the N-monodesmethylated derivative was found to be some 80% as inhibitory as metoxuron, the N-bisdesmethylated and aniline derivatives were ineffective as photosynthetic inhibitors. It would therefore appear that N-demethylation of metoxuron does represent an effective detoxication mechanism and offers an explanation for the recovery of the resistant variety from metoxuron treatment observed in the present study.

In the course of the present investigation it was noted that not all of the ^{14}C-labelled metoxuron taken up from the treatment solutions could be accounted for in the total radioactivity present in the methanolic extracts and the remaining solid residues. Since the metoxuron used was labelled with ^{14}C in the 4-methoxy group it was realised that an ability

in wheat to O-demethylate the herbicide might result in some of the absorbed radioactivity being lost as $^{14}CO_2$.

There are a considerable number of reports indicating that various methoxylated aromatic compounds, in particular methoxylated cinnamic and benzoic acids, may undergo O-demethylation when administered to plant tissues (Towers, 1974). Other workers have demonstrated O-demethylation of syringin (Kratzl, 1960) and other polyphenols (Barz & Grisebach, 1967; Ebel, Achenbach, Barz & Grisebach, 1970) in plants. Because of considerable O-demethylation of the precursor, Barz & Grisebach (1967) encountered difficulty in demonstrating the incorporation of p-methoxycinnamate (methyl-^{3}H) into the 4'-methoxyisoflavones of Robinia pseudoacacia. That the O-demethylation activity resides in the plants themselves rather than in contaminating microorganisms is indicated in experiments with sterile tissues. Sterile cultures of wheat seedlings (Harms & Priess, 1973) and cell suspension cultures of Phaseolus aureus (Harms, Haider, Berlin, Kiss & Barz, 1972) have been found to O-demethylate various methoxybenzoic acids such as anisic, veratric and 3,4,5-trimethoxybenzoic acids to form phenolic products in each case. Though no reports of O-demethylation by any isolated plant sub-cellular organelle fractions were encountered by the present author, such reactions are well documented in mammalian microsomes which oxidatively cleave aryl-alkyl ethers to produce the corresponding phenols (Brodie et al, 1958; Shigematsu et al, 1976). In addition, O-demethylating enzymes responsible for the demethylation of para- and meta-substituted methoxybenzoic acids have been identified from species and strains of Pseudomonas (Bernhardt, Ruff & Staudinger, 1971; Cartwright & Buswell, 1967; Cartwright & Smith, 1967; Ribbons, 1970, 1971). Pseudomonas putida grown in the presence of

p-anisic acid has been shown (Bernhardt et al, 1971) to contain a monoxygenase enzyme which O-demethylated the substrate to produce p-hydroxybenzoic acid and formaldehyde. The enzyme system apparently consisted of a flavoprotein, an iron-sulphur protein and a terminal oxidase and thus possesses certain similarities to the membrane-associated monoxygenase systems of mammalian microsomes (Gunsalus, Pederson & Sligar, 1975).

To test whether O-demethylation of metoxuron represented an additional degradative route in the present study with wheat, metabolism of (methoxy-^{14}C)metoxuron was investigated in leaf disc segments of both resistant and susceptible plants in the presence of a CO_2 trap. Though studies reported above with Pseudomonas and with mammalian microsomal systems demonstrated that the methyl groups involved in O-demethylation reactions are removed as formaldehyde, the present experiments were carried out on the basis of the premise that any formaldehyde generated by an analogous plant enzymic system would be incorporated into the general metabolism of the plant cell via 1C-transfer reactions to be released ultimately as respiratory CO_2. The results of this study show that leaf tissue of both susceptible and resistant wheat is capable of N-demethylation of metoxuron though the extent of this metabolism is seen to be more pronounced in the latter variety. That the 4-methoxy group of metoxuron may be cleaved is indicated by the evolution of appreciable quantities of $^{14}CO_2$ from the leaf discs of both varieties. A clear distinction between resistant and susceptible varieties is again apparent, however, in that leaf discs of the former variety evolved a five to six-fold greater quantity of $^{14}CO_2$ than those of susceptible wheat over the duration of the experiment. If, indeed, the observed evolution of $^{14}CO_2$ results from an initial O-demethylation of metoxuron, then an initial

product of this reaction pathway might be expected to be the corresponding phenol, N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea. However, this latter compound when formed from (methoxy-^{14}C)metoxuron would be non-radioactive and consequently would have escaped detection in the earlier experiments described in the present thesis had it represented an additional component of the methanolic extracts examined. It should, however, be possible to detect the presence of the phenolic product of metoxuron O-demethylation by treatment of the chromatograms of the methanolic extracts with a locating agent specific for the phenolic group such as diazotised p-nitroaniline (Swain, 1953).

Fig. 41 presents a summary of the results obtained in the present thesis on the metabolic fate of metoxuron in wheat. Though the existence of the N-demethylation pathway has been clearly demonstrated a further study will be necessary in order to confirm the simultaneous operation of an additional degradative route based on an initial O-demethylation of the herbicide. If the latter pathway can be confirmed it would be particularly interesting to carry out a substrate specificity study for the enzyme system involved to see whether the various intermediates of the N-demethylation pathway can also be O-demethylated to even less phytotoxic products. The radioautograms prepared from chromatograms of methanol extracts of resistant wheat in particular also indicate that a substantial amount of radioactivity absorbed as (methoxy-^{14}C)metoxuron is associated with polar metabolites remaining at or adjacent to the origin. These components are likely to be conjugated derivatives of the parent herbicide and/or its metabolites, and further studies should be carried out to examine the number of conjugates produced and to

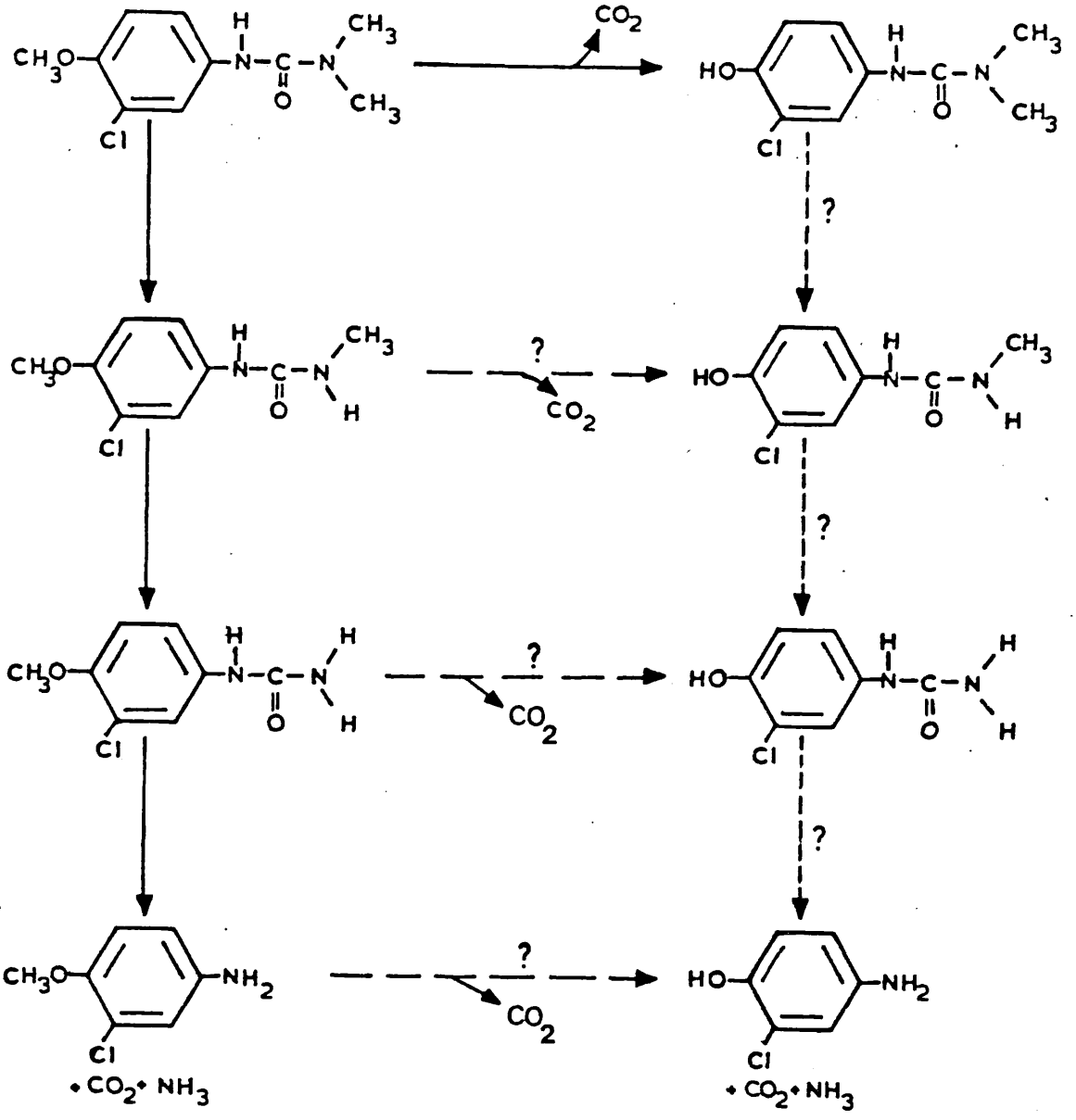


Fig. 41: Proposed pathways for the metabolism of (methoxy-^{14}C)-metoxuron in wheat plants

In addition to the confirmed N-demethylation pathway shown on the left of the scheme, experimental data presented in the present study suggest that an O-demethylation route may also exist. It has not, however, been established whether the intermediates in the latter pathway serve as substrates for the microsomal N-demethylase, and whether they exist free or as conjugates.

elucidate their chemical nature. A number of O-glycosides have previously been identified as metabolites of monuron (Frear & Swanson, 1972; 1974; Lee & Fang, 1973) and chlortoluron (T. Laanio, personal communication) in a number of plant species.

An interesting result relevant to the present discussion is that of Higuchi & Brown (1963) who found that O-demethylation of methoxybenzoic acids in wheat seedlings is specific for para-methoxy groups, this being the position of the methoxy substituent on the phenyl ring of metoxuron. In addition, Ebel, Barz & Grisebach (1970) have also demonstrated in studies with Robinia pseudoacacia that O-demethylation is much less rapid in the younger leaves of this species. If this situation can also be shown to exist in wheat, the experimental material used in the present study, then this would provide a plausible explanation for the observation that metoxuron is unsafe for weed control in even resistant varieties until the crop has reached the three-leaf stage of development (R. Jones, Sandoz Ltd. - personal communication). It has been shown previously for other substituted phenylurea herbicides that phytotoxicity varies with the stage of plant growth (Carlson & Wax, 1970). Consequently, it would be of considerable interest to examine the fate of metoxuron in wheat plants of varying age to see whether behaviour in the field can be correlated with rate of degradation of the herbicide and/or with the nature of the metabolites formed.

In addition to O-demethylation reactions, a number of reports have indicated that O-demethoxylation of aromatic ring substituted methoxy groups can occur in plant tissues. Studies related to the biosynthesis of lignin (El-Basyouni, Neish & Towers, 1964; Shimada, Fushiki & Higuchi, 1972) have shown that ferulic acid used in precursor studies could be

O-demethoxylated to p-coumaric acid whilst studies on the biosynthesis of hydroxybenzoic acids in plants revealed that sinapic acid was both O-demethylated and O-demethoxylated to give caffeic acid (Steiner, 1970; El-Basyouni, Chen, Ibrahim, Neish & Towers, 1964). These latter observations allow for the alternative possibility that the ^{14}C-label lost as $^{14}CO_2$ from (methoxy-^{14}C)metoxuron in the present studies may have resulted from an O-demethoxylation of the herbicide by the leaf disc preparations used. The product of such a reaction would by analogy with the above results be N'-(3-chlorophenyl)-N,N-dimethylurea, which does not possess a reactive phenyl substituent and consequently would be difficult to detect on chromatograms by procedures other than autoradiography. The question as to whether cleavage of the 4-methoxy group of metoxuron occurs by O-demethylation or O-demethoxylation might therefore only be resolved by repeating some of the metabolism studies discussed using a sample of ^{14}C-metoxuron labelled in a position other than the 4-methoxy group, either in the phenyl ring or in the carbonyl group.

In agreement with the present results, evolution of $^{14}CO_2$ from whole wheat plants treated with (methoxy-^{14}C)metoxuron has been reported recently by Müller & Sanad (1975). Ability to mediate this transformation was reported to be greater in metoxuron-resistant plants than in susceptible types, and the conclusion was drawn that this metabolic difference probably accounts for the different varietal reactions to the herbicide. This conclusion does not, however, provide an explanation for the observation (Hubbard & Livingston, 1974) that wheat varieties resistant to metoxuron are similarly tolerant of the related phenylurea, chlortoluron. The latter herbicide has a methyl group substituted for the 4-methoxy group of metoxuron and is therefore not subject to metabolism via an O-

demethylation or O-demethoxylation pathway. Studies on the metabolism of chlortoluron by various animal tissues (Hinderer & Menzer, 1976b) indicated that although the ring-methyl group was oxidised to carboxyl via the hydroxymethyl intermediate, there was no evidence for subsequent decarboxylation of the carboxylic acid group. Results of a recent study on the metabolism of chlortoluron in wheat (T. Laanio, personal communication to W.J. Owen) indicate that the ring-methyl group also plays an important role in the metabolism of the herbicide in plants. Although stepwise N-dealkylation was used to some extent, oxidation of the ring-methyl group yielding benzyl alcohol derivatives apparently represents the main mechanism of chlortoluron degradation in young wheat plants. Although these primary metabolites were found to be readily conjugated, the radioactivity of the mature plants was mainly present in the form of 4-carboxyphenyl derivatives, indicating a lack of ability in young wheat plants to oxidise the benzyl alcohol metabolites to benzoic acid derivatives. No comparison between resistant and susceptible wheat cultivars was attempted in this latter study. Consequently, it would be particularly interesting to examine the ring-methyl oxidation and N-demethylation degradative routes in the two types of wheat to see which pathway represents the more important detoxification mechanism.

In their study of the evolution of $^{14}CO_2$ from wheat plants treated with (methoxy-^{14}C)metoxuron, Müller & Sanad (1975) also detected the presence of metoxuron metabolites retaining ^{14}C-label in both aqueous and chloroform extracts. Though the levels of radioactive metabolites were found to be greater in extracts obtained from resistant wheat cultivars,

no characterisation and phytotoxicity data for the separated metabolites were presented in support of their conclusion that O-demethylation of metoxuron represents a major factor responsible for resistance to the herbicide. Data presented in the present thesis, however, show that both N-demethylation and O-demethylation of metoxuron constitute effective detoxication mechanisms. Studies of the Hill activity of isolated chloroplasts incubated with the various metabolites *in vitro* demonstrated that N'-(3-chloro-4-hydroxyphenyl)-N,N-dimethylurea, the O-demethylation product of metoxuron, and N'-(3-chloro-4-methoxyphenyl)-urea, the product of N-bisdesmethylation, both caused only some 10% inhibition of DCPIP photoreduction when applied at a final concentration of 3.3×10^{-5}M in the reaction cuvettes.

Having demonstrated that the metoxuron molecule is subject to two different types of metabolic attack, it should now be possible to quantitate the two pathways in order to assess which route has the major significance in relation to varietal resistance to the herbicide.

In a final series of experiments an attempt was made to locate the site(s) of metoxuron metabolism within the plant cell. The existence of an *in vitro* cotton leaf microsomal oxidase system which N-demethylated monuron to form the N-monodesmethylated derivative was reported previously by Frear (1968). The results obtained in the present study with wheat clearly demonstrate the association of a metoxuron N-demethylase activity with the microsomal fraction of the leaves of plants of both resistant and susceptible varieties. In addition, the specific activity of the N-demethylase was shown to be somewhat higher in the microsomal fraction of resistant compared to susceptible wheat, though it is difficult to

conceive how such an apparently small difference could alone account for the observed differences in varietal response to metoxuron treatment. Attempts to detect larger differences between the two varieties with respect to activity of the N-demethylation enzyme system have, to date, met with little success. Results discussed above, however, showed that O-demethylation of metoxuron may also be effected by the leaf tissue of wheat. If this latter metabolic reaction can also be ascribed ultimately to the microsomal fraction of the wheat cell then it would clearly result in an interference with the N-demethylase assay if it occurs simultaneously in the microsomal preparations used. The product of any O-demethylation reaction involving either metoxuron or N-monodesmethylated metoxuron would be non-radioactive, and since cleavage of the 4-(^{14}C)methoxy group was demonstrated previously in the present studies using leaf discs to occur at a five to six-fold greater rate in resistant wheat, the amount of N-monodesmethylated metoxuron detected in the microsomal assays for this latter variety may have been reduced relative to that measured for the susceptible variety by a corresponding order of magnitude. Consequently, these studies on the sub-cellular location of metoxuron-N-methylation activity should be repeated using a metoxuron sample labelled with ^{14}C in an alternative position so that this particular point may be clarified. Furthermore, it would be particularly interesting to know whether the enzyme system responsible for metabolism of metoxuron is a novel one or whether the observed activities can be attributed to enzymes of known metabolic pathways in plants. An attempt should also be made to obtain more information on the specific activities of the detoxifying enzyme systems in susceptible and resistant wheat plants from different parts of the world (to avoid

misinterpretation of data from such a study involving only single examples of resistant and susceptible wheat varieties) and also in the cereal weeds, Avena spp and Alopecurus myosuroides, in an attempt to correlate enzyme activity with susceptibility or resistance to the phenylurea in field trials. Inheritance studies (Tottman et al, 1975) have demonstrated a distinct segregation in the progeny of crosses between resistant and susceptible wheat varieties, implying that herbicide tolerance is simply inherited and that it should be possible to include selection for tolerance of metoxuron and chlortoluron in a wheat breeding programme. If such a correlation can therefore be shown to exist it should be possible to develop a rapid screening test based on enzyme levels to assist in such plant breeding programmes.

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