PHYTOTOXICITY OF PHTHALATE PLASTICISERS

by

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ABSTRACT

This project arose from problems of phytotoxicity caused by some samples of polyvinyl chloride (PVC). The toxin was identified as butyl phthalate plasticisers and it was shown that radish plants exposed to 3 $l.min^{-1}$ of air containing 160-180 $\mu g.l^{-1}$ of dibutyl or diisobutyl phthalate (DBP or DIBP) developed chlorotic new leaves in 3 days and died within 10-12 days.

Novel damage to glasshouse tomato plants was diagnosed as due to DIBP plasticiser volatilising from PVC glazing strip. Following laboratory bioassays it was recommended that this strip be replaced with one plasticised with diisodecyl phthalate. A case history during seasons 1980-84 show how crop losses valued at around £20000 per acre per year of tomatoes was remedied when the glazing strip was changed.

Chloroplasts of DBP-treated plants were swollen, possessed many plastoglobuli and lacked starch grains. In young chloroplasts the grana failed to develop, while in mature chloroplasts they appeared to be pushed to one side.

Measurements of CO_2 exchange in young plants, using an IRGA, demonstrated that photosynthetic rates were reduced in plants treated with DBP-plasticised PVC, or with the pure liquid plasticiser. Respiration was virtually unaffected.

CO_2-dependent O_2 evolution, measured using a leaf disc electrode, was reduced in both young and mature leaves after treatment with DBP, but results were variable in the latter.

Chlorophyll a_2 fluorescence yields increased in DBP-treated leaves. Quenching of this fluoresence was lost. State 1 - State 2 changes in treated leaves were often impaired.

Electron transport in isolated spinach thylakoids, measured using an oxygen electrode, was severely inhibited by DBP and DIBP - 50% inhibition by 44µM DBP and 42µM DIBP. Inhibition by other dialkyl phthalates was very weak. Electron transport was inhibited at a site near the reducing side of photosystem 2.

These results suggest that a major site of action of the butyl phthalates lies within the chloroplast and that they inhibit photosynthesis. This conclusion is discussed in relation to the work and ideas of other researchers.

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ABBREVIATIONS

ADAS	Agricultural Development Advisory Service
ATP	Adenosine 5'-triphosphate
DBP	Dibutyl phthalate
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
DEHP	Diethylhexyl phthalate
DIBP	Diisobutyl phthalate
DIDP	Diisodecyl phthalate
DIOP	Diisooctyl phthalate
DMP	Dimethyl phthalate
DNA	Deoxyribonucleic acid
DOP	Dioctyl phthalate
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscope
ESBO	Epoxidised soya bean oil
F	Yield of fluorescence emission
FER	Fluorescence enhancement ratio
GC	Gas chromatograph
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
ICI	Imperial Chemical Industries
IRGA	Infrared gas analyser
I_{50}	µM concentration required for 50% inhibition of chloroplast electron transport
LHCP	Light Harvesting Chlorophyll Protein
MeOH	Methanol
MeV	Methyl viologen

NVRS	National Vegetable Research Station
PQ	Plastoquinone
PVC	Polyvinyl chloride
Q	Quinone
Q_E	Energy-dependent chlorophyll fluorescence quenching
Q_Q	Q-dependent chlorophyll fluorescence quenching
PAR	Photosynthetically active radiation
PS 1	Photosystem 1
PS 2	Photosystem 2
SE	Standard error

1. INTRODUCTION

1. INTRODUCTION

1.1 Plastics And Society

Plastics play an important role in our lives, since most things we use contain plastic of some sort. Plastics find uses in construction, car and household products, children's toys, packaging, clothes, scientific equipment, medical and horticultural products and many others. By far the most common plastic is polyvinyl chloride (PVC).

PVC is available in rigid or flexible forms. The project reported in this thesis was concerned with the observation that some flexible PVC formulations are phytotoxic.

1.2 History

The earliest reference to the phytotoxicity of some flexible polyvinyl chloride (PVC) compositions was noted in the annual report of the National Vegetable Research Station (NVRS) Wellesbourne in 1968 (Austin *et al.*, 1968). Severe chlorosis and stunting occurred in cauliflower seedlings, grown in Saxil controlled environment cabinets following the introduction of some ordinary plastic hosepipe. Since such cabinets contain many other plastic components it was decided to test all these plastics for

phytotoxicity.

A series of experiments were undertaken to examine this problem. These took the form of a) vapour tests - in which growing cauliflowers and pieces of plastic were placed in glass flasks plugged with cotton wool and b) solution tests - in which the plastic under test was mixed with vermiculite. A number of common plasticisers and stabilisers - exactly which is not disclosed in the report - were tested in similar experiments. These workers "established that the commonly-used plasticisers in flexible PVC were non-phytotoxic in these tests." They also concluded "that neither the PVC itself nor the commonly-used plasticisers were the cause of the symptoms observed in these tests, and that either some other additives or degradation products of PVC were the phytotoxic agents." Similar experiments, using a variety of plastics demonstrated that rigid PVC, polyethylene, polypropylene, nylon, ABS and certain other flexible PVC products were not phytotoxic.

During 1974, undergraduate students at Imperial College engaged in pollution experiments to assess the effects of sulphur dioxide (SO_2) and ozone (O_3) on radish plants, inadvertently rediscovered the phytotoxic property of certain flexible PVC compositions. Groups of two-week-old test plants were grown in separate, perspex growth chambers (2'x 2'x 2') through which air was passed at 200 $l.min^{-1}$ from an air compressor. Pollutant gases were added

to the incoming air of certain growth chambers, the control receiving none. Within seven days it was apparent that all plants, including those in the control growth cabinet, were unhealthy - chlorosis being observed in the younger leaves. Most plants had died by the fourteenth day. The source of this phytotoxicity was eventually attributed to lengths of clear, flexible PVC wide-bore tubing (4ft x 1.5") which were conducting air from the compressor to the growth chambers. This observation was important because it implied that the phytotoxin was a volatile substance, since the plastic itself never came into physical contact with the test plants. This tubing was still emitting a phytotoxin in 1981 when it was retested; the sample was then at least 10 years-old so the volatile constituent is not quickly given off - the toxicity is likely to last throughout the useful life of the plastic.

Around 1976 B.A.S.F. published an undated advertising leaflet entitled -"Plasticised PVC in Horticulture"- which only came to our notice in 1980, following Dr. Hannay's note in The Grower. This information leaflet for horticulturists describes experiments in which plants were grown in temporary greenhouses, covered with PVC film plasticised with different phthalates. A similar greenhouse was covered with polyethylene sheeting. The original test plants were a mixture of cauliflower, radish, kohlrabi and lettuce; followed by a crop of tomatoes. The trials showed that Cruciferae (radish,

kohlrabi and cauliflower) were severely damaged by only one particular plastic. This plastic had been plasticised with DBP. The lettuce appeared to be immune. Yields of tomatoes appeared to be reduced under PVC sheeting containing DBP but no replication nor statistical data were included. Requests to B.A.S.F. for further information on these results elicited the reply that there was no further data, since the original experiments were only done in response to litigation following claims of damage to plants from plasticised PVC. Apparently the work had not been published anywhere except in the advertising leaflet - many years later (personal communication, 1980). B.A.S.F. concluded that the phytotoxicity of flexible PVC was due to particular plasticisers. This conclusion conflicted with that of the N.V.R.S.(Austin *et al.*, 1968).

1.2.1 Undergraduate Projects on PVC Phytotoxicity

The findings of the N.V.R.S. and the undergraduate pollution experiments were followed up by a series of short student projects. The results from these were not conclusive in identifying the compound(s) responsible for the observed phytotoxicity. This was due, in part, to a lack of certainty about the formulations of some of the PVCs tested; the different sensitivities of different plant species to the phytotoxin; and occasional contradictory evidence. These projects have emphasised that it is only certain formulations of flexible PVC which are phytotoxic. Moreover some species of plants appear

immune to the phytotoxin, while members of the Cruciferae appear to be particularly susceptible.

A warning, aimed primarily at commercial horticulturalists, was published in the Grower (Hannay, 1980) in order to alert them to potential problems arising from the use of certain PVC materials. This followed a serious crop failure suffered by a North Humberside grower in March 1979 who had to uproot 8500 four-month-old tomato plants in one of his greenhouses. This house contained a new plant training and support system. Subsequent tests demonstrated that a black, flexible PVC strip was responsible. This PVC strip lined grooves in the metal strips which were being used to support the plants.

The experiments reported in this article consisted of exposing plants - radish (var. Cherry Belle) or tomato (var. Sonatine) - to the vapour from two samples of PVC. The black PVC from the North Humberside grower was very toxic to both radish and tomato, while the other, older, clear PVC sample only proved injurious to the radish plants. Moreover the visible damage to radish plants was different to that sustained by tomato. Radish leaves which grew in the presence of the black plastic showed marked yellowing after five days exposure, whilst new emergent leaves were almost white after fourteen days. Treated tomato plants showed little or no chlorosis but the young leaves went a brownish or khaki-green colour. Also growth of these plants was reduced to about one-fifth that of

untreated control plants. These experiments failed to establish whether the results were due to differing sensitivities of the test plants to a single toxin or whether the plastics contained different toxins. Further experiments again demonstrated marked differences between species. All Cruciferae tested - cabbage, cauliflower, sprout and turnips as well as radish - proved to be sensitive to the old, clear tubing; severe chlorosis being followed by death over a period of three to four weeks. In a simultaneous exposure to this tubing cucumber, tobacco, rye grass and nettle appeared to be unaffected.

A computer search of the literature in 1975 revealed no references to PVC effects on plants, but a further search in 1981 was more productive because it also included phthalates as a keyword.

Gudin and Harada (1974) reported that certain fractions isolated from petrol had the ability to inhibit the elongation of the coleoptile of wheat and the mesocotyl of oats. In addition these fractions retarded the senscence of the leaves of *Rumex obtusifolius*. Use of mass spectrometry identified two substances - *n*-dibutyl phthalate and *n*-dioctyl phthalate.

A report by Buta (1975) describes the effects of spraying emulsions containing substituted phthalate esters on several plant species. Twenty-nine compounds were tested. A number of these compounds exhibited the classical PVC

toxicity symptoms seen in our work - namely stunting of growth and chlorosis in the developing part of the plants.

Another piece of work which has only come to light in 1983, although not via the computer search because these do not include most Experimental Station Reports, is that by Inden and Tachibana (1975). These workers undertook an extensive investigation into the toxicity of PVC formulations used in horticultural environments in Japan. Nowadays the Japanese use over 1/4 million tons of plasticised PVC film each year in greenhouses and for horticultural mulching. However it seems very likely that in the early 1970's they too became aware of the liability to toxic side effects from certain samples of PVC. The work involved the laboratory testing of an enormous variety of samples of PVC film of differing formulation together with individual sampling of the numerous components which go to make this film. Also the degree of polymerisation in the PVC was varied. Plasticisers tested included phthalates, adipates, sebacates, phosphates, together with a miscellaneous selection. A correspondingly wide variety of stabilisers was also tested: metal soaps and inorganic stabilisers, organometallic stabilisers, composite stabilisers and a miscellaneous selection. Phytotoxicity was attributed to vapours from commercial samples of diisobutyl phthalate (DIBP), dioctyl adipate, diisodecyl adipate, butyl oleate and dibutyl phthalate (DBP) plasticisers; triphenyl phosphite, monolauryl diphenyl phosphite and composite organic stabilisers. PVC

sheet compounded with these materials also caused phytotoxicity. Most plasticisers and stabilisers which did not injure crops at normal temperature caused damage when they were heated to 100 - 200°C in a fumigation chamber. Again this work was not published for wide circulation in a journal of international circulation but was 'buried' in an obscure experimental station bulletin - presumably because it had commercial sensitivity.

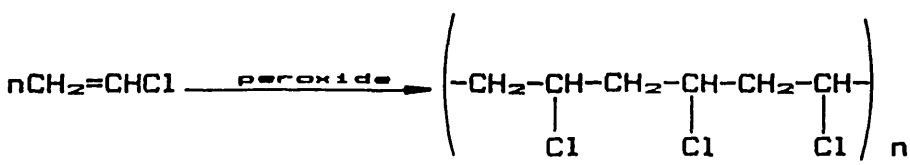
Very recent work undertaken in Sweden (Virgin *et al.*, 1981) again implicates DBP (or an impurity) as a phytotoxic agent. Test plants - blue bells (*Browallia speciosa* L. cv. Major) and radish seedlings (*Raphanus sativus* L. cv. Cherry Belle) - were subjected to the vapour from samples of liquid DBP. The visible phytotoxic symptoms seen in these experiments were that leaves formed during treatment were white while older leaves appeared not to be affected. However after a longer period of treatment - more than one week - the whole plant showed chlorotic symptoms. The plants then finally died. Chlorosis of older, fully expanded leaves has not been observed in experiments undertaken at Imperial College, except after prolonged exposure of three or more weeks in very dilute toxic atmospheres. Then the effect may well have been due to natural or slightly accelerated senescence, rather than a direct affect of the toxin on the leaves.

1.3 Composition of Flexible PVC

The components essential to all flexible PVC formulations are polyvinyl chloride resin, a plasticiser and a stabiliser. Numerous other additives may be included in the complete formulation. The precise nature of these additions will be determined by the intended use and environment of the finished PVC article. Some are added to protect the PVC from degradation during manufacture and to aid processing. These additions include lubricants, pigments, dyes, anti-oxidants, ultraviolet absorbers, fillers, flame retardants, blowing agents, antistaining agents, fungicides, bactericides, pesticides, optical brighteners and antistatic agents (Sears & Darby, 1982).

1.3.1 Polyvinyl Chloride Resin

The polyvinyl chloride polymer is composed of a vinyl chloride monomer repeating unit:- $[CH_2\text{-}CHCl]_n$ where n (reflecting the degree of polymerisation) normally has a value between 500 and 2000. The monomer is converted by free radical polymerisation to the polymer; any number of free-radical producing initiators may be used to start or to carry out the polymerisation as desired.



The terminating groups of the polymerised molecule are fragments of the initiator molecule, chain transfer agents, or miscellaneous impurities deliberately or inadvertently present (Penn, 1971). Examples are:

$-CH=CHCl$; $-CCl=CH_2$; $-CH_2OH$;

$-CH_2OCC_6H_5$; $-CH_2OSO_2OR$ (where R =alkyl group)

A small amount of branching is normally present, the degree depending in part on the polymerisation temperature. Branching is increased with the use of different polymerisation initiators and by chain transfer agents such as chloroform. Commercial PVC homopolymers normally contain impurities, which may be up to at least 10%, such as surfactants, protective colloids, and stabilisers.

Many commercial PVC resins are copolymers with vinyl acetate (by far the most commonly used), vinylidene chloride, diethyl maleate, di-2-ethylhexyl fumarate, maleic anhydride, itaconate esters, ethene, or related comonomers. The ester comonomers promote internal plasticisation by side chain effects, while the nitrile group increases the hydrogen bonding ability for fiber formation. Comonomers such as ethene and propene add internal plasticisation by increased flexibility in the polymer backbone.

The PVC resin is purchased in the form of small particles. The polymerisation process used determines the size of these particles. The process used also determines the surface characteristics and porosity of these particles. The nature of these physical properties will in turn contribute to differences in the finished plastic article even though all subsequent additives may be identical.

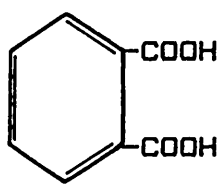
1.3.2 Plasticisers

Plasticisers, as the name suggests, are used to convert the hard rigid PVC resin into a compound which is both flexible and comparatively soft. This enables the PVC to be worked i.e. extruded, moulded and ca lendered. Plasticisers also modify or confer new properties on the PVC resin. Their widespread use and large production (2 million tonnes yearly; US Environmental Protection Agency 1980) together with their ubiquitous nature has raised concern about possible environmental effects.

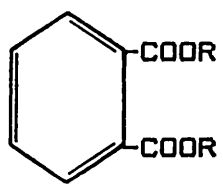
The most commonly used plasticisers are the family of dialkyl phthalates. These are esters of phthalic acid (1,2-dibenzocarboxylic acid).

The alkyl group may be straight chained or branched. Di-2-ethylhexyl phthalate (DEHP), the best general purpose plasticiser for PVC, accounts for about a quarter of the world's production. A further quarter of the market is cornered by the closely related plasticisers diisooctyl

phthalate (DIOP) and diisodecyl phthalate (DIDP). In recent years the linear and near-linear phthalates have come into favour by offering superior performance at only slightly increased prices. The latest and most popular of these is heptyl nonyl undecyl phthalate (Sears and Darby, 1982).



phthalic acid



dialkyl phthalate

(R = alkyl group derived from an alcohol)

Industrially, phthalate esters are prepared from phthalic anhydride and the appropriate alcohol.The purity of esters from modern esterification processes range from 99.70% to 99.97% (Graham, 1973).



The plasticiser may constitute up to 40% of the total weight of the plastic.

1.3.3 Stabilisers

Both rigid and flexible PVCs are very susceptible to degradation by heat, light and oxidation. The mechanisms of degradation are complex and not yet fully understood. Stabilisers are used to prevent, or at least retard, these processes. A stabiliser's properties should include the ability to adsorb HCl; react with and inactivate degradation products and impurities; provide antioxidant protection and ultraviolet screening. No single stabiliser possesses all these properties and therefore they are normally used in combination. Some of these combinations have been found to have synergistic properties. The stabilisers employed in a particular formulation will depend in part on the other compounds being used, together with the intended use of the finished article - both the mechanical properties required of it and the type of weathering it will have to withstand. Stabilisers sometimes have lubricant properties.

Many stabilisers are metallic salts of organic acids or phenols. Most commonly employed are mixtures of barium and cadmium salts sometimes with a small amount of zinc. Mixtures of calcium and zinc salts are used when the stabilising system needs to be non-toxic, e.g. 'food grade' PVC film. Lead soaps and other lead salts including phosphites are used when good electrical insulation properties are required. Organo-tin compounds have a measurable plasticising ability in addition to their

stabilising properties. More rarely used are the compounds of other metals such as lithium, aluminium, magnesium and antimony. Epoxidised fats and oils, such as soya and linseed, epoxidised fatty-acid esters and epoxy "resins" such as bisphenol-A-diglycidyl ether are frequently used in conjuction with the metal compounds mentioned above as they confer additional synergistic properties on the stabilising system. Some of the compounds above possess other properties in addition to their stabilising activity. The metal salts frequently act as lubricants while the epoxidised natural oils and the tin compounds have the ability to aid plasticisation of rigid PVC. These compounds are normally present at a concentration of 2% or less in the finished plastic. These stabilisers are usually bought from the manufacturers as a package.

1.4 Aim of Project

The primary objective of this research project was to establish the identity of the phytotoxin present in some formulations of flexible PVC. The proposed method involved screening a number of PVC compounds, of precisely known formulation, for phytotoxicity. The components involved in compounding these samples would also be screened for phytotoxicity. The remainder of the project was to be devoted to elucidating the site and mode of action of the identified phytotoxin.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Screening Of Plastics For Phytotoxicity

2.1.1 Plastics

The plastics tested in the experiments described in this thesis came from one of two sources. Plastics of known formulation were kindly provided by Imperial Chemical Industries (ICI), Plastics and Petrochemicals Division. The origins of the other plastics were varied. Some were received from Agricultural Development Advisory Stations (ADAS), while most of the others were provided by commercial horticulturalists whose crops appeared to be suffering damage due to an unknown phytotoxin. Many of these plastics were chemically analysed by the Technical Services Department at ICI.

2.1.2 ICI Plastics

The various formulations of flexible PVC rod, prepared for this project by ICI, were made to our specifications. Samples of each of the individual components, from the identical batches used for the formulation of the plastics, were also supplied for separate assessment.

The table below makes use of the plastic's industry's

Table 2.1 Formulations of plastics supplied by ICI. A to G - first batch; H to M - second batch. Plastic supplied as rods, approximately 6mm in diameter.

	PLASTIC						
FORMULATION	A	B	C	D	E	F	G
S67/111	100	100	100	100	100	100	100
DBP	-	42	-	-	42	-	-
DOP	-	-	42	-	-	42	-
ESBO	-	-	-	42	-	-	42
Stearic Acid	0.5	0.2	0.2	0.2	-	-	-
Barium Cadmium-1	1	1	1	1	-	-	-
Barium Cadmium-2	1	1	1	1	-	-	-
Calcium Zinc	-	-	-	-	2.5	2.5	2.5

	PLASTIC					
FORMULATION	H	I	J	K	L	M
CORVIC S71/102	100	100	100	100	100	100
DIBP	60	-	-	-	-	-
DIOP	-	69	-	-	-	-
DIDP	-	-	76	-	73	-
DNP	-	-	-	75	-	74
ESBO	-	-	-	-	3.0	3.0
Calcium Zinc	-	-	-	-	2.0	2.0
Stearic Acid	-	-	-	-	0.2	0.2
Tri-basic Lead Sulphate	4.5	4.5	4.5	4.5	-	-

ESBO - Epoxydised Soya Bean Oil

standard system for quantifying formulations. The formulation of a compound is expressed on the basis of 100 parts by weight of polymer. The numbers for the rest of the ingredients refer to parts per hundred (phr) of resin. Plastics A to G comprised the first batch of PVC samples supplied by ICI. Plastics H to M the second batch.

2.1.3 Plants

Radish seeds - *Raphanus sativus* L. cv Cherry Belle - were germinated and subsequently grown in a 50:50 mixture of Levingtons Potting Compost and John Innes No.2 Compost. After seven days the seedlings were transplanted to small seed trays (220mm. x 165mm. x 50mm., Transatlantic Plastics). Each tray accommodated 9 plants in three rows of three. These plants were grown in a greenhouse at 25°C under natural light conditions. During winter months natural light was supplemented using 400W mercury vapour lamps.

2.1.4 Exposure Of Plants To PVC Vapour

The apparatus is shown in Fig.2.1 The air flow to each chamber was provided by an oil-less rotary vane air compressor (Gast Model 0240, Gast Manufacturing Company Ltd). Organic pollutants were removed by passing the compressed air through an activated charcoal filter. The air line was then divided and passed to two flowstats (Platon Flostat MNBM, Platon Flowbits Ltd) with flowmeters

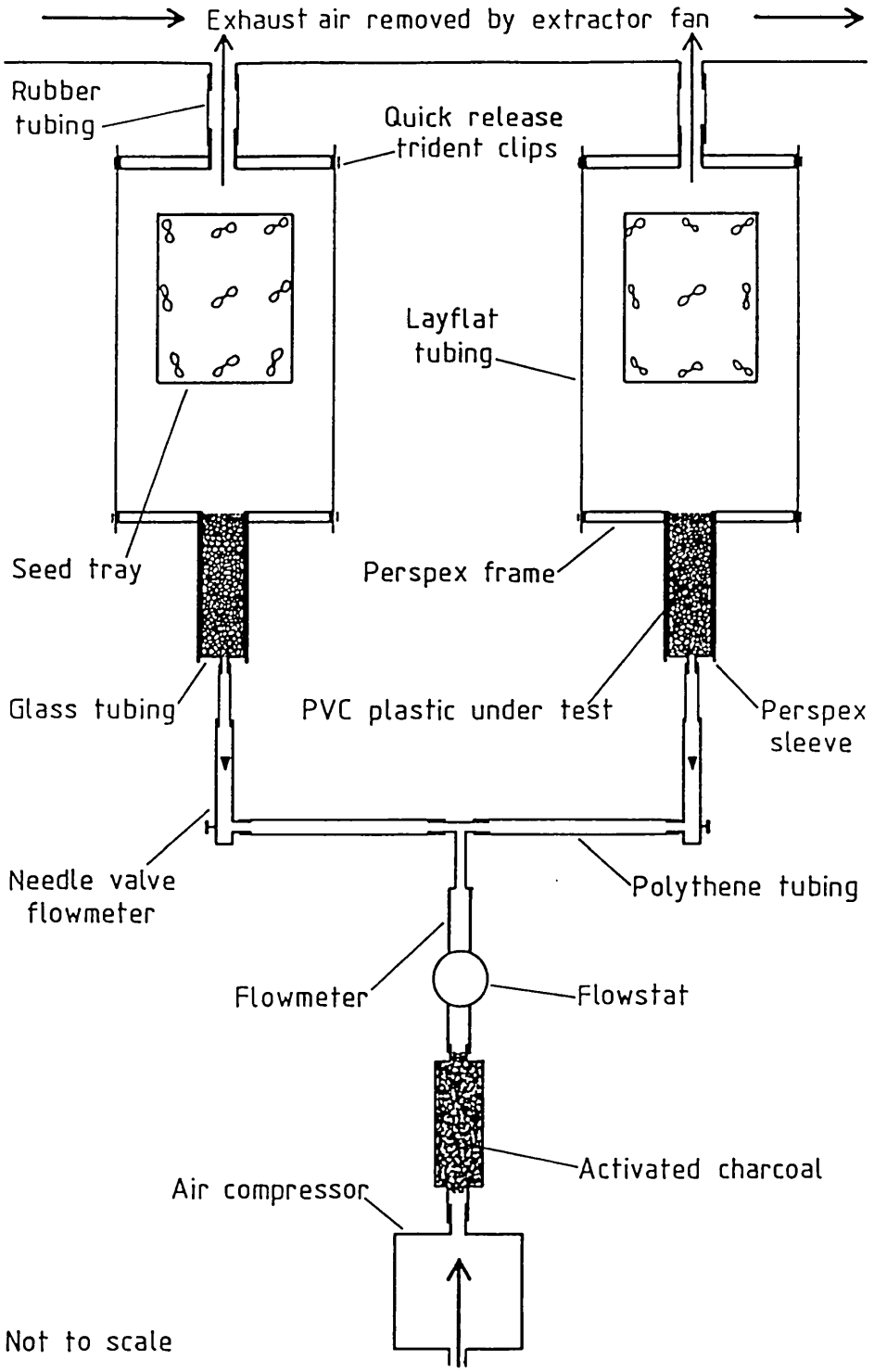


Fig.2.1 Diagram of the bioassay equipment used to expose test plants to the vapours of plastics and the individual components of plastics. This apparatus was situated in a constant temperature growth room at 22±2°C. Air flow through each growth chamber was 3 l.min^{-1}. Volume of growth chambers was approximately 15 l.

attached. Airflow at this point was approximately 15 $l.min^{-1}$. Each airline then went to one of two benches where it was divided into four air lines. These were connected to four needle valve flowmeters (Platon Gapmeter GSAV TPX, Platon Flowbits Ltd). At this point air flow was reduced to 3 $l.min^{-1}$. The purified air then flowed through a glass tube packed with the plastic under test and then passed immediately over the test plants. The plants were contained within a 'flexible tunnel' made from polythene layflat tubing (12" wide, Transatlantic Plastics) - see Fig.2.1. Situated at each end of the layflat tubing were discs of perspex. The disc at the inlet end also incorporated a perspex cylindrical sleeve which covered the glass containing the plastic under test. A rubber O-ring sealed the gap between the glass and perspex. The disc at the other end had a central hole through which the contaminated air left the growth chambers and was ducted directly to an extractor fan exhaust system and thence outdoors. The positive pressure provided by the air flow from the compressor prevented the layflat tubing from collapsing on the plants. All the flexible tubing used on the inlet side of the layflat tubing growth chambers was made of polythene; while the Y-adaptors were made of polypropylene. Rubber tubing and polypropylene adaptors were used on the exhaust side of the chambers. The polythene 'tunnels' were renewed each experiment to eliminate the possibility of spurious results arising from contamination. Also the perspex discs were washed with a weak detergent solution between experiments.

A photograph of the equipment used to expose test plants to the vapour volatilising from PVC plastics is shown in Fig.2.2 below.



Fig.2.2 Photograph of the bioassay growth chambers.

The plant growth chambers were arranged in groups of four and illuminated with nine 8 ft fluorescent tubes and four 60W tungsten bulbs. These provided a photon fluence rate of 185 $\mu E.m^{-2}.sec^{-1}$ (photosynthetically active radiation - PAR) inside the layflat tubing growth chambers. Photoperiod was of 18 hours duration. The temperature within these growth chambers was 27°C during the light

period. This dropped to 22°C in the dark. Air was drawn over the enclosed lights by an extractor fan to reduce heating effects.

The apparatus was located in a 22±1°C constant temperature room.

2.1.5 Growth Analysis

2.1.5.1 Sampling Regime

The trays of test plants were numbered from left to right, front to back; the front of the tray being that end closest to the plastic under test. At each sampling time three plants were selected for analysis - one plant from each row and column. Hence: day 3 - plants 1,6 and 8 were selected; day 6 - 2,4 and 9; day 10 - 3,5 and 7. This order of sampling was employed for all experiments. The idea of sampling from each row and column was to reduce to a minimum the effects of the plants being different distances from the plastic under test.

2.1.5.2 Fresh weights

The plants to be sampled were removed from their seed tray, the tap root rinsed in tap water, then gently dried with tissue paper. The remains of the fine root system were removed before drying. The plant, including the attached tap root were then weighed in a pre-weighed piece

of aluminium foil which was then folded loosely around the plant to form a packet.

2.1.5.3 Dry Weights

Following fresh weight measurement the plants, in their aluminium foil jacket, were placed in a 60°C incubating oven to dry to constant weight, normally 48 to 96 hours.

2.2 Porometry

Stomatal measurements were made using a Delta T Device's Porometer Mk 2.

2.3 PAR Measurement

Measurements of photosynthetically active radiation were made with a Li-Cor Quantum Meter, Model LI 185.

2.4 Gas-Liquid Chromatography

This technique was employed to determine the atmospheric concentration of phthalate plasticisers within the growth chambers.

2.4.1 Gas Chromatograph

A Pye Series 104 Chromatograph Model 4 fitted with a flame ionisation detector was used. The chromatographic column was a glass column (0.5M x 2 mm i.d.) packed with 3% Dexsil 300 on 80-100 mesh Chromosorb G. The oven temperature was normally 230°C.

Carrier gas was oxygen-free nitrogen - flow rate 40 $ml.min^{-1}$; hydrogen flow rate - 40 ml.min-1. Both the nitrogen and hydrogen gas cylinders were fitted with a gas purifying bottle.

The electrical output from the gas chromatograph was fed to one channel of a Servogor 120 dual channel recorder.

2.4.2 Sampling Tubes

These consisted of thoroughly cleaned glass tubes (90 x 4 mm i.d.). Cleaning was achieved by first rinsing the tubes in cyclohexane (all cyclohexane used was Fisons Spectrograde) and then heating the tubes to approximately 300°C in the gas chromatography (g.c.) oven for 4 hours. One end of each glass tube was marked with a diamond marker.

2.4.3 Tenax GC

Tenax GC (poly(2,6-diphenyl-*p*-phenylene oxide) 60-80 mesh

(Phase Sep) was used to pack the cleaned glass tubes. The Tenax was preconditioned prior to use by heating at 300°C. in a g.c. column while flushing with oxygen-free nitrogen gas (40 $ml.min^{-1}$) for a period of 24 hours.

2.4.4 Preparation of Sampling Tubes

A plug of quartz wool (Thermal Syndicate Ltd., Wallsend, Tyne and Wear) was placed 20 mm from the unmarked end of the tube. 200 mg of preconditioned Tenax was loaded into the tube. The tube was then gently tapped in order to encourage the packing to settle. The other end (marked end) was plugged with quartz wool.

The tubes were purged with a nitrogen flow of about 100 $ml.min^{-1}$ at 300°C for 10 minutes immediately prior to use. The tube ends were capped with flexible caps fashioned from polythene tubing.

2.4.5 Regeneration of Used Sample Tubes

Following desorption (see Section 2.4.7) the tubes were purged at room temperature with a nitrogen flow of about 200 $ml.min^{-1}$ for 15 minutes. The tubes were then purged at 300°C for about 10 minutes with a nitrogen flow of about 100 $ml.min^{-1}$ and then recapped. If stored, the tubes were further purged at 300°C for 10 minutes with a nitrogen flow of about 100 $ml.min^{-1}$ before use.

2.4.6 Sampling of Growth Chamber Atmosphere

The volatile phthalate esters were collected by drawing known volumes of air through the Tenax packed glass tube. The growth chamber atmosphere was normally sampled for a period of 48 hours. At the end of the sampling period the total volume, flow rate and the time were recorded. Following removal from the growth chamber the ends of the tube were capped. The phthalate was then desorbed with a solvent and the quantity collected determined by gas chromatography.

A diagram of the apparatus used to sample the growth chamber atmosphere is shown in Fig.2.3. The glass tube, which was packed with the adsorbent, was situated in a hole in the perspex disc at the exit end of the growth chamber just below the exhaust hole. It was connected to an externally placed vacuum pump via silicone rubber tubing. The vacuum pump was an aquarium pump (Petcraft, Model 4400) which had been modified to provide a nozzle for air uptake. The pump was attached to the unmarked end of the sampling tube (see Section 2.4.2). Located after the pump was equipment to monitor and adjust the air flow rate, together with a meter to record the total volume of air which passed through the tube during a sampling period. A dry type gas meter located immediately after the vacuum pump was used to record the total volume of air which passed through the tube. The meter measured the volume in cubic metres to three decimal places. Flow rate

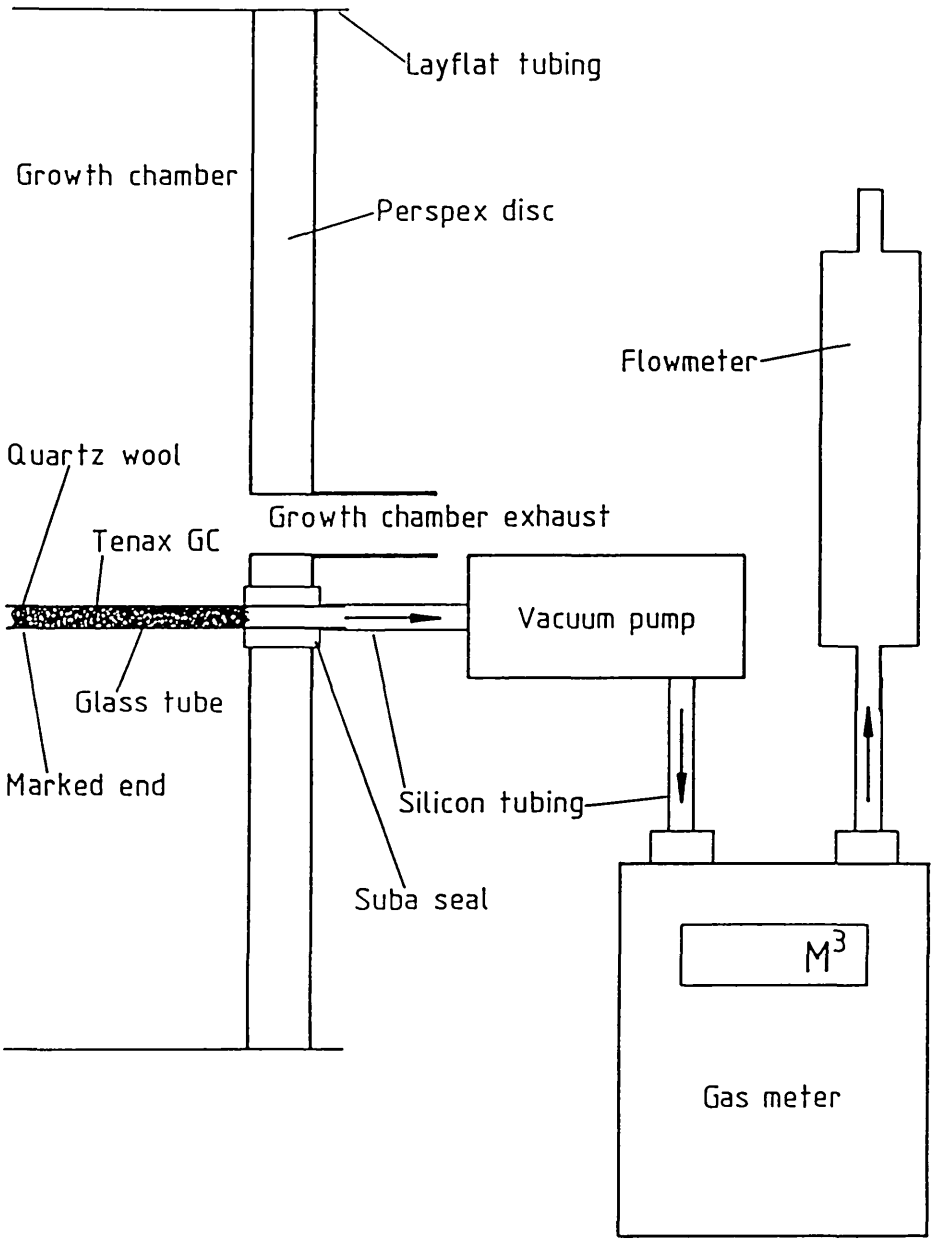


Fig.2.3 Diagram of the apparatus used to sample the atmosphere of the growth chambers. (Not to scale).

was monitored by a flowmeter (range 0-600 $ml.min^{-1}$, Platon Flowbits). Adjustments to the flow rate were made using two Hoffman clips on the outlet side of the gasmeter. The flow rate was adjusted so that it was of the order of 180-200 $ml.min^{-1}$.

2.4.7 Desorbtion of Phthalate

With the end caps removed the tubes were positioned with the marked end pointing vertically down. Into the open upper end 0.5 ml of cyclohexane was poured. This was allowed to percolate completely, the eluate being collected in a graduated sample vial (micro-vials, Camlab Limited). This operation was then repeated using two further 0.5ml portions of cyclohexane. The total volume of eluate collected was not allowed to exceed 1 ml. The vial was immediately stoppered to prevent loss of contents from evaporation. Desorbed tubes were regenerated as described in Section 2.4.5.

2.4.8 Calibration

A standard 5% w/v solution was prepared for each phthalate in cyclohexane - this will be referred to as Solution C in the calculations shown later in this section. Each of the four sampling tubes were fitted, in turn, with an injection adapter at the marked end of the tube. While the tube was purged with nitrogen at about 200 $ml.min^{-1}$, 1µl, 2µl, 5µl, and 10µl aliquots of Solution C were injected

into the individual tubes respectively via the injection adapter. The nitrogen purge was maintained for 5 minutes after which the adapter was removed and both ends of the tube were capped. The phthalates in the tubes were then desorbed with cyclohexane as described in Section 2.4.7. A 5µl aliquot of each calibration solution was injected in turn into the gas chromatograph.

The peak areas (A_c) on the resulting chromatograms were measured in standard peak area units. Each peak area was then divided by the respective volume (v) originally injected into the tubes. For the calibration graph (see Appendix 1) this value (A_c/v) was plotted against the volume (v). The line of best fit was determined using regression analysis. The plotted graph line was extrapolated to the y-axis. The ordinate value, Y, was the mean area response for 1µl of the standard phthalate solution in 1 ml eluate solution with a 5µl injection into the gas chromatograph.

2.4.9 Calculations

Where P = grams of phthalate per 100ml Solution C;
and under the above conditions
1 Area Unit corresponds to $\frac{P \times 10^{-2}}{Y}$ mg phthalate
= calibration factor = f

Samples collected from the growth chambers were solvent desorbed with cyclohexane in the same way and a 5µl

aliquot of each sample was injected into the gas chromatograph.

Amount of phthalate injected into gas chromatograph = area of peak (A_P in standard peak area units) x f = mg of phthalate

Aerial concentration of phthalate in growth chamber:

$$\frac{A_P \times f \times 10^6}{V} = ng.l^{-1}$$

Where V is the atmospheric sample volume, in ml.

2.4.10 Precautions

Great care was exercised in ensuring that no contamination occurred during the procedure. Problems with phthalate ester contamination are widely reported in the literature, during: analysis of biological samples (Giam *et al.*, 1975a); analysis of herbicide residues (Giam *et al.*, 1975b); pesticide research (Crosby and Singmaster III, 1973); and in aerospace technology (Gross and Colony, 1973). All glassware was washed in cyclohexane (Fisons Spectrograde) and heated in the chromatograph oven at 230°C for a period of several hours immediately prior to use. Polythene end caps for the sampling tubes were left in a 60°C incubator oven until used.

2.5 Solution Culture

2.5.1 Plants

Radish seeds - *Raphanus sativus* L. cv. Cherry Belle were germinated on wet filter paper in an inverted seed box. After 4 days the young radish plants were transferred to solution culture. The plants were then grown in a greenhouse.

2.5.2 Solution Culture Apparatus

Hewitt's solution culture (Hewitt, 1964) - 'low' nitrogen formula - was used as the growth medium. The apparatus to hold the plants consisted of small Kilner jars which had previously been painted matt black. A stainless steel mesh was used in place of the lid. This mesh was covered with tin foil. Small holes were punctured in the tin foil to allow the roots of the radish seedlings to pass through - four radish plants were placed in each jar. The Kilner jar was filled with Hewitt's medium (approximately 380 mls). The young radish plants were left to grow for a period of 6 days after which drops of pure DBP (either 2,5,10,20,40 or 60) from a teat pipette were added to the solution.

2.6 Electron Microscopy

2.6.1 Plants

Radish plants were grown as described in Section 2.1.3. The plants were then exposed to phthalate plasticiser vapour in the bioassay system described in Section 2.1.4. Plants were exposed to the DBP plasticiser volatilising from approximately 150g of plastic E.

2.6.2 Sampling Of Specimens

Leaves were removed from treated or untreated plants and then a small piece of leaf (approximately 2-3 mm^2) was excised from a region halfway along the lamina between the midrib and the leaf margin. This was placed immediately in a solution of 2.5% glutaraldehyde (EM grade, EM-scope) fixative in a 0.1M cacodylate buffer. The exised piece of leaf was then cut into smaller pieces under the glutaraldehyde (approximately 0.25 mm^2 in area). The cut pieces of leaf were then fixed for 24 hours at room temperature in embryo cups.

2.6.3 Specimen Preparation

Specimens for electron microscopy were prepared by the following method subsequent to fixation in glutaraldehyde:

1) Three 20 minute washes in 0.1M cacodylate buffer.

2) Post fixation for 90 minutes in 1% osmium tetroxide in 0.1M cacodylate buffer.

3) Dehydration in ethanol:

   i) series of 10 minute washes in increasing concentrations of ethanol (10% to 90% in 10% increments).

   ii) Two 15 minute steps in 95% ethanol

   iii) Three 20 minute steps in 100% ethanol.

4) Embedding:

   i) Impregnation with 50% epoxypropane in ethanol for 30 minutes

   ii) 100% epoxypropane for 30 minutes

   iii) 100% epoxypropane : Epon* (1:1). Cups were covered for 60 minutes and then left open for a further 60 minutes at room temperature. Then left overnight at 4°C.

   iv) Four changes of resin (Epon) over 3 days at 4°C.

   v) Specimens were then placed in fresh resin which was allowed to polymerise at 60°C for 48 hours.

5) Sectioning. Sections, cut on a LKB 4801A ultramicrotome, were 60 nm thick and were picked up on formvar coated 200 mesh grids.

6) Positive Staining. Sections were stained with 2% uranyl acetate for 30 minutes at 60°C. The sections were then washed in glass distilled water for 3 minutes, then dried by touching the edge of the grid against filter paper. This was followed by staining in lead citrate for 10

minutes at room temperature. Sections were then washed in glass distilled water and finally dried by touching the edge of the grid against filter paper.

The specimens were examined on a Philips 301 electron microscope.

*Epon 812 (1A:1B) (Luft, 1961)

2.7 Measurement Of CO_2 Exchange

2.7.1 Plants

Radish plants were grown as described in Section 2.1.3. After 6 days the young radish plants were transplanted into small plastic pots. Care was taken to ensure that about 3 cm of hypocotyl could be seen above the top of the pot. At the start of CO_2 exchange experiments the plants possessed four leaves and were about 10 days old.

2.7.1.1 Leaf Area

The leaf areas of plants were measured at the start and end of experiments. A piece of perspex with a grid of 25 mm^2 squares was used to estimate the area of a leaf. The grid was placed on a leaf and the number of grid junctions covering the leaf was noted. The area of the leaf was

calculated by multiplying the number of intersections counted by the area of each square. This was repeated a further four times with the grid placed at different angles on the leaf. The average area could then be calculated.

2.7.2 Infrared Gas Analyser

CO_2 exchange was measured using an infrared gas analyser (IRGA), type G.C.225.2A, manufactured by The Analytical Development Company Ltd., England.

The IRGA was incorporated in the experimental set up shown in Fig 2.4.

2.7.2.1 IRGA Calibration

The IRGA was calibrated on the absolute scale using two air cylinders of known CO_2 concentration.

2.7.3 Air Supply

Air flow was supplied by a British Oxygen Company air cylinder. Three flowstats with flowmeters (G.A. Platon Ltd.) were used to maintain an airflow of 200 $ml.min^{-1}$ through each of the three growth chambers.

2.7.4 Plant Growth Chambers

The growth chambers are illustrated in Fig.2.4. A detailed

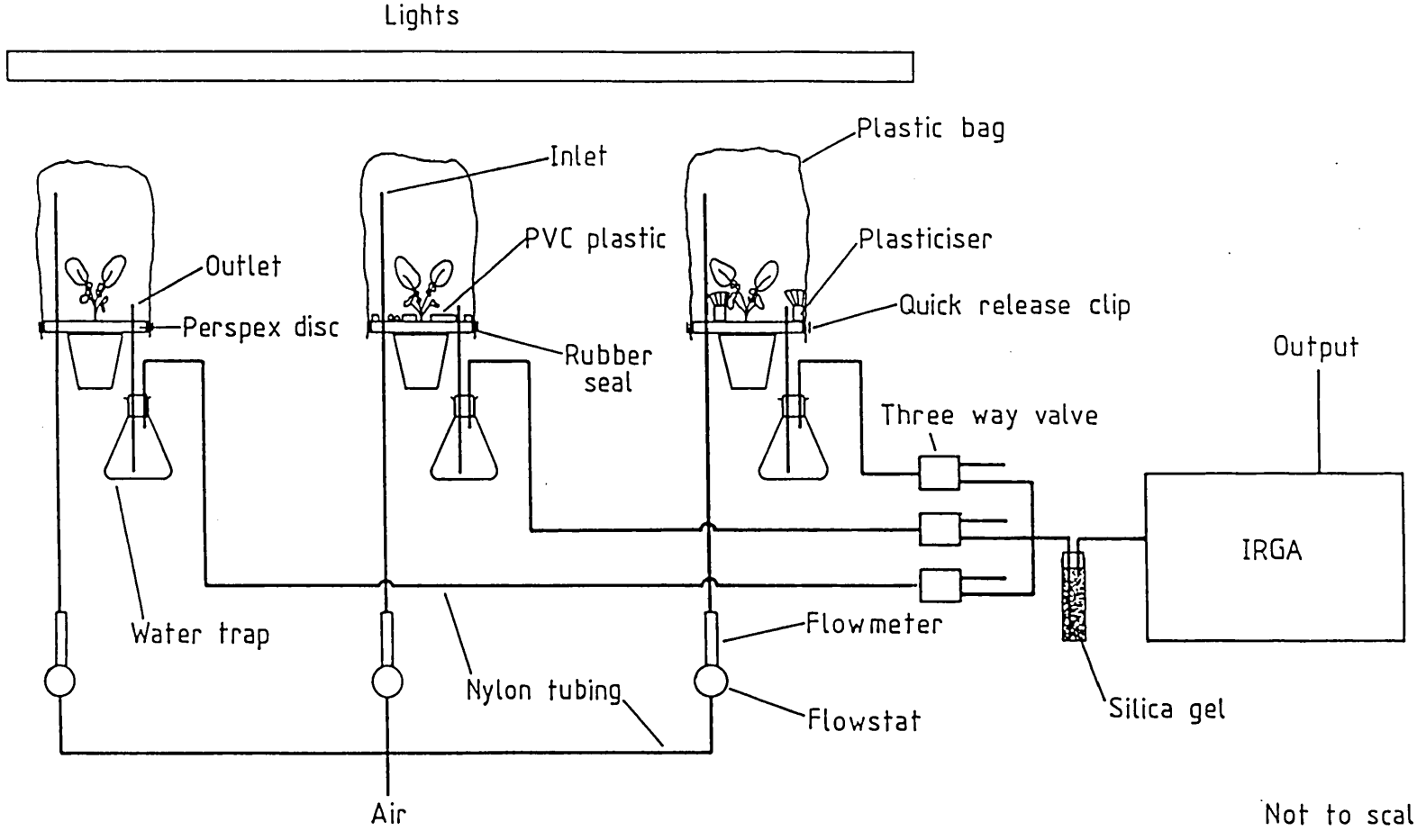


Fig.2.4 Schematic diagram of the experimental set-up to monitor CO_2 exchange using an infrared gas analyser. Air flow was 200 ml.min^{-1} through each growth chamber.

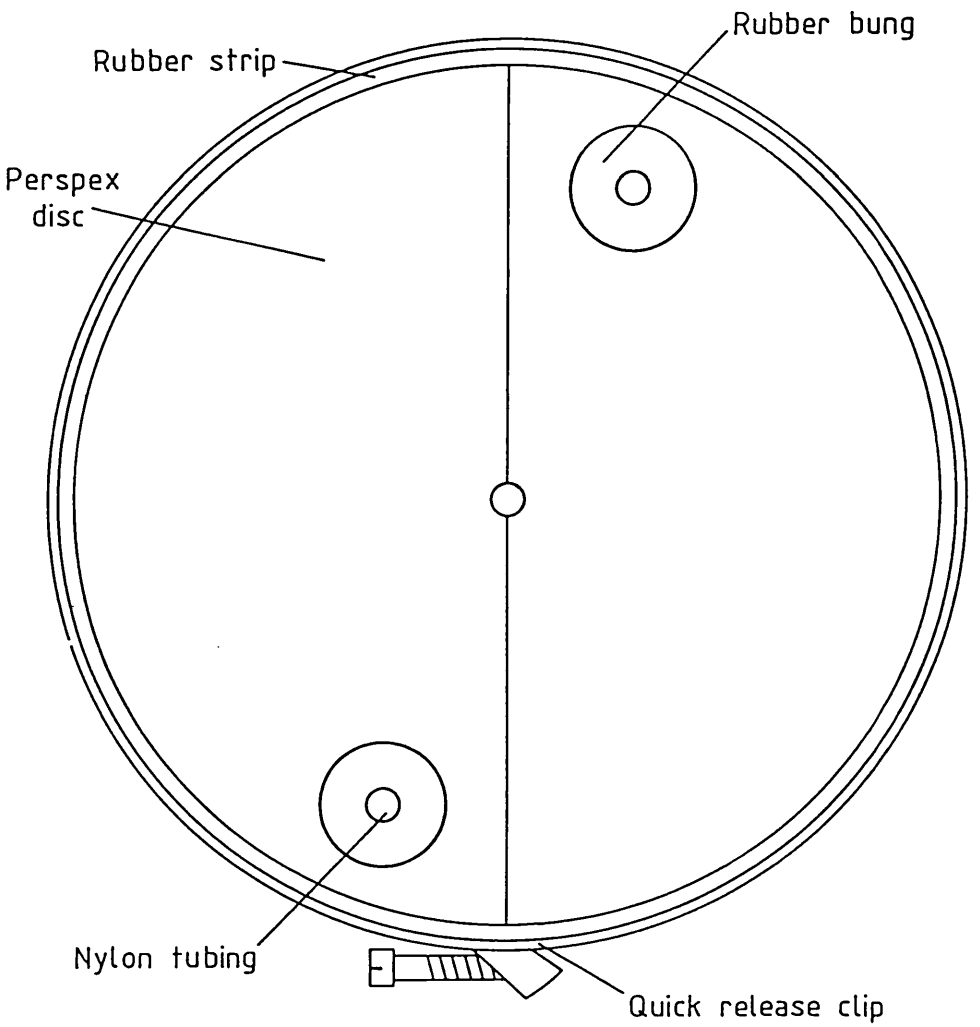


Fig.2.5 Diagram of the perspex base (18cm diameter x 2cm) of the growth chambers in Fig.2.2. (Not to scale).

diagram of the base of these growth chambers is shown in Fig.2.5. Each growth chamber consisted of a circular perspex base which could be separated into two halves. This disc of perspex surrounded the hypocotyl of the test plant and sat on the top of the plant pot. The join in the perspex halves and the space surrounding the hypocotyl were sealed using lanolin. A small plastic bag was clamped over the plant at the perspex disc. This join was sealed by a rubber strip which ran round the edge of the perspex disc. The bag was held against the rubber strip by a quick release clip. The polythene tubes which conducted air to and from the growth chambers passed through tightly fitting bungs in the perspex disc. The approximate volume of the chamber was 600 ml. All tubing and adapters were made of polythene or polypropylene.

2.7.5 Light Source

Illumination of the growth chambers was provided by four 5 ft. 80W white light fluorescent tubes and two 25W tungsten bulbs. The photon fluence rate at the mid-height of the growth chambers was 135 $\mu E.m^{-2}.sec^{-1}$ (PAR).

2.7.6 Air Sampling

The air leaving each growth chamber passed through a water trap to flow through a valve-switching assembly. By means of a multi-channel switching unit, air could either be passed through a valve to the IRGA or it could be

exhausted to the atmosphere. The timing of the opening and shutting of these valves was such that air from each growth chamber in turn passed through a separate valve to the IRGA for a period of 10 minutes, while the air from the remaining two chambers was exhausted. The air being sampled then flowed to the IRGA via a small silica gel column which removed the remaining traces of water vapour and phthalates. The lengths of tubing following the valves was kept to an absolute minimum to ensure that the air passing from the growth chambers to the IRGA at 200 $ml.min^{-1}$ could reach equilibrium within the ten minute sampling period.

2.7.7 Treatments

One plant was exposed to the DBP vapour emitted from 62g of plastic E (see Table 2.1). This DBP-plasticised rod was cut into lengths of 3-4 cm. with a total surface area of approximately 220 cm^2.

Another plant was exposed to the vapour from a sample of pure liquid DBP. This DBP was a sample of the plasticiser used in the formulation of plastic E. Two small glass jars were used to hold the plasticiser and a piece of fluted Whatman No1 filter paper was placed in each jar to increase the surface area of DBP. The total surface area of the two pieces of filter paper was similar to that of the plastic rod.

The third chamber held the control plant.

2.7.8 Recording Of Results

CO_2 concentration was recorded as the electrical output from the IRGA. The chart recorder was a Servoscribe S model.

Calibration of the IRGA enabled the electrical output to be directly converted to vpm CO_2. A sample calculation to convert these figures to mg CO_2 exchanged per unit leaf area per unit time is shown below.

Assuming CO_2 concentration was depleted by 120 vpm, at a flow rate of 12 $l.min^{-1}$ by a radish plant of 40 cm^2 then:

$$\text{reduction in } CO_2 \text{ concentration} = \frac{120}{40} = 3 \text{ vpm}.cm^{-2},$$

$$\text{and since 1 vpm} \triangleq 1\ \mu l.l^{-1} \qquad = 3\ \mu l.l^{-1}.cm^{-2}$$

Air flow rate was 12 $l.hr^{-1}$, therefore:

$$\text{change in } CO_2 \text{ concentration} = 36\ \mu l.cm^{-2}.hr^{-1}$$

At room temperature, 44.0g CO_2 occupies 24 l., therefore:

$$1\mu l = \frac{44.0}{24.0}\ \mu g$$

$$\text{Change in } CO_2 \text{ concentration} = 66\ \mu g.cm^{-2}.hr^{-1}$$

$$= \underline{6.6\ mg.dm^{-2}.hr^{-1}}$$

2.8 Oxygen Exchange Measurements

2.8.1 Plants

Radish plants were grown as described in Section 2.1.3. The plants were then exposed to phthalate plasticiser vapour in the bioassay system described in Section 2.1.4. Plants were exposed to the phthalate plasticisers volatilising from approximately 170g samples of plastic.

2.8.2 Leaf Disc Electrode

Oxygen exchange in leaf discs was measured using a Hansatech D.W. oxygen electrode. Details of the construction, calibration and use of this electrode may be found in the paper by Delieu and Walker (1981). Illumination was provided by a slide projector or an alternative light source called an Intralux 2000 (Volpi). The light from the projector passed through a 250 cm^3 conical flask containing a 1% $CuSO_4$ solution. The light was then reflected from a heat reflecting mirror. The Intralux 2000 was used in conjunction with two heat absorbing filters of 2mm and 3mm thickness. The photon fluence rate at the surface of the leaf disc chamber was 650 $\mu E.m^{-2}.sec^{-1}$ (PAR) with the projector and 725 $\mu E.m^{-2}.sec^{-1}$ (PAR) with the Intralux 2000.

The leaf chamber was maintained at a temperature of 25°C

by circulating water through it's water jacket from a constant temperature water bath and a cooler.

The electrical output from the oxygen electrode was amplified and fed to a Servoscribe S model chart recorder.

2.8.3 Chlorophyll Determination

Following O_2 exchange measurements leaf discs were torn by hand into small fragments. These leaf fragments were then placed in a small hand grinder with about 10 mls of 80% acetone and ground until no green colour was visible in the fragments. The volume was made up to 50 mls in a volumetric flask using 80% acetone. This solution was filtered through Whatman No1 filter paper and measured according to the calculations of Arnon (1949). Alternatively, after tearing the leaf into fragments they were placed in a pestle and mortar to which a small amount of liquid nitrogen was added. The frozen leaf fragments were then ground. Eighty per cent acetone was used to remove the chlorophyll from the pestle and mortar. The volume was made up to 50 mls and treated as before.

2.9 Chlorophyll a Fluorescence Measurements

2.9.1 Plants

Radish plants were grown as described in Section 2.1.3.

The plants were then exposed to phthalate plasticiser vapour in the bioassay system described in Section 2.1.4. Plants were exposed to the phthalate plasticisers volatilising from approximately 170g samples of plastic.

2.9.2 Chlorophyll Fluorescence Measuring Apparatus

Changes in the yield of chlorophyll $a_{\approx}$ fluorescence of radish leaves were measured using the apparatus illustrated in Fig.2.6.

Modulated blue-green light (light 2), intensity 5.9 $\mu E.m^{-2}.sec^{-1}$ (PAR) transmitted through Schott BG18 (4 mm) and BG38 (2 mm) filters was used for photosystem 2 (PS2) absorption. The modulated fluorescence emission at 686±11 nm was detected by a photomultiplier connected to a lock-in amplifier. Non-modulated light (light 1) at 710±12 nm (transmitted through a Balzer interference filter) and an intensity of 34 $\mu E.m^{-2}.sec^{-1}$ (PAR) was employed for preferential absorption by photosystem 1 (PS1). The incoming lights were mixed and the outgoing light was measured using a three-way fibre optic system. The optic fibre mixing head was held vertically over the surface of the radish leaf. During measurements the leaf was situated in a closed metal chamber whose upper surface possessed a single hole in which the fibre optic head could be placed.

Experimental material consisted of either small, whole radish leaves or parts cut from larger leaves. Chlorophyll

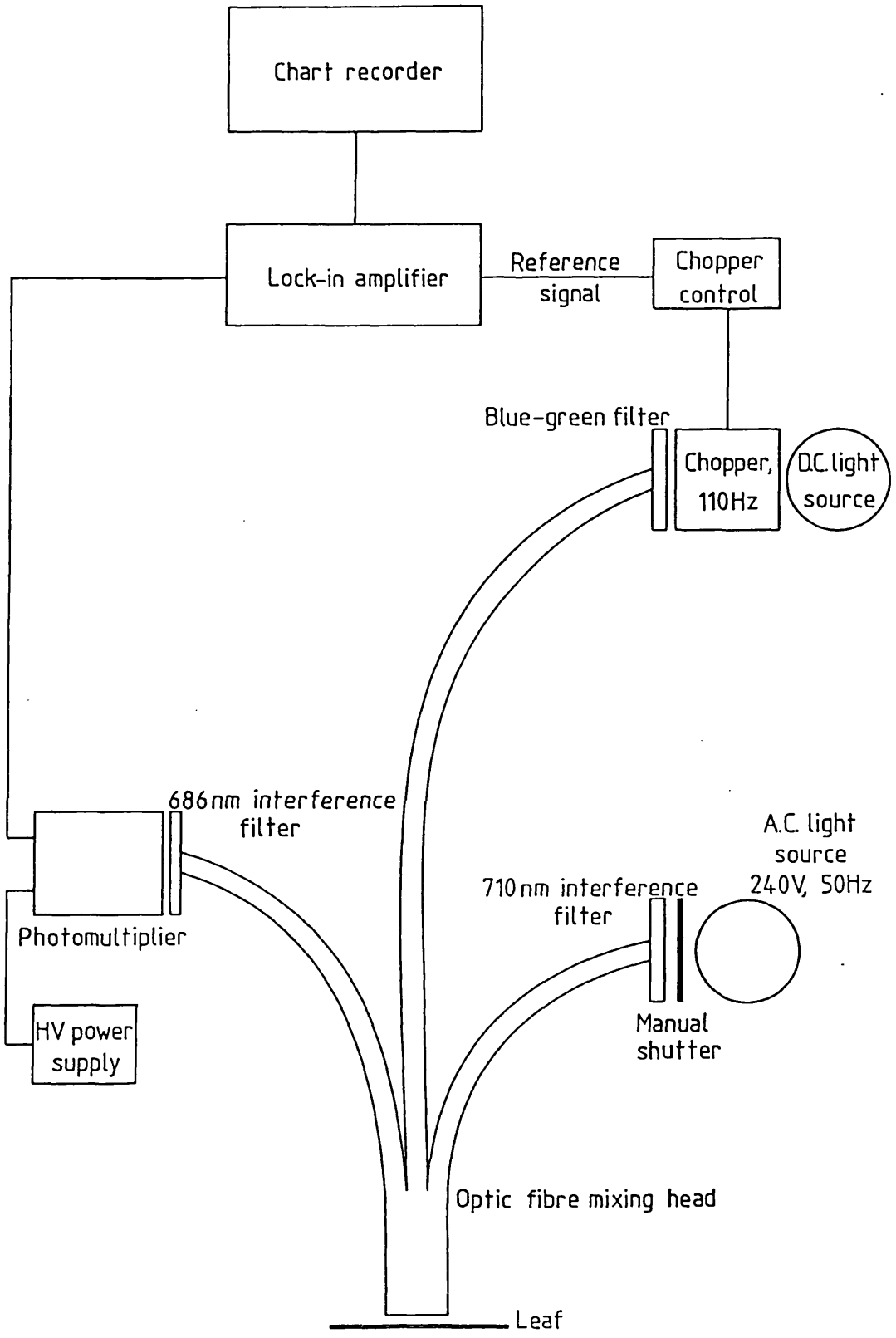


Fig.2.6 Block diagram of the *in vivo* modulated chlorophyll a_2 fluorescence measuring apparatus. (Adapted from Barber *et al.*, 1981)

fluorescence was measured immediately after the leaf was detached from a plant.

2.10 Electron Transport Reactions

2.10.1 Spinach Chloroplasts

Chloroplasts were prepared from market spinach using the method of Nakatani and Barber (1977). They were finally resuspended in reaction medium (see Section 2.10.3).

2.10.2 Oxygen Electrode

The oxygen electrode used possessed a glass reaction chamber (Hansatech Ltd.). This enabled the chamber to be washed with methanol and then water. Use of a glass reaction chamber avoided the possibility of the dialkyl phthalates causing permanent contamination and damaging the chamber by stress cracking. Total reaction volume was 2.5 ml.

2.10.3 Electron Transport Activity

Electron transport activity was measured in thylakoids. Aliquots of chloroplasts were osmotically shocked in distilled water for ten seconds. Double strength reaction medium was then added to bring the final concentration of

constituents to 10 $\mu g.ml^{-1}$ chlorophyll, 0.33M sorbitol, 1mM $MgCl_2$, 1mM $MnCl_2$, 20mM HEPES, 20mM KCl, pH adjusted to 7.6 with KOH. A modified reaction medium was used for some experiments. The final concentration of constituents being the same as before save for the addition of 2mM EDTA. Full chain electron transport (PS2 + PS1) activity was generally measured as oxygen uptake with methyl viologen (MeV) as primary electron acceptor. Reaction medium then additionally contained 60µM MeV and 600µM sodium azide (NaN_3). For some experiments ferricyanide was used as the primary electron acceptor. Final concentration of K_3FeCN_6 in the reaction medium was 800µM. In uncoupled experiments 1×10^{-3} µM nigericin and 1×10^{-3} µM valinomycin were also present in the reaction medium. PS1 activity was measured with MeV as the primary electron acceptor. Additionally the reaction medium included 8µM DCMU, 40µM DCPIP and 1200µM sodium ascorbate.

2.10.4 Chlorophyll Determination

Chlorophyll concentration was determined according to Arnon (1949).

2.11 Chemicals

All chemicals used were of analytical quality and were purchased from Sigma or BDH unless stated otherwise.

3. RESULTS

3 RESULTS

3.1 Screening Of Plastics And Plasticisers

3.1.1 Preliminary Experiments

Preliminary screening experiments were undertaken to test the PVC plastics provided by ICI. It was hoped that by testing a series of plastics of known formulation it would be possible to identify the toxic principle present in certain plastic formulations.

The new bioassay system was initially tested using plastics known to be toxic from previous experiments. The plastics tested included a black PVC strip supplied by a tomato grower from North Humberside and samples of the original clear PVC tubing found to be toxic during undergraduate experiments at Imperial College (Hannay, 1980). Analysis by ICI showed that the black PVC strip contained a 31.8% mixture of DIBP (30% of extract) and DOP (37% of extract) the rest of the plasticiser being made up from ESBO and Cereclor. The clear PVC tubing contained 29.4% DBP. Bioassaying young radish plants with the black plastic caused faint signs of chlorosis in as little as two days. Obvious signs of chlorosis were seen after three days and all plants were dead after 10 days exposure to the black plastic.

Plastics with the formulations A-G given in Table 1.1 - the first batch provided by ICI - were then screened. The different formulations were intended to incorporate most, if not all, the components present in the plastics we had screened up to that time. Essentially these are the dialkyl phthalates: dibutyl phthalate and dioctyl phthalate; stabilisers: a barium-cadmium mixture and calcium-zinc mixture. Stearic acid, a lubricant was incorporated in some formulations, as was epoxidised soya bean oil (ESBO) - which can be used as both a plasticiser and a stabiliser and is also a lubricant. Different combinations of these components were used to formulate the plastics tested.

Experiments quickly demonstrated that only those plastics formulated with DBP as their plasticiser were toxic, irrespective of the stabiliser used. The time-course for the appearance and development of phytotoxic symptoms was slightly slower than that of the black plastic from Humberside.

There are a number of possible explanations for these differences. The most obvious is that different surface areas were available for volatilisation. It might be expected that different concentrations of toxic plasticiser in the plastic would be an important factor but this is not necessarily so. The two main factors controlling the loss of a plasticiser from a plastic are the resistance of the plasticiser to move to the surface

of the plastic and resistance to loss from that surface. Either of these two factors may control the other (Sears and Darby, 1982). Moreover it has been found that the volatilisation of a particular plasticiser from a plastic also depends on the resin and stabilisers used as these factors will determine in part the compatibility and diffusion rate of the plasticiser (Mellan, 1961). Since these experiments were undertaken, it has been found that the black Humberside plastic was plasticised with DIBP which is of greater volatility than DBP. Differences in the intrinsic toxicity of these phthalates may also exist.

Further preliminary experiments involved screening the individual components of the plastics for phytotoxicity. Initial experiments testing the plasticisers and stabilisers used shallow aluminium foil trays with a chromatography paper "sail" to give a surface area of plasticiser roughly equal to that of the plastic rods. The first experiments showed that DBP was toxic, although the toxicity, on the crude equal surface area basis, did not appear to be as great as an equal area of plastic. Plants exposed to DOP also showed very faint signs of toxicity in these experiments. The appearance of the plants was not that of typical PVC phytotoxicity as from the early stages of treatment the plants looked unhealthy. The cadmium-barium stabiliser killed all the test plants within twenty-four hours. This was thought to be caused by the solvent in which the stabiliser was dissolved, which was phenolic in nature. No typical chlorotic symptoms were

seen in these plants; they rapidly became shrivelled and died.

During these experiments problems arose because of the plasticisers' tendency to 'creep' which increased their surface area and, more seriously, caused worries about contamination of equipment. In addition to this the perspex tubes which normally held the plastic to be screened were subject to stress cracking when they contained trays of liquid plasticisers (Sears & Darby 1982). For this reason the perspex tubes were each lined with a glass tube. In subsequent experiments a gas washing bottle with a sintered glass bulb on the distribution tube was used to hold the liquid plasticiser. This bottle was situated immediately prior to the glass lined perspex tube. The testing of liquid plasticisers was finally discontinued due to the worry of contamination of the equipment and the room in which it was situated.

From the results of these experiments it was apparent that DBP was the toxic principle present in the flexible PVC rods. During the course of this project many PVC samples supplied by various commercial growers and government research stations were tested. In conjunction with chemical analysis by ICI it soon became evident that an isomer of DBP - diisobutyl phthalate (DIBP) - was also phytotoxic. These results were confirmed after we tested a sample of PVC formulated by ICI which contained DIBP as it s plasticiser (Plastic H - second batch of plastics

received from ICI).

3.1.2 Growth Analysis

3.1.2.1 ICI Plastics

A series of growth analysis experiments were used to quantify the effects of the phytotoxin on the growth of radish plants. In the first experiments ICI plastics containing either DBP, DIOP and DIDP as their plasticiser were tested. Figure 3.1 shows that there are differences in fresh weight between those plants exposed to DBP and the other treatments in as little as 5 days. By the tenth day the DBP treated plants showed a decrease in fresh weight. A similar trend is seen in the graphs of dry weight (Fig.3.2), however there were no decreases in dry weight. Figures for the percentage dry weight/fresh weight show a marked increase towards the end of these experiments indicating that the treated plants were suffering from water loss (see Appendix 2). The accompanying photographic evidence confirmed these findings (Figs.3.3 - 3.5).

3.1.2.1.1 Photographic Record

The photographs show the appearance of the plants which were used for the fresh weight measurements in Fig.3.1, second graph, on days 0, 3, 6 and 10. The younger radish leaves showed definite signs of chlorosis by day 3. The

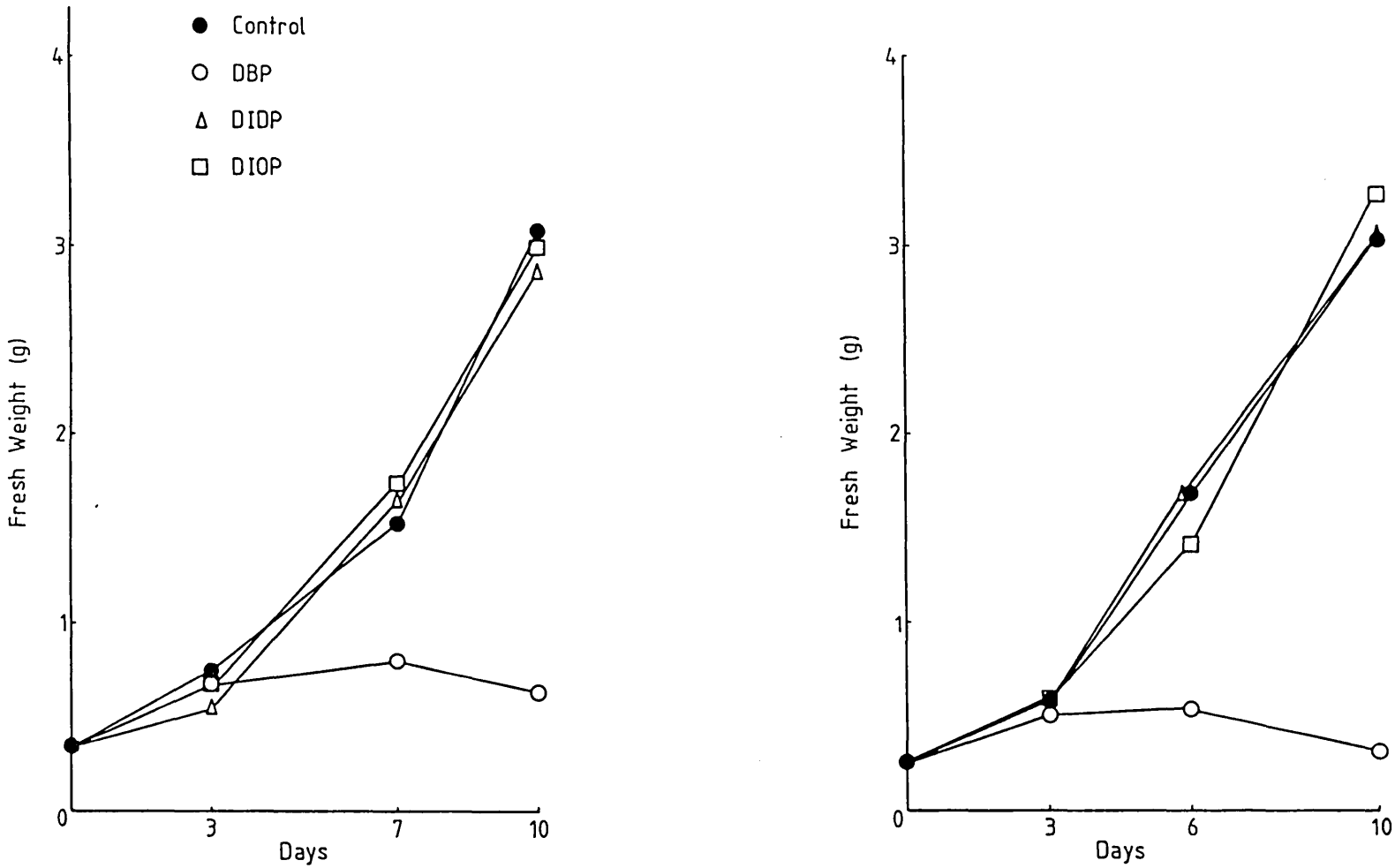


Fig.3.1 Changes in fresh weight of radish plants exposed to the vapour from PVC formulations. Results of two individual experiments. Each point represents the mean of three weights.

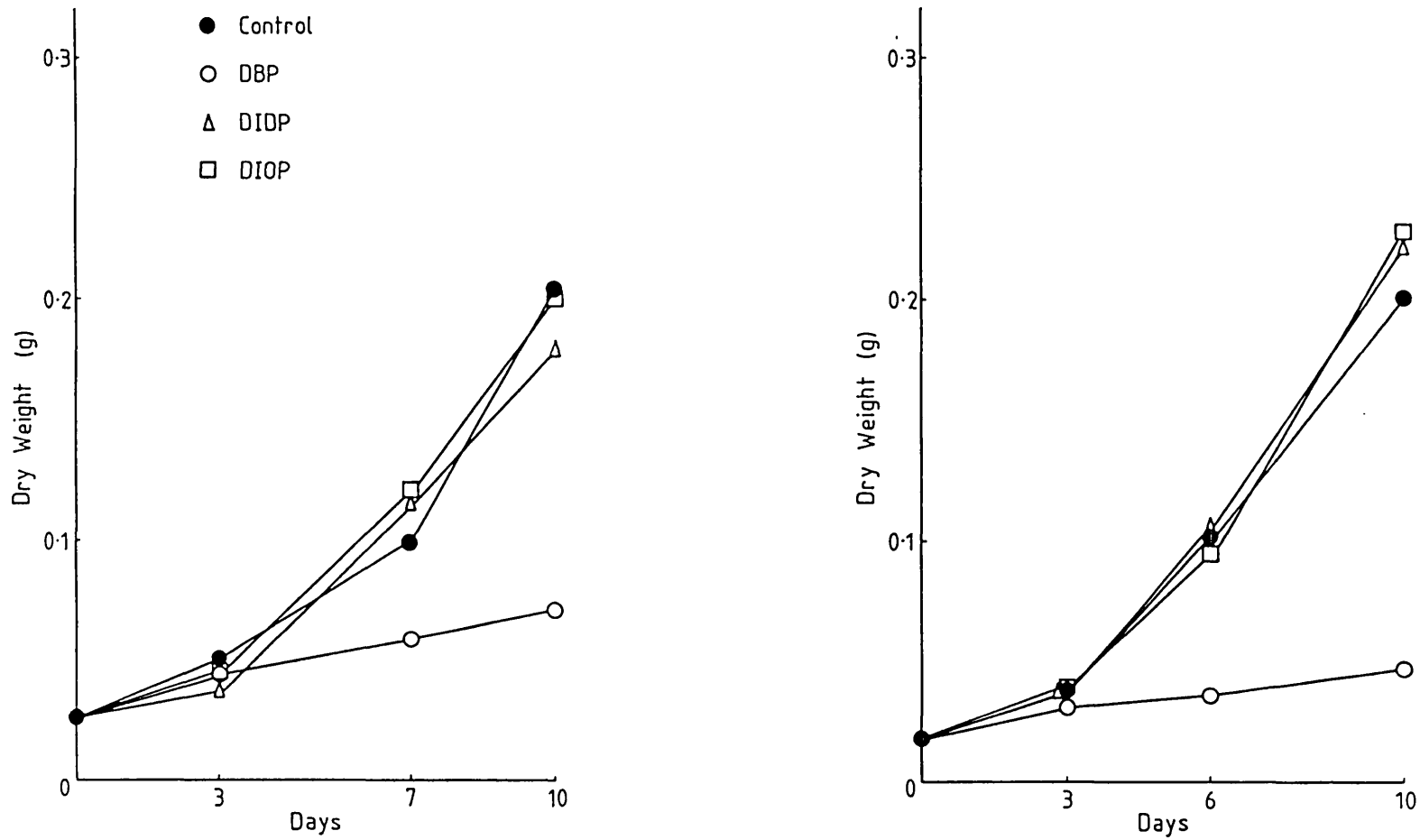
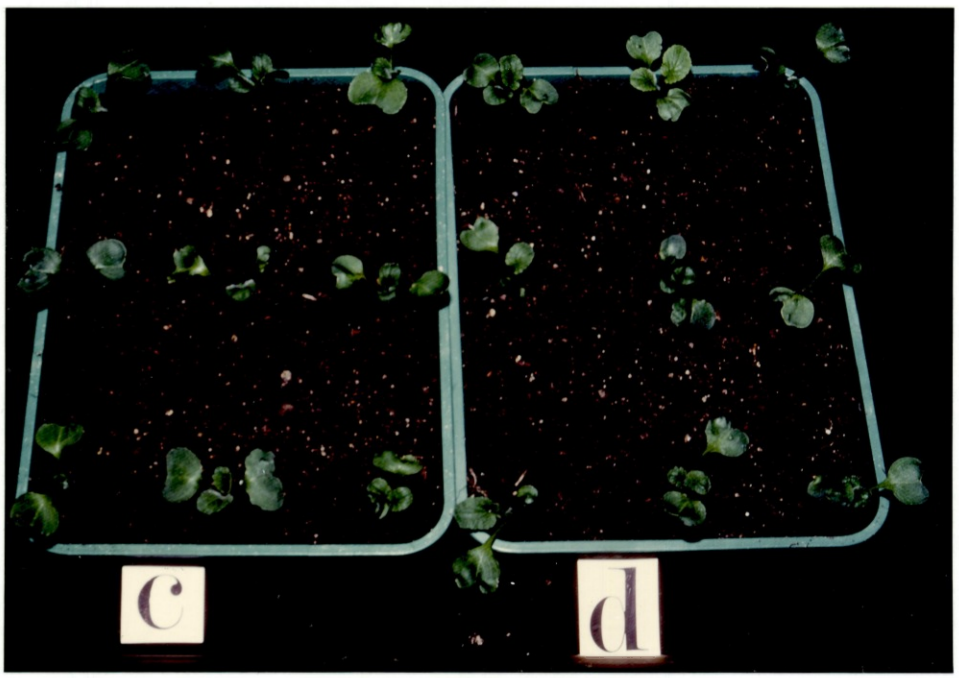
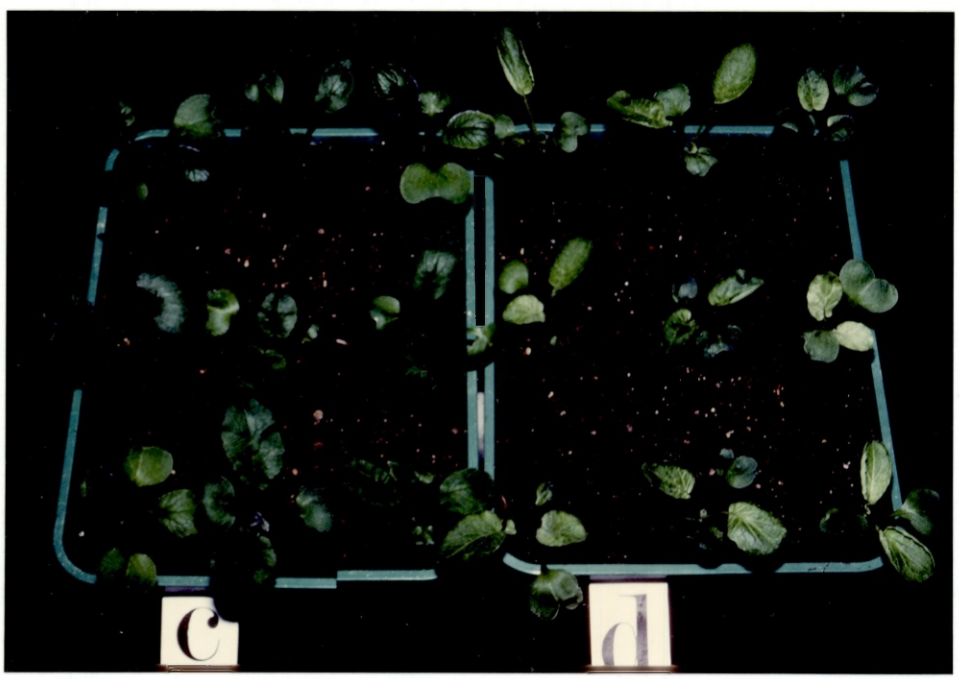


Fig.3.2 Changes in dry weight of radish plants exposed to the vapour from PVC formulations. Results of two individual experiments. Each point represents the mean of three weights.

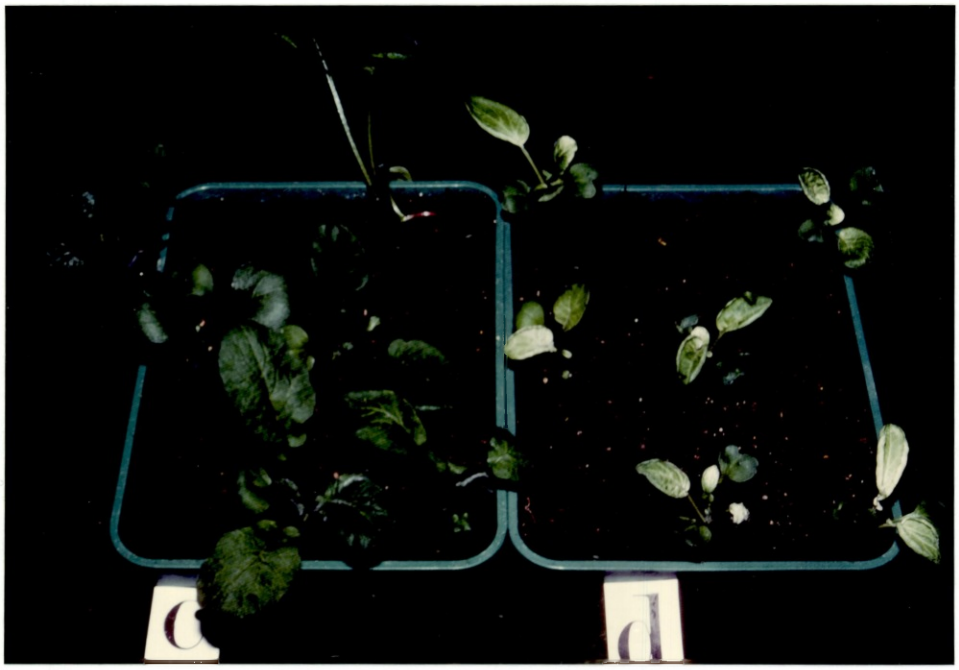


i)

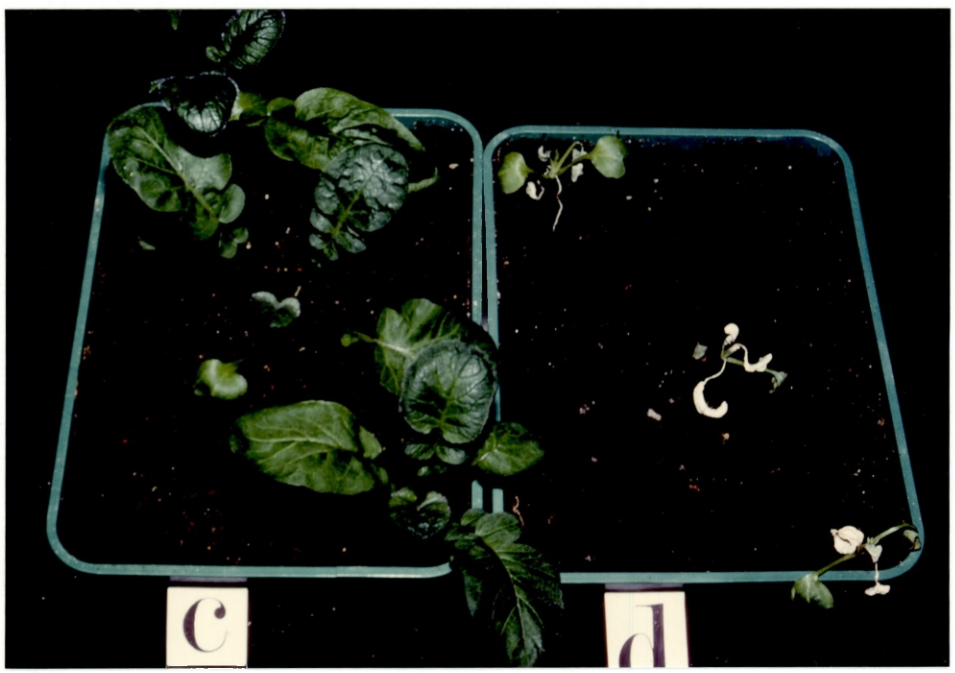


ii)

Fig.3.3 i) Radish plants before exposure to PVC plastics. ii) Radish plants following 3 days exposure. Control - tray c; DBP treated - tray d.



i)



ii)

Fig.3.4 i) Radish plants following 6 days exposure. ii) Radish plants following 10 days exposure. Control - tray c; DBP treated - tray d.

margins of some leaves of the treated plants showed signs of curling. No signs of chlorosis were seen in the cotyledons. By day 6 these effects were much more severe. Chlorosis was still only seen in the younger leaves - which had continued to grow during exposure to DBP vapour. On day 10 the appearance of the remaining plants was in marked contrast to the vigorously growing control plants. All the leaves of the treated plants had dried and shrivelled; they were also almost white in colour. Some of the cotyledons had also started to collapse and dry but they did not appear to have become chlorotic.

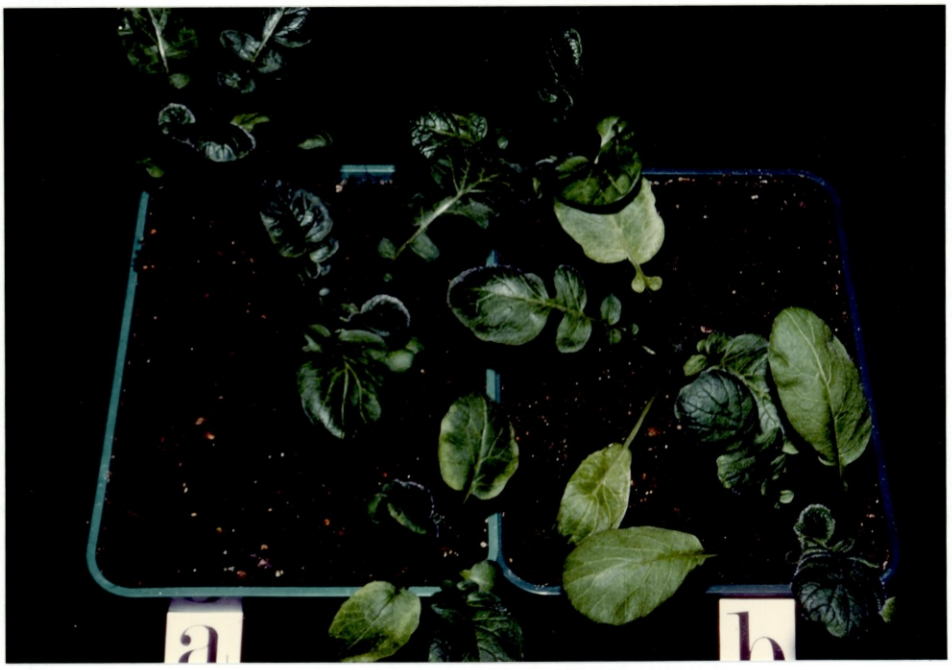


Fig.3.5 Radish plants following 10 days exposure. DIDP treated - tray a; DIOP treated - tray b.

These photographs show the typical appearance of radish plants exposed to volatilised DBP. One point of interest about the appearance of treated plants, which is not obvious from the photographs, is that leaves remained fully turgid even when they were totally chlorotic.

Other species of plants, such as cabbage are affected in a similar way : the young leaves grown in the presence of the DBP vapour showing evidence of chlorosis. In some species however the appearance and development of symptoms is different. Tomatoes are a good example. The following description is of plants growing in a contaminated greenhouse. They were observed in late March 1983. Due to ventilation commencing about 4 weeks prior to this inspection and description the upper parts of the plants were growing normally as the toxin was diluted. As the leaves become older chlorosis becomes more marked with the tips and edges of leaves showing browning. Little clear "windows" also developed on these leaves, the area of windowing increasing with increasing duration of exposure to DBP vapour. The leaves eventually turn brown and then whither and die. Stem diameter towards the base of the plants is reduced. The overall appearance of the exposed plant is a healthy growing top part with brown, withered leaves on the lower part of the plant. The most important visual difference is the absence of the type of marginal chlorosis seen in radish plants. Instead initial chlorosis is interveinal.

3.1.2.2 Greenhouse Glazing Strip

Similar experiments to those described above made use of PVC glazing strips supplied by a grower of glasshouse tomatoes, V.W.C. Bailey and Partners of Penzoy Nurseries, Hampshire. In addition a new non-toxic glazing strip, plasticised with diisodecyl phthalate (DIDP), supplied by Econopak was also tested. One type of glazing strip supplied by the growers contained approximately 30% by weight of DOP as it's only phthalate plasticiser whilst the other contained approximately 15% each of DIBP and DOP. From the two graphs of fresh weight (Fig.3.6) it is apparent that the glazing strip containing DIBP severely inhibited the growth of the test plants. The strips containing only DOP or DIDP produced no differences from the control. Again the young leaves of affected plants turned chlorotic and were dying by day 10. This was confirmed by the dry weight measurements as seen in Fig.3.7 although there seemed to be some loss of dry weight by day 10 in the first experiment using the glazing strip.

These results confirm that PVC plasticised with DBP or DIBP is phytotoxic. Under these experimental conditions it appears that DOP, DIOP and DIDP were not toxic. However it should be remembered that the volatility of the octyl and decyl phthalates is much lower than that of the butyl phthalates and the results may be a reflection of this.

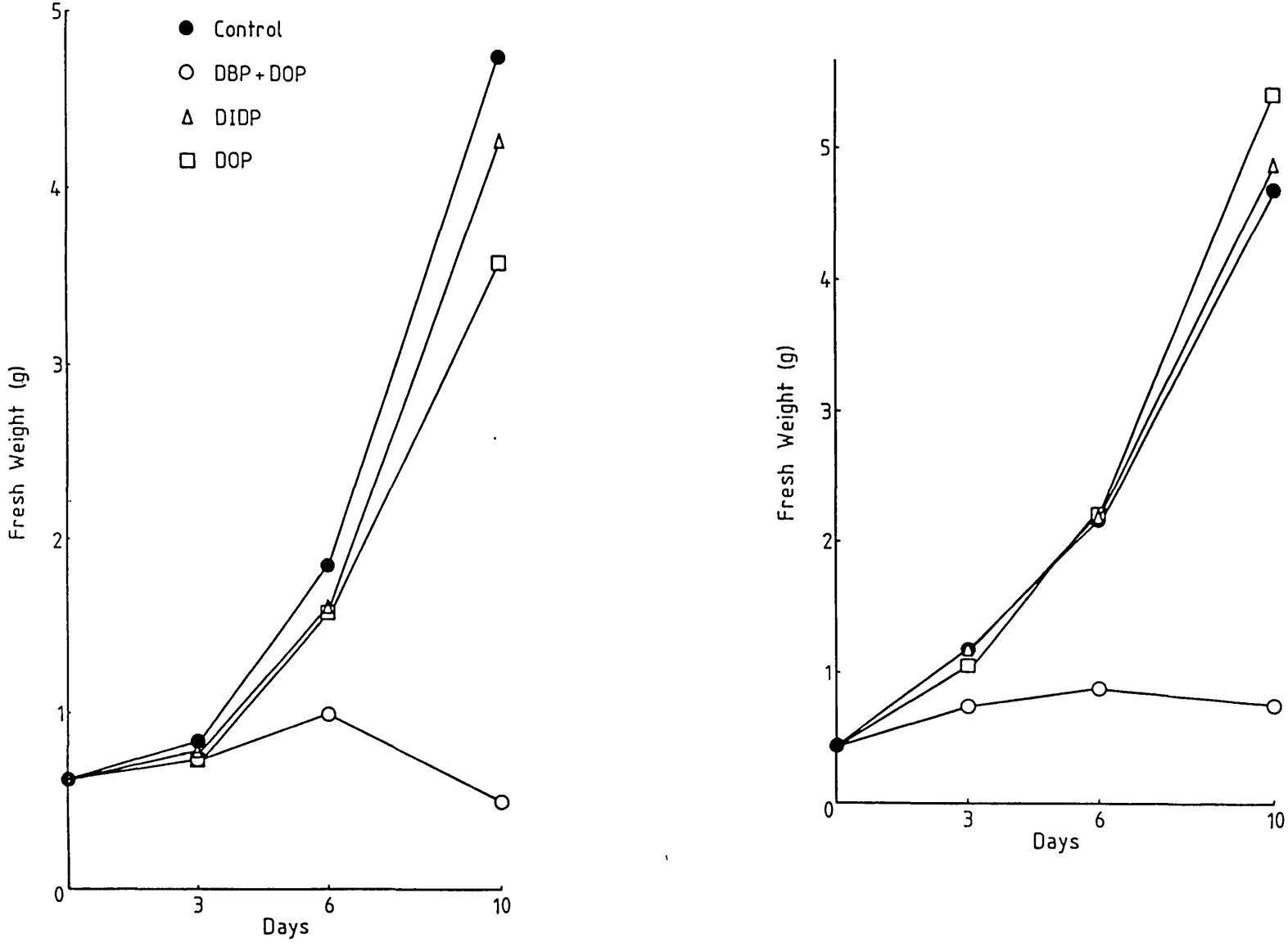


Fig.3.6 Changes in fresh weight of radish plants exposed to the vapour from PVC glazing strip. Results of individual two experiments. Each point represents the mean of three weights.

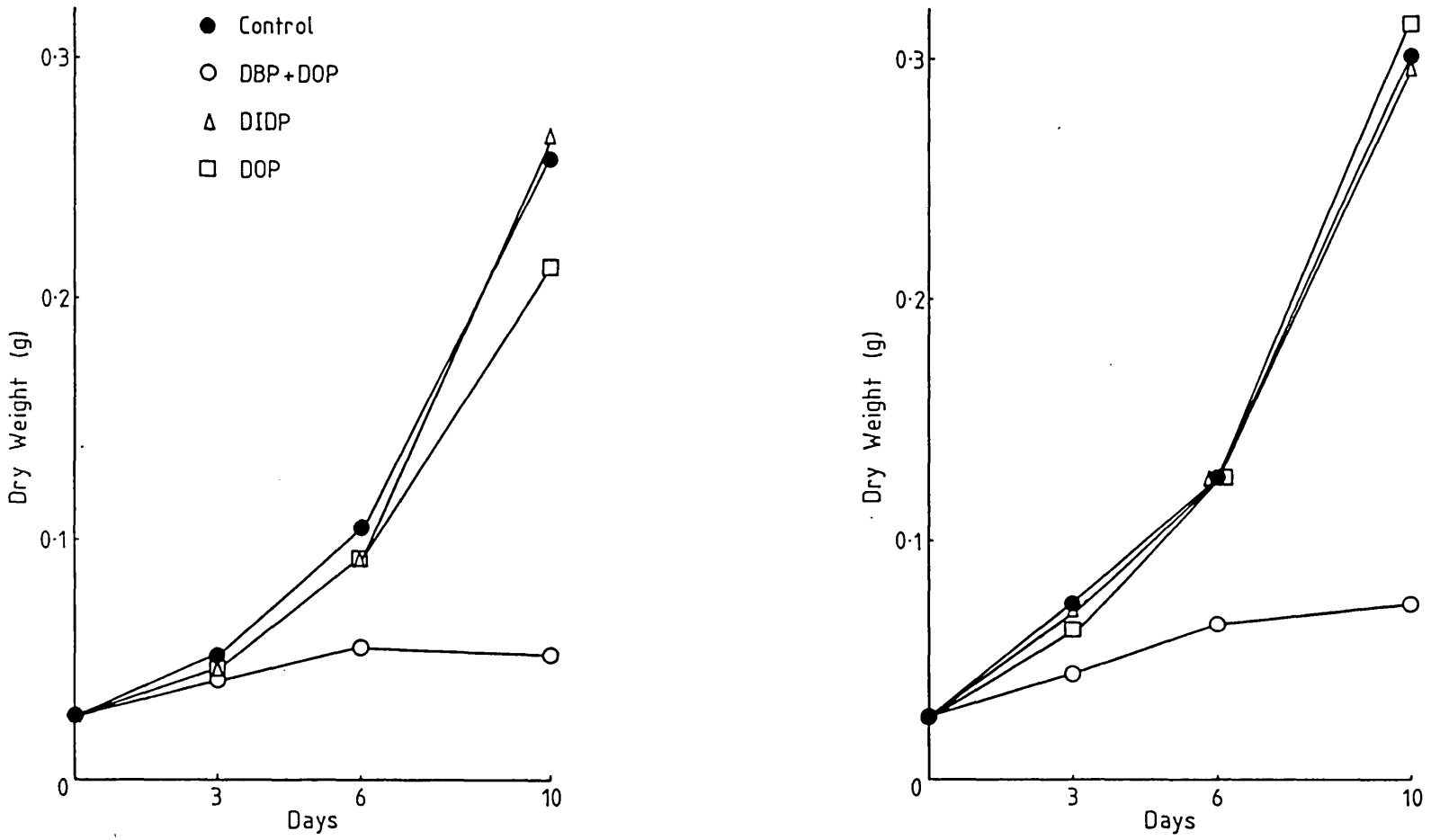


Fig.3.7 Changes in dry weight of radish plants exposed to the vapour from PVC glazing strip. Results of two individual experiments. Each point represents the mean of three weights.

3.1.3 Glasshouse Crop Losses

The results from the experiment above can be favourably compared to the problems experienced by the growers who supplied the PVC glazing strip for the bioassay. The two samples of glazing strip they provided came from two different greenhouses. One greenhouse, No 3, incorporated a DOP plasticised glazing strip. The growers have not had any problems with this greenhouse since it was erected in 1979. The other glazing strip containing DIBP plasticiser came from a greenhouse (No 7) erected in 1981. This greenhouse has incurred crop losses, as compared to House No 3, in the 1981-82 and 1982-83 growing seasons. In autumn 1983 the DIBP plasticised glazing strip was removed from House No 7 and replaced with a black, DIDP plasticised glazing strip - to the formulation recommended by a joint committee of manufacturers, ADAS representatives and research workers in May 1983 (Anon, 1983).

Figure 3.8 shows the accumulated total tomato yields for each of the three growing seasons. Figures are given on a tons per acre basis to enable comparison of the two greenhouses which were of different size. The growing season which finished in October 1983 is of the most interest to this problem. Between March and April the yield from each greenhouse diverged markedly with No 7 being the lower of the two. The figures for April to June show that the monthly yields for each greenhouse were not

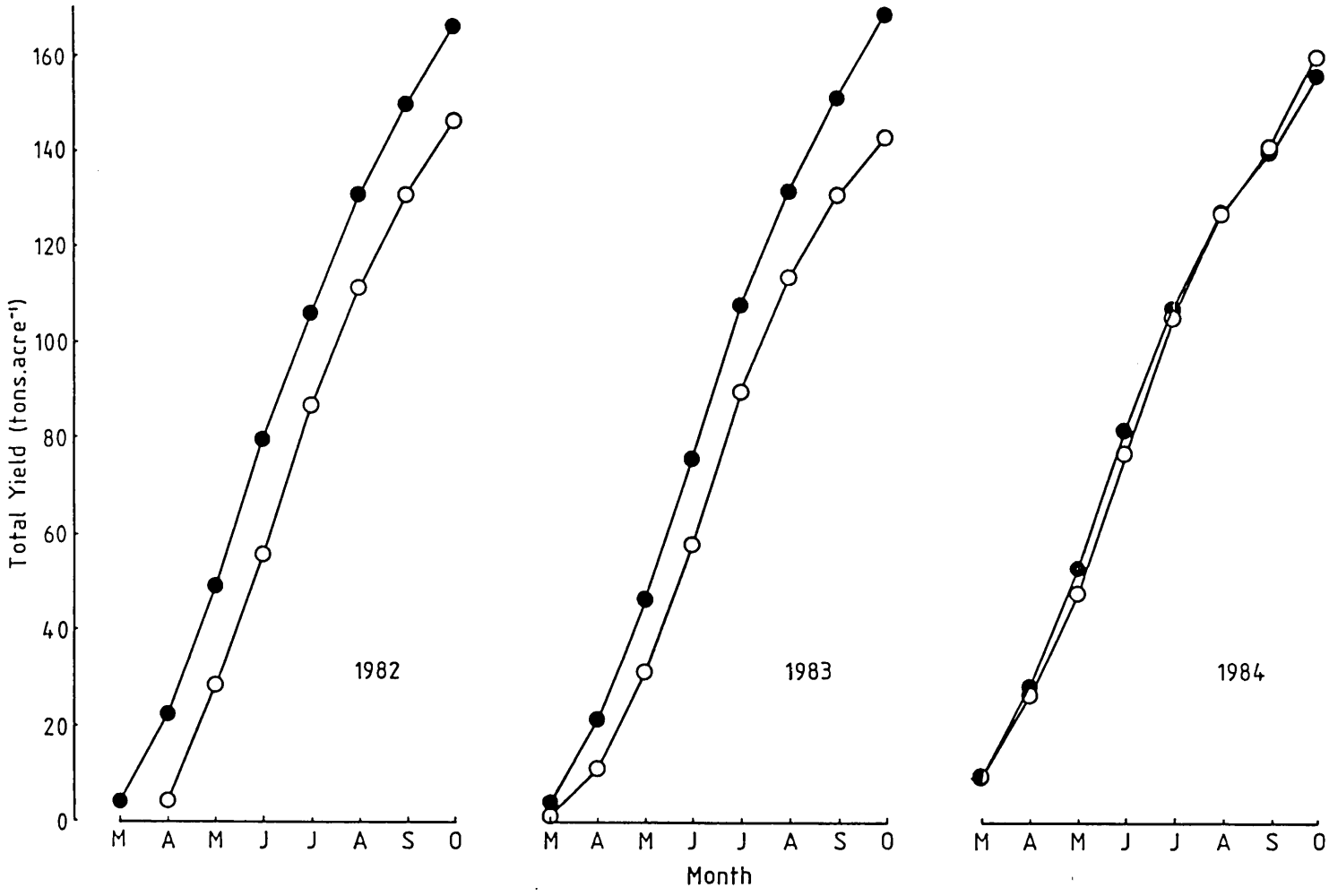


Fig.3.8 Cumulative tomato yield from two glasshouses. Closed circles - toxic greenhouse - DIBP + DOP glazing strip. Open circles - DOP glazing strip.

as different as the previous months. From June to August the tomato yield was nearly equal. From August to the end of the growing season in October the yields from each house increasingly diverged again.

The initial difference in yields was due to the high concentrations of DIBP which may be expected to have been present in greenhouse No 7 as vents in the house would be closed to conserve the heat. The differences in yields in the following months decreased as the frequency of venting increased as temperatures rose. The concentration of DIBP within the greenhouse therefore decreased. Towards the end of the growing season two factors probably contributed to the decreased yields from House No 7. One would be a reduction in the frequency of venting due to colder weather, thereby permitting an increase in the DIBP concentration. Secondly, and possibly of more significance at this stage in the growing season, is the fact that the plants in House 7 were not as healthy and vigorous as those in House 3 after six months exposure to DIBP vapours, even if at times the aerial concentrations had been low. Differences in the tomato yields from these two greenhouses may be artificially low as all plants grown in the houses were held for 8 weeks, from mid-October to mid-December, in House No 7 prior to the final planting out in the nutrient film system. The growers felt that this contributed to the delay in picking and the poor quality of early fruit from both houses.

In the season 1981-82 the results are complicated by the fact that the construction of glasshouse No 7 was not completed until after the growing season had started. Thus the lower yields at the beginning of the season from House No 7 would be mainly due to the delayed planting. However, towards the end of the season a slight divergence in yields occurred. As the plants in House No 7 were younger than those in House No 3 they might have been expected to outyield the senescing plants in House No 3, from August onwards. It seems likely that the observed decrease in yield was caused by DIBP accumulating as the temperatures dropped and venting decreased.

The graph for 1984 shows the accumulated yields after the DIBP glazing strip on House No 7 was exchanged for DIDP plasticised strip. The yields for each of the greenhouses were similar. It is evident from the graph that House No 7 had a reduced yield at the beginning of the growing season, mainly due to a phytophthera attack in this house during April and May. From July onwards the yields in House No 3 were depleted as a consequence of a severe infestation of whitefly. These problems may account for the slightly lower total yields from both greenhouses that year.

The following graph (Fig.3.9) looks at the tomato yields from a commercial point of view. On a £1000 per acre basis the difference in yields of the two Houses becomes more significant. This is a result of the high prices commanded

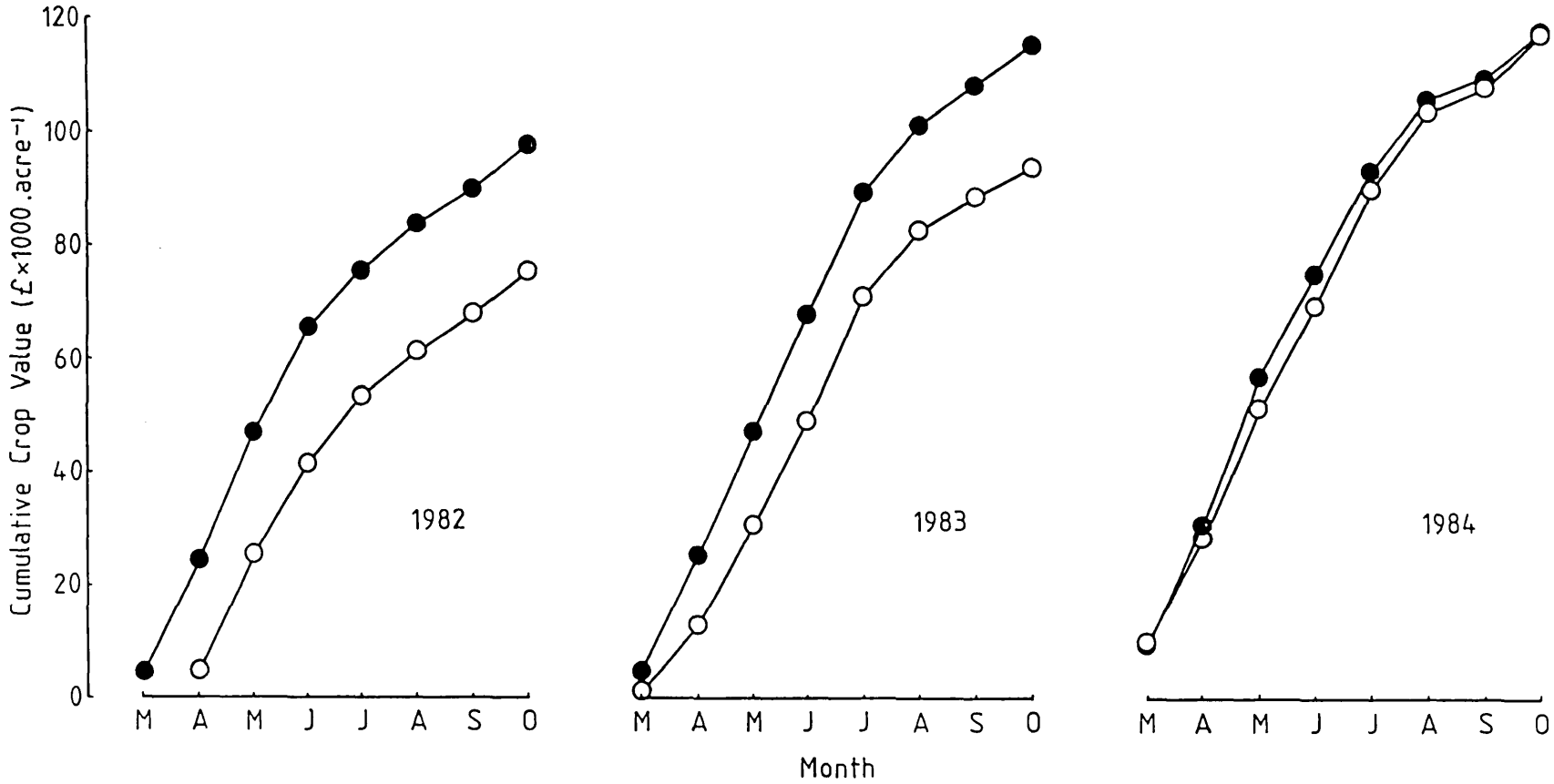


Fig.3.9 Cumulative tomato crop value from two glasshouses. Closed circles - toxic greenhouse - DIBP + DOP glazing strip. Open circles - DOP glazing strip.

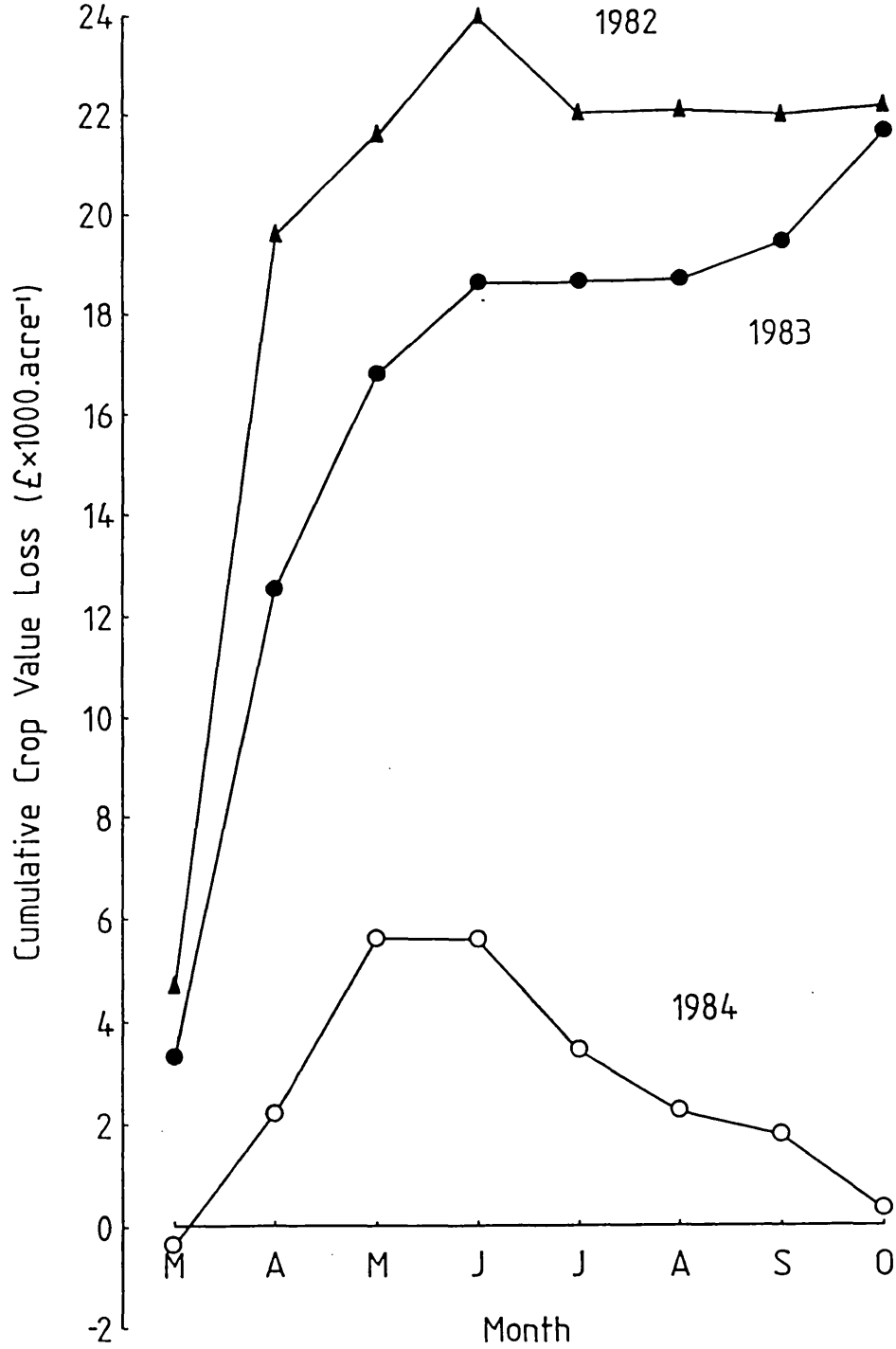


Fig.3.10 Cumulative crop value loss (safe greenhouse - toxic greenhouse)

by the early tomatoes - a time when this grower was experiencing his greatest problems with DIBP phytotoxicity.

Figure 3.10 shows the cumulative crop value loss suffered by House No 7 as compared to House No 3. Again 1983 is the best representative year for losses due to DIBP vapour damage. The largest financial loss occurred at the beginning of the season; essentially in the first two months. Towards the end of the season there was another addition to crop value loss but the lower price of tomatoes at this time of year helped to make it less severe than the early season losses.

3.2 Aerial Concentrations Of Phthalates

Due to the differing degree of phytotoxicity observed, using equal surface areas of plastics containing dibutyl phthalate and the corresponding liquid plasticiser, it was evident that a method to determine the aerial concentration of phthalate was required. The analytical services at ICI provided the basis of a method to collect and analyse samples of airborne phthalates in our bioassay system.

The method of collecting samples and preparing them for analysis by gas chromatography is described in the Materials and Methods, Section 2.2.

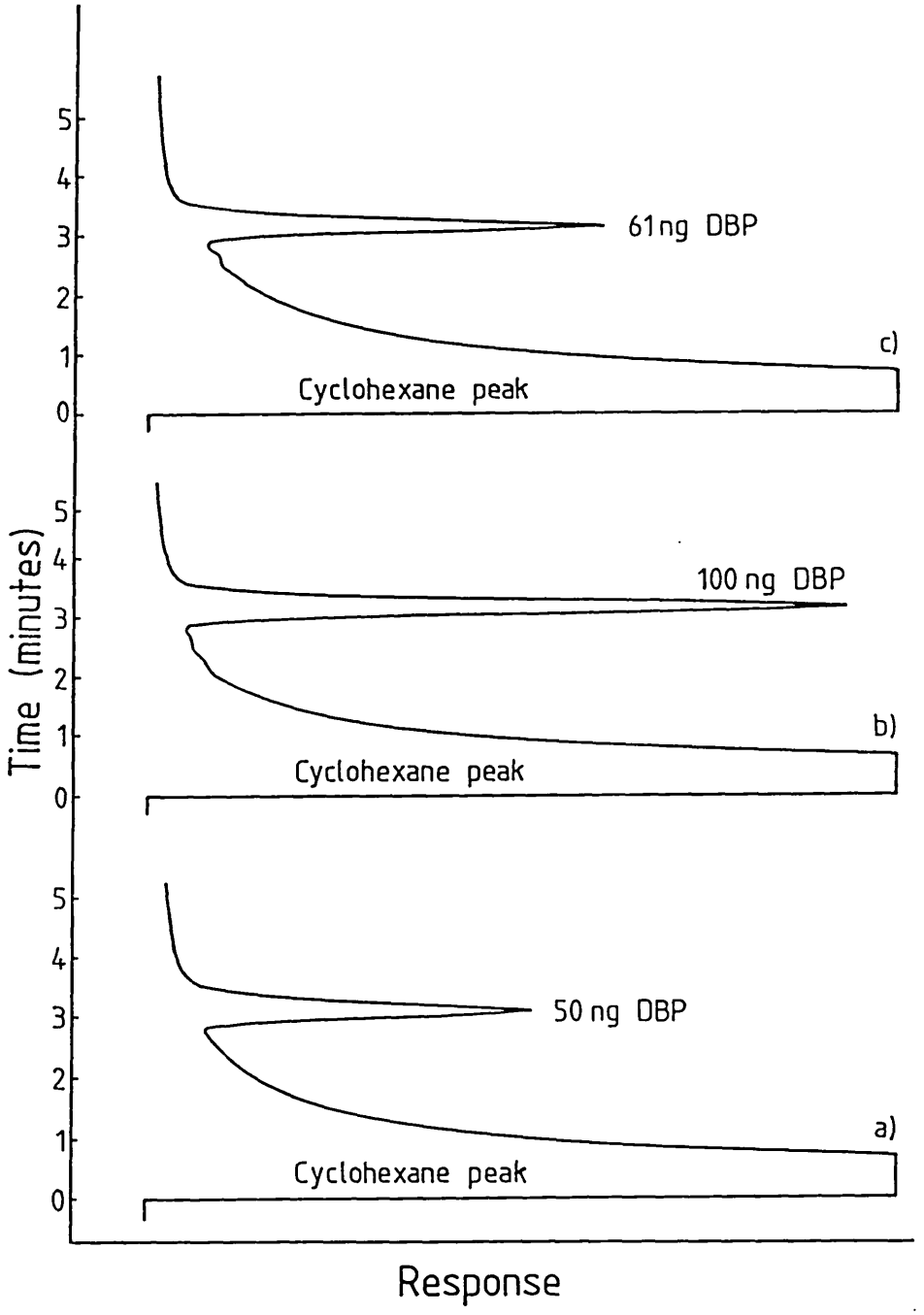


Fig.3.11 Gas chromatography traces. a) calibration trace; b) calibration trace; c) air analysis of layflat tubing growth chambers.

Figures 3.11a & b show typical traces of the calibration of the GC for quantifying DBP.

Figure 3.11c shows a typical trace from the gas chromatographic analysis of the atmosphere within the plant growth chambers of the bioassay system.

In Table 3.1 the results of analysing the atmosphere in different chambers containing different plastics is presented. DBP and DIBP were found to be present only in the atmosphere of those plastics which were toxic in the bioassay. Moreover these were also the plastics supplied by ICI which contained butyl phthalates or which have been analysed by ICI and shown to have DIBP present in their formulation. The figures given are the average concentrations over a 48 hour period as the temperature in the growth chamber varied by about 5°C, depending on whether the lights were on or off, thereby altering the volatilty of the plasticer. The new DIDP-plasticised glazing strip appeared to carry trace amounts of DIBP and DBP. This result is in agreement with the findings of Hardwick *et al.* (1984), who also found traces of butyl phthalates in the DIDP-plasticised granules used to manufacture the strip.

The table shows that an estimation of the aerial concentration of DBP was made when a tray of young radish plants were present in the growth chamber (Table 3.1,i). Measurements of the atmospheric DBP concentration were

Table 3.1 Estimates of aerial DBP plasticiser concentrations during 48 hour sampling periods of growth chamber atmospheres.

Plastic	Weight (g)	Aerial Concentration ($ng.l^{-1}$)
control	-	none
plastic E (DBP plasticiser)	150	69 (i)
plastic E (DBP plasticiser)	150	104 (ii)
plastic E (DBP plasticiser)	171	121 (iii)
plastic I (DIOP plasticiser)	169	none (iii)
plastic J (DIDP plasticiser)	170	none (iii)
DIBP+DOP-plasticised glazing strip (Bailey's, House 7)	77	169 (iii)
DOP-plasticised glazing strip (Bailey's, House 3)	76	none (iii)
New glazing strip DIDP-plasticised	79	trace (iii)

(i) Analysis of atmosphere for DBP was undertaken with a tray of 9 radish plants in the growth chamber.
(ii) Without the tray of plants.
(iii) Estimates of DBP or DIBP concentration in the growth chambers during growth analysis experiments.

also made with the same plastic in the absence of a tray of plants (Table 3.1,ii). Volatiles were always collected at the end of the growth chamber remote to the PVC plastic source. When plants were present they were situated between the plastic and the Tenax containing trap with which volatiles were collected. These figures give some indication of the amount of butyl phthalates removed from the atmosphere by the test plants. The figures show that in this particular example the plants removed approximately 33% of the phthalate present in the growth chamber atmosphere during the 48 hour sampling period.

Included in the table are the figures of the expected concentrations (iii) of phthalates in the atmosphere during the growth experiments described above.

3.3 Solution Culture

Generally additions of liquid plasticiser to the nutrient solutions in which radishes were grown produced variable results. A mottled chlorosis was evident in some of the leaves of both treated and control plants. It is probable that the DBP volatilised from the treated solution cultures in sufficient quantities to induce chlorosis in the control plants. At the same time some plants growing in higher concentrations of DBP in solution showed no signs of chlorosis. It was noted however that the radishes growing in the higher concentrations of DBP had

considerably less well developed root systems when compared to the control plants. The implication from this work is that chlorotic symptoms develop from the volatilised DBP vapour being absorbed by unknown means through the leaves. Reduction in the growth of the root system may contribute towards the stunting of growth of the whole plant. These experiments were discontinued, partly because of problems isolating the aerial parts of the plant from the roots, but also as a result of the apparent lack of translocation from the roots as evidenced by the lack of chlorosis in some plants in nutrient medium of high DBP concentration. It seems likely that all the chlorosis seen in these experiments arose from DBP volatilising from the nutrient solution. The implication from these experiments is that the DBP is not translocated to other parts of the plants once it has entered by the root system.

3.4 Electron Microscopy

The work described here is not a comprehensive study. It was undertaken with a view to clarifying the results of earlier work, as there had been some problems in ascertaining the precise age and history of the material used in previous electron microscopical studies.

A few representative photomicrographs are presented in this thesis to illustrate the major effects of dibutyl

phthalate on the ultrastructure of radish chloroplasts. The age and history of the plant material is of great interest as the appearance of treated chloroplasts varies greatly depending on the stage of development of the leaf at the time it was subjected to DBP vapour.

3.4.1 Untreated Leaves

The appearance of the untreated chloroplast, Fig.3.12, is typical of a healthy chloroplast. There is a well organised, evenly distributed granal system. There is no swelling of the intra-thylakoid space. A starch grain is present. The white patches with a diffuse outline in the stroma are probably DNA. There are few plastoglobuli; those which are present are comparatively small compared to those in treated chloroplasts. The chloroplast is elongated in shape.

3.4.2 Immature Treated Leaves

Figure 3.13 shows the appearance of chloroplasts from a young radish leaf treated with DBP vapour for 3 days. All the chloroplasts in this section are of the typically rounded shape seen in treated plants. This does not seem to be an artifact introduced as a result of the plane of the section as this rounding is seen in all sections of treated chloroplasts, often to a greater degree than in Fig.3.13. Some chloroplasts also show the increased numbers of plastoglobuli often seen in treated

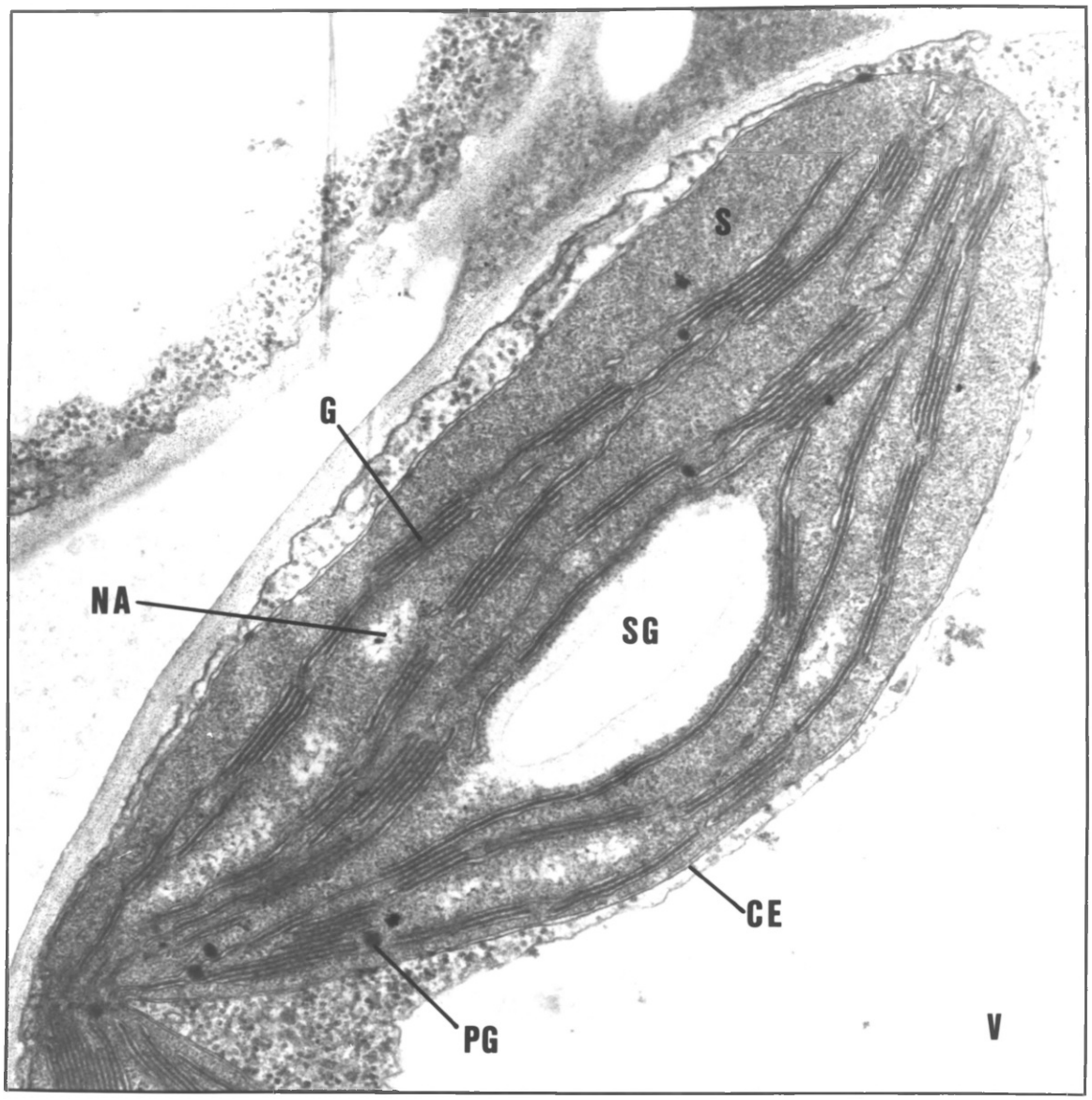


Fig.3.12 Electron micrograph of a cross section through a chloroplast from a mature radish leaf. G - granum; SG - starch grain; PG - plastoglobuli; Th - thylakoid; CE - chloroplast envelope; NA - nucleic acid (DNA); V - vacuole. (x 43000)

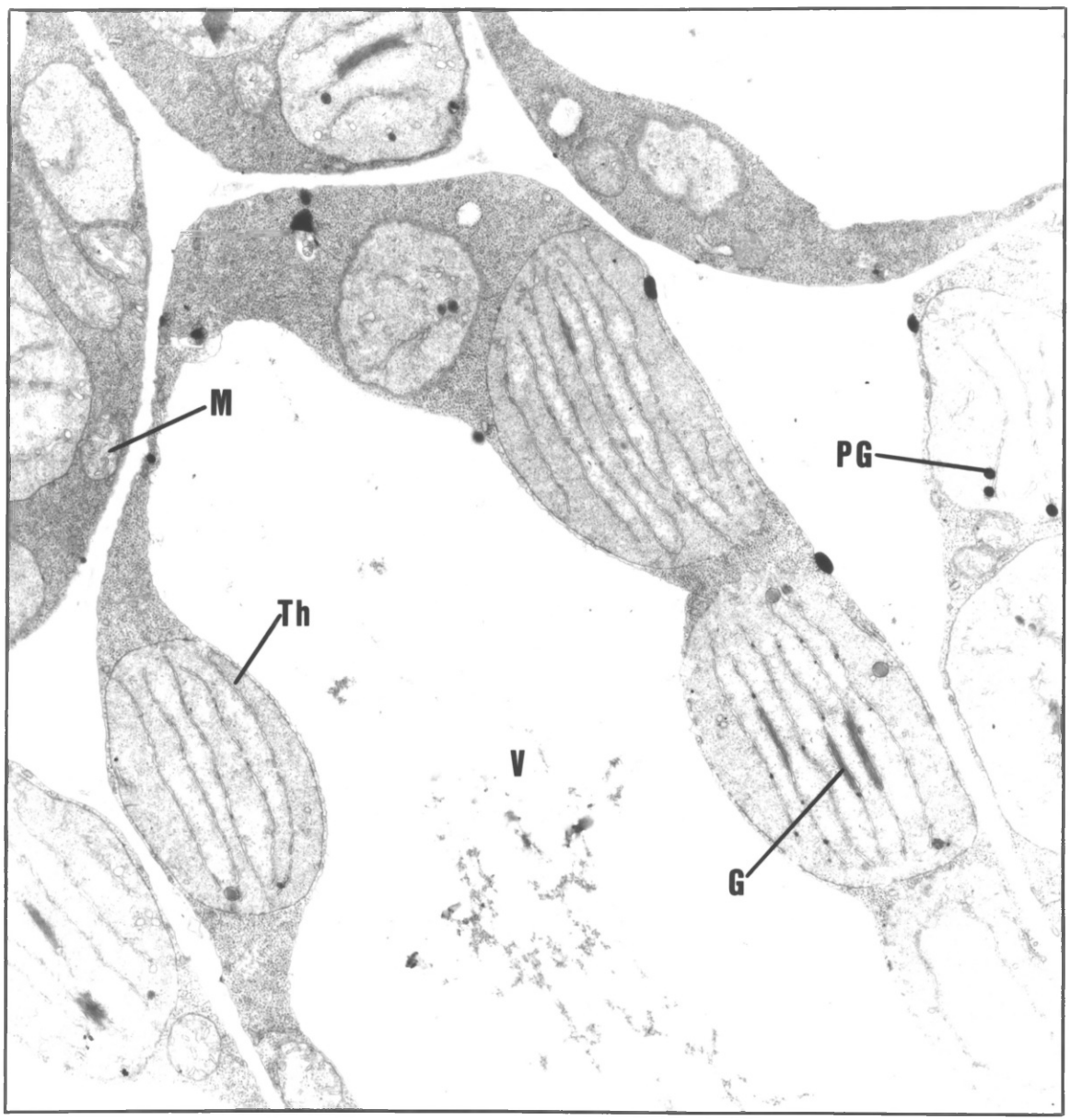


Fig.3.13 Section through a young radish leaf treated with DBP vapour for 3 days. G - granum; Th - thylakoid; PG - plastoglobuli; M - mitochondrion; V - vacuole. (x 12000)

chloroplasts. Some of these - usually the larger ones - are lightly stained and a bounding membrane may be seen. A number of generally smaller, very intensely staining plastoglobuli are also present. Curiously, these are usually associated with the thylakoid membranes. One other point to note about the structure of these treated chloroplasts is the slight swelling of the intra-thylakoid spaces. This swelling does not appear to occur along the whole length of the thylakoid but at various localised spots. In some sections this swelling has been much greater and often seems to be associated with the ends of thylakoids. The young leaf from which this material was taken was completely chlorotic and not yet fully expanded. The mitochondria in this section are of normal appearance.

Figure 3.14 shows a higher magnification of one chloroplast from the section in Fig.3.13. The most striking difference between this chloroplast and the control, Fig.3.12, is the lack of grana in the treated chloroplast. Another notable difference is the absence of starch grains in this and other treated chloroplasts. A comparison of this chloroplast with the untreated one in Fig.3.12. again demonstrates the differences in the overall shape. It is clearly demonstrated that the treated chloroplast has a more rounded shape when compared to the elongated shape of the control chloroplast. One of the reasons for choosing this particular view of a treated chloroplast is the presence of long roughly parallel lines of broken thylakoids. This is similar in appearance to a

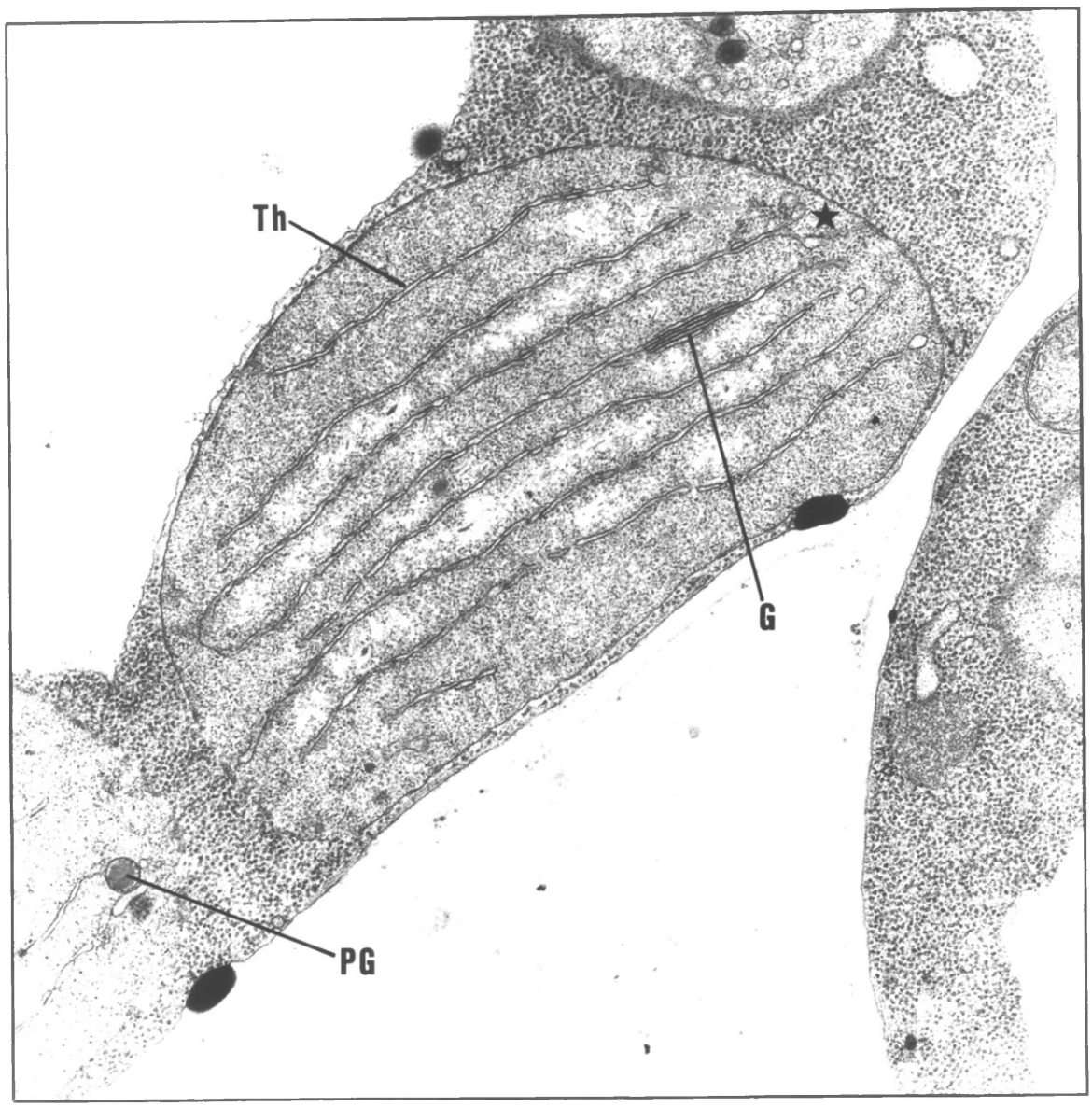


Fig.3.14 Higher magnification of one chloroplast from Fig.3.13. G - granum; Th - thylakoid; PG - plastoglobuli. (x 29000)

developmental stage in the differentiation of proplastids and etioplasts into chloroplasts. In developmental terminology they are referred to as perforated or fenestrated sheets. The ends of these thylakoids are very swollen (marked by a star in the photomicrograph) and milder swelling and apparent discontinuities may be seen along their entire length.

3.4.3 Very Immature Treated Leaves

The next photomicrograph (Fig.3.15) shows sections of chloroplasts from a leaf which has been exposed to DBP vapour for a period of 5 days. The degree of structural organisation within all four of the chloroplasts seen in this section is less than that seen in the leaves exposed for only 3 days. This may be due to the increased duration of exposure, however the results may be explained by supposing that at the initiation of exposure to DBP the chloroplasts were at an earlier developmental stage than those seen in Figs.3.13 and 3.14. Granal or even thylakoidal structures are virtually absent from these chloroplasts. In chloroplast (a) the remains of a few thylakoidal membranes are present. There appears to be no organisation to these membranes. Two large and a number of smaller plastoglobuli are also present in this chloroplast. In chloroplast (b) only a few membranous structures remain, together with a few lightly staining plastoglobuli. This may be a result of the plane of the section. The chloroplast (c) of the photomicrograph has

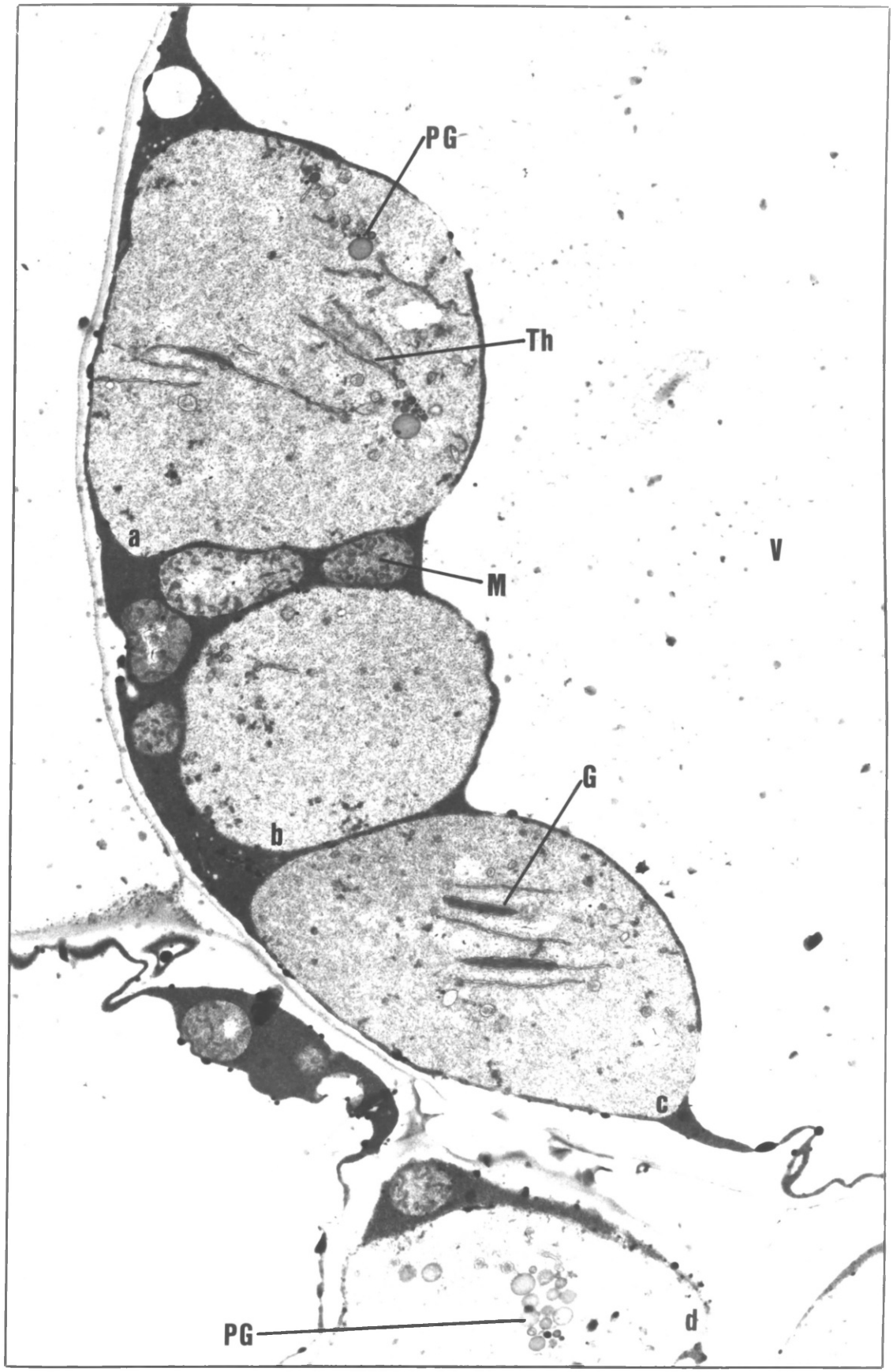


Fig.3.15 Chloroplasts from a young radish leaf treated with DBP vapour for 5 days. G - granum; Th - thylakoid; PG - plastoglobuli; M - mitochondrion; V - vacuole. (x 14000)

two grana but no intergranal thylakoids. There are three disconnected thylakoids and a number of light or very lightly staining plastoglobuli. The internal structure of chloroplast (d) in this photomicrograph has been reduced to small groups of lightly staining plastoglobuli. These treated chloroplasts were typical of others seen in other sections. Again the appearance of mitochondria is normal.

3.4.4 Mature Treated Leaves

The final two photomicrographs, Figs.3.16 and 3.17 show the effects of DBP vapour on the ultrastructure of chloroplasts which were mature and green from the start of exposure. The plant material used for these photographs was exposed to DBP vapour for a period of 5 days. One of the most striking aspects of Fig.3.16 is the roundness of the chloroplasts and how distended into the vacuole of the cell they are. The other remarkable phenomenon is the way in which the grana and the intergranal thylakoids are displaced to the vacuole side the chloroplast. It is possible that the grana are displaced in this direction due to the restrictions of the cell wall. What is curious is the way in which the white patches of DNA and most of the plastoglobuli have also been swept to one side of the chloroplast. This may be more clearly seen in Fig.3.17. The large size of the grana, in terms of the number of stacked thylakoids, is probably a consequence of this movement towards one side of the chloroplast. Beyond the increased numbers of large, darkly staining plastoglobuli

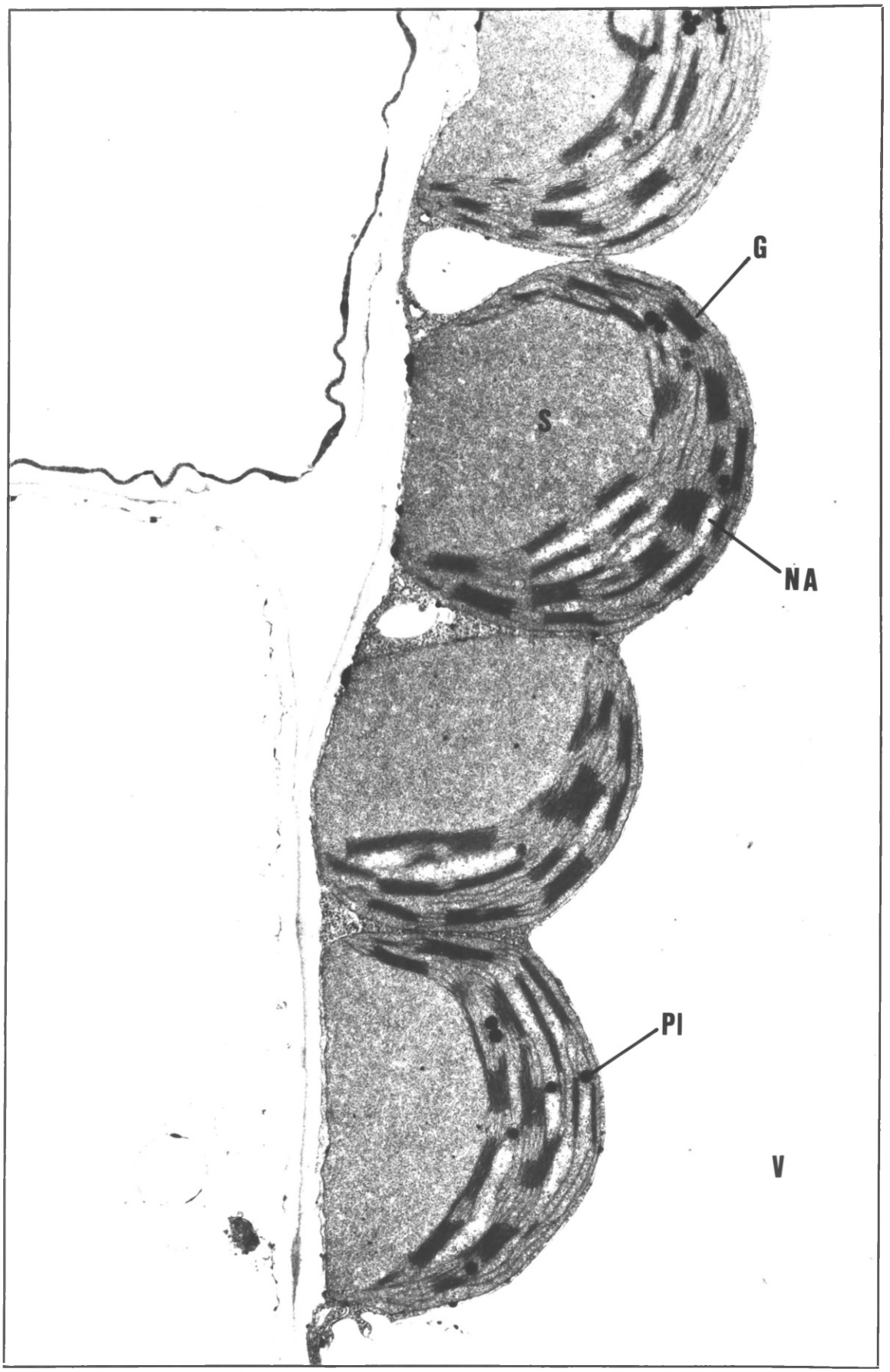


Fig.3.16 Section through chloroplasts from a mature leaf exposed to DBP vapour for 5 days. G - granum; PL - plastoglobuli; NA - nucleic acid (DNA); S - stroma; V - vacuole. (x 16000)

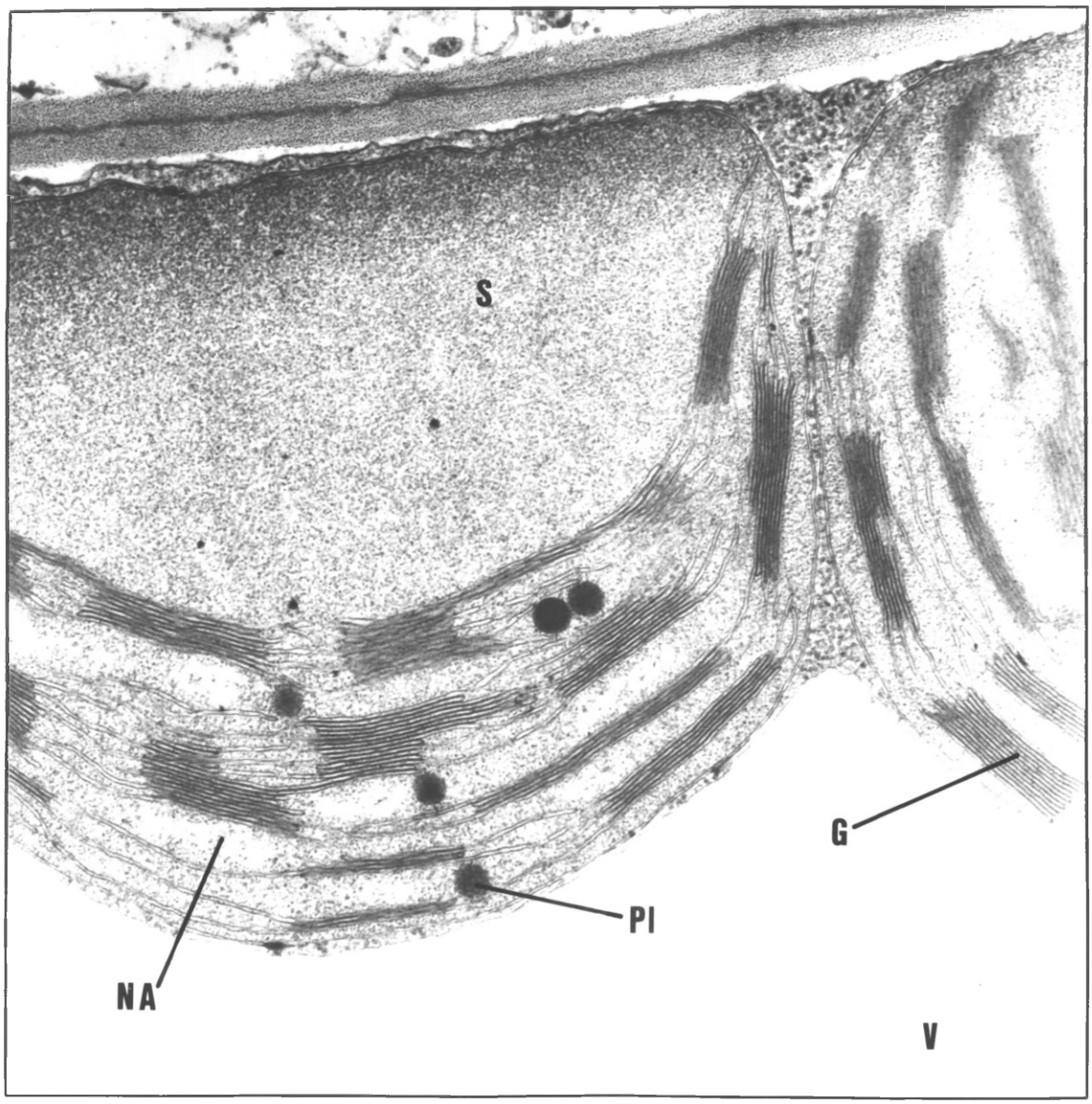


Fig.3.17 Higher magnification of one chloroplast from Fig.3.16. G - granum; PL - plastoglobuli; NA - nucleic acid (DNA); S - stroma; V - vacuole. (x 40000)

there appears to be no indication of thylakoid membrane breakdown. As in the treated chloroplasts of young leaves there is an absence of starch grains.

3.5 Effect Of Dibutyl Phthalates On CO_2 Exchange

The growth experiments described in Section 3.1 show that one effect of the butyl phthalates is to inhibit growth. This result coupled with the visual evidence provided by the photomicrographs indicate that one of the primary sites of action of DBP may lie somewhere in the chloroplasts, possibly affecting the fixation of CO_2.

In order to explore this possibility it was decided to measure the CO_2 exchange of radish plants using an infrared gas analyser. Three treatments were looked at: exposure to samples of DBP-plasticised PVC; exposure to the pure liquid DBP plasticiser; and a control.

3.5.1 Preliminary Experiment

The plants tested were 10 days old, each plant possessed four leaves and similar leaf areas at the beginning of the experiment. Leaf area was measured immediately prior to the start of the experiment and at the end when the plants were removed from the treatment chambers. At the start of this experiment the control plant had the lowest

photosynthetic rate per unit area. By the sixth day it's rate was the highest. Photosynthesis was reduced in both treated plants, the rate being more reduced in the plant exposed to the DBP-plasticised plastic. Large fluctuations of photosynthetic rates were recorded during the mornings. This was a result of the sun striking the growth chambers at certain times of the day. This was prevented in subsequent experiments by surrounding the growth chambers with large sheets of white cardboard. The other major problem which arose was leaking growth chambers. This usually occurred at the junction of the bag and the perspex disc. Great care was needed in setting up these experiments to ensure that readings were not inaccurate due to a leak. After a time it was possible to recognise the occurrence of a leak by variations in the shape of the traces obtained from the IRGA.

3.5.2 Measurements Of CO_2 Exchange

The stomatal resistances of all plants were measured at the start and end of this experiment to see if the reduction in photosynthetic rates could be attributed to the closing of stomata.

Figure 3.18 shows typical chart recorder traces of variations in CO_2 concentration obtained with the infrared gas analyser.

The various steps seen in the trace represent the

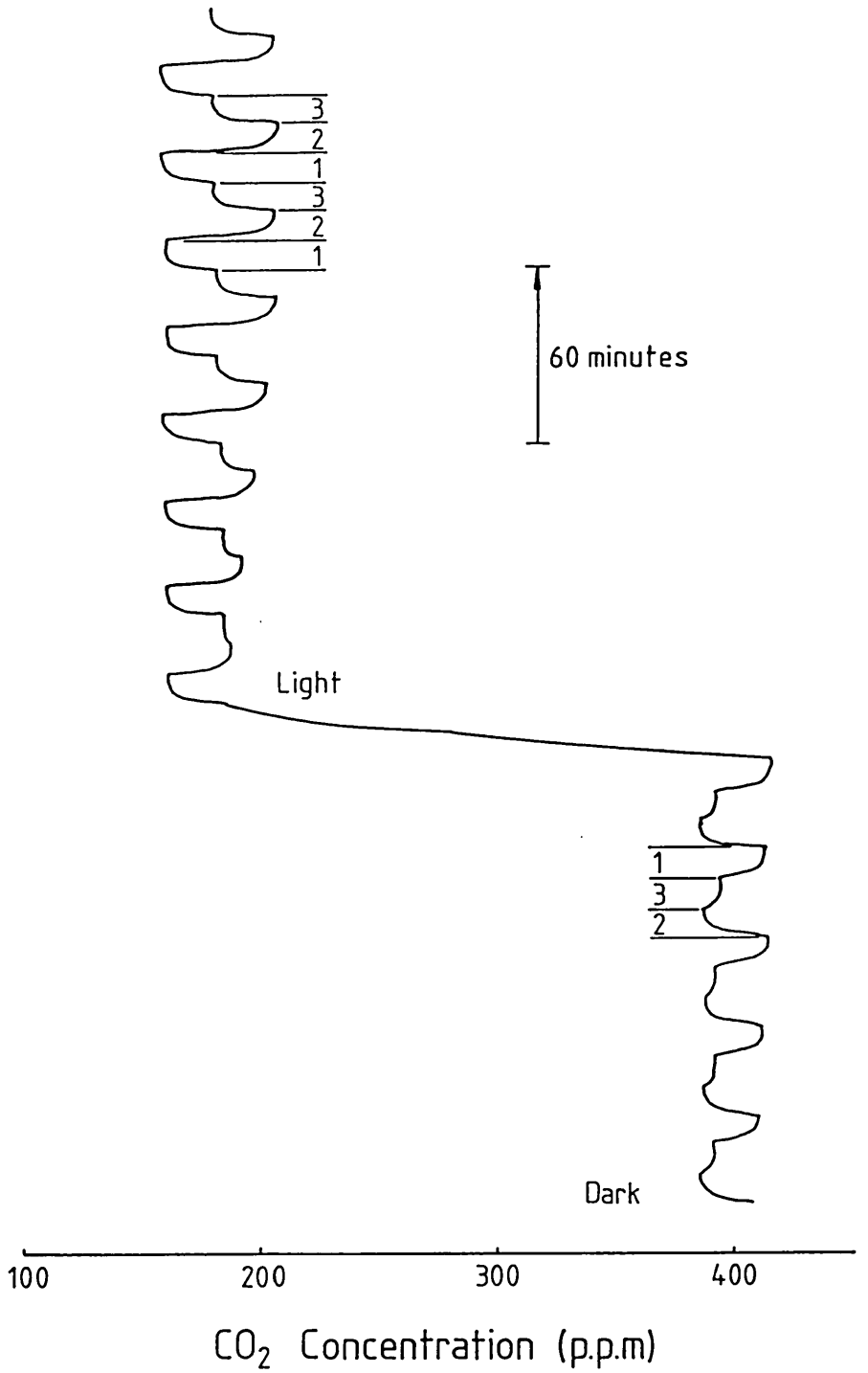


Fig.3.18 Infrared gas analyser traces of CO_2 exchange in the aerial parts of radish plants. 1. Control; 2. DBP-plasticised plastic; 3. Liquid DBP plasticiser.

concentrations of CO_2 in each of three growth chambers - one being the control (1), another containing the DBP-plasticised plastic (2), whilst the third held two small glass vials of liquid DBP (3). The atmosphere of each chamber was sampled, in turn, for a ten minute period. Concentrations of CO_2 for each chamber were calculated using the IRGA reading at the end of each of these sampling periods, to ensure that the CO_2 concentration measured in the IRGA was the same as that in the growth chambers.

The next graph (Fig.3.19) shows the variation of CO_2 concentrations plotted for each of the three treatments. Leaf area was measured at the start and end of the experiment. Leaf areas were not measured more frequently than this because of the problems in ensuring the apparatus was sealed after measurements. There was also the danger of damaging the plant during removal from the growth chambers for measurements.

Calculations of CO_2 exchange are made on a leaf area basis assuming log growth between the start and end of the experiment. The average CO_2 concentration of three readings between 1pm and 2.30pm each day were plotted against the number of days the experiment had been running. These times were chosen as the experimental plants would have been able to fully adjust to the switch over from dark to light by this stage of the day. They would also have had time to adjust after any effects of

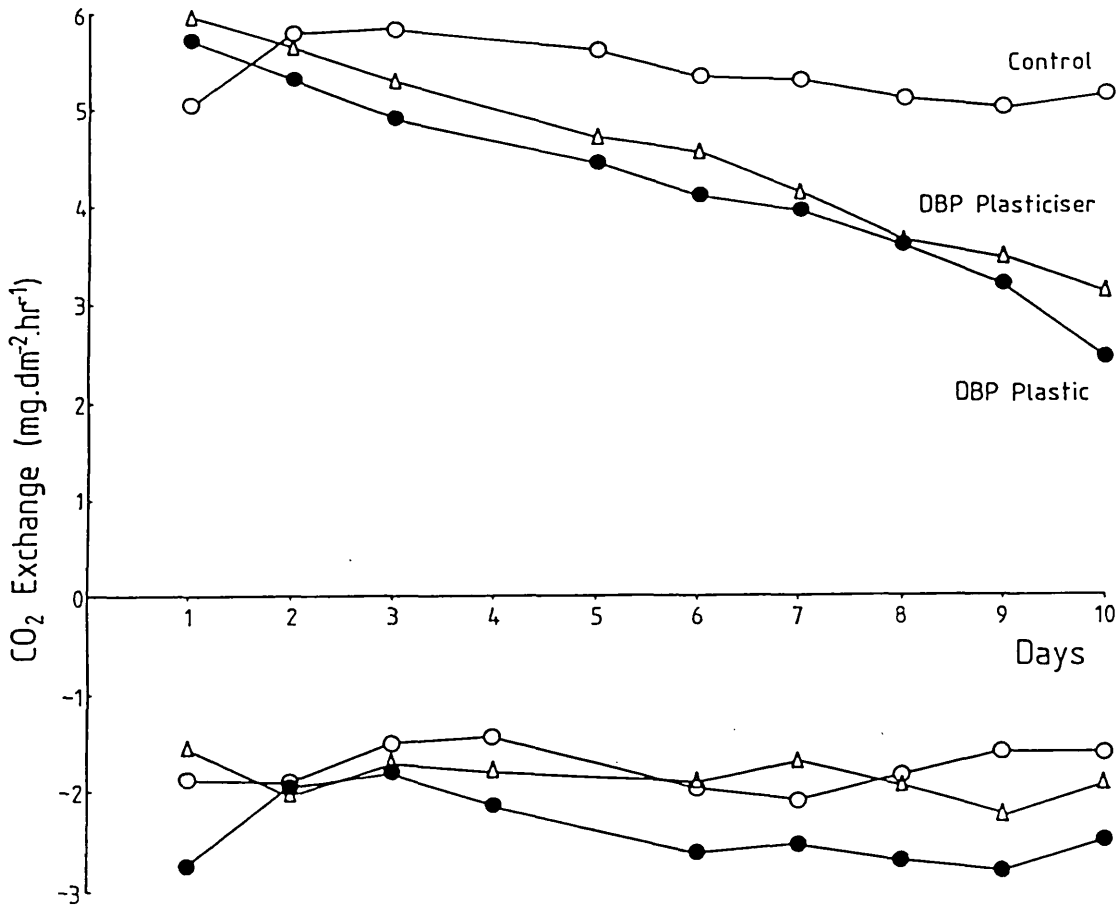


Fig.3.19 Graph showing the CO_2 concentration during the light period (calculated as the mean of three readings between 1pm and 2.30pm) and during the dark period (calculated as the mean of three readings between 1am and 2.30am). No measurements taken on day 0.

replenishing the silica gel - a task which was normally performed around 9am and 6pm each day.

As with the preliminary experiment, the control, although initially possessing the lowest photosynthetic rate of the three plants, maintained it's CO_2 uptake rate throughout the duration of the experiment. In the meantime the other two plants are seen to suffer from their treatments as evidenced by their reduced photosynthetic rates during the course of this experiment. As in other experiments the equal surface area of DBP-plasticised plastic reduced photosynthesis to a greater degree than an equal area of liquid DBP plasticiser. The atmospheric concentration of DBP was probably higher in the DBP-plastic growth chamber.

Respiration rates were also calculated assuming log growth and again the figures provided are the average of three readings between 1am and 2.30am. Although respiration rates were slightly different for each of treatments the three plants had maintained their respiration rates by day 10. This result is in line with the ultrastructural work as no obvious damage of mitochondria was seen in sections from the young leaves of treated plants after 5 days exposure to DBP vapour.

The measurements of stomatal resistances (Table 3.2) indicate that the reduction in the photosynthetic capability of treated plants was not due primarily to the closure of stomata but to some more direct effect on

photosynthesis. The fact that there was little or no effect on respiration also tends to confirm this.

Table 3.2. Total leaf areas and stomatal resistances at the start and end of CO_2 exchange measurements. (Mean of three leaves ± S.E.)

	Treatment	Leaf area cm^2	Stomatal Resistance (±S.E.) $sec.m^{-1}$
start	1. control	39.5	7.3±0.84
	2. DBP plastic	45.0	6.9±1.16
	3. DBP plasticiser	43.75	8.2±1.75
end	1. control	73.6	2.4±0.01
	2. DBP plastic	50.00	2.1±0.36
	3. DBP plasticiser	55.10	2.9±0.45

3.6 Leaf Disc Electrode

A leaf disc electrode was used to measure the O_2 exchange in whole young leaves and 10 cm^2 discs cut from mature leaves.

Radish plants were exposed to the plastics provided by ICI, using the growth chambers described in Section 2.1.3. The trays of plants were removed from the growth chambers and either a young or an old leaf was cut from a plant. Whole young leaves were used for the photosynthesis measurements, while a 10 cm^2 disc was cut from the larger, mature leaves. The tray of plants was replaced in the growth chamber immediately after sampling.

3.6.1 O_2 Exchange In Young And Old Leaves

The graph in Fig 3.20 shows the effect of DBP vapour (from 150g of plastic E) on photosynthesis and respiration in young leaves over a period of 7 days. The results are presented on a mg O_2 per unit leaf area basis. After a single day of exposure to the DBP, photosynthetic CO_2-dependent O_2 evolution was reduced but the young leaves showed no chlorosis at this stage. Over the next 6 days O_2 evolution continued to fall until by the seventh day it was below the gas exchange compensation point. Therefore there was net respiration in the light. The leaves towards the end of the experiment were increasingly chlorotic and so the decreased O_2 evolution rates may be a reflection of reduced assimilatory apparatus per unit area.

Respiration rates of the control and treated plants differed little during the experiment although in the

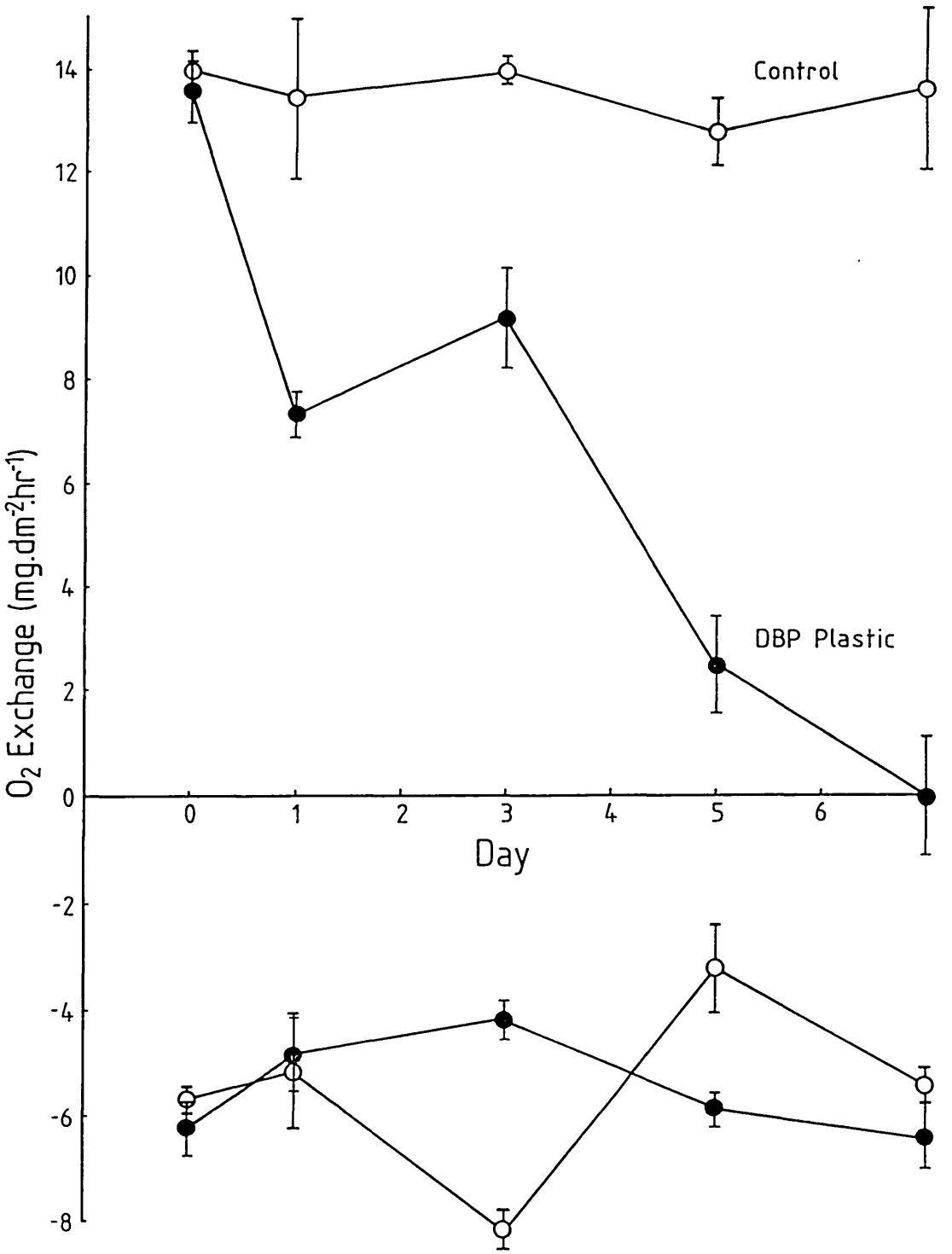


Fig.3.20 Changes in oxygen evolution in young radish leaves following exposure to dibutyl phthalate plasticisers. Each point is the mean rate of three leaves, ±SE.

later stages the rate was slightly higher in the treated plants.

The explanation of the results of oxygen evolution in mature, fully expanded leaves was less clear. Figure 3.21 shows the oxygen evolution of leaf discs cut from mature leaves. Again the results are expressed on a mg O_2 per unit leaf area basis. Although fairly large fluctuations were present, photosynthetic oxygen evolution in the control changed little overall. Photosynthesis was reduced over a period of 11 days in the leaf discs from treated plants, although the reduction was not as great as that seen in the young leaves.

Respiration rates of control and treated plants fluctuate considerably during the experiment but no overall trend is seen with either treatment.

Stomatal resistances fluctuated in both the control and treated plants during the experiment (Table 3.3). Overall the resistances of the treated plants were higher. It should be pointed out that closure of stomata offer relatively little resistance to the high concentration of CO_2 (Zelitch, 1971) present within the leaf disc electrode and hence the present measurements of O_2 evolution should not be affected.

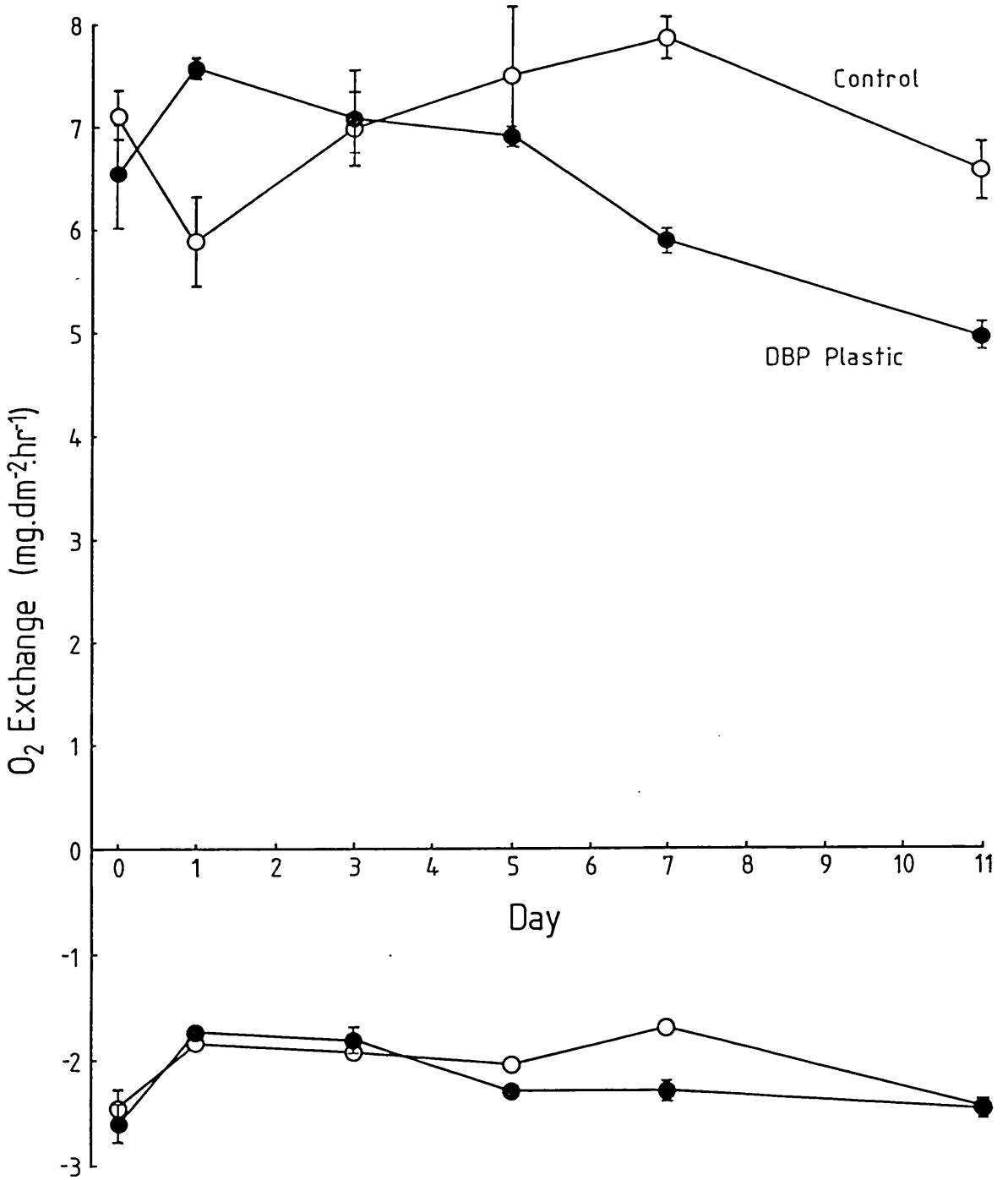


Fig.3.21 Changes in oxygen evolution in mature radish leaf discs following exposure to dibutyl phthalate plasticisers. Each point is the mean rate of three leaves, ±SE.

Table 3.3 Stomatal resistances of young and mature leaves of DBP-treated and control radish plants. (Mean of three leaves ±S.E.)

Day	Stomatal resistances ($sec.cm^{-1}$)			
	Young leaves		Mature leaves	
	Control	DBP	Control	DBP
1	2.4±0.6	3.6±0.4	7.9±3.1	8.8±2.6
2	2.7±0.2	2.8±0.7	5.4±0.9	3.7±1.0
4	3.2±0.3	3.9±1.1	7.2±1.1	4.6±0.8
6	2.3±0.4	7.2±1.1	9.8±1.3	12.1±1.9
8	4.0±0.3	9.6±2.1	4.2±0.7	9.3±1.2
12	-	-	6.4±1.0	10.6±2.1

3.6.2 Oxygen Evolution Of Mature Leaves

As the reduction in photosynthesis of mature leaves exposed to DBP vapour was not as clear cut as that in young leaves further experiments were carried out using mature leaves only. Oxygen evolution measurements were made in conjunction with the chlorophyll fluorescence readings described in Section 3.7.5.

Figure 3.22 shows examples of traces of the leaf disc electrode response to the CO_2-dependent O_2 evolution of a leaf disc from a radish plant previously exposed to DBP plasticisers for a period of 13 days compared to an

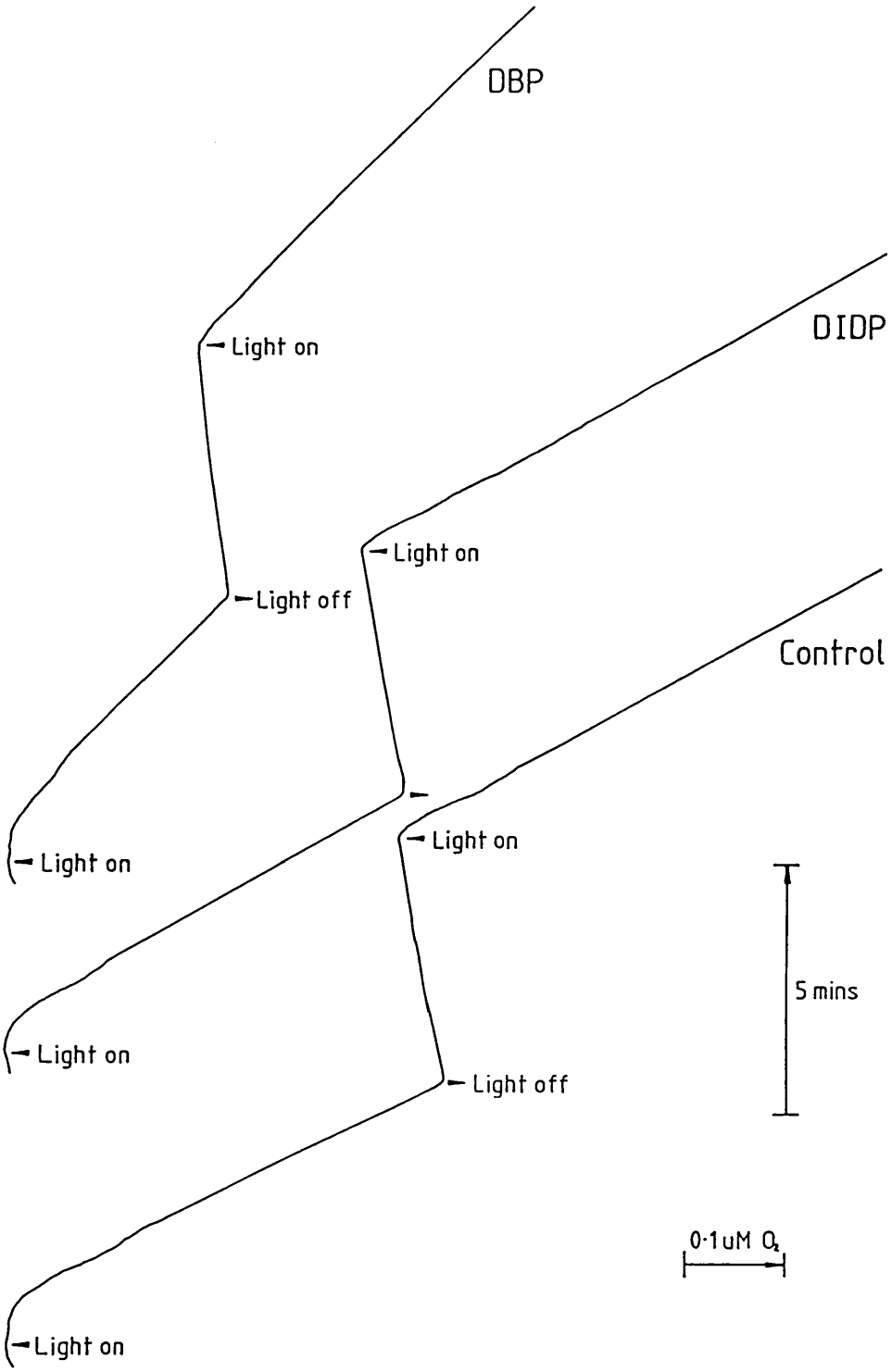


Fig.3.22 Traces of the electrode response to the CO_2-dependent oxygen evolution of: untreated, DIDP- and DBP-treated 10 cm^2 leaf discs cut from mature leaves.

untreated control. The rate of oxygen evolution in the DBP treated leaf disc was obviously reduced.

Table 3.4 Rate of CO_2-dependent O_2 evolution from radish leaf discs from the mature leaves of plants exposed to the vapour of various plasticisers.

	Rate of O_2 evolution ($\mu mol.mg^{-1}chl.hr^{-1}$)	
Treatment	Day 0	Day 13
Control	181.1	196.4
DBP	179.3	122.1
DIDP	182.2	230.4
DIOP	181.5	263.8

Oxygen evolution was calculated on a μmol O_2 per mg chl per hour basis. The results in Table 3.4 show that oxygen evolution was substantially reduced in the mature leaves of plants exposed to DBP vapour for 13 days when compared to the control rate. The rates of the other two treatments, DIDP and DIOP, were slightly higher than the control. It is not realistic to compare O_2 evolution rates at the start and end of the experiments as the large differences probably reflect adaptations in the morphology of the leaf to cope with the new environment of the growth chambers. It is pertinent that only the O_2 evolution rate

of the DBP-treated leaf disc declined over the 13 days of the experiment, while the rates in all the other treatments increased substantially.

3.7 Chlorophyll Fluorescence Measurements

Chlorophyll a_2 fluorescence can be used as a very sensitive intrinsic probe of the primary reactions of photosynthesis. In the *in vivo* system the factors affecting the yield of fluorescence are very complex and therefore difficult to interpret (Heinrich & Weis, 1984). The yield is inversely related to the yield of photosynthesis providing nothing else changes. The physiological state of both the chloroplast and the cell have an important effect on the kinetics of fluorescence emissions. Moreover these physiological states need not be influenced by the functioning of the photosynthetic apparatus.

As the theories underlying fluorescence emissions are complex, it would seem appropriate at this stage to briefly explain the current views held on chlorophyll fluorescence and the associated kinetics.

3.7.1 Chlorophyll Fluorescence

Chlorophyll fluorescence at room temperature is almost entirely due to emission from the chlorophyll a molecules

associated with photosystem 2 (PS2). The chlorophyll species (chlorophyll a_2) which acts as the light harvesting antennae for PS2 has a maximum red peak of absorption at 678nm and a peak of emission about 685nm.

Light absorption by chlorophyll molecules induces a first excited singlet state. Excited chlorophyll molcules have several ways of dissipating this energy to return to the ground state, namely - photochemical reaction (electron transport), excitation transfer to neighbouring non-excited pigments, non-radiative (thermal) de-excitation and fluorescence emission. The fluorescence yield from a sample excited by a given quantity of incident light is determined by the rate constants for these competing processes and the concentration of open reaction centres (PS2 traps).

The equation for the *in vivo* chlorophyll a_2 fluorescence yield is

$$F = \frac{k_f}{k_f + k_h + k_t + k_p[P]} \qquad (3.1)$$

where k_f, k_h, k_t and k_p are rate constants for fluorescence emission, radiationless de-excitation to heat, transfer to neighbouring non-excited pigments and photochemical de-excitation. [P] is the concentration of open PS2 reaction centres.

From Equation (3.1) it is clear that any changes in the rate constants or in [P] will result in changes in the fluorescence yield.

3.7.2 Fluorescence Emission Kinetics

When a dark treated leaf is exposed to light, the yield of chlorophyll a_2 fluorescence will vary with time. These changes can be divided into two major components; fast (~<1 second) and slow changes (seconds to minutes). The work in this thesis utilised the slow fluorescence changes as a probe to examine the effects of dibutyl phthalates on the primary reactions of photosynthesis. The fast changes are closely related to primary photophysical and photochemical events.

The time course of slow fluorescence changes *in vivo* are very complex but they are thought to be essentially independent of [P] (Barber, 1976).

3.7.2.1 Fluorescence Quenching

The effects of the dibutyl phthalates on slow fluorescence changes were examined in two ways. The simplest method was to expose a dark-treated leaf to modulated light of a wavelength which was preferentially absorbed by photosystem 2 (Light 2) and observe the subsequent modulated fluorescence kinetics. An idealised trace from an untreated control leaf is shown in Fig.3.23a. A trace

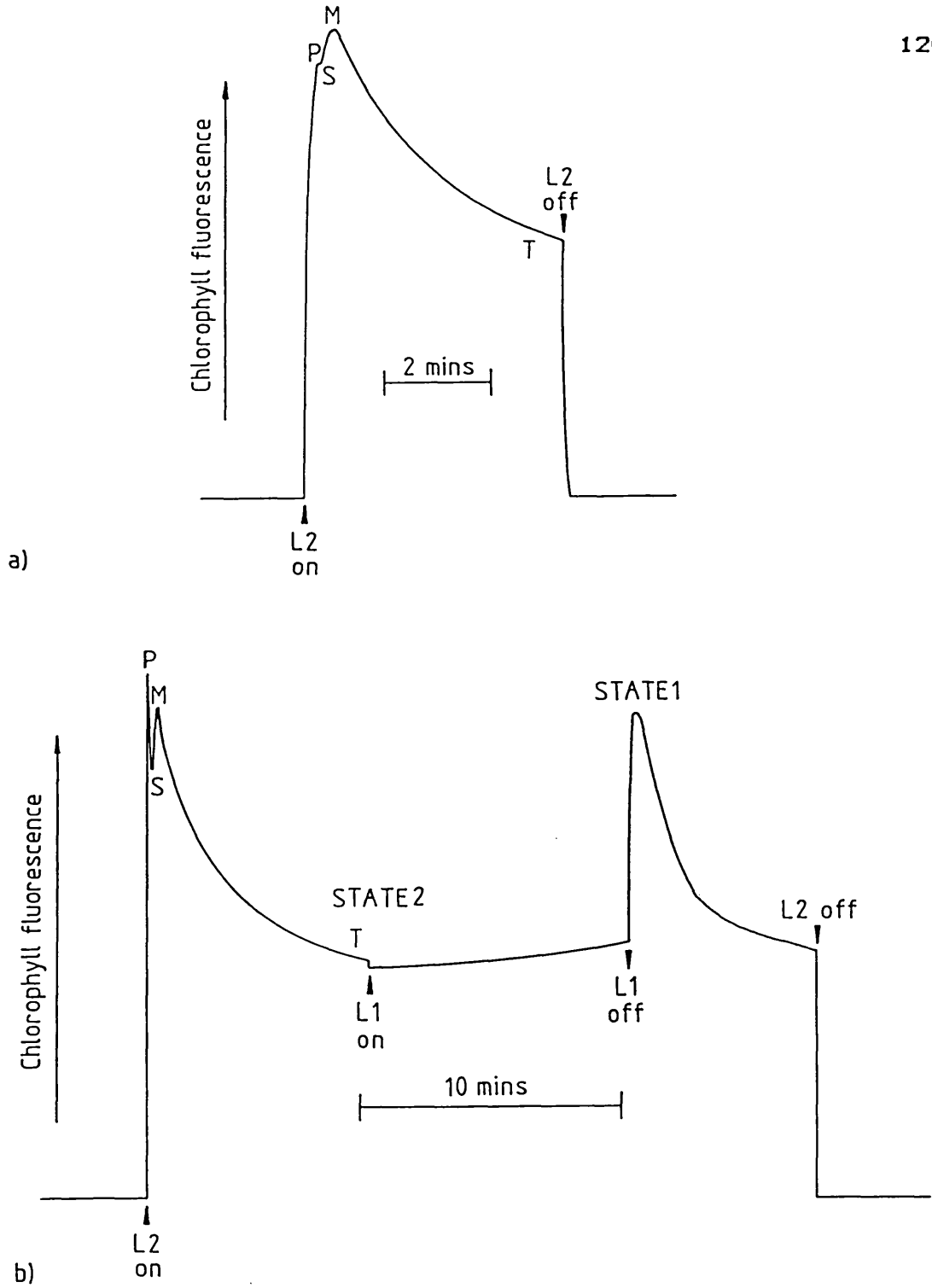


Fig.3.23 Typical changes in the yield of chlorophyll a fluorescence from detached radish leaves dark pretreated for 5 minutes: a) Kinetics of chlorophyll fluorescence from a radish leaf exposed to light 2 - light preferentially absorbed by PS2; b) State 1 - State 2 changes. Light 1 - light preferentially absorbed by PS1.

such as this shows a rapid rise in fluorescence to a level P. In an intact leaf the rate and magnitude of the fluorescence rise to P depends on the intensity of the light and the pre-conditioning of the leaf. With a light intensity which saturates photosynthesis only the pre-conditioning of the leaf is important. This initial rapid rise is thought to occur primarily as a result of the reduction of the plastoquinone (PQ) pool. A decline in fluorescence then occurs from P to S. Slower variations in fluorescence level then occur which vary depending on the leaf. The first is often a rise in fluorescence from S to M. This is followed by a marked decrease to a low final level T. This quenching of fluorescence is the consequence of several processes. Re-oxidation of PQ and Q make a major contribution to this quenching. Other factors responsible for quenching include changes in the proton gradient across the thylakoid membranes and stromal ATP concentrations.

3.7.2.2 State 1 - State 2 Changes

The second method used observed changes in fluorescence yield as a leaf was exposed to modulated blue-green light (Light 2) - preferentially absorbed by PS2 - and/or non-modulated far-red light (Light 1) - preferentially absorbed by PS1 - in various sequences. These sequences were employed to examine both fluorescence quenching and State 1 - State 2 changes.

This technique is based on the findings of Bonaventura and Myers (1969), and by Murata (1969), who showed that the excitation of PS2 and PS1 by a fixed wavelength of light was variable. If there is an imbalance in the wavelength of incident light striking a leaf in favour of PS2 there will be a slow change in the excitation of PS2 and PS1 so that a better balance exists between the two. When a leaf is illuminated for several minutes solely with blue-green light which preferentially excites PS2, additional illumination with far-red light, which preferentially excites PS1, will induce only a small change in fluorescence yield. This suggests that electron flow through PS2 and PS1 was already at a maximum and addition of light 1 made little or no difference to the rate. However, if light 1 is left on for 10 minutes and then switched off a large increase in fluorescence yield occurs. This indicates that light 1 has driven the system into a state where enhancement is at a maximum if the photosystems are illuminated by light 2. (Enhancement is the term given to the observation that the rate of oxygen evolution with certain pairs of wavelengths of monochromatic light was greater than the sum of the rates obtained using each light acting alone). A large fluorescence yield is also obtained if a leaf is kept in the dark for several minutes, or illuminated with light 1, before it is illuminated with light 2. This state of maximum fluorescence and maximum enhancement is termed State 1, while the state of low fluorescence and low enhancement is called State 2.

In order to illustrate these ideas Fig.3.23b shows a typical trace from an untreated control leaf. A 5 minute dark period is used to adapt the leaf to State 1. Illumination with blue-green light induces a large fluorescence increase. The complex fluorescence changes which follow are the result of a number of competing processes which are described above, including adaptation to light 2. Continued exposure to light 2 decreases fluorescence as the system is driven into State 2. After 7 minutes exposure to light 2, light 1 is switched on and a small change in fluorescence yield is observed. The leaf is therefore in State 2 - addition of light 1 making little or no difference to the rate of electron flow. Following a period of 10 minutes exposure to lights 2 and 1 during which excess light 1 drives the system into State 1, light 1 is switched off. A substantial increase in fluorescence occurs as a result of the system being in State 1.

3.7.3 Preliminary Experiments

For these experiments radish leaves were treated with light 1, for 6 minutes to drive them into State 1. In the first trace (Fig.3.24a) the fluorescence yield from a mature control leaf is shown. After the 6 minute period of light 1 illumination light 2 was added. On addition of light 2 the yield of chlorophyll fluorescence rose to a certain level and remained there. Following a further 7 minutes illumination with light 1 it was turned off. The

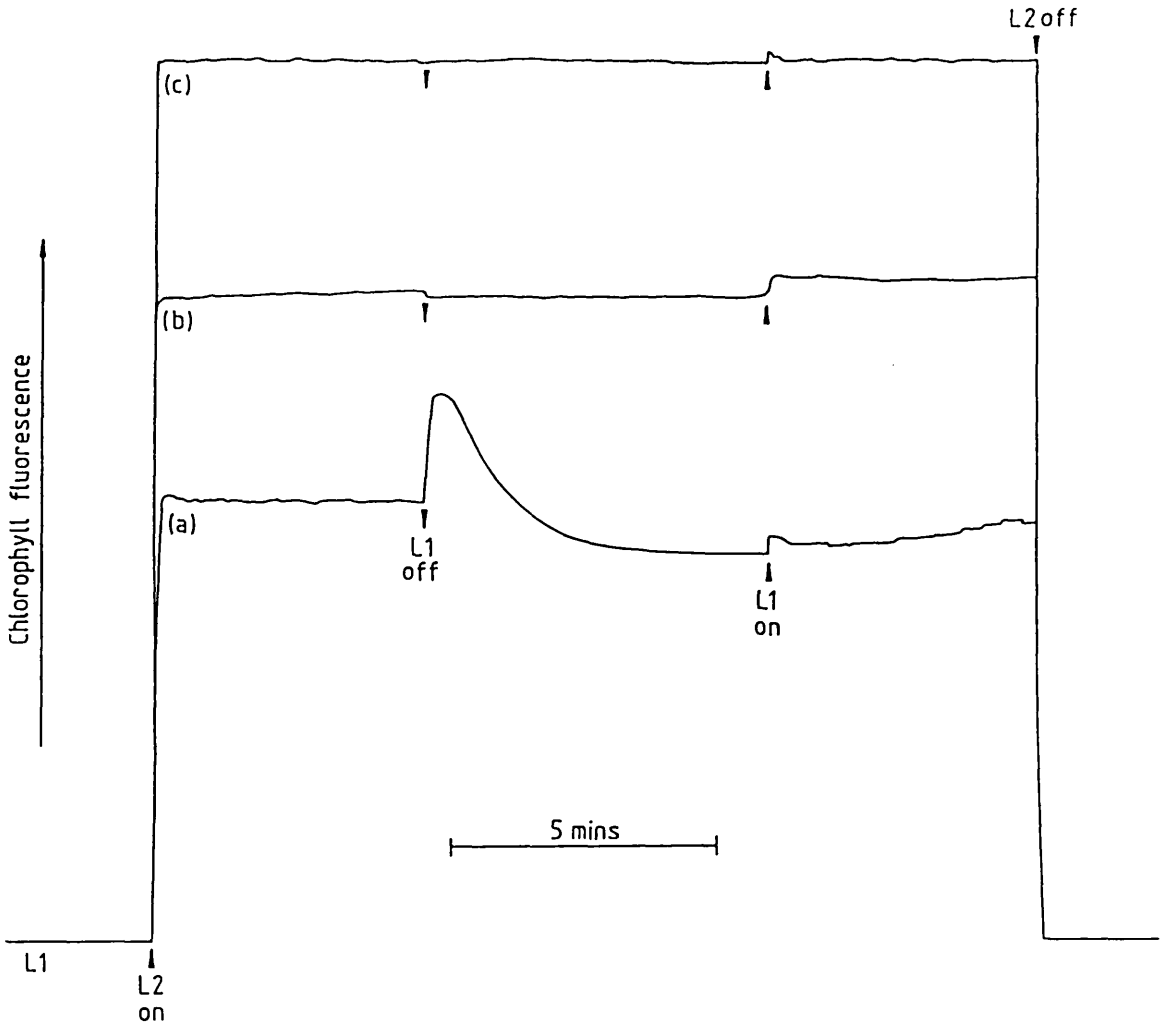


Fig.3.24 Yield of chlorophyll a_2 fluorescence from radish leaves attached to their 25-day old parent plants. (a) Mature control leaf after 15 days; (b) mature leaf exposed to DBP vapour for 15 days; (c) young leaf exposed to DBP vapour for 15 days.

large increase in fluorescence shows that the leaf was in State 1. Following a further 6 minutes exposure to light 2, light 1 was turned on again. Only a small change in fluorescence level was observed indicating that light 2 had driven the leaf into State 2. This trace contrasts strongly with trace (b) shown in Fig.3.24. This trace shows the effect that 15 days exposure to DBP had on the chlorophyll fluorescence yield of a leaf. The intensity, order and length of exposure to lights 2 and 1 was the same as for the control leaf. Comparison of the traces shows that the initial rise in fluorescence on addition of light 2 to light 1 was much greater following exposure to DBP. More significantly there was no decrease in the fluorescence yield during the period when the leaf was exposed solely to light 2. Apparently the leaf had lost the ability to adapt to the wavelength of the absorbed light as a result of exposure to DBP. The greater yield of total fluorescence also suggested that one or more of the fluorescence quenching mechanisms was being inhibited. When light 1 was turned off, leaving light 2, there was only a very small change in the high level of fluorescence. When light 1 was turned on again after a period of 6 minutes a small increase in fluorescence could be detected. The neglible effect that the addition of light 1 had on the high fluorescence yield implied that one fluorescence quenching mechanism inhibited by the DBP was the photochemical one. If another mechanism were inhibited a greater decrease in chlorophyll fluorescence would be expected when light 1 was turned on. These

results indicate that DBP has the ability to inhibit State 1 - State 2 transitions. They also imply that DBP has some inhibitory effect on the photosynthetic mechanisms of a plant.

Trace (c) in Fig.3.24 illustrates the effect of 15 days exposure to DBP vapour on the chlorophyll fluorescence of an immature chlorotic leaf. The story is very similar to that of the mature leaf except that the fluorescence yield is even greater. It could be argued that the loss of State 1 - State 2 transitions in a young DBP-treated leaf are not totally unexpected as a reduction in the total amount of chlorophyll is evident. It is probable that much of the light measured as chlorophyll fluorescence was reflected light 2. The loss of State 1 - State 2 transitions in the mature leaf is of greater interest as the leaf had the appearance of an untreated leaf - there were no chlorotic symptoms.

3.7.4 Chlorophyll Fluorescence Quenching

For this experiment 13-day old radish plants were exposed to the vapour of a DBP-plasticised plastic prepared by ICI (150g Plastic E - see Table 2.1). After 5 days treatment the cotyledons of the control appeared fully expanded, the first and second leaves were well formed and the beginnings of the third and fourth leaves were starting to appear. No chlorosis was seen in these plants. The treated plants were much smaller in size. Cotyledons on these

plants were not fully expanded and the first and second leaves were small and chlorotic. The cotyledons had remained green. The leaves of some plants were removed for electron microscopy. The remaining plants were not exposed to any further DBP vapour. Measurements of fluorescence quenching were taken on detached cotyledons of intact plants the following day; six days after commencing the treatment with DBP. Additional chlorophyll fluorescence measurements were taken a further six days after the last measurements.

Figures 3.25 a,b,c & d give examples of the traces obtained from this experiment. For each reading the cotyledon was exposed to light 1 for 10 minutes, in order to drive the cotyledon into State 1. Substitution of light 1 with light 2 induced a rise in fluorescence yield. On the first day of the experiment, before exposure to volatilised DBP, the initial rise in fluorescence was fairly low and then much of it was quenched (data not shown). The fluorescence yield traces obtained on the day after the plants were removed from the growth chambers showed a different story. The control plants had similar fluorescence kinetics to the plants before they were treated with DBP vapour (Fig.3.25a). Cotyledons from treated plants show a marked initial rise in fluorescence (Fig.3.26b) when light 2 was substituted for light 1. There was no fluorescence quenching during the subsequent exposure of the cotyledons to light 2. The results obtained on the seventh day after exposure to DBP ceased,

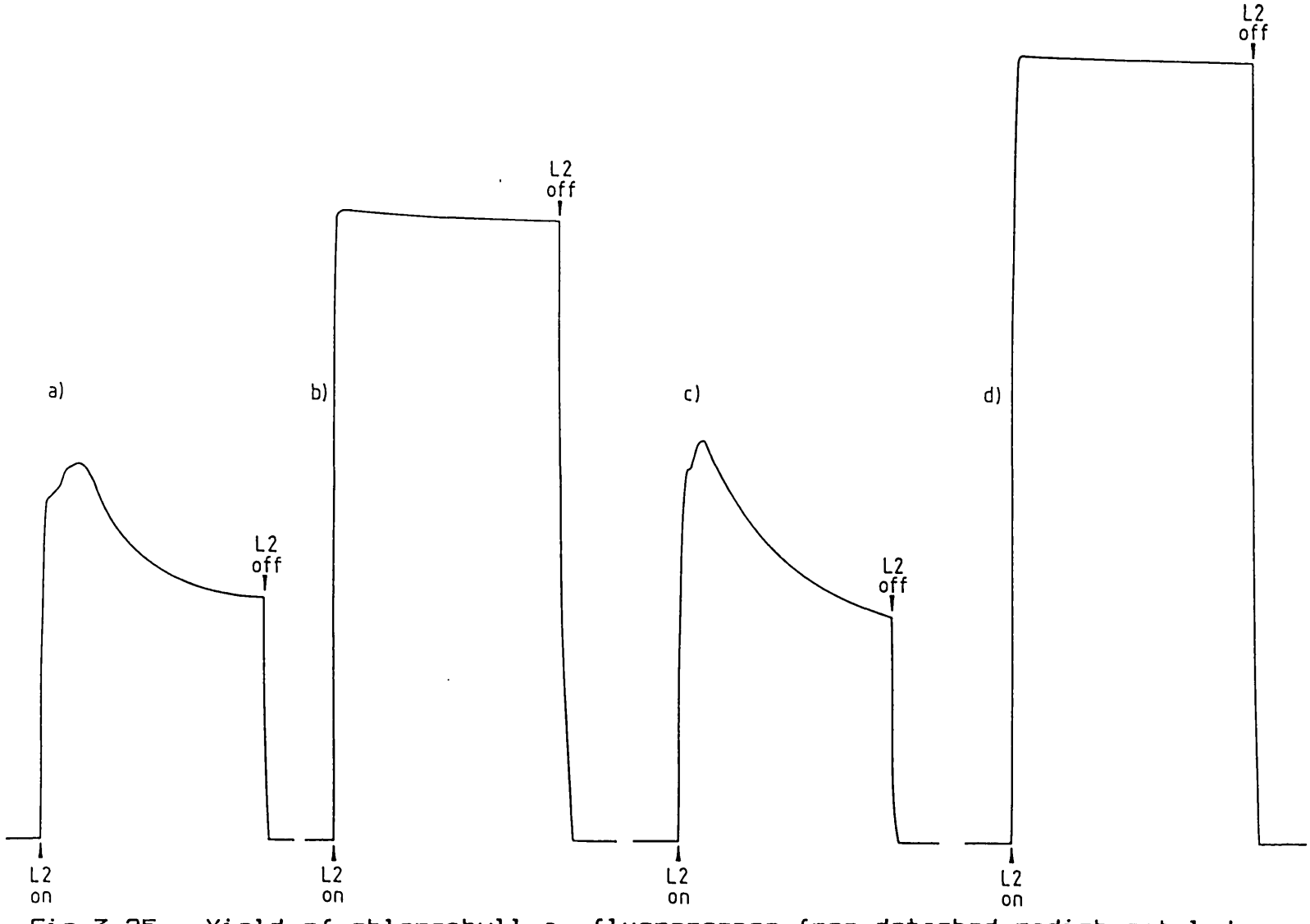


Fig.3.25 Yield of chlorophyll a_2 fluorescence from detached radish cotyledons. a) control after 6 days; b) exposed to DBP vapour for 5 days, fluorescence measurements taken after a further day; c) control after 12 days; d) exposed to DBP vapour for 5 days, fluorescence measurements taken after a further 7 days.

showed a large increase in the overall fluorescence yield (Fig.3.25d). No quenching of this fluorescence was seen.

This experiment confirmed the indications of the previous experiment. The large increase in fluorescence yield suggested that DBP inhibits photosynthesis directly or indirectly. The lack of any quenching of fluorescence following continued exposure to light 2, could also result from an inhibition of photosynthesis.

3.7.5 State 1 - State 2 Transitions

The purpose of this experiment was to examine and confirm the effects of DBP vapour on the older (mature) leaves of a plant. The reason for this interest stems from the observation that during exposure of plants to DBP no macroscopic symptoms of phytotoxcity could be seen on those leaves which were mature at the onset of exposure to DBP. Also previous experiments, such as growth analysis and CO_2 exchange measurements, have implied that some reaction(s) associated with photosynthesis have been inhibited. Virgin *et al.* (1981) have advanced the opinion that the mechanism underlying the observed phytotoxicity is attributable to an inhibition of carotenoid synthesis. Carotenoids are thought to protect the chlorophylls from photodegradation in the light and so their absence permits the breakdown of chlorophyll - hence the chlorosis observed in young leaves of treated plants. If it could be shown that there are effects on the photosynthetic rate in

older, mature leaves then the inhibition of carotenoid synthesis may not be the only factor responsible for the phytotoxicity of DBP.

For this experiment a 10 cm^2 disc was cut from the middle of a detached leaf to measure O_2 exchange, while a further sample of the same leaf, towards the tip, was used for chlorophyll fluorescence analysis. The measurements of O_2 exchange using a leaf-disc electrode are described in Section 3.6.2.

The traces of chlorophyll fluorescence are shown in Fig.3.26. Leaf discs from fully mature leaves were given a 5 minute dark period before being subject to a 7 minute exposure of light 2. The effect of this was to induce Kautsky slow transients. The fluorescence was then slowly quenched during the remaining period of exposure to light 2. When light 1 was switched on there was a small decrease in the amount of fluorescence. It is probable that fluorescence would have decreased to this level had the length of time of exposure to light 2 been increased. Following this small decrease there was a slow but steady rise in fluorescence as light 1 drove the leaf into State 1. Proof that light 1 drove the leaf into State 1 was the large increase in fluorescence when light 1 was turned off. Again the fluorescence was quenched to a level similar to that seen before.

The next chlorophyll fluorescence traces were obtained 13

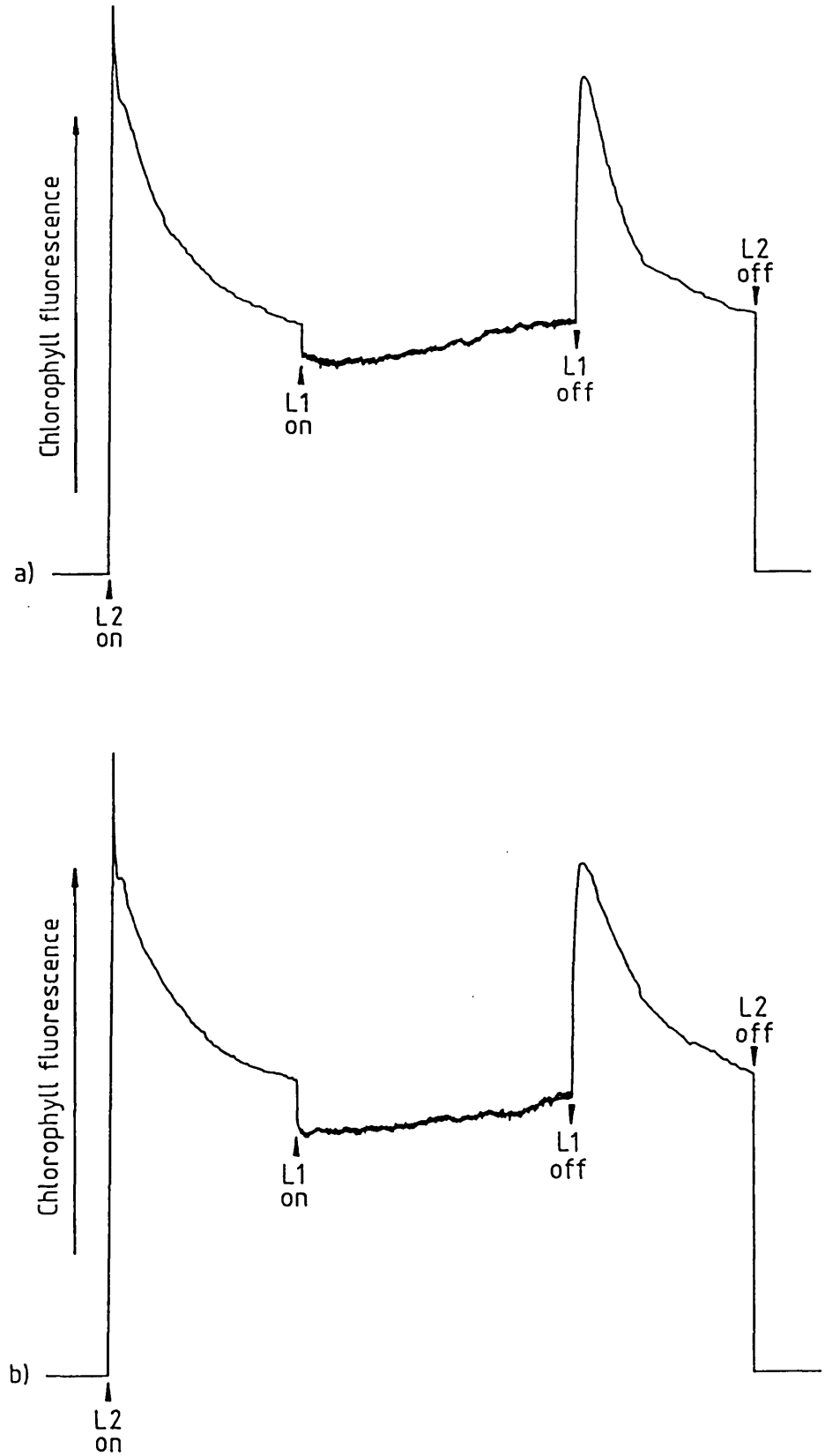


Fig.3.26 Yield of chlorophyll a_2 fluorescence from detached, mature radish leaves prior to commencement of treatment. a) Control; b) DBP.

days after the commencement of exposure to DBP vapour. Figs.3.27a and 3.28a show the traces from the leaves of a control plant which was not subjected to DBP fumes. In general the trace was similar to those obtained previously. When light 2 was turned on after the dark period, the Kautsky transients were seen to fluctuate a great deal more than in previous measurements. At the end of the light 2 exposure, fluorescence was quenched to a comparable level to that seen in the original traces. After the 10 minute illumination with light 1 the increase in fluorescence was not as great as before when this light was switched off. Whether these small differences are important is doubtful, since the plants were now much larger and older. Figures 3.27b and 3.28b show the fluorescence transients of leaves exposed to DBP vapour for a 13 day period. The major difference between these traces and the controls is that the yield of fluorescence was greater throughout the duration of the measurement.

It is difficult to quantify fluorescence emissions for leaves because of differences in chlorophyll content and slightly differing physiological conditions. This in turn makes direct comparison of fluorescence traces difficult, however it is possible to examine fluorescence ratios and compare these. Table 3.5 shows the fluorescence enhancement ratios (FER) and the F_{red}/F_{ox} for this experiment.

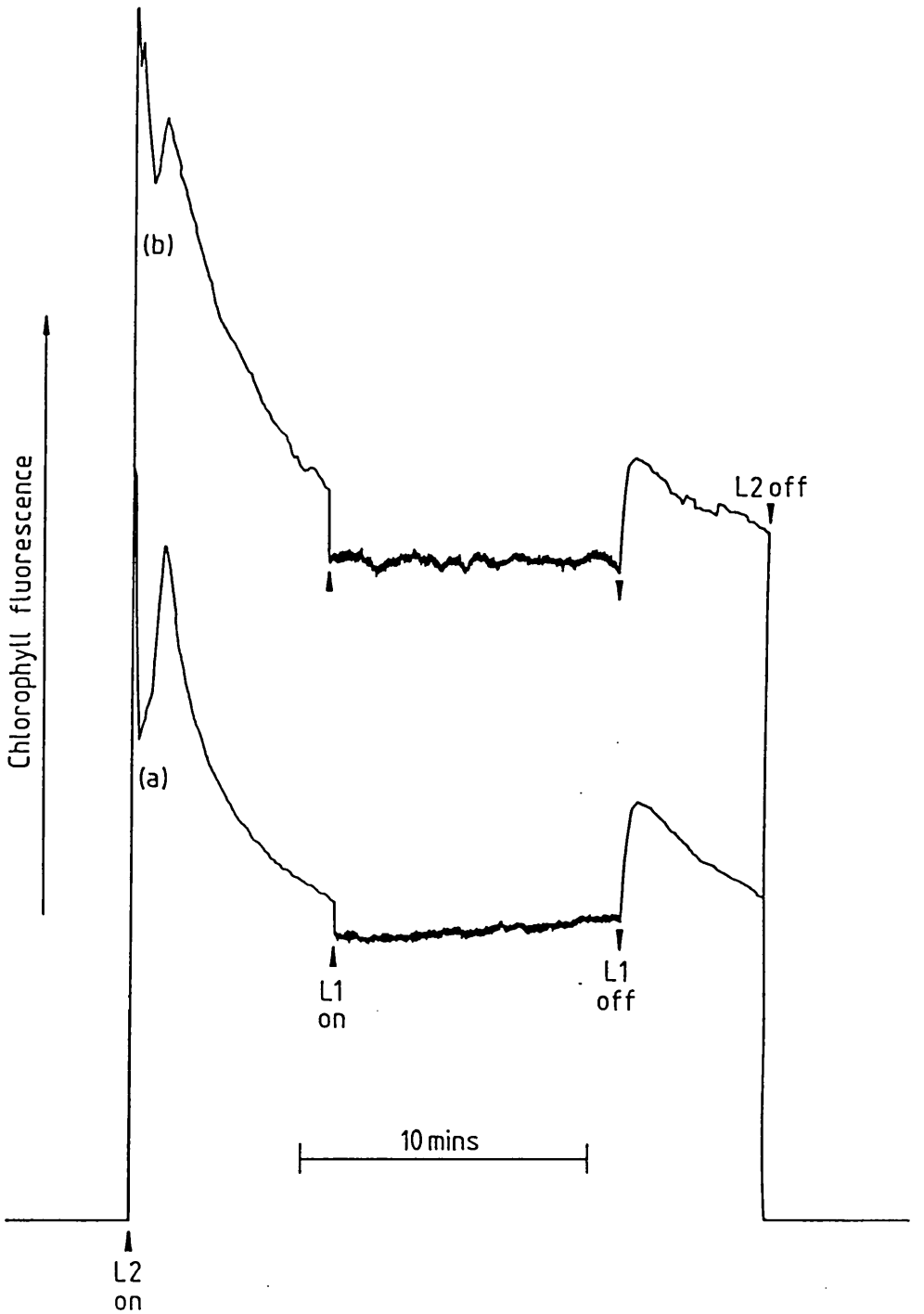


Fig.3.27 Yield of chlorophyll a_2 fluorescence from detached, mature radish leaves after 13 days. (a) Control; (b) DBP.

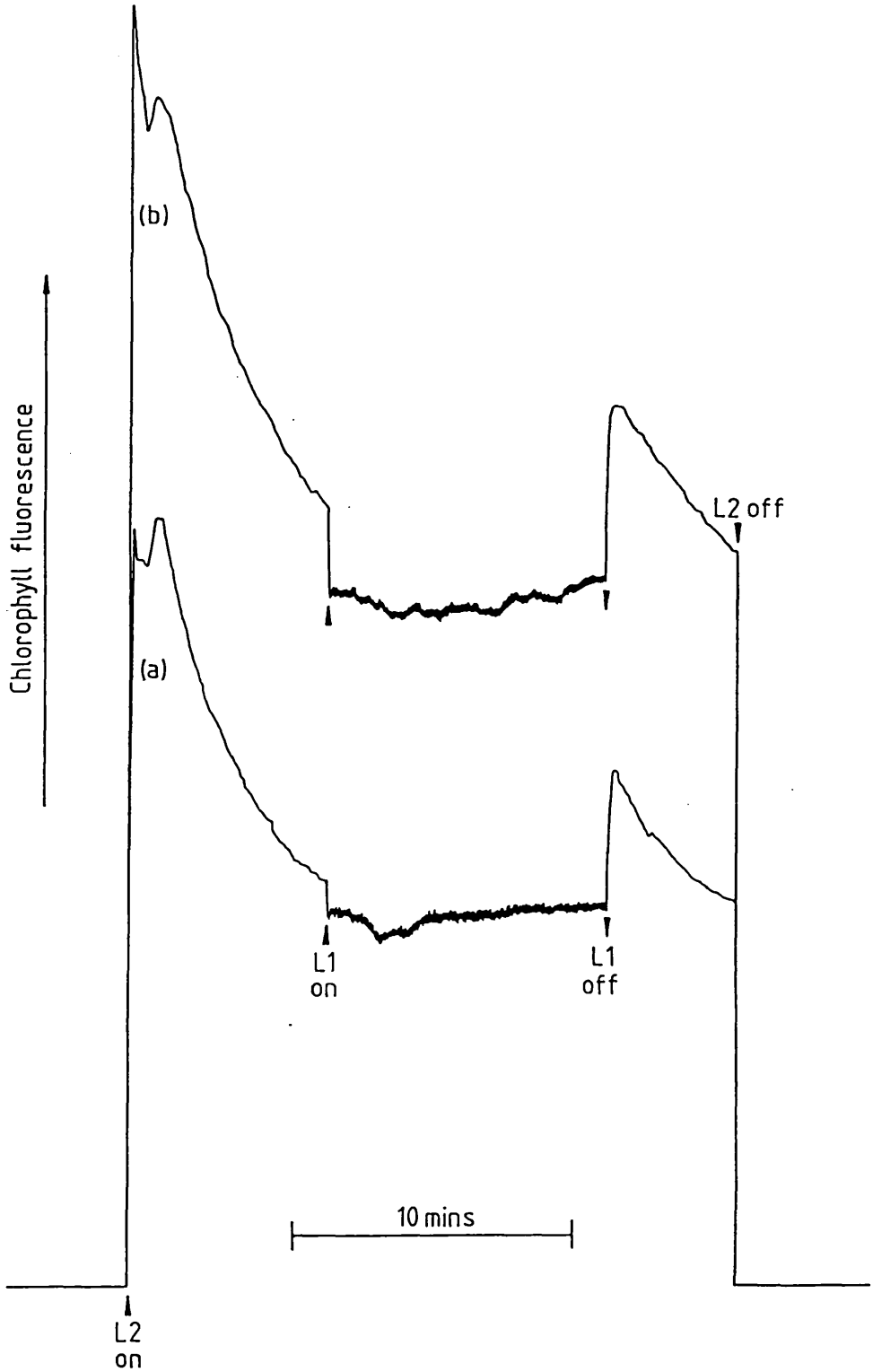


Fig.3.28 Yield of chlorophyll a_2 fluorescence from detached, mature radish leaves after 13 days. (a) Control; (b) DBP.

Table 3.5 Fluorescence enhancement ratios (FER) and F_{red}/F_{ox} ratios of detached radish leaves. FER is the ratio of the fluorescence yield when the leaf was in State 1 and light 1 had been switched off divided by the fluorescence yield before light 1 was switched off (State 1 fluorescence enhancement ratio). F_{red}/F_{ox} where F_{red} = fluorescence yield with excess light 2, (e.g. Q is reduced); F_{ox} = fluorescence yield with light 1 on, (e.g. Q is oxidised) State 1.

Treatment	Fluorescence Enhancement Ratio		$\frac{F_{red}}{F_{ox}}$	
	Day 0	Day 13	Day 0	Day 13
Control	2.00	1.39	-	3.91
DBP	1.86	1.21	-	1.98
DIDP	1.86	1.67	-	3.79
DIOP	1.86	1.31	-	3.71

FERs give an indication of the ability of the photosynthetic apparatus of a leaf to undergo State 1 - State 2 changes. High ratios will be obtained when this ability is not impaired. Ratios will be reduced in those leaves which are unable to undergo State 1 - State 2 transitions. The ratios in Table 3.5 are reduced in all treatments by day 13 but the lowest ratio was seen in the DBP treated plant.

A F_{red}/F_{ox} ratio of around 4 is expected in a healthy plant. The ratio for the treated plant following 13 days exposure to DBP was 1.98 - about half the ratio expected. The ratios for the other treatments were slightly less than 4. This result suggests that DBP inhibits photosynthesis at some point. In this particular experiment there was only a mild inhibition when compared to the results shown in Fig.2.24.

Concern has been expressed in previous sections of this report that some results may have arisen primarily as a consequence of enhanced senescence and not a direct effect of DBP on the parameter being measured. Chlorophyll fluorescence is drastically reduced in senescent bean leaves (Baker and Bradbury, 1981). The effect of DBP in the present work increases fluorescence emission. This fact would argue for an effect of DBP which occurs independently of any increased senescence.

A problem with these experiments was the size of the radish plants. Large plants were required as the effects of DBP on mature leaves were to be examined. Unfortunately these plants needed four mature leaves when they were initially placed in the growth chambers. Subsequently they were too large for the chambers after a period of exposure. This meant that the experiments did not run for as long as required. The large size of the plants also made it difficult to be sure whether all the leaves were

subject to the DBP vapour. The variability of results among the treated plants indicated that shielding of some leaves from the DBP vapour occurred. This is not unexpected as there was evidence that the small young plants nearest the plastic in the growth experiments were more affected than those at the back. During the present experiments there must have been a great deal of shielding because of the large size of the plants.

3.8 Oxygen Electrode

3.8.1 Radish Chloroplasts

When this work was started an attempt was made to isolate chloroplasts from radishes. Unfortunately the activity of these chloroplasts was very low, even after a series of modifications to the isolation technique. To overcome this problem thylakoids from spinach chloroplasts were used as an alternative system to examine the *in vitro* effects of the dialkyl phthalates.

3.8.2 Spinach Chloroplasts

In order to improve the relevance of this work young spinach plants were exposed to the vapour of DBP for a period of 9 days. The macroscopic symptoms were not exactly the same as seen in the radish plants. Growth was

severely stunted in the treated plants as in the radish. The chlorosis in these plants was not as marked as radishes, the chlorosis being confined to the tips of the young growing leaves. There was also some curling of leaves and by day 9 some had started to dry. No effects were seen in the control plants growing in the growth chambers. Shown below is a photograph of spinach plants after 9 days treatment (Fig.3.29).



Fig.3.29 Photograph of spinach plants following 9 days treatment. Tray c - control; tray d - DBP.

3.8.3 Effects Of Phthalates On Electron Transport

The first experiments utilising isolated thylakoids from spinach examined the effects of a variety of dialkyl phthalates on full chain (PS1 + PS2) activity. Methyl viologen was used as the artificial electron acceptor, while nigericin and valinomycin were used to uncouple the system for the experiments described in this section. Phthalates were added as a solution in methanol (MeOH). The concentraton of methanol in the reaction chamber never exceeded 0.2% in this and all subsequent experiments.

From Fig.3.30 it can be seen that at a concentration of 100µM, DBP and DIBP had the greatest inhibitory effect. The inhibition exerted by DMP, DIDP and especially DIOP was, comparatively, very low.

The next experiments looked at the effects of varying the concentrations of the phthalates, tested above, from 10µM to 1000µM. The results are shown in Fig.3.31. Both DBP and DIBP showed an inhibition at 10µM which rose rapidly as the concentration was increased to about 100µM. The degree of inhibition - about 80% - remained the same when the concentration was increased ten fold to 1000µM. DIOP effectively showed no inhibition while DIDP had a maximum inhibition of about 6% at 200µM and DMP had a maximum inhibition of about 10% at 1000µM.

The trace shown in Fig.3.32 illustrates the source of some

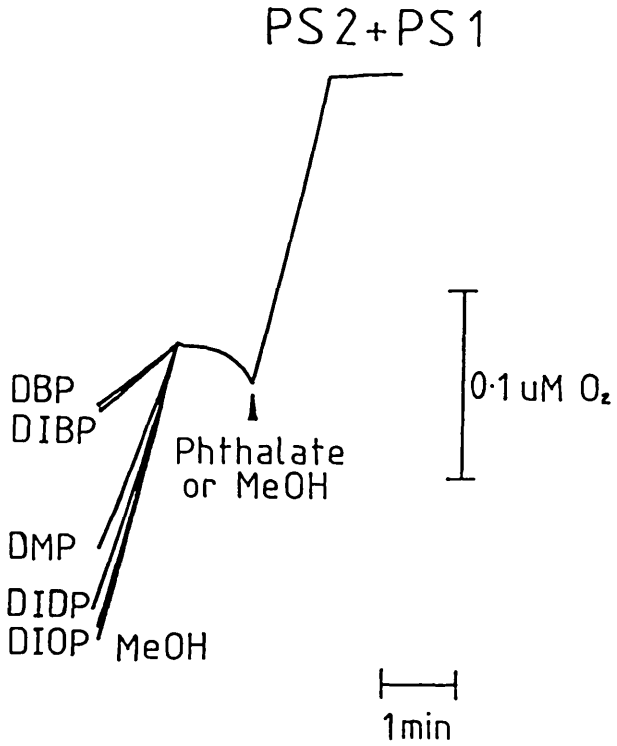


Fig.3.30 Polarographic traces showing the activity of (PS2 + PS1) in isolated spinach thylakoids, measured as oxygen uptake in the presence of MeV. Each composite curve consists of a series of superimposed traces of individual experiments in which the dialkyl phthalate was varied. Time of addition of the phthalate is indicated by the arrow. Thylakoids were kept in the dark for 1 minute after which they were illuminated for 1 minute prior to addition to dialkyl phthalate, after which they were incubated in the dark for 1 minute, before being illuminated.

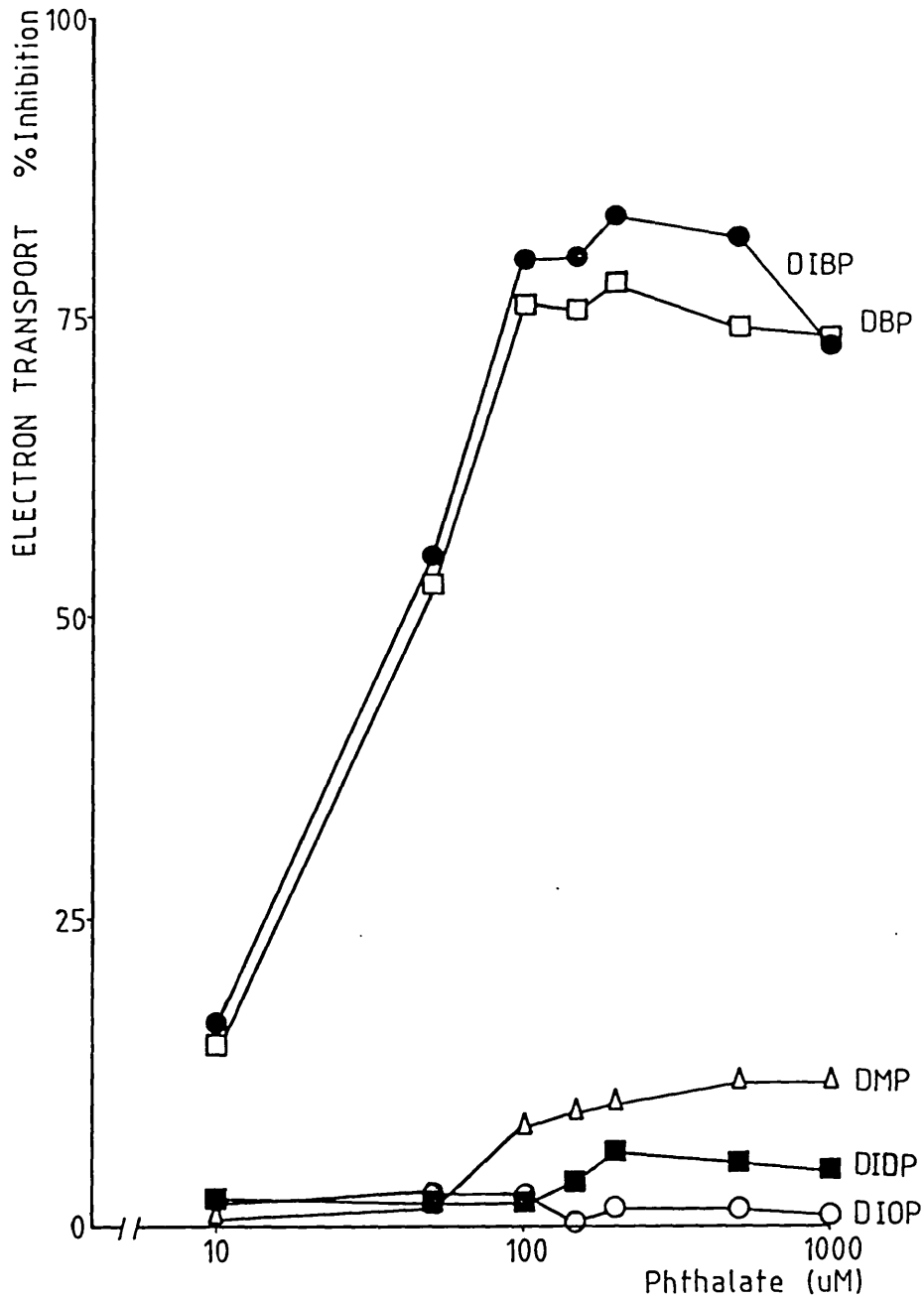


Fig.3.31 Inhibition of uncoupled electron transport (PS2 + PS1) by dialkyl phthalates in isolated spinach thylakoids. Control rates of MeV induced O_2 uptake varied between 520-580 $\mu M\ O_2.mg^{-1}$ chl.hr^{-1}.

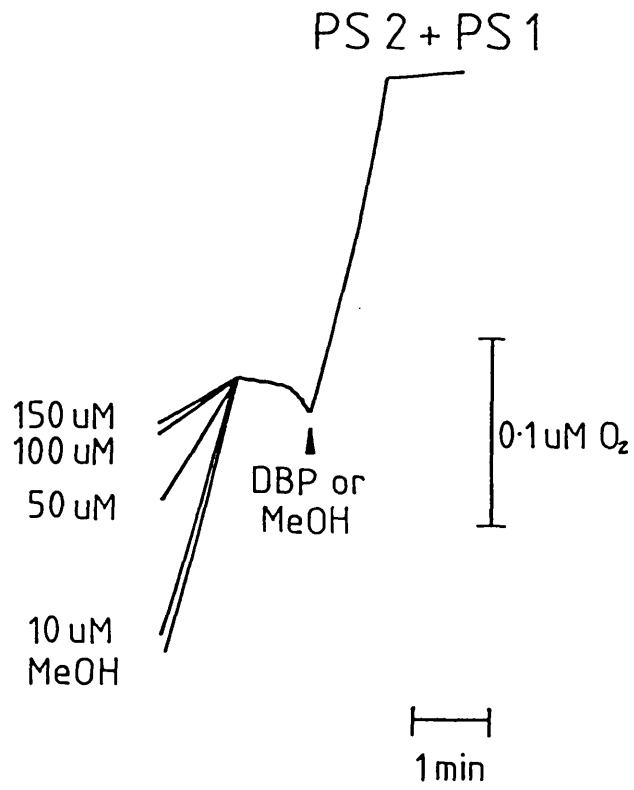


Fig.3.32 Polarographic traces showing the activity of (PS2 + PS1) in uncoupled, isolated spinach thylakoids, measured as oxygen uptake in the presence of MeV. Each composite curve consists of a series of superimposed traces of individual experiments in which the concentration of the dibutyl phthalate was varied. Time of addition of the phthalate is indicated by the arrow. Thylakoids were kept in the dark for 1 minute after which they were illuminated for 1 minute prior to addition to dibutyl phthalate, after which they were incubated in the dark for 1 minute, before being illuminated.

of the results used in Fig.3.31. Inhibition at a DBP concentration of 10µM was low. With increased DBP concentrations up to 100µM inhibition of electron transport rapidly increased. Above this concentration there was little change in the degree of inhibition.

3.8.4 Coupled And Uncoupled Electron Transport

During these experiments methyl viologen was used as the electron acceptor. Nigericin and valinomycin were used as the uncouplers when required.

Figure 3.33 shows the extent of inhibition by DBP and DIBP on coupled and uncoupled electron transport rates. It is evident from the graph that the uncoupled system is more sensitive to the toxic phthlalates than the coupled system. The I_{50} for the uncoupled system for DBP and DIBP was 44µM and 42µM respectively. The coupled system was less sensitive to the phthalates with an I_{50} of 87µM for both DBP and DIBP. As with uncoupled electron transport, the inhibition of the coupled system rapidly increased as the concentration of toxic phthalate was increased from 10µM to 100µM. Above this concentration there was no increase in the degree of inhibition. The maximum inhibition exerted on the coupled system was approximately 60% as compared to about 80% for the uncoupled electron transport. Coupled electron transport rates remained unchanged when DMP, DIOP and DIDP were tested.

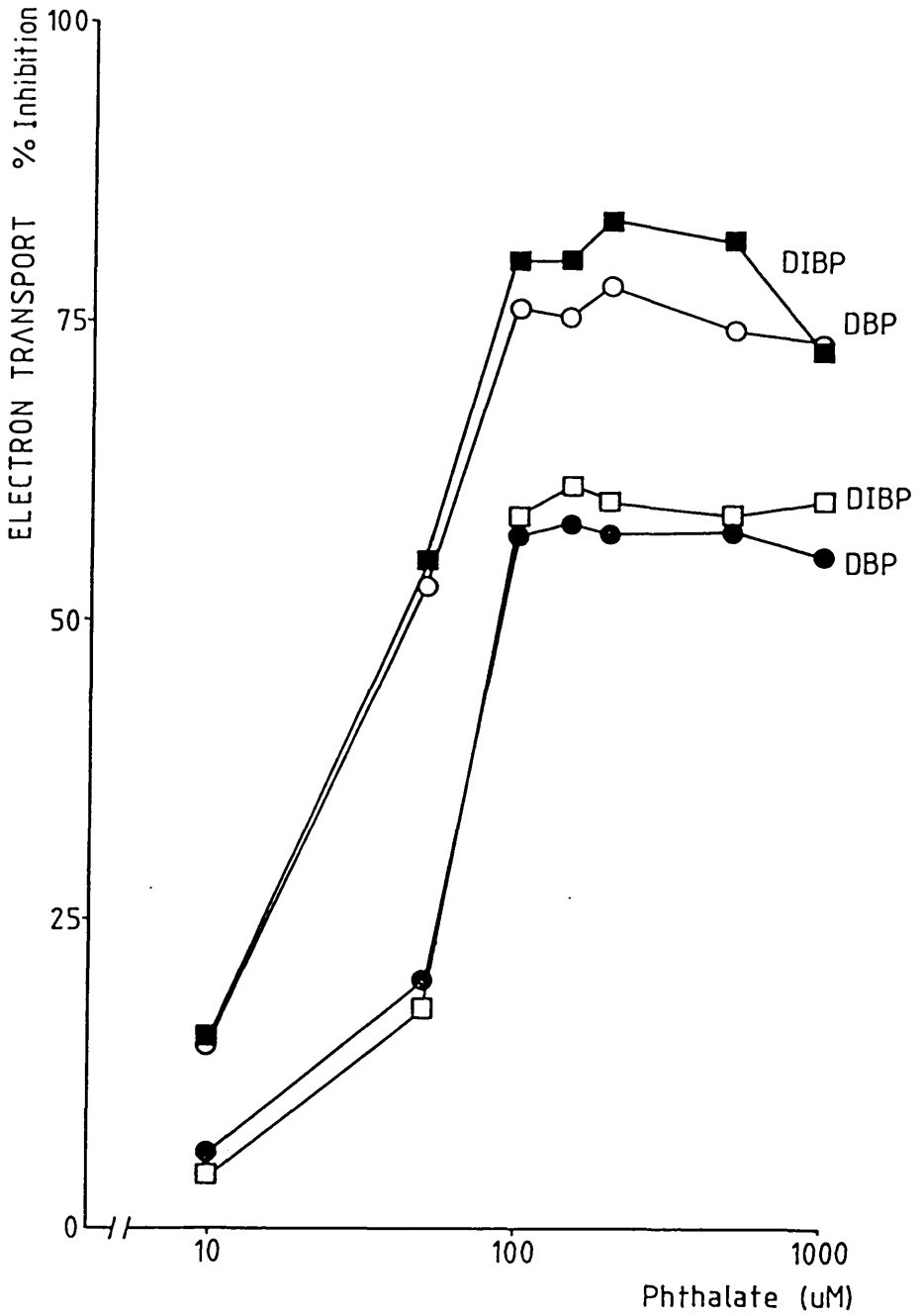


Fig.3.33 Inhibition of coupled (lower pair of curves) or uncoupled electron transport (PS2 + PS1) by dibutyl or diisobutyl phthalate in isolated spinach chloroplasts. Control rates of MeV induced O_2 uptake varied between 525-580 $\mu M\ O_2.mg^{-1}$ chl.hr^{-1} in uncoupled electron transport, and 130-165 $\mu M\ O_2.mg^{-1}$ chl.hr^{-1} in basal electron transport.

3.8.5 Assay Of Photosystem 1

In order to localise the site of action of the butyl phthalates more precisely a photosystem 1 assay was undertaken. Methyl viologen was used as the electron acceptor and dichlorophenol indophenol plus ascorbate were used as the electron donor couple. Nigericin and valinomycin were used to uncouple the system. DCMU was used to inhibit the flow of electrons from water through PS2 to the plastoquinone pool.

In Fig.3.34 typical traces are shown for the full chain reaction (PS1 and PS2) and the partial reaction (PS1) treated with DBP or methanol (MeOH). It is evident that inhibition of the electron transport rate in the PS1 assay is much lower than for a given DBP concentration in the full chain reaction. These results are presented graphically in Fig.3.35. At butyl phthalate concentrations as high as 1000µM, inhibition of PS1 electron transport only reached 40%. The full chain assay showed 80% inhibition at a phthalate concentration as low as 100µM. These results indicate that the main site of action of the butyl phthalates lies at a point prior to or on the DCMU sensitive site.

3.8.6 Ferricyanide Induced Electron Transport

During early experiments measuring electron transport rates a different reaction solution was used to the one

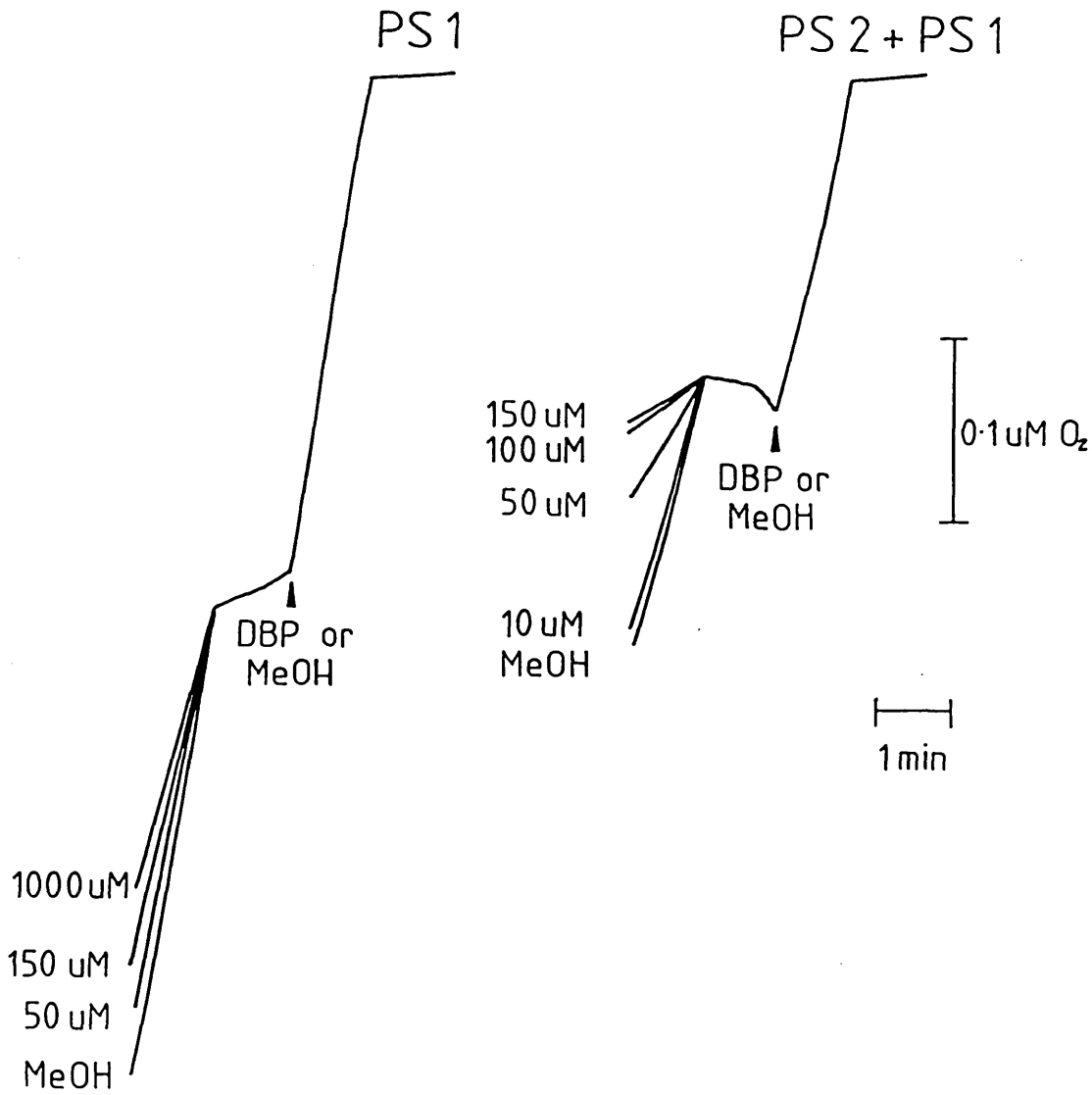


Fig.3.34 Polarographic traces showing the activity of (PS2 + PS1) or PS1 in isolated spinach chloroplasts, measured as oxygen uptake in the presence of MeV. Each composite curve consists of a series of superimposed traces of individual experiments in which the concentration of the dibutyl phthalate was varied. Time of addition of the phthalate is indicated by the arrow. Thylakoids were kept in the dark for 1 minute after which they were illuminated for 1 minute prior to addition to dibutyl phthalate, after which they were incubated in the dark for 1 minute, before being illuminated.

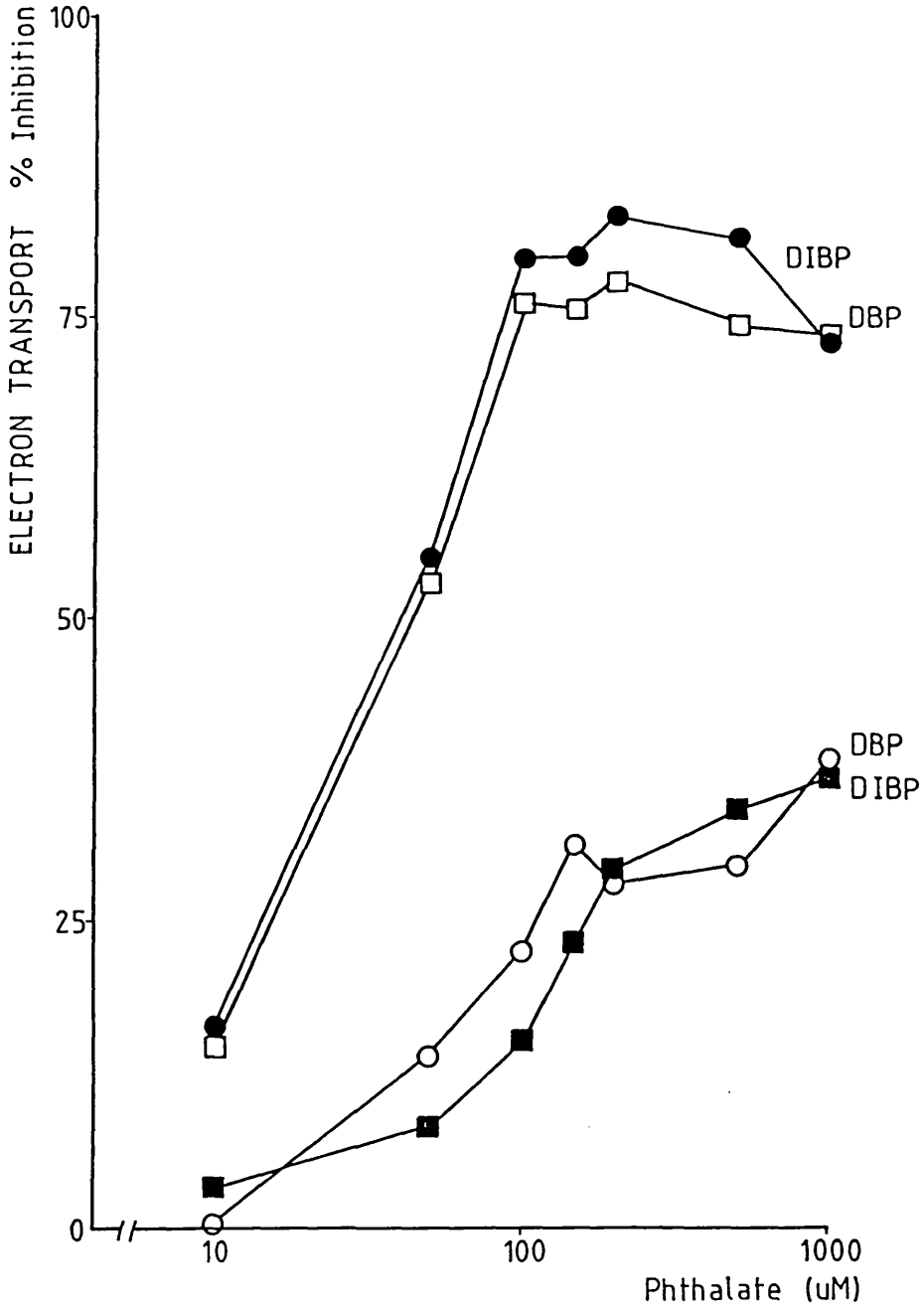


Fig.3.35 Inhibition of (PS2 + PS1) and PS1 uncoupled electron transport by *dibutyl or diisobutyl phthalate in* isolated spinach chloroplasts. Control rates of MeV induced O_2 uptake varied between 525-580 µM $O_2.mg^{-1}$ chl.hr^{-1} in uncoupled (PS2 +PS1) electron transport, and 620-715 µM $O_2.mg^{-1}$ chl.hr^{-1} in uncoupled PS1 electron transport.

employed for the experiments described above. This reacton medium did not include EDTA. During the use of this reaction mixture it was noted that maximum inhibition of electron transport occurred at a concentration of approximately 150µM for DBP. Inhibition then appeared to decrease as the concentration of phthalate was increased to 1000µM. As there was a possibility that the methyl viologen, which was used as the terminal electron acceptor, was the cause of these unusual results it was decided to substitute it with ferricyanide.

The results shown in Fig.3.36 demonstrate that this effect could also be obtained using ferricyanide as the artificial electron acceptor. In fact the decrease in inhibition seen as the concentration of phthalate was increased from 150µM to 1000µM was greater than that seen when using methyl viologen.

3.8.7 Use Of Filters

Another possible explanation for the apparent decrease in electron transport inhibition, as phthalate concentration was increased above a certain level, was oxidation of membrane lipids. Certain wavelengths of light - usually high energy, in the blue-green part of the spectrum - may cause this effect. In an attempt to resolve this problem an orange filter was used in the incident light path which removed these wavelengths of light. Methyl viologen and

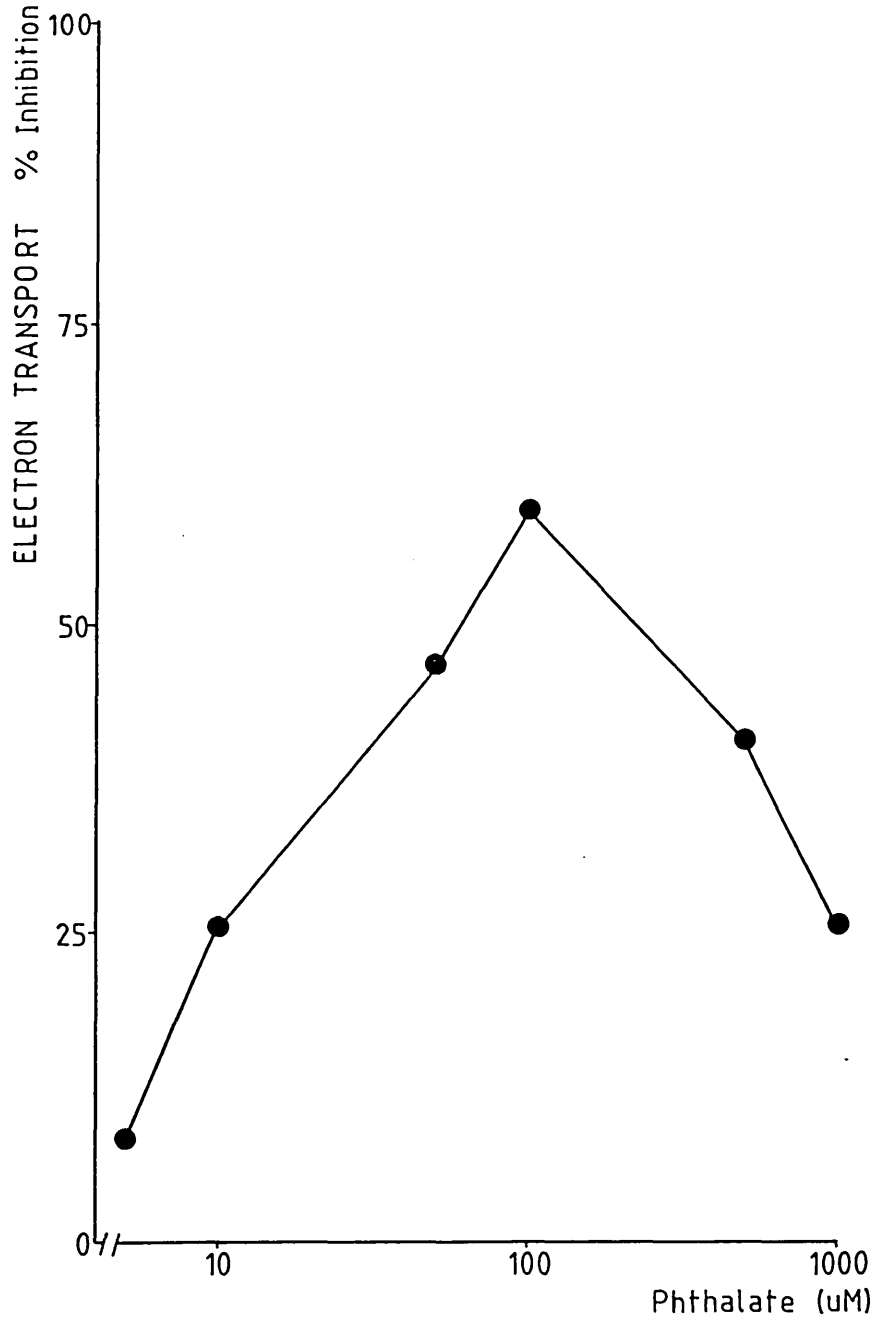


Fig.3.36 Inhibition of (PS2 + PS1) uncoupled electron transport by dibutyl phthalate in isolated spinach chloroplasts. Electron transport measured as oxygen evolution; ferricyanide used as electron acceptor. Control rates of O_2 evolution varied between 225-285 µM $O_2.mg^{-1}$ chl.hr^{-1} in uncoupled electron transport.

nigericin and valinomycin were used as electron acceptor and uncouplers respectively.

The results from this experiment are shown in Fig.3.37. As in previous experiments, maximum inhibition occurred at a DBP concentration of approximately 150µM when the orange filter was used. Above this concentration there was a decrease in inhibition. The inhibition at a concentration of 1000µM, using the orange filter, was similar to that seen at 10µM. These results suggested that the observed decrease in inhibition at increased phthalate concentrations was not a result of oxidation of the lipid membrane by high energy wavelengths of light.

The lower inhibition at all phthalate concentrations when using the orange filter was probably a consequence of the lower light intensity.

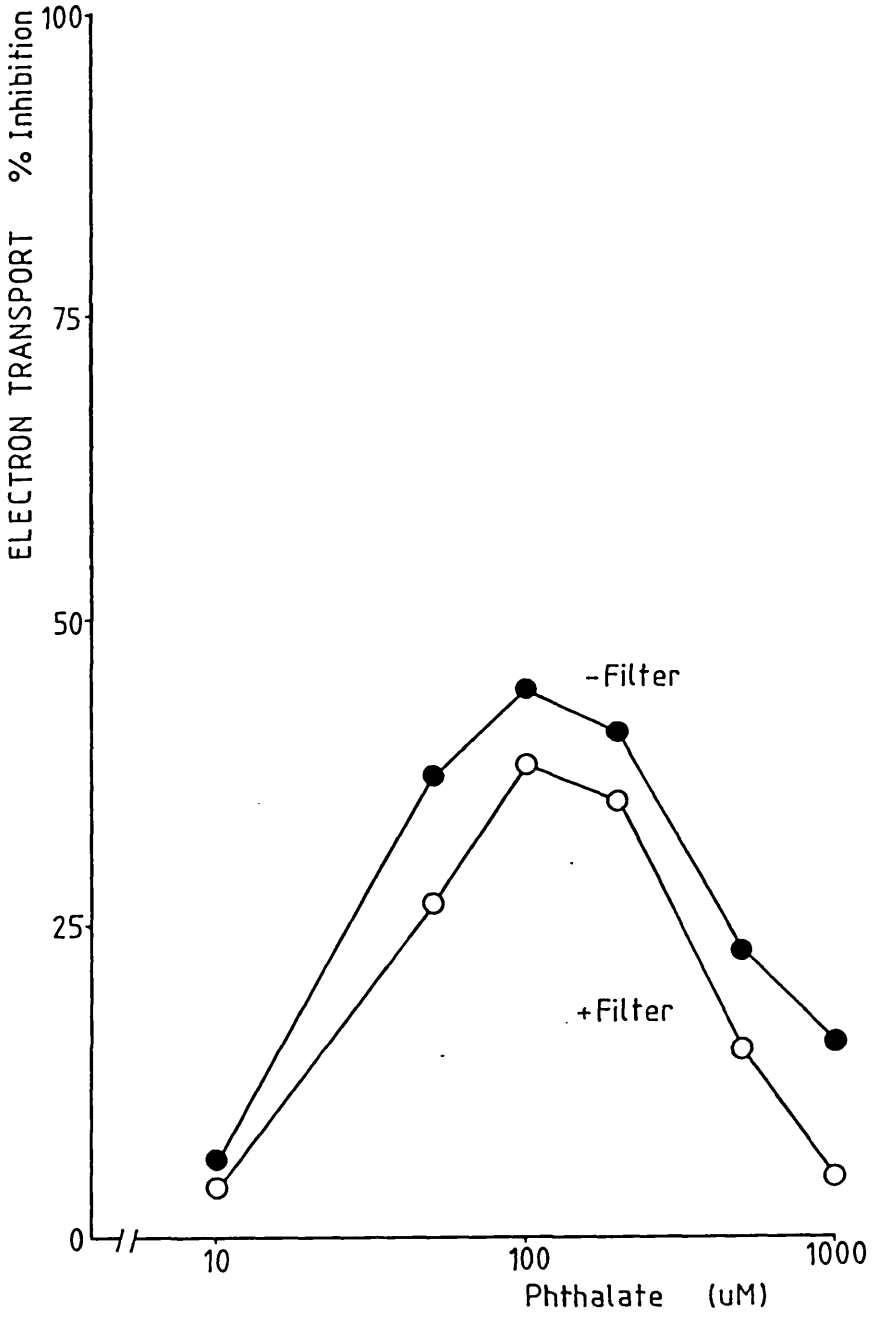


Fig.3.37 Inhibition of (PS2 + PS1) uncoupled electron transport by dibutyl phthalate in isolated spinach chloroplasts. An orange filter was used in the incident light path. Control rates of MeV induced O_2 uptake varied between 325-395 $\mu M\ O_2.mg^{-1}$ chl.hr^{-1} without the orange filter and 125-145 $\mu M\ O_2.mg^{-1}$ chl.hr^{-1} with the orange filter.

4. DISCUSSION

4. DISCUSSION

This discussion will be divided into five sections. The first will deal with the identification of the phytotoxins present in some formulations of flexible PVC. This section will include discussion of the difficulties in identifying the toxin prior to this present work; the new bioassay system; and the commercial aspects of this project. The second section will discuss attempts to localise the site of action of the toxin through morphological and physiological studies of treated plants. The third section will discuss the initial attempts to identify the mode of action of the phytotoxin. The penultimate section will discuss the results of this project in the light of the work of other researchers. The fifth and final section will suggest directions for future research.

4.1 Identification Of The Phytotoxin

Prior to the start of this project it had been established that some flexible PVC formulations contained a phytotoxin which volatilised slowly over a long period of time (Hannay, 1980). In retrospect it is clear that the identification of this toxin was seriously hindered due to some curious experimental results and inadequate analytical information.

The first of these were the results of simple bell-jar tests which indicated that the plasticisers were not responsible for the phytotoxicity. Similar results and conclusions had been obtained by workers at the National Vegetable Research Station at Wellesbourne (Austin *et al.*, 1968). This suggested that the stabilisers were the source of toxicity or that a new product was formed from the original constituents. The main problem with the idea that the stabiliser was the source of the toxicity was that it was only present as about 2% or less of the plastic, and usually consisted of metal salts of fatty acids - which seemed very unlikely to volatilise at normal temperatures. Nevertheless cadmium is commonly used in stabilisers and was known to be present in both of our toxic plastics - the original clear tubing and the black plastic rod from Humberside. The researchers at NVRS had also tested a stabiliser and found it to be very toxic. Also toxic plastics had been shown to be toxic many years after first being tested. This would imply that the stabiliser was toxic at extremely low concentrations otherwise the plastic would have lost it s toxicity over such a long period of time. The second problem concerning the idea of a toxic stabiliser, is the mechanism by which the stabiliser could reach the plants. A possible explanation was that another molecule - perhaps the solvent in which the stabilisers are dissolved or the plasticiser - 'carried' them to the plants. Cadmium-barium stabilisers which were tested in liquid form were found to be extremely toxic - plants collapsed and died within 24

hours. However the symptoms in these tests were not typical of plastic toxicity. When tested in liquid form the solvent for the stabiliser was probably responsible for the collapse of the plants. However when stabiliser was applied to filter paper, which was then left for 24 hours in a ventilated fume cupboard to dry at room temperature, radishes still collapsed and died within a day or so when placed under the bell jar with the filter paper suspended above the plants. Experiments were undertaken with a new sample of stabiliser. The stabiliser was dried as before but test plants showed no signs of toxicity. However it should be pointed out that these experiments were conducted in large (2' x 2' x 2') perspex growth chambers.

When it was possible to test specially prepared samples of PVC rod, made by ICI, it became obvious that plastics which contained DBP in their formulation were toxic while those with DOP were not; regardless of whether they had cadmium stabilisers or not.

There was one important exception to this. The sample of black PVC rod which had been used by a tomato grower in North Humberside as part of a support system and was found to be extremely toxic in bioassay. When this was first analysed by ICI it was found to contain mainly DIOP as its plasticiser, probably with small amounts of other plasticisers. It was suggested that as the plastic was black it was very likely that the firm which had extruded

the rod had added odd bits of scrap plastic which could account for the presence of other phthalates. The stabiliser contained cadmium.

The other puzzle concerned plastic sheeting obtained from another grower (Rafeenan Nurseries, Eire). This grower was unable to grow tomatoes in his novel greenhouse constructed from 1 metre square panels of two sheets of PVC with a 1 inch gap between the sheets, to gain heat insulation associated with double glazing. In bioassay at Imperial College the plastic sheeting could not be shown to be toxic and chemical analysis by ICI showed that the sheeting contained DOP as it's plasticiser. There was no cadmium in the stabiliser.

These contrasting findings, prior to the start of this project, indicated the need to bioassay a series of PVC plastics of precisely known chemical composition and uniform surface to weight ratio. To this end ICI kindly agreed to supply a series of plastics made to our specification.

4.1.1 Bioassays Of PVC Plastics

Bioassaying the first batch of plastics provided by ICI demonstrated conclusively that only those plastics having DBP in their formulation produced the symptoms of phytotoxicity observed in previous work. The nature of the stabilisers did not appear important. The most obvious

effects of this phytotoxicity was to severely stunt growth and induce the formation of chlorotic leaves. These results are in line with those reported by Hannay (1980). The effects of the vapour from pure liquid DBP on radish plants were found to be the same as those seen with DBP-plasticised plastic - namely stunted growth and chlorosis of younger growing leaves. Similar results on the effects of DBP have been reported by Gudin and Harada (1974); Inden and Tachibana (1975); Lokke and Bro-Rasmussen (1981); Virgin *et al.* (1981). Furthermore, Gudin and Harada (1974) also reported that DOP inhibited the elongation of the coleoptile of wheat and the mesocotyle of oat. DBP had a greater inhibitory effect than DOP on the growth of wheat coleoptiles. The senescence of the leaves of *Rumex obtusifolius* L. was also retarded. Phthalates were applied as an emulsion to the leaves in these experiments. The work of Inden and Tachibana (1975) is the most extensive and the vapour from an enormous variety of different plasticisers and stabilisers, both as part of plastic sheeting and individually, were found to exhibit varying degrees of phytotoxicity. Many of the remaining plasticisers and stabilisers were toxic if heated. These effects were examined on many different species of plant. They also looked at the differences in the inter-varietal effects of DIBP on sweet pepper. The differences in damage were quite considerable. In contrast to these findings Lokke and Bro-Rasmussen (1981) working with *Sinapis alba* L. (white mustard) found that although DBP caused chlorosis of

leaves at a dosage of 1.5 $\mu g.cm^{-2}$ of leaf surface the other dialkyl phthalates tested, DIBP and DEHP, produced no adverse effects. The plasticisers were sprayed on to the foliage of the plants as a solution (phthalates, acetone, Tween 20 and distilled water). Chlorosis developed in new leaves on the third day after application of the solution, but subsequent young leaves did not develop chlorotic symptoms. Foliar applications were also made with pure phthalates - again, only DBP induced chlorotic leaves. Emulsions of DBP, DIBP and DEHP applied to *Lapsana communis* L. and *Achillea millefolium* L. produced no visible adverse effects. Buta (1975) examined the plant growth regulating activity of a series of substituted phthalate esters. Of these, dipropyl 3-methyl phthalate inhibited growth of the apical meristems of *Nicoti ana tabacum* L. by 100%, while with dipropyl phthalate it was only 48%. Both compounds caused chlorosis in the developing parts of the treated plants.

In retrospect, the lack of symptoms in earlier bell jar tests were probably due to the atmospheric concentrations of DBP never reaching a toxic level since the surface area of plasticiser was much lower than that of the plastic. Subsequent experiments have shown that a given surface area of DBP-plasticised plastic tends to be more toxic than an equal area of pure liquid DBP. This is probably due to the phthalates volatilising at different rates from the two surfaces.

Subsequent information from ICI with regards to the composition of the black PVC rod from the grower in Humberside revealed that this plastic did contain a significant amount of DIBP. The second analysis provided by ICI indicated that 31.8% of the plastic was plasticiser of which 9.5% was DIBP and 12% was DOP the rest being made up from ESBO and Cereclor.

The grower in Ireland recently (November 1983) volunteered the information that he suspected that the cause of his problems might have been due to oedema. This is a physiological disorder often associated with high humidity in the environment. He saw colour photographs of this syndrome in tomato which very closely resembled the appearance of his dying plants.

These explanations for the apparently curious results of some experiments, together with additional information concerning the Irish grower justify the view that DBP and DIBP plasticisers are the two components responsible for the phytotoxicity encountered with some flexible PVC plastics.

4.1.2 Growth Analysis

The growth analysis experiments showed that the fresh weight of treated plants was only 10% of the controls while dry weight was only 20% at the end of the ten day period. Moreover within this period an actual loss in dry

weight was not seen whereas a loss in fresh weight always occurred with DBP. The photographic evidence shows that the reason for this was drying of leaves; presumably due to loss of osmotic control following damage to cell membranes. The dry weight measurements do show that the accumulation of dry matter is greatly reduced in plants exposed to DBP vapour.

Growth analysis experiments which tested the effects of the glazing strips obtained from horticultural growers were useful in establishing that the phytotoxicity encountered in commercial greenhouses could be repeated in our bioassay system. The results obtained using the glazing strip are similar to those using the plastic rod provided by ICI.

4.1.3 Glasshouse Crop Losses

The figures for tomato yields presented in this section give an indication of the commercial importance of this problem. The results demonstrate that it is the loss of crops at the start of the season which are the most significant financially because of the high value of tomatoes at market. This is also a time when the air vents have spent, and still spend, the great majority of the time closed - a factor which favours the accumulation of toxic phthalates.

There is another point which emphasises the seriousness of

this problem for a tomato grower. The figures for the yields from each glasshouse in 1984 varied very little from each other even though the plants in one house suffered a bad attack of phytophthera from March to early May, while the plants in the other house were severely infested with whitefly from July onwards. Comparison with the 1983 growing season demonstrates how much more dramatic was the inhibitory effect of the plasticiser. Plasticiser toxicity reduced early yields to a far greater extent than the phytophthera attack the following year, in spite of the fact that in 1983 even the 'control' plants had spent their first eight weeks in the closed, toxic greenhouse. Towards the end of the growing season, when the vents were closed more often as the temperature dropped, yields in the toxic house were reduced. Again, more severely than by the bad attack of whitefly in the 1984 season.

4.2 Aerial Concentrations Of Phthalates

This was an important part of the work as it provided an indication of the concentration of phthalates reaching the plants from a sample of plastic. It was unfortunate that experiments involving the liquid plasticisers had to be abandoned, due to fear of contamination of the equipment, before it was possible to measure aerial concentrations.

Nevertheless the results enable a comparison to be made of

the aerial concentrations of phthalates in our bioassay system and the concentrations found in a greenhouse glazed with DBP or DIBP glazing strip. Within our system an average DBP concentration of approximately 120 ng.litre^{-1} was encountered compared to the figure of 1.2 ng.litre^{-1} which was the mean concentration seen in a toxic greenhouse at NVRS, Wellesbourne (Hardwick *et al.*, 1984). The maximum concentration measured was 2.0 ng.litre^{-1}. Therefore the average concentration of DBP in the atmosphere of our bioassay system was 100 times greater than that encountered in the glasshouse environment. This seems to be reasonable since visible phytotoxic damage occurred in our bioassay system in as little as two days and plants were dead or drying after ten days. This is in contrast to the glasshouse environment where typical symptoms took three weeks to appear in the sensitive cabbages used for the experiments and growth continued for months, though the plants looked small and very unhealthy.

Some measurements of the aerial concentrations of DBP within the growth chambers were made with and without a tray of 9 radish plants. These plants were 25 days old and possessed 3 leaves which were still expanding with a fourth emerging. The air flow through a 150g sample of DBP-plasticised plastic was 3 l.min^{-1}. The results showed that without the tray of plants the aerial concentration of DBP was approximately 104 ng.l^{-1} while the concentration was reduced to less than 70 ng.l^{-1} when plants were present. These results suggested that a

substantial amount of the phthalate came into contact with the plant surface. Presumably this phthalate was able to penetrate the lipophilic cuticle of the radish plant otherwise it would be expected to evaporate again from the plant surface due to its high volatility.

4.3 Solution Culture

Although these experiments were not entirely successful they did, nevertheless, yield some useful information.

Firstly the reduced growth of the root system of radishes growing in nutrient solution culture suggests that the roots are able to take up DBP. Moreover this was not a result of DBP absorbed through the leaves as some of these plants showed no signs of chlorosis. Also as these plants had a poorly developed root system, and yet some had suffered no chlorosis, it is probable that DBP entering by the roots is not subsequently translocated to the leaves of a plant. We have found that large, treated radish plants with a few chlorotic leaves will produce young green leaves if removed from the source of DBP vapour. Similar results were reported by Lokke and Bro-Rasmussen (1981) working with *Sinap is alba* L. This may indicate a lack of translocation; or that DBP is rapidly metabolised. However Buta (1975) also found no evidence of translocation of substituted phthalates when the compound was placed within a lanolin ring on the surface of a

tobacco leaf. With plants grown in soil, such as those in our bioassay system, absorption of DBP through the roots is probably not as important as that entering by the leaves. It should be noted however that Ogner and Schnitzer (1970) have reported the isolation of dialkyl phthalates from humus in the form of fulvic acid - dialkyl phthalate complexes. These complexes are soluble in water. Whether the dialkyl phthalates retain their biological activity in these complexes is not known. However it may be that the roots of tomatoes grown in commercial greenhouses using a nutrient film technique suffer some inhibition of growth which in turn would reduce aerial growth. This may be a more serious problem if DBP containing plastics are used in the irrigation system for the plants as the plasticiser will leach from the plastic. With this in mind it is important to appreciate that the amount of plasticiser extracted from a plastic does not depend on the solubility of that plasticiser in water. Research has shown that water diffuses into the plastic and a plasticiser-water blend exudes. This process does not stop when the water is saturated but, as shown by a closed system, continues until droplets of plasticiser can be seen in the water (Sears and Darby, 1982). Problems of leaching of phthalates from plastic tubing used in milk collection have been reported (Wildbrett, 1973) and from plastic medical devices, including the PVC bags used to store blood (Jaeger and Rubin, 1973; Kevy and Jacobson, 1982). The extraction of plasticiser is increased when the plastic comes into contact with solutions which contain

lipophilic molecules such as blood and milk. Leaching of phthalates from plastic packaging is a problem that has been recognised by the food industry and the health authorities in several countries (Shibko and Blumenthal, 1973). It has been found that certain phthalate plasticisers will leach out of plastics when in contact with fatty foods while others leach out when in contact with foods with a high water content. For this reason some plasticisers are not used in the packaging of fatty foods, while others cannot be used to package foods with a high water content. Generally it is expected that the migration will be kept to a minimum by using the least amount possible for food packaging material (Shibko and Blumenthal, 1973).

4.4 Electron Microscopy

The only published work on the ultrastructural changes induced by dialkyl phthalates in plants known to the author is contained in the paper by Virgin *et al.* (1981). These workers examined the ultrastructural damage caused by exposure to DBP on the chloroplasts of *Browallia speciosa* L. They presented a photomicrograph from partly green leaves in which the grana of chloroplasts were seriously disorganisd. In the chloroplasts of completely chlorotic leaves there was a more or less complete disinte gration of the granal system with few distinguishable structures. Traces of a lamellar system

could be seen in one photomicrograph while in another only an irregular maze-like network of lamellae was present. Plastoglobuli, which were small and densely staining in a chloroplast from a green leaf appear to become more swollen and less densely staining in the chloroplasts from chlorotic leaves. The green leaf chloroplast probably came from a plant which had been exposed to DBP vapour although this was not stated. A number of similarities exist between Virgin's work and the present work using radishes. The most obvious of these is the disruption of the granal system. In our photomicrographs of the chloroplasts of chlorotic leaves, various stages of disintegration can be seen. In the early stages the number of grana is drastically reduced leaving a number of lamellae which appear to run roughly parallel for the length of the chloroplast. These lamellae appear to be broken along their length. In later stages even this degree of structure and organisation is not apparent. Only a few small pieces of lamellae can be seen. The most seriously affected chloroplasts appear to have no lamellar structure present although this may be a reflection of the plane of sectioning. In contrast to the work on *Browallia* in which there was no visible effect on the granal structure of chloroplasts from green leaves, the grana in radishes, although showing no obvious visible structural damage, were swept to one side of the chloroplast. This appears to be a result of the swelling of the chloroplast which in the treated plants are distinctly rounded in shape instead of the more usual elongated shape. DBP appears to increase

the number and size of the plastoglobuli of both radish and *Browallia*. In radish leaves which were green and mature at the beginning of their treatment, these plastoglobuli were densely staining and appeared to be attached to the thylakoid membranes. In younger chlorotic tissue the numbers of plastoglobuli increased still further but many were larger and lighter staining than seen in the green treated material.

Large numbers of plastoglobuli are generally taken as a sign that there is something wrong with the metabolism of a chloroplast - usually being considered to be stores for excess lipid. Their presence in large quantities may indicate that lipid is not being incorporated into membranes in the normal way - this seems a reasonable explanation for their appearance in young chlorotic tissue. Alternatively their appearance may suggest that lipid is being lost from the chloroplast membrane - a more reasonable explanation for their presence in the chloroplasts from green leaves. The present EM study does not provide conclusive evidence as to whether pre-existing structures are broken down as a consequence of DBP toxicity or whether it is the development of new chloroplasts which is arrested. The latter would seem more likely as the most seriously affected chloroplasts are found in young expanding leaves. The appearance of the membranes within these chloroplasts is somewhat similar to that found in proplastids and etioplasts. Moreover, there is an increase in the number of plastoglobuli associated

with extensive membrane proliferation in developing etioplasts. In etioplasts this store of lipid is used for membrane synthesis. In treated chloroplasts the utilisation of this lipid may be prevented. In chloroplasts which were at a later stage of development at the commencement of treatment, the lack of chlorophyll will contribute to the disruption of thylakoid stacking.

Many of these observations may be seen in the chloroplasts of senescing leaves (Barton, 1966) and it could be argued that DBP was acting to promote the onset of senescence. Other compounds have been found which will do this, e.g. abscissic acid. However this would go against the results of Gudin and Harada (1974) who found that DBP and DOP isolated from crude oil delayed the senescence of *Rumex obtusifolius* leaves.

A feature of treated radish chloroplasts which was not reported by Virgin *et al.* (1981) is the complete absence of starch grains. Starch grains are a means of storing excess photosynthate. Their absence, in treated plant tissue, as compared to the control chloroplasts, could indicate that photosynthesis was impaired. In young tissue this would be expected from the lack of chlorophyll in DBP treatments, but the lack of starch grains in mature tissue suggests that DBP can act directly on some part of the photosynthetic mechanism, subsequent to light harvesting.

The swelling of chloroplasts in both young and old treated

leaves may indicate that a change in permeability of the chloroplast envelope has occurred. However there was no evidence of any swelling of mitochondria of treated leaves, nor in Golgi bodies, nuclei or vacuoles - so the effect is specific to chloroplasts. Ohyama (1977) demonstrated that phthalate esters induced swelling of mitochondria isolated from rat livers. Of the phthalate esters tested di-*n*-butyl phthalate exhibited the greatest activity. The mechanism responsible for this swelling was not known.

The rounding of the chloroplasts could also be a result of a decrease in size of the bounding membrane. This could occur by loss of membrane constituents which would contribute to the increase in size and number of plastoglobuli. Barton (1966), looking at the senescence of cells in *Phaseolus* noted that the chloroplasts were smaller than those in normal cells and that they also possessed a spherical shape. These chloroplasts seemed to have lost the ability to become flattened in the light. Instead they appeared to remain in the more rounded shape expected in the dark (Kirk and Tilney-Bassett, 1978).

The swelling of chloroplasts, together with the structural changes in the grana, demonstrates that DBP has an effect on all the membrane systems of the chloroplast. The work of Jamieson and Reid (1971) is of interest here. They surveyed the occurrence of hexadeca-7,10,13-trienoic acid in the leaf lipids of angiosperms. This fatty acid (C_{16})

is a lower homologue of linolenic acid (C_{18}). O'Brien and Benson (1964) had previously demonstrated that monogalactosyl and digalactosyl diglycerides, the major constituents of the chloroplast lipids of higher plants, have a high linolenic acid content. In spinach it was shown that both linolenic acid and hexadecatrienoic acid were concentrated in the galactolipids and that the latter was found mainly in the monogalactosyl diglyceride fraction (Allen *et al.*, 1966). However this fatty acid does not occur in all species of plants but Jamieson and Reed (1971) found it in the chloroplast lipids of spinach, water cress, tomatoes and cabbage, accounting for up to 17.5% of the fatty acid composition. All of these plants are sensitive to the effects of DBP, whereas pea, lettuce and nettle which appear to be resistant to the effects of DBP have none of this fatty acid present. DBP does not appear to have any effect on the cereal crops tested and barley is known to contain no hexadecatrienoic acid (Harwood and Russell, 1984). It is interesting to speculate whether the sensitivity of these species to DBP is in some way connected to the presence of hexadeca-7,10,13-trienoic acid. The simplest explanation for this is that this particular fatty acid permits the access of DBP into the chloroplast. This would explain why other organelles and membrane structures appeared to be unaffected by DBP in EM photomicrographs. Virgin *et al.* (1981) also noted that the mitochondria of DBP-treated leaves were normal.

4.5 CO_2 Exchange

Measurements of CO_2 using an infrared gas analyser showed that DBP vapour, from both DBP-plasticised plastic and liquid DBP, reduced CO_2 uptake in young radish plants. CO_2 evolution in the dark differed little from that of the control plant.

These results are compatible with the EM study which showed damage to chloroplasts but not to mitochondria.

Reduced photosynthetic rates (mg $CO_2.dm^{-2}$ leaf area $.hr^{-1}$) in plants suffering from chlorosis is not unexpected - presumably the chlorotic areas are unable to contribute to the overall rate of photosynthesis. It should be pointed out that the extent of chlorosis in these plants was quite small. This was probably a result of the small quantities of PVC used in the experiment, together with the fact that an open system was used in which air was flowing through the small growth chambers at a rate of 200 $ml.min^{-1}$. The duration of these experiments was fairly short as well, due to the constraints placed on maximum plant size by the small growth chambers. It is doubtful whether the chlorosis seen in these plants could be entirely responsible for the reduction in CO_2 uptake. It would have been useful to express photosynthesis on a unit chlorophyll basis on the final day of the experiment to check this idea.

4.6 Leaf Disc Electrode

Early experiments which utilised the leaf disc electrode to measure O_2 exchange, examined the effect of DBP vapour on young and old radish leaves. The results clearly show that DBP reduces photosynthetic rates in the young leaves. After one days exposure to DBP vapour, the young leaves showed signs of reduced photosynthetic rates even though the leaves appeared to show no visible signs of chlorosis. As the experiment progressed the photosynthetic rates in the leaves decreased while at the same time more chlorosis of the leaves was evident. At the end of the experiment photosynthesis was below the gas exchange compensation point. It should be remembered that these rates in the early experiments were calculated on a leaf area basis. Reduced levels of chlorophyll will obviously reduce the rate of photosynthesis when measured on a leaf area basis. The rate of O_2 uptake in the dark (respiration) by young detached radish leaves varied little throughout the duration of the experiment, even on a leaf area basis.

Measurements of the photosynthetic rates of discs cut from mature, fully expanded leaves were less conclusive in establishing the effects of DBP on photosynthesis. Rates of photosynthesis were marginally reduced in mature leaves after 11 days exposure to DBP vapour. However these rates were also calculated on a leaf area basis, and therefore the lower photosynthetic rates could also be a reflection

of reduced chlorophyll levels in the leaf.

Further experiments were undertaken which examined the effects of DBP vapour on photosynthesis in mature, fully expanded leaves only. These measurements were made in conjunction with the chlorophyll fluorescence readings discussed in Section 4.7. Oxygen evolution measurements using the leaf disc electrode again showed variability between different leaves but the results of oxygen evolution and fluorescence yield from any one leaf were self consistent. Thus when the oxygen evolution was reduced in a leaf disc, fluorescence measurements taken from another part of the same leaf showed an increased fluorescence yield. The O_2 evolution rates were also calculated on a unit chlorophyll basis, which would be expected to minimise differences in rates arising from different chlorophyll concentrations in the leaf disc samples.

Although the degree of inhibition of oxygen evolution rates of treated leaf discs varied, no inhibition was ever seen in the control discs. This variation probably arose for the same reasons as those outlined in the fluorescence measurements. Again shielding by other leaves was the main problem.

The purpose of measuring the oxygen evolution rates in mature, DBP-treated leaves was to establish whether DBP acted by directly affecting the functioning of the

photosynthetic apparatus in a leaf which had a known photosynthetic capacity at the start of an experiment. This is in contrast to the possibility that DBP acts primarily by preventing the acquisition of a fully functional photosynthetic apparatus in leaves which undergo development and expansion in the presence of the toxin. In order to overcome the problems of shielding in the present bioassay system smaller plants need to be used. The partially expanded leaves on these plants would develop chlorotic areas around their edges following longer periods of exposure to DBP vapour. But the measurements of oxygen evolution on a unit chlorophyll basis would distinguish whether DBP was able to directly reduce the photosynthetic capacity of a leaf or prevent the development of a functional photosynthetic apparatus. The possibility would still remain that reduced photosynthetic rates were a consequence of an accelerated senescence. It is known that a number of herbicides induce changes in a leaf which are similar in many respects to those which occur naturally in senescence (Moreland and Hilton, 1976).

Approaching the problem from a different angle - it would be informative to measure the CO_2 exchange in the individual leaves of plants previously exposed to DBP vapour. Plants could be exposed for various periods to DBP vapour and then removed from the growth chamber for measurements of CO_2 exchange in individual leaves using an IRGA. In this way it would be possible to measure the

effect of DBP on the photosynthetic capacity of young and old leaves individually.

Melin and Egneus (1983) examined the effect of DBP on the photosynthesis of isolated barley protoplasts and algae. CO_2-dependent oxygen evolution was measured and found to be inhibited at DBP concentrations greater than 10^{-5}M in protoplasts. Although DBP affected photosynthesis of *Chlorella* at a concentration of 10^{-5}M, inhibition was weak and at the maximum concentration tested only reached about 50%.

4.7 Chlorophyll Fluorescence Measurements

As was stated in the results section - the analysis of *in vivo* chlorophyll fluorescence is complex and difficult to interpret (Heinrich and Weis, 1984). Thus, any interpretation of the traces of chlorophyll fluorescence must be more qualitative than quantitative. Nevertheless the measurement of *in vivo* chlorophyll fluorescence is a very sensitive, and potentially non-destructive, technique for examining the primary photochemical reactions of photosynthesis.

It may be appropriate at this juncture to show a simple diagram of the photosynthetic electron transport chain in order to illustrate the position of the electron transport carriers referred to later in the text.

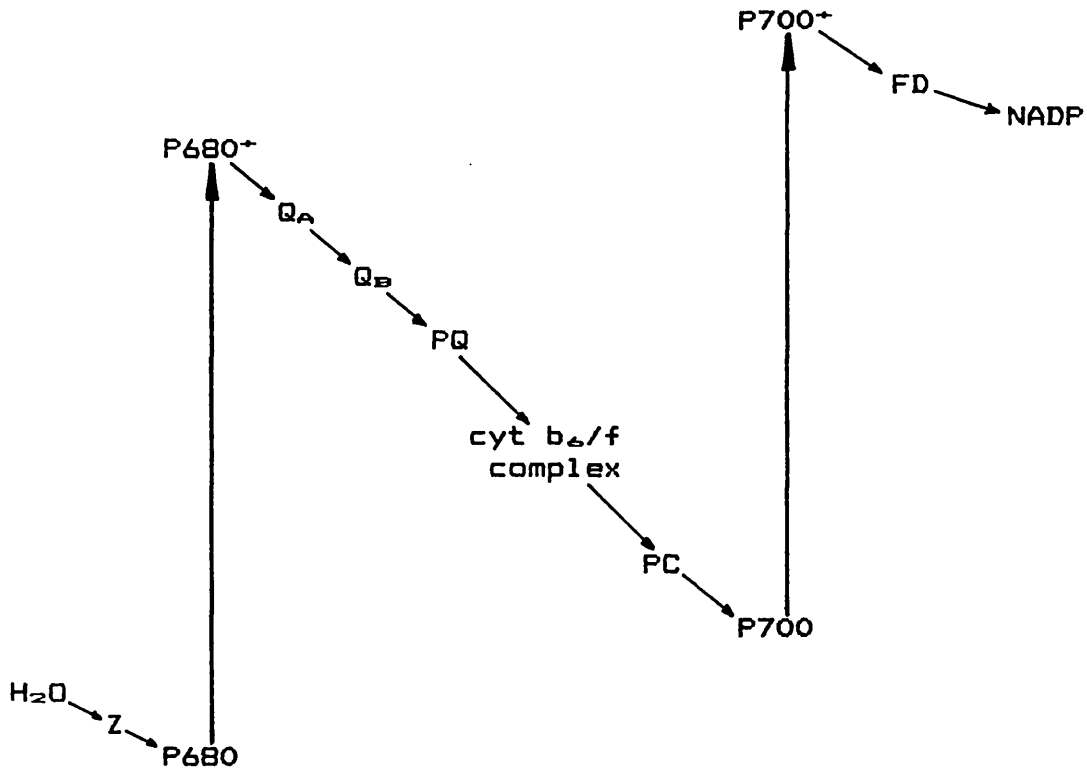


Fig.4.1 Z-scheme representation of the photosynthetic electron transport chain. Z - hypothetical carrier; P680 - PS2 reaction centre; Q_A, Q_B - quinones; PQ - plastoquinone; cyt b_6/f - cytochrome b_6/f complex; PC - plastocyanine; P700 - PS1 reaction centre; FD - ferredoxin; NADP - nicotinamide adenine dinucleotide phosphate.

4.7.1 Chlorophyll Fluorescence Quenching

During the early experiments the interest was to examine the effects of DBP on the maximum yield of chlorophyll fluorescence and on fluorescence quenching. It quickly became apparent that exposure to DBP vapour increased the

total yield of fluorescence and also inhibited fluorescence quenching. These particular experiments, in which plants were removed from an environment of DBP vapour and fluorescence readings taken following a day and seven days with no further exposure to DBP showed that the plants did not recover from their exposure to the toxin. The increased fluorescence yield after seven days may be partly due to enhanced senescence, arising from exposure to the toxin, as well as a direct result of interference with primary photosynthetic reactions.

The quenching of chlorophyll fluorescence *in vivo* is complex and *in vitro* may be brought about by a number of competing processes, such as light-induced fluorescence quenching, energy-dependent quenching (a function of pH), ATP-dependent quenching, Mg^{2+} depletion of thylakoids and quenching related to photoinhibition. Changes in temperature may also affect fluorescence quenching. The degree to which these different processes are responsible for the observed quenching of fluorescence emitted from a leaf will depend on the experimental conditions. Work involving intact chloroplasts or *Chlorella* cells seems to show that there are two main components of fluorescence quenching. One is Q-dependent quenching (Q_Q) - this is dependent on the redox state of the electron transport chain component Q (a quinone) (see Fig.4.1). The other is energy-dependent quenching (Q_E). The relative contribution from these two processes will vary considerably. Under conditions of fast reoxidation of NADPH and utilisation of

ATP in the Calvin cycle Q_a quenching will predominate. Low rates of ATP turnover (low Calvin cycle activity) will promote Q_E-dependent quenching. However Q_a quenching will be reduced when electron transport is restricted (due to $NADP^+$ exhaustion). In view of the reduced growth of DBP-treated radish plants and the ultrastructural damage to the chloroplasts it is possible that the absence of fluorescence quenching is a reflection of reduced electron transport rates. The fluorescence traces obtained with DBP poisoned leaves were very similar to those caused by treatment with DCMU. This electron transport inhibitor acts between Q and PQ in the electron transport chain. However infiltrating a leaf with a solution of DCMU induced a greater maximum fluorescence yield than that obtained with leaf material treated with DBP. This may reflect the more complete electron transport inhibition by DCMU.

4.7.2 State 1 - State 2 Changes

The purpose of these experiments was to examine further the effect of DBP vapour on the kinetics of fluorescence yield changes. Early experiments examined the State 1 - State 2 changes in both young and old leaves. Because the effects of DBP vapour on fully expanded leaves, both in the leaf disc electrode and the fluorescence measurements, were not as conclusive as those seen with younger leaves, subsequent experiments were devoted to

analysing the yields of chlorophyll fluorescence from mature leaves.

Experiments showed that State 1 - State 2 changes in young leaves ceased to occur within two days exposure to DBP vapour. There was also an even earlier increase in the maximum yield of fluorescence. Considering the ultrastructure of the chloroplast as revealed by electron microscopy this result is not entirely suprising. The lack of State 1 - State 2 changes was also seen in leaves which were chlorotic at the time of the fluorescence measurements and they also showed a rise in the maximium fluorescence yield. This apparent increase in fluorescence was probably a result of increased reflectance of incident light on the leaf.

Measurements of State 1 - State 2 changes in leaves which were mature at the start of exposure to DBP were of more interest as they appeared to have suffered much less damage ultrastructurally, compared to young leaves, and there was no evidence of chlorosis.

Unfortunately the results from these experiments varied considerably. Some treated leaves appeared to be unaffected by the DBP vapour, while other leaves exhibited increased maximum fluorescence. Examination of fluorescence enhancement ratios (FER) shows them to be most reduced in the DBP-treated leaves which demonstrates that State 1 - State 2 transitions were inhibited.

The yield of fluorescence was greater in the treated leaves throughout these State 1 - State 2 changes. Moreover F_{red}/F_{ox} ratios were considerably reduced in the DBP-treated leaves - due to increases in the F_{ox} level. Therefore the plastoquinone pool was not fully oxidised by light 1. This implies that DBP inhibits at some site beyond Q_A - which is the first accepter in the electron transport chain after PS2. It is not possible to distinguish from these results whether inhibition occurs in the electron transport chain or in CO_2 fixation. It should be remembered that DBP is lipophilic and would partition into the chloroplast membrane. Nazir *et al.* (1973) reported large amounts of DEHP - 60% of the total fatty acids - associated with mitochondrial triglycerides. Likewise Morris (1970) found unusually large percentages of phthalic acid (a dialkyl phthalate metabolite) during analysis of jellyfish lipids. Therefore it seems more reasonable to expect DBP to inhibit electron transport reactions. Measurements of the fluorescence yields of leaves infiltrated with DCMU during these experiments showed that the maximum fluorescence was 50% higher than that obtained from leaves exposed to DBP for 8 days. DCMU is more inhibitory than DBP. It should be stressed that although the results with DBP-treated plants showed variation, no enhancement in fluorescence yield was ever encountered in control plants.

Whilst these results were not conclusive in determining

the site or mode of action of DBP, they did show that DBP had an effect on the primary photosynthetic reactions of mature leaves. This inhibition of electron transport would result in reduced levels of ATP available for repair of existing structures within the chloroplast. In the results section it was suggested that the reason for the variability in yields of fluorescence seen in mature plants was a consquence of the size of the plants and the problems arising from leaves shielding others from the effects of the phytotoxin. This problem could have been compounded by the fact that the penetration of DBP might be reduced in older leaves, since Sargent and Blackman (1972) showed that penetration of herbicides into both the abaxial and adaxial surfaces of a leaf declined as the leaf matured. Shading of leaves was also a problem in our experiments and it has been shown that penetration of herbicides into a leaf is greatly enhanced by light (Sargent and Blackman, 1965). High humidity generally favours foliar absorption of herbicides, possibly reflecting the increased drying time of spray droplets. During our experiments the humidity was always high. Workers at Shell's Sittingbourne Research Centre found that mustard seedlings exposed to glazing strip plasticised with DIBP showed no ill effects when the plant's atmosphere was of low humidity (personal communication).

It is interesting to speculate on whether DBP is acting primarily as an electron transport inhibitor - the

increased yields of chlorophyll fluorescence being a reflection of this because of reduced Q_o quenching. At the same time, the loss of State 1 - State 2 changes may only result when DBP concentrations are high. The loss may be an indirect effect due to a lack of energy in the form of ATP to do essential repair work to membranes etc., or to phosphorylate the light harvesting chlorophyll *a/b* protein. As a consequence of this, fluorescence quenching may not occur by spillover of energy from PS2 to PS1 due to damage to the membranes containing these particles.

Until recently the explanation of the mechanism underlying State 1 - State 2 changes was that phosphorylation of the PS2 light harvesting chlorophyll *a/b* protein (LHCP) normally occurred when the light energy absorbed by PS2 was greater than PS1 (Bennett *et al.*, 1980). The excess PS2 light results in the plastoquinone pool being reduced which in turn activates a protein kinase located on the thylakoid membrane (Horton and Black, 1980). This kinase then phosphorylates the LHCP. It has been supposed that this phosphorylation alters the surface properties of the protein (i.e. van der Waals, coulombic and chemical) in such a way that its interaction with PS1 complexes is altered. This enables a redistribution of light energy which was preferentially absorbed by PS2 to be more equally utilised by PS1. The phosphorylation of the LHCP should allow it to migrate to non-appressed regions of the thylakoid because of the increased surface charge. The degree of granal stacking should be reduced as the

phosphorylated LHCP moves from appressed regions of the thylakoid to non-appressed regions (Telfer, 1984). However the EM photomicrographs suggest that granal stacking was increased in mature DBP-treated leaves. This may explain why State 1 - State 2 changes are inhibited as presumably phosphorylated LHCP is unable to migrate from appressed to non-appressed regions of the thylakoid.

With this theoretical mechanism in mind other explanations for the loss of State 1 - State 2 changes are suggested. From a knowledge of the physical properties of DBP - specifically its lipophilic nature - it could be supposed that the DBP molecules are incorporated into the thylakoid membrane and prevent or alter the interaction between PS2 and PS1 thereby inhibiting spillover. Another possibility is an effect on the kinase which phosphorylates the LHCP. Either by directly inhibiting the kinase itself or by preventing it from phosphorylating the LHCP.

In order to establish that the increase in fluorescence yield and loss of State 1 - State 2 changes was not a consequence of enhanced senescence in old leaves it would be of great value to examine the effects of DBP on the chlorophyll fluorescence of isolated chloroplasts or thylakoids. An experiment of this type would also permit measurement of the chlorophyll fluorescence arising during partial reactions from thylakoids or chloroplasts isolated from DBP-treated plants.

4.8 Oxygen Electrode

The third part of this discussion examines the *in vitro* effects of the dialkyl phthalates, in an attempt to determine their mode of action. This work is discussed in the light of the *in vitro* work on animal mitochondria and other biochemical aspects of animal cells treated with dialkyl phthalates.

All the *in vitro* work discussed in this section was performed on thylakoids isolated from spinach leaves. Unfortunately the electron transport activity of thylakoids isolated from radish leaves was far too low for the kind of experiments envisaged. However young spinach plants were bioassayed in our system and were found to suffer from the effects of DBP vapour. Chlorosis in these plants did not seem as marked as that seen in radish plants. As with radishes, the growth of these plants was severely stunted. These results help to justify the use of spinach thylakoids for *in vitro* measurements of electron transport rates.

The earliest experiments using the isolated spinach thylakoids examined the effects of a 100µM concentration of a variety of dialkyl phthalates on the combined, uncoupled activities of the two photosystems (PS2 + PS1). It was soon evident that only dibutyl and diisobutyl phthalate inhibited the activity of the full electron chain to any great extent. The inhibition exerted by DMP,

DIDP and DIOP was very low at concentrations of 100µM

These results correspond with the early screening experiments which demonstrated that only those plastics containing either DBP or DIBP as part of their formulation were phytotoxic. They also confirm the work of Melin and Egneus (1983) who demonstrated that DBP inhibited the activity of the full electron transport chain in isolated spinach thylakoids. These findings are also similar to those obtained from mitochondrial electron transport work. Ohyama (1976), examining the effects of a series of phthalate esters found that of those tested, DBP was the most potent inhibitor of mitochondrial electron transport. The degree of inhibition exerted by the other phthalate esters tested on mitochondrial electron transport was greater than that seen in the experiments with isolated thylakoids when compared to DBP.

These preliminary experiments were expanded and a range of concentrations of these phthalates were tested on full chain electron transport in isolated thylakoids. Inhibition of electron transport could be seen at a phthalate concentration as low as 10µM. The degree of inhibition rose rapidly to a maximum at approximately 100µM. Increasing the concentration to 1000µM produced no appreciable rise in inhibition. Melin and Egneus (1983) found that increasing DBP concentration up to 1000µM caused increased inhibition of electron transport in thylakoids isolated from spinach. The percentage

inhibition at this concentration was 80%. In our experiments this degree of inhibition was achieved at a concentration of 100µM. Melin and Egneus (1983) reported an inhibition of 18% at 100µM DBP. The I_{50} for DBP and DIBP were 44µM and 42µM respectively. Melin and Egneus (1983) obtained an I_{50} of 300µM. The results of the present work compare favourably with the mitochondrial work of Ohyama (1976) who reported that a DBP concentration of 180µM gave a 50% inhibition of State 3 oxygen uptake by sonicated mitochondria. Measurements of CO_2 evolution, O_2 uptake and photomicrographs have shown that plant mitochondria are more resistant to the effects of DBP than chloroplasts. The differences between Melin's work and the present may be a result of different assay media. Certainly the control rates of oxygen uptake in our experiments were far higher than those obtained by Melin and Egneus. In addition the concentration of chlorophyll in our experiments was very low - 10 $\mu g.ml^{-1}$ - which gave a high DBP to chlorophyll ratio compared to the 33 $\mu g.ml^{-1}$ chlorophyll concentration used by Melin and Egneus. The I_{50} may be overestimated because DBP readily attaches itself to different surfaces (Sodergren, 1982). Increasing the concentrations of DMP, DIDP and DIOP to 1000µM produced a small increase in inhibition of electron transport - the maximum of 11% inhibition was achieved by 1000µM DMP.

Further experiments concentrated on the effects of the most potent inhibitors of electron transport - DBP and

DIBP.

The effects of DBP and DIBP on the basal full chain electron transport activity of isolated thylakoids was examined. Inhibition of electron transport was found to increase with increasing phthalate concentration. At the maximum concentration tested - 1000µM - inhibition was approximately 60% for both DBP and DIBP. Melin and Egneus (1983) attained an inhibition of 44% with 1000µM DBP. It is evident that uncoupled electron transport is more sensitive to the action of DBP and DIBP than the coupled system. There is no evidence from these results that DBP acts primarily as an uncoupler i.e. that it dissociates electron transport from ATP formation. These results do not discount the possibility that DBP may act as an inhibitory uncoupler.

Earlier in this discussion it was suggested that a reason for the differences between the results of the present work and that of Melin and Egneus (1983) - both electron transport rates and degree of inhibition for a given DBP concentration - might be due to the different assay media used. During our early experiments we found that not only was the inhibitory effect of DBP not as marked as in the experiments described above but also a maximum inhibition was seen at a DBP concentration of 150µM, after which it appeared to decrease as the phthalate concentration was increased to 1000µM. A different assay medium was used in these early experiments. For these reasons it was decided

to substitute ferricyanide for methyl viologen as the final electron acceptor in an attempt to establish that the apparent decrease in inhibition was not an artifact due to the chemical nature of methyl viologen. The decrease in inhibition of the electron transport rate above a DBP concentration of 150µM was still obtained using ferricyanide as the terminal electron acceptor.

Another possible explanation for the apparent decrease in inhibition was that oxidation of the lipid membrane occurred due to a combination of the high light intensity - hence energy - and the higher DBP concentrations. Use of an orange filter, which removed the high energy blue - green wavelengths of light, demonstrated that this was not the case.

Subsequently the assay solution was modified and included EDTA together with different concentrations of cations. This modification yielded substantially increased electron transport rates and no decrease in inhibition as the phthalate concentration was increased above 100µM.

Photosystem 1 was assayed in an attempt to localise the site of action of the phthalates more precisely. Inhibition of PS1 was low compared to that seen for full chain activity (PS2 + PS1) - reaching a maximum 40% at a phthalate concentration of 1000µM. The full chain was inhibited to 80% with a phthalate concentration of 100µM. Therefore the main site of action of DBP and DIBP lies at

a point prior to or at the DCMU sensitive site (between Q_B and PQ - see Fig.4.1). Melin and Egneus (1983) reported no effect on PS1 with a concentration of DBP up to 1000µM. Moreover in isolated thylakoids potassium ferricyanide is thought to accept electrons from some point interconnecting PS2 and PS1 (Trebst 1972). The experiment which used ferricyanide as the terminal electron acceptor fortuitously confirmed that DBP acts at a site in the electron transport chain between the water splitting reaction and some point prior to PS1.

The mechanism by which the toxic phthalates inhibit electron transport is not known from the present work. Considering the high concentrations of phthalates required, it may be that inhibition results from a spatial separation of the electron transport components within the thylakoid membrane. Another possibility is that phthalates interfere with an active site involved in the electron transport chain.

4.9 Mechanism Of DBP And DIBP Action

The penultimate section of this discussion will relate the present work on the mechanism of phthalate toxicity to the findings and theories held by other workers in this field.

The area of research covered by the present work strongly suggests that an important site of action of the

phthalates lies within the chloroplast. The evidence for this comes from both physiological and biochemical experiments. Measurement of CO_2 uptake demonstrated that this was reduced in whole radish plants exposed to DBP vapour. Similarly the O_2 evolved from radish leaf discs from treated plants showed a reduction. More interestingly, leaf discs from mature leaves sometimes showed inhibited O_2 evolution rates. Both these results suggest that a primary site of action of DBP lies at a photosynthetic level - hence, within the chloroplast. Electron micrographs of the chloroplasts of treated plants support this view as their appearance was altered by the presence of the phytotoxin. The chloroplast structure in young leaves was the more dramatically altered. This might be expected as these leaves suffered from chlorosis and chlorophyll is required for the development of the grana. However definite alteration of the structure of chloroplasts from mature leaves was also observed.

The effects of DBP on chlorophyll fluorescence confirmed that a site of action lay within the chloroplast and more specifically that DBP was capable of inhibiting photosynthetic reactions.

In vitro work on isolated spinach thylakoids demonstrated that DBP and DIBP inhibited electron transport and that this inhibition was localised primarily at a site close to PS2. These findings are similar to those of Melin and Egneus (1983). It is interesting to note that most

herbicidal electron transport inhibitors are thought to have a site of action closely associated with PS2 (Moreland, 1980). Although these phthalates were found to inhibit *in vitro* electron transport it was not established whether they could also act as uncouplers. Work with animal mitochondria has suggested that DBP, together with other phthalates, could act as electron and energy transport inhibitors but not as uncouplers (Ohyama, 1976); while Takahashi (1977) suggested that they acted mainly as energy transfer inhibitors. In contradiction to these findings Inouye *et al.*, (1978) concluded that DBP acted primarily as an uncoupler of oxidative phosphorylation. Melnick and Schiller (1982) also assigned a role as an uncoupler of oxidative phosphorylation to DBP. They also found that electron transport was inhibited.

At present it is established that DBP inhibits the *in vitro* electron transport of chloroplasts and animal mitochondria. The concentration of DBP required for 50% inhibition of State 3 respiration in sonicated animal mitochondria was 180 µM (Ohyama, 1976). This value is approximately 4.5-fold higher than the concentration required for 50% inhibition of chloroplast electron transport. Therefore higher concentrations of DBP may inhibit plant mitochondria. The work to date does not justify assigning a role of electron transport inhibitor to DBP in the case of whole plants.

Moreland and Hilton (1976) suggested that photosynthesis

could be inhibited in several ways. One was by the interference with the reproduction, development, structure and integrity of the chloroplast. The second was by inhibiting the light reactions involved in the conversion of light energy to chemical energy. And the third was by inhibiting the biosynthetic pathways concerned with the production of photosynthetic products. The work presented in this thesis demonstrates that DBP probably affects the development of chloroplasts in young leaves and inhibits the light reactions of isolated thylakoids.

With this in mind, the work and ideas of others researching the biological effects of phthalates will now be discussed.

Virgin *et al.* (1981), examining the effects of DBP vapour on the pigments of *Browallia speciosa* L. cv. Major and *Raphanus sativus* L. cv. Cherry Belle looked at absorbance spectra of hexane extracts of leaves. From the spectra it was apparent that in chlorotic leaves there was a lack of chlorophyll, carotenes and xanthophylls. Absorption peaks in the ultraviolet region of the spectrum indicated the build up of a precursor of carotene, most probably phytoene, in treated leaves. This observation, together with the fact that light was required to induce chlorophyll and carotene deficient leaves, suggested to them that DBP acted by inhibiting carotene biosynthesis. They suspect that the loss of chlorophyll is a secondary phenomenon. Carotenes are thought to protect the

chlorophyll from photooxidation by quenching reactive, singlet oxygen species. These workers found that prolonged exposure to DBP vapour caused symptoms of chlorosis in leaves which were green at the onset of exposure. This was contrary to our findings. On no occasion did chlorotic regions develop on leaves which were green and mature at the start of exposure to DBP. It is possible that the symptoms of chlorosis observed by Virgin *et al.* (1981) in mature leaves were due to the senescence of the leaf in question. The absence of chlorosis in the mature leaves may be explained by assuming that there is no turnover of carotenes. Treharne *et al.* (1966) demonstrated that turnover of carotenes is sluggish if it happens at all. Carotenoid levels, although stable in mature leaves, will be reduced following the onset of senescence and enzyme systems which can destroy these pigments have been extracted from these leaves (Goodwin, 1980). It has also been demonstrated that there is little or no turnover of chlorophyll in non-expanding leaves (Aronoff, 1963). Ogawa *et al.* (1973) suggested that the rate of β-carotene synthesis was limited by the rate at which it could be incorporated into a chlorophyll-carotene-protein complex. If the rate of formation of this complex is linked to the rate of chlorophyll formation this would account for the action spectrum of β-carotene synthesis corresponding to the action spectrum of chlorophyll a and the absorption spectrum of protochlorophyllide in intact, etiolated wheat leaves. This suggests that the absence of carotene from chlorotic leaves arises directly from the lack of

chlorophyll, rather than the lack of carotene causing the destruction of chlorophyll - as Virgin *et al.* (1981) proposed.

Inhibition of electron transport in a leaf will seriously deplete energy reserves in the form of ATP, and reducing power in the form of NADPH. Reduced levels of these substrates will have serious detrimental effects on many biosynthetic pathways including those of chlorophyll and carotene synthesis. Mature, fully expanded leaves do not suffer from chlorosis as they possess a full complement of carotenes and chlorophylls at the onset of exposure to DBP and it is only newly developing parts of the leaf which lack these pigments. Presumably they were always absent.

Recently it has been suggested that a major metabolite of DBP - phthalic acid - acts by chelating certain cations (Hardwick *et al.*, 1985). Phthalic acid is a dibasic acid which can chelate copper ions. Copper ions are essential for the activity of some enzymes such as super-oxide dismutase and ascorbic acid oxidase. Reduction of the activity of these enzymes would eventually lead to photobleaching of chlorophyll. Work with animal systems has shown that dialkyl phthalates can alter zinc metabolism (Gangolli, 1982; Thomas *et al.*, 1982) and calcium metabolism (Mayer *et al.*, 1972). Serious depletion of the cation concentration in the chloroplast stroma could also prevent the stacking of grana (Izawa and Good, 1968).

One final explanation for DBP toxicity is suggested by the work of Lichtenthaler and Thiess (1974) who demonstrated that low concentrations of ethanol inhibited the light induced formation of chlorophyll, carotenoids and anthocyanins in *Raphanus sativus* seedlings. The fate of the toxic phthalates is not known once they have entered a plant but it is possible that they are hydrolysed which would result in the presence and possible accumulation of butanol. *In vivo* work with many animal species has shown that DEHP is hydrolysed first to mono-2-ethylhexyl phthalate and then to phthalic acid (Stalling *et al.*, 1973; Albro *et al.*, 1973; Albro and Moore, 1974; Peck and Albro, 1982). Also Albro and Thomas (1973) have demonstrated the *in vitro* hydrolysis of DEHP by lipases from a variety of rat tissues.

Biochemical work with animal systems has demonstrated that DBP can have a wide variety of effects. In addition to the effects on mitochondria reported above, investigators have found that dialkyl phthalates alter the activity of many enzymes both *in vitro* and *in vivo*. The most frequently tested phthalate with animal systems is DEHP; since this is both the most widely used plasticiser and the most ubiquitous environmental phthalate contaminant.

Seth (1982) has reported that DEHP alters the activity of aminopyrine N-demethylase, aniline hydroxylase, alcohol dehydrogenase and high and low K_m aldehyde dehydrogenases

under *in vivo* conditions. However no effect was produced on aminopyrine N-demethylase or aniline hydroxylase under *in vitro* conditions. Bell (1982) reported that feeding DEHP to animals produced diverse biochemical effects, including inhibition of cholesterolgenesis, decreased synthesis of hepatic phospholipid and triglyceride and increased fatty acid oxidation in mitochondria isolated from the liver of treated animals. *In vitro* effects included inhibition of cholesterolgenesis. Phthalate plasticisers have been shown to have measurable effects on a variety of other enzymes (Lake *et al.*, 1975; Ohyama, 1977a,b; Srivastava *et al.*, 1977, 1978) and biosynthetic pathways (Bell, 1976; Bell *et al.*, 1978a, 1978b, 1979). Differences have also been shown in the sensitivities of enzymes depending from which organ they originated (Srivastava *et al.*, 1977; Ohyama, 1977a; Osumi and Hashimoto, 1978).

Ultrastructural studies of chloroplasts in DBP-treated plants in this project demonstrated an absence of starch grains. Starch grains were absent from the photomicrographs of DBP-treated chloroplasts in the paper by Virgin *et al.* (1981). Yanagita *et al.* (1977) found that the levels of liver glycogen were reduced in rats fed a diet containing 0.5% DEHP for 10 days. These results are consistent with the electron transport inhibition of chloroplasts and mitochondria.

The fact that some species of plants are resistant to the

toxin may enable some more progress to be made in elucidating the primary mechanism underlying DBP toxicity. Of the limited number of plants tested, only certain species are sensitive to DBP; all Cruciferae tested so far being particularly sensitive, whilst lettuce and pea appear to be totally resistant to DBP. Intraspecific variations also occur, as shown by Hardwick *et al.* (1984) for cabbage and by Inden and Tachibana (1975) for sweet pepper. Earlier in this discussion it was suggested that an explanation for the differences in sensitivity to the toxin may be explained by variations in the fatty acid compositions of certain chloroplast lipids. The basis for this was a correlation between sensitivity to DBP and the presence of the fatty acid, hexadecatrienoic acid. This fatty acid is found almost exclusively in the monogalactosyl diglyceride fraction of chloroplast lipids (Jamieson and Reed, 1971). Measuring the quantity of hexadecatrienoic acid in the chloroplasts of a selection of different species and cabbage cultivars of varying sensitivity to DBP would establish whether this idea was valid.

Similarly, marked differences in the susceptibilities of animals to phthalate plasticisers have been reported. There are several reviews of this work - by Autian (1973) and Gesler (1973). Other work has shown that there are differences in the ability of organs to accumulate plasticisers. Intravenous injections in rats demonstrated that DEHP disappeared rapidly from the bloodstream and was

localised primarily in the liver (Schulz and Rubin, 1973; Tanaka *et al.*, 1975). This localisation appears to occur at a subcellular level also. Work by Nazir *et al.* (1971, 1973) has shown in bovine heart muscle that of the total DEHP recoverable from these cells 99.5% was present in the triglceride fraction of mitochondria. Other tissue specific differences appear to exist. Bell and Gillies (1977) have shown that in rats fed 0.1% DEHP for 2 to 3 days the oxidation of ^{14}C-palmitoyl CoA to $^{14}CO_2$ increased two to three fold in hepatic mitochondria. In contrast to this, the oxidation of ^{14}C-palmitoyl CoA by heart mitochondria was decreased by about 40%.

Assuming that the explanation for the different sensitivities of plants to DBP is due to the hexadecatrienoic acid present in the chloroplast lipids it remains to be answered why. It may be that this particular fatty acid allows the entry of DBP through the chloroplast envelope into the stroma or that it permits the accumulation of DBP molecules in the lipid of the chloroplast membranes especially those of the thylakoid as the proportion of MGDG to DGPG is ~2:1 as opposed to ~0.9:1 in the envelope (Goodwin and Mercer, 1983). The absorption and accumulation of the herbicide, propanil by isolated spinach chloroplasts is influenced by their lipid, but not protein, content (Fletcher and Kirkwood, 1982). Most hexadecatrienoic acid is located in the MGDG fraction. The most plausible alternative to these explanations is that DBP somehow alters the synthesis of

hexadecatrienoic acid. Yanagita *et al.* (1977) found that the percentage of stearic and arachidonic acids was increased at the expense of palmitic and docosahexaenoic acids in hepatic phosphatidylethanolamine in young rats fed a diet including 0.5% DEHP. The percentage of major fatty acids of hepatic phosphatidylcholine and triglycerides were also altered significantly. These results indicate that phthalates do have the ability to alter the fatty acid composition of animal lipids. Moreover, it is suspected that some chlorosis-inducing herbicides may act by an inhibition of the synthesis of essential membrane lipids which would limit the capacity for thylakoid formation which in turn would decrease the capacity of the membrane to incorporate chlorophyll (Feierabend and Schubert, 1978).

The results of the work undertaken with both plant and animal systems indicate that the effects of DBP are many and varied. Evidently DBP is not very specific in it's action - a view held by Melin and Egneus (1983) who found that the DBP to chlorophyll ratio required for inhibition of photosynthesis was high compared to that for herbicides. It is not possible at the present time to attribute any particular mechanism of DBP action to the main cause of phytotoxicity. However from the evidence presented in this thesis an important site of action lies within the chloroplast. It is quite possible that subsequent work will demonstrate that it is by a combination of mechanisms that DBP exerts it's

phytotoxicity.

4.10 Future Work

The final section of this discussion will be used to suggest future lines of research; firstly for commercial considerations and secondly for mode of action studies.

4.10.1 Commercial

Further screening of glasshouse crops has high priority. There may be several more species sensitive to the toxic phthalates amongst the numerous house plants, bedding plants etc., grown under glass. It may be worth selecting for resistant cultivars amongst *Cruciferae* such as alyssum and ten-week stocks so that they will tolerate phthalates as well as most of the other common bedding plants appear to do.

The effects of phthalates in solution need to be examined more fully than they have at present as leaching of plasticisers may reduce crop yields.

It would be logical to seek the reasons for the difference between resistant and sensitive species alongside such screening work as the two topics are closely interrelated. (See Section 4.10.2 for specific suggestions).

A Britsh Standard for horticultural PVC plastics is urgently needed and a clearly understood mark of approval should appear on all such plastics since PVC hose-pipes, trickle irrigation tubing and even some plant pots have been found to be toxic in a few instances, as well as the glazing strip. Dose response curves for individual plasticiser toxicities will be required and a standardised method for headspace analysis of PVC plastics needs to be agreed.

4.10.2 Mode Of Action

The refinement of some of the present techniques has been referred to previously in the appropriate sections of the discussion. A full EM study on mature green leaves of plants following extended periods of exposure to DBP would establish whether or not the chloroplasts of mature leaves are damaged.

Measurements of photosynthesis, based on unit chlorophyll content, are needed for young, partially chlorotic leaves to establish whether DBP inhibits photosynthesis *in vivo* as well as preventing chlorophyll formation.

Chlorophyll fluorescence experiments using isolated spinach chloroplasts could be used to correlate *in vitro* results with those obtained *in vivo*. Also the effects of DBP on the chlorophyll fluorescence of thylakoids during partial reactions would allow a more precise localisation

of the site of action - as would further experiments using the O_2 electrode; such as a PS2 assay. It would also be useful to determine whether DBP inhibits electron transport in intact chloroplasts (with a double limiting membrane) in order to demonstrate that the molecule is able to reach the inhibitory site, in a system closer to that found *in vivo*. Examination of the effects of DBP on electron transport rates in the isolated thylakoids of resistant species such as pea or lettuce is an additional approach.

The work of Virgin *et al.* (1981) which indicated a critical role for carotenoid pigments in DBP-treated plants needs to be confirmed and extended to other species in which chlorosis is less obvious than in radish.

Variations in sensitivity to phthalates may be due to any of the following:

a) Differing hexadecatrienoic acid content of chloroplasts - this is relatively straightforward to check.

b) Differing access of phthalate to the sensitive site - this could be directly correlated with (a) above but other aspects could be investigated by exposing plants to radiolabelled DBP and then measuring the radioactivity present in different sub-cellular fractions. Alternatively electron microscopy of leaf sections coupled with autoradiography of the sections would give a more detailed analysis. Approaching the problem from a molecular angle,

the lipids from various cell structures could be analysed for the presence of DBP.

c) Differences in metabolism of the phthalates - either as detoxification, or as a mechanism causing toxicity by the release of toxic metabolites. This could be examined by following the fate of specifically labelled DBP in resistant and non-resistant plants.

Now that the main guidelines have been established by the current work, it is important that future investigations should concentrate on finding the reason for the difference between sensitive and resistant plants. This wil ensure that research will be aiming at the primary mechanism of toxicity rather than some interesting but less relevant side effect.

SUMMARY

1. The phytotoxicity of certain samples of plasticised polyvinyl chloride (PVC) is caused by particular plasticisers, either dibutyl phthalate (DBP) or diisobutyl phthalate (DIBP). Originally there was a strong suspicion that the phytotoxicity might involve the stabilisers but there was no evidence that they were toxic when incorporated in the plastic.

2. The susceptibility to the toxic phthalate vapours varies between species and between varieties. Affected plants usually show chlorosis of the younger leaves which develop in the presence of the toxin. The *Cruciferae*, seem to be particularly susceptible and provide good bioassay material. In the bioassay used in this project, young radish plants showed early signs of chlorosis when exposed to around 160-180 ng DBP.l^{-1} for 2-3 days and died after about 10-12 days exposure.

3. A major toxicity arising from phthalates in glasshouse glazing strip was identified. New formulations were suggested and tested, as a result of which the diisobutyl phthalate + dioctyl phthalate plasticised plastic which was causing the trouble was substituted with one plasticised with diisodecyl phthalate. This has proved to be satisfactory after more than a year in commercial use. Previous losses in tomato yield were remedied during the 1984 season by replacing the

toxic glazing strip with the new formula glazing strip. It was also established that dioctyl phthalate produced no toxic symptoms when used as sole plasticiser in PVC glazing strip.

4. Use of diisodecyl phthalate as a plasticiser produced no toxic symptoms and was recommended for use as a safe plasticiser in horticultural plastics.
5. When DBP was added to the nutrient solution in which radish plants were grown there appeared to be little uptake and no translocation to the leaves.
6. Ultrastructural studies showed that chloroplasts were the most susceptible organelle to the effects of DBP. Damage was seen as a swelling of the chloroplast, absence of grana and starch grains and increased numbers of plastoglobuli.
7. DBP inhibited photosynthesis, as measured by CO_2 uptake, in young radish plants treated with DBP-plasticised PVC or pure liquid DBP. Respiration was virtually unaffected.
8. DBP inhibited photosynthesis, as measured by CO_2-dependent O_2 evolution of young radish leaves detached from treated plants. Photosynthesis was also reduced to varying degrees in leaf discs cut from the mature leaves of treated plants.
9. The *in vivo* yield of chlorophyll a_2 fluorescence was increased following exposure to DBP. In some plants no quenching of this fluorescence was observed. The ability of some leaves to undergo State 1 - State 2 changes was impaired.

10. Of the dialkyl phthalates tested only DBP and DIBP severely inhibited electron transport in isolated spinach thylakoids I_{50} for DBP and DIBP respectively were 44 µM and 42 µM.
11. The main site of electron transport inhibition appears to be located near the reducing side of photosystem 2, at or before the site of DCMU inhibition.

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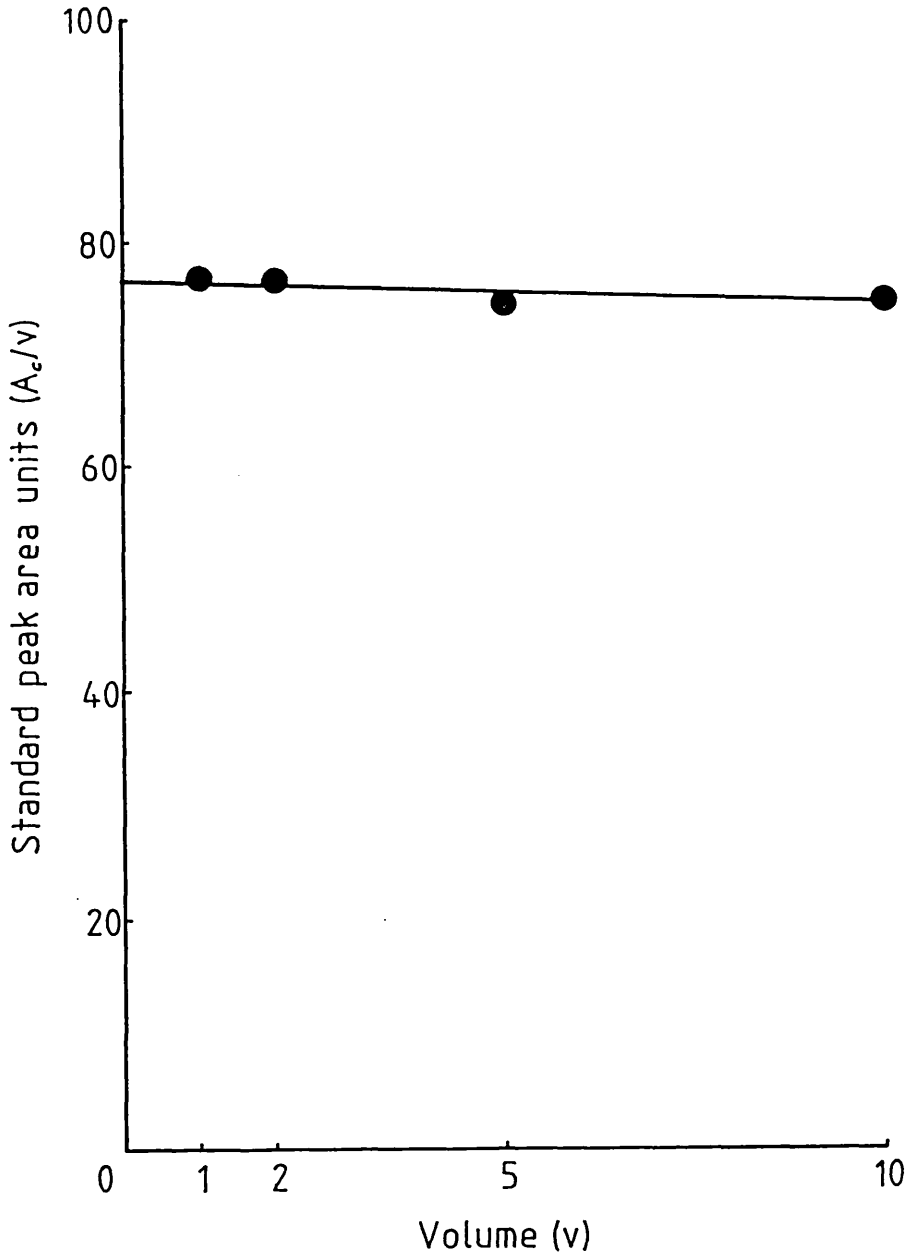
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APPENDIX 1. Sample of a calibration curve for the estimation of the atmospheric concentrations of dibutyl and diisobutyl phthalates using a GC. Line of best fit $y = -0.2x + 76.41$.

APPENDIX 2

ICI Plastics

Experiment 1

Day	Treatment (n)	Mean fresh weight (g) ±SE	Mean dry weight (g) ±SE	DW% FW
0	control (9)	0.351±0.029	0.026±0.002	7.38±0.16
3	control (3)	0.756±0.084	0.051±0.005	6.83±0.13
	DBP (3)	0.679±0.052	0.044±0.002	6.49±0.34
	DIDP (3)	0.562±0.051	0.038±0.003	6.82±0.24
	DIOP (3)	0.670±0.102	0.045±0.008	6.62±0.33
7	control (3)	1.534±0.271	0.099±0.013	6.69±0.43
	DBP (3)	0.803±0.125	0.059±0.003	7.90±1.11
	DIDP (3)	1.658±0.054	0.116±0.005	6.99±0.18
	DIOP (3)	1.741±0.134	0.121±0.008	6.97±0.30
10	control (3)	3.011±0.030	0.206±0.013	6.74±0.25
	DBP (3)	0.633±0.025	0.071±0.006	11.24±1.03
	DIDP (3)	2.874±0.101	0.181±0.009	7.02±0.31
	DIOP (3)	2.995±0.741	0.220±0.046	6.80±0.17

Experiment 2

Day	Treatment (n)	Mean fresh weight (g) ±SE	Mean dry weight (g) ±SE	DW% FW
0	control (9)	0.258±0.016	0.018±0.001	7.03±0.22
3	control (3)	0.584±0.019	0.038±0.001	6.57±0.12
	DBP (3)	0.506±0.020	0.031±0.002	6.04±0.24
	DIDP (3)	0.599±0.051	0.038±0.003	6.34±0.40
	DIOP (3)	0.594±0.062	0.040±0.004	6.69±0.02
6	control (3)	1.685±0.033	0.102±0.008	6.07±0.44
	DBP (3)	0.545±0.076	0.036±0.005	6.73±0.63
	DIDP (3)	1.691±0.303	0.107±0.019	6.36±0.08
	DIOP (3)	1.411±0.162	0.095±0.010	6.72±0.10
10	control (3)	3.033±0.246	0.201±0.009	6.74±0.54
	DBP (3)	0.315±0.073	0.047±0.003	17.00±2.90
	DIDP (3)	3.072±0.430	0.222±0.035	7.17±0.17
	DIOP (3)	3.279±0.249	0.228±0.016	6.95±0.08

Glazing Strip

Experiment 1

Day	Treatment (n)	Mean fresh weight (g) ±SE	Mean dry weight (g) ±SE	DW% FW
0	control (9)	0.374±0.020	0.026±0.001	6.83±0.11
3	control (3)	0.843±0.074	0.051±0.004	6.04±0.12
	DBP (3)	0.741±0.066	0.041±0.004	5.59±0.16
	DIDP (3)	0.791±0.160	0.046±0.006	5.98±0.34
	DIOP (3)	0.734±0.081	0.045±0.003	6.18±0.30
6	control (3)	1.857±0.046	0.105±0.004	5.67±0.08
	DBP (3)	0.995±0.128	0.055±0.007	5.56±0.17
	DIDP (3)	1.614±0.137	0.092±0.006	5.73±0.14
	DIOP (3)	1.583±0.068	0.092±0.002	5.81±0.16
10	control (3)	4.758±0.572	0.258±0.034	5.41±0.07
	DBP (3)	0.503±0.131	0.052±0.004	11.72±1.80
	DIDP (3)	4.281±0.156	0.268±0.005	6.28±0.16
	DIOP (3)	3.581±0.201	0.213±0.006	5.99±0.24

Experiment 2

Day	Treatment (n)	Mean fresh weight (g) ±SE	Mean dry weight (g) ±SE	DW% FW
0	control (9)	0.442±0.023	0.026±0.001	6.00±0.08
3	control (3)	1.192±0.057	0.074±0.004	6.24±0.09
	DBP (3)	0.744±0.046	0.044±0.004	5.94±0.24
	DIDP (3)	1.189±0.045	0.072±0.003	6.05±0.06
	DIOP (3)	1.059±0.088	0.063±0.006	5.91±0.14
6	control (3)	2.176±0.034	0.127±0.004	5.82±0.09
	DBP (3)	0.882±0.146	0.065±0.008	7.76±1.04
	DIDP (3)	2.212±0.084	0.127±0.008	5.73±0.17
	DIOP (3)	2.220±0.164	0.127±0.010	5.72±0.09
10	control (3)	4.690±0.641	0.302±0.045	6.42±0.31
	DBP (3)	0.750±0.040	0.074±0.005	9.94±0.84
	DIDP (3)	4.890±0.409	0.297±0.022	6.10±0.07
	DIOP (3)	5.422±0.308	0.316±0.029	5.81±0.31