THE UNIVERSITY OF MANITOBA

AN INVESTIGATION OF SOME METABOLIC EFFECTS OF THE INFECTION OF *HORDEUM VULGARE* BY *HELMINTHOSPORIUM TERES*, WITH SPECIAL REFERENCE TO THE NUTRITION OF THE HOST-PARASITE COMPLEX.

A Thesis

Submitted to The Faculty of Graduate Studies

in Partial Fulfilment of

The Requirements for the Degree of

Doctor of Philosophy

by

Janet Rowe

Department of Botany

Winnipeg, Manitoba

May, 1973

ACKNOWLEDGEMENTS.

The author has very greatly appreciated the advice and guidance of Dr. James Reid throughout this project, and wishes especially to record her thanks for his unflagging encouragement.

The experimental work was largely carried out in the laboratory of Dr. E. R. Waygood, Department of Botany, and the author is grateful for the facilities made available to her.

She also gratefully acknowledges the assistance of Michael Bryan in the photographic work; the interest and help of her friends and colleagues in the Botany Department; the invaluable support of Valerie Paape; and, in financial assistance, the awards of a Graduate Fellowship by the University of Manitoba and a Post-graduate Scholarship by the National Research Council of Canada.

ABSTRACT.

A quantitative study was made of some of the metabolic effects of Helminthosporium teres infections of barley varieties Parkland (susceptible) and C.I.5791 (resistant): first leaves of seedlings were inoculated.

In Parkland, RuDP carboxylase activity and carbon fixation in the light fell over the seven-day infection cycle (from inoculation to sporulation) to ca. 50% of control levels, though the net accumulation of carbon in the leaves over the 24-hour diurnal cycle was reduced by only 25%. The activity of PEP carboxylase and the malic enzyme, and carbon fixation in the dark, rose to 200 - 400% the levels of controls, peaking just before pathogen sporulation, though, in vivo, fixation in the dark was only 0.5 - 2.0% that in the light. Analytical studies of the early products of $^{14}CO_2$ fixation indicated that carbon entered metabolism predominantly via the Calvin cycle in the light in both healthy and infected plants (a comparative experiment with rust-infected barley giving similar results) but that β-carboxylation in all samples was higher in the light than in the dark; and a significant increase in β-carboxylation occurred upon H.teres-infection, so that perhaps 15% of the total CO_2 entered metabolism via this route in infected leaves. Export of photosynthate was greatly reduced by infection, but import from younger leaves was unaffected.

The metabolic effects in vitro and in vivo of the hypersensitive reaction of C.I.5791 were generally similar to those of Parkland until the development of the pathogen was inhibited (ca. four days after inoculation), after which metabolism returned to normal: the degree of metabolic alteration was apparently related to the extent of fungal growth.

The effects of infection on carbon-fixation in situ could be reproduced to a limited extent by mechanical or chemical damage, but there were indications that the response to pathogenesis was more specific e.g. effects on uninvaded susceptible tissue. The possible origins of the metabolic effects of infection in Parkland and C.I.5791 are discussed and some suggestions made as to their significance in the development of the pathogen in susceptible tissue.

TABLE OF CONTENTS.

TABLE OF CONTENTS CONT'D.

PAGE

TABLE OF CONTENTS CONT'D.

PAGE

TABLE OF CONTENTS CONT'D.

TABLE OF CONTENTS CONT'D.

PAGE

TABLE OF CONTENTS CONT'D.

PAGE

TABLE OF CONTENTS CONT'D

PAGE

LIST OF ABBREVIATIONS.

ADP	-	Adenosine diphosphate
Ala	-	Alanine
Asp	-	Aspartic acid
Asp^n	-	Asparagine
ATP	-	Adenosine triphosphate
Cit	-	Citric acid
2,4 D	-	2,4-dinitrophenol
DHAP	-	Dihydroxyacetone phosphate
EDTA	-	Ethylene diamine tetra-acetic acid
FDP	-	Fructose 1,6-diphosphate
F6P	-	Fructose-6-phosphate
Fru	-	Fructose
Fum	-	Fumaric acid
3PGA	-	3-phosphoglyceric acid
G1P	-	Glucose-1-phosphate
G6P	-	Glucose-6-phosphate
Glu	-	Glutamic acid
Gluc	-	Glucose
Glu^n	-	Glutamine
Gly 3 P	-	Glyceraldehyde-3-phosphate
Glycer	-	Glyceric acid
Glycol	-	Glycolic acid
Glyox	-	Glyoxylic acid
H	-	Healthy
hexose Ps	-	hexose phosphates
I	-	Infected
Mal	-	Malic acid
Mlt	-	Maltose
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	" " " , reduced form
NADP	-	" " " phosphate
NADPH	-	" " " " , reduced form
NAD(P)	-	NAD & NADP
OAA	-	Oxalacetic acid
O.D.	-	optical density
Pi	-	inorganic phosphate

LIST OF ABBREVIATIONS CONT'D.

PEP	-	Phosphoenolpyruvate
Phe	-	Phenylalanine
Pyr	-	Pyruvate
Rib	-	Ribose
R 5 P Rib 5 P	-	Ribose-5-phosphate
RNA	-	Ribonucleic acid
RuDP	-	Ribulose 1,5-diphosphate
S7P	-	Sedoheptulose-7-phosphate
Ser	-	Serine
Suc	-	Sucrose
Tar	-	Tartaric acid
Thr	-	Threonine
TLC	-	thin layer chromatography
TLE	-	thin layer electrophoresis
Tris	-	Tris(hydroxymethyl)aminomethane
Tyr	-	Tyrosine
UDPG	-	Uridine diphosphate glucose
Val	-	Valine
Xyl	-	Xylose

LIST OF TABLES.

LIST OF TABLES CONT'D.

LIST OF TABLES CONT'D.

LIST OF TABLES CONT'D.

LIST OF TABLES CONT'D.

LIST OF TABLES CONT'D.

LIST OF FIGURES

LIST OF FIGURES CONT'D.

LIST OF PLATES.

LIST OF PLATES CONT'D.

LIST OF PLATES CONT'D.

INTRODUCTION.

The organs primarily responsible for photosynthesis in the green plant are the leaves, and through photosynthesis they supply most of the chemical energy and the organic substrates required by the plant for its growth, development and reproduction. Hence any factor affecting the leaves, be it mechanical, physiological or biochemical, may have a more or less profound effect on the plant because of the concomitant effects on photosynthesis.

Fungal leaf parasites may affect leaves mechanically, physiologically and biochemically, and they always render leaves less photosynthetically useful to the plant, not only by tapping the available products of photosynthesis for their own growth and development, but also by creating a complex tissue (host plus parasite) with abnormally high rates of respiration and synthesis, and therefore an abnormal substrate-energy demand. However, despite the increased demand for substrates in the infected leaf, the leaf as a whole is less photosynthetically efficient: the invading parasite has caused physiological disruptions, some of which result in loss of chlorophyll, necrosis and flaccidity, and may have mechanically reduced the area of the leaf surface available for photosynthesis by an overgrowth of mycelium and spores as well as by necrosis.

How then is the increased substrate demand met? Some fungal parasites can bring about alterations in the translocation patterns of infected plants so that there is abnormal retention of photosynthate by infected leaves and redirection of photosynthate from other parts of the plant to the sites of infection, at the expense of the growing apices. Some obligate parasites affect chlorophyll distribution in infected leaves so that the elaborate patterns known as 'green islands' are produced, and

these may be more than merely sites of accumulation of metabolites and capable of actively supplying substrates to the host-parasite complex through photosynthesis. However, the ways in which the increased substrate-energy demand of the host-parasite complex is met are generally far from clear, and especially the role played by primary fixation of carbon in situ.

Relatively little research into the carbon fixation of host-parasite complexes has been carried out, and most of that accomplished has been limited to investigations of the changes in photosynthetic rates in plants infected with obligate parasites. It would appear that in such infections, a fine balance may be achieved between obligate parasite and host so that during much of the life-cycle of the parasite the level of photosynthesis of the host in situ is maintained and photosynthate is readily available to the fungus. However, in the later stages of obligate parasite infections, and immediately after the infection of leaves by most other parasitising fungi, chlorophyll and photosynthesis decline rapidly, and the death of colonised cells soon ensues. While many non-obligate parasites could probably derive sufficient substrate from the tissue immediately colonised for their reproduction, it is obviously to the advantage of the parasite to reproduce as prolifically as possible, especially if it is only facultatively saprophytic and depends on chance infection of a susceptible host for the completion of its life-cycle.

With the carbon input to the infected tissue becoming increasingly reduced as infection progresses, a high substrate demand by the host-parasite complex would have to be met from other sources. Translocated photosynthate from healthy areas of the plant which have maintained their photosynthetic rate is one obvious source; another is non-photosynthetic fixation of carbon. There are several systems in the plant which can bring about carboxylation in the absence of chlorophyll, utilising reducing power of general metabolic

origin; and it is conceivable that these could contribute increasing amounts of carbon to the host-parasite complex in infected leaves where chlorophyll and photosynthesis are diminishing. Such non-photosynthetic fixation of CO_2 has been little studied in host-parasite complexes, and the importance of its contribution to the complex has not been established.

It was the object of this investigation to further the understanding of the nutrition of the host-parasite complex, and in particular that of a complex involving a facultative saprophyte in an attempt to determine whether such a parasite might be capable of significantly altering the metabolism of the host, either directly or indirectly, in order to obtain a maximal reproductive yield from the infection. The disease chosen for the investigation was net blotch of barley, caused by Helminthosporium teres Sacc., because in this disease there is little compatibility: the growth of the pathogen is rapid, the fungal life-cycle is short, and the infected leaf quickly loses the capacity for normal metabolism, so that for any abnormal carbon demands by the host-parasite complex to be met, a rapid metabolic response in the host tissue would be necessary. The disease develops simply in that there is no 'green island' effect, and necrotic spots lead to spreading lesions and subsequent leaf collapse as the fungus begins to sporulate, which, with a moderate inoculum, occurs at about seven days after inoculation. As far as the author knows, this study represents the first phsiological investigation of this disease.

Barley variety Parkland C.I.10001 was used as the susceptible host. As well as in vivo studies of photosynthetic and non-photosynthetic carbon dioxide fixation in whole plants, the products of photosynthetic carbon

fixation were examined, and the responses of carbon-fixing enzymes to infection were analysed *in vitro*. Parallel experiments were carried out on a variety of barley resistant to net blotch in the belief, with Samborski and Shaw (1956), that such comparative data are essential for an understanding of host-parasite relations, and also with the aim of gaining some insight into the nature of metabolic resistance to this disease. It was originally intended that the resistant variety should be a hybrid (C.D.A. B.T.201) between Parkland and a resistant Ethiopian variety C.I.5791, namely C.I.5791 x Parkland^2, so that the genetic background would be as similar as possible in the susceptible and resistant plants. However, the resistance of individual plants of the hybrid was found to vary, possibly due to gene-dosage effects (Omar *et al.*, 1971) and therefore C.I.5791 itself was used as the resistant host. In one series of experiments, a comparison was made between the early products of carbon fixation in the light in infection of *H.teres* and those of an obligate parasite, *Puccinia graminis tritici* Erikss. & Henn. Race 56, to which Parkland and C.I.5791 barleys respectively are resistant and susceptible.

As described above, this study involved investigations of various aspects of both photosynthetic and non-photosynthetic fixation of carbon dioxide in barley. Non-photosynthetic fixation is referred to repeatedly in the literature pertaining to the physiology of host-parasite complexes as 'dark' fixation of CO_2, and the enzymes involved as the enzymes of 'dark fixation' (e.g. Livne, 1964; Rick and Mirocha, 1965; Daly and Livne 1966). In the author's view, this terminology is somewhat misleading, since non-photosynthetic (or non-autotrophic) fixation occurs both in the light and the dark (Bassham and Kirk, 1960; Walker, 1962; Tamas *et al.*, 1970) and indeed, since photosynthetic and non-photosynthetic carbon

fixation share some intermediates and co-factors, it is very possible that the so-called 'dark' fixation is enhanced in the light: e.g. Bassham et al. (1964) found that the formation of malate in Scenedesmus and soybean was greater in the light; and Tolbert and Gailey (1955) showed that the rates of synthesis of some of the compounds usually considered to be the products of 'dark' fixation increased upon the illumination of etiolated wheat. Neales et al. (1971) have suggested a small but significant correlation between PEP carboxylase and photosynthesis in the leaves of C_3 plants. Carboxylation as measured in the light by the uptake of CO_2 certainly comprises both photosynthetic and non-photosynthetic fixation. In this work, then, while carboxylation in the light and the dark may be referred to as such, the β-carboxylation enzymes (Walker, 1962) will not be termed the enzymes of 'dark' fixation, nor their reactions 'dark' reactions, the latter especially since all carboxylation reactions are 'dark' reactions; these aspects of carboxylation will be referred to as non-photosynthetic, while bearing in mind the possible influence of photosynthesis upon them.

After a general review of the relevant literature, this report is divided into four sections. The first covers general materials and methods used throughout the work, and includes details of the disease cycle in susceptible and resistant plants; and each of the following sections comprises the descriptions, results and a discussion of the results of one of the three series of experiments, namely, the enzyme analyses; the investigations of $^{14}CO_2$ fixation by intact plants; and the analyses of the products of short-term fixation of $^{14}CO_2$ in the light. In each sectional discussion the results presented in that section are compared with those in foregoing sections; and the sections are followed by a general discussion.

REVIEW OF LITERATURE.

I. THE DISEASE AND THE PATHOGEN.

Net blotch of barley has been known since 1881 when Saccardo collected diseased barley plants in Italy and named the organism parasitising these plants Helminthosporium teres (Saccardo, 1882). The disease has commonly been referred to as net blotch since 1920 (Atanasoff, 1920). The net blotch organism was identified by Drechsler (1934) as the imperfect state of the Pyrenomycete Pyrenophora teres Drechs. In addition to H.teres, the imperfect state has been referred to as H.hordei Eidam. (Dickson, 1956) and was re-classified as Drechslera teres (Sacc.) by Shoemaker (1959). H.teres is not specific to barley: it may infect many species of Gramineae including wild grasses (Singh, 1956).

Net blotch is frequently a serious disease in North America and reductions in yield as high as 17% have been reported (McDonald and Buchannon 1964). H.teres is a very variable organism and physiologic and pathogenic races have been demonstrated (Pon, 1949; Peterson, 1956; Singh, 1962).

II. PHYSIOLOGICAL STUDIES OF HOST-PARASITE COMPLEXES.

Of all the aspects of the physiological effects of fungal infections which have been studied, only those which have a more or less direct bearing on the nutrition of the host-parasite complex need be considered here. These most obviously include photosynthetic and non-photosynthetic fixation of carbon, and translocation to and from infected organs; effects on respiration and nitrogen metabolism are discussed more briefly since they are often manifestations of an increased carbon demand. These aspects of metabolism, for the susceptible reaction to infection, are presented under separate headings; since such information pertaining to metabolic resistance is scarce, it has been collected under a separate heading on p.26.

A. EFFECTS ON PHOTOSYNTHESIS.

Studies of photosynthesis in infected plants have emphasised changes in overall rates rather than specific effects at the metabolic level. Declining overall photosynthetic rates as infection progresses were early noted in apple leaves infected with Gymnosporangium juniperi-virginianae Schw. (Reed and Crabhill, 1915) and sunflower and clover leaves infected with Puccinia helianthi Schw. and Erysiphe martii Lev. respectively (Iljin, 1923). In general, the higher the infection intensity, the more abrupt the decline in photosynthesis (e.g. Allen, 1942; Livne, 1964) as would be expected if metabolic disruption is a result of the extent of fungal invasion. Diminishing photosynthetic capacity is generally associated with destruction of the chloroplasts (Moore and McAlear, 1961; Ehrlich and Ehrlich, 1962; Peyton and Bowen, 1963) and loss of chlorophyll, with chlorophylls a and b not necessarily disappearing at the same rate, as demonstrated by Dabala and Munteanu (1969) in barley leaves infected with Helminthosporium gramineum Rb. Photosynthetic efficiency, in terms of photosynthesis per unit chlorophyll, may be maintained after the onset of the decline in total photosynthesis, as Allen first showed in 1942 in mildewed wheat when comparing photosynthetic rates on the bases of chlorophyll and fresh weight.

Though infection always brings about an eventual loss of photosynthetic capacity, in the early stages it may be stimulatory (Montemartini, 1904; Kourssanov, 1928; Grecusnikov, 1936; Yarwood and Childs, 1938; Allen, 1942; Akai and Tanaka, 1955; Livne, 1964; Scott and Smillie, 1963, 1966). Such stimulation of photosynthesis appears to be related to the intensity of infection, and this factor may account for the variability of the results of different researchers seeking to document the early changes in

photosynthesis in host-parasite complexes. Livne (1964) found a stimulation of photosynthesis in bean leaves lightly but not heavily infected with Uromyces phaseoli (Pers.)Wint., but no such stimulation in lightly rusted wheat; he proposed that this latter result was due to the short duration rather than the absence of the phenomenon, but Zaki and Mirocha (1965) also failed to show stimulation in this host-parasite complex. Allen (1942) found slight stimulation of photosynthesis in the early stages of infection in heavily but not lightly mildewed wheat, a result borne out by Sempio (1950), who showed a biphasic stimulation, with the second rise in photosynthesis occurring at the time of differentiation of conidiophores and conidia of the mildew organism. Yarwood (1967) states that of twelve host-parasite complexes where increases in photosynthesis in the early stages of infection were reported, eight involved rusts; two, powdery mildews; one, a smut; and one, a species of Cochliobolus Drechs.

It appears from the literature that photosynthetic stimulation in the early stages of infection has usually been noted in host-parasite interactions where 'green islands' are formed. Green islands were first reported by Cornu (1881) on leaves of several deciduous plants infected with both obligate and facultative parasites: the term describes the localised concentrations of chlorophyll around infection sites in leaves which have elsewhere become chlorotic as a result of infection. The phenomenon is of some significance because the green islands could, if photosynthetically active, provide a source of carbon readily available to the fungus, thus playing an important part in its nutrition. Allen (1942) suggested that in mildewed leaves, chlorophyll was first destroyed upon infection during a phase of rapidly changing host metabolism, and subsequently reformed to produce green islands which, since the photo-

synthetic efficiency of infected leaves dropped during this latter phase, were photosynthetically inactive. However Wang (1961), upon studying the green islands induced by U.phaseoli in bean leaves, came to different conclusions: he found the green islands to be the result of pigment retention in the host tissue, so that while the chlorophyll levels of control bean leaves diminished through the experimental period due to the onset of senescence, those of rusted leaves were maintained or even rose above the initial levels after the appearance of the first symptoms; photosynthetic levels in the rusted leaves were the site of de novo synthesis of starch, with starch accumulating in them to a greater extent than in the surrounding tissue (confirming an observation in 1926 by R.Allen). Shaw and Samborski (1956) had also found that, though photosynthesis might be depressed at the sporulation sites of rust on wheat and mildew on barley, it could be greater in the surrounding green island zone than in the healthy areas of the leaves if these areas had a low photosynthetic rate. Wang's (1961) hypothesis of pigment retention and active photosynthesis in the green islands gained support from the results of Harding et al. (1968) who found using electron microscopy that infection of detached radish cotyledons with Albugo candida (Hook.)O.Kuntze resulted in the retention of the internal grana structure of the chloroplasts and the maintenance of chlorophyll levels after the onset of senescence and concomitant chlorophyll breakdown in uninfected detached cotyledons, and moreover that the levels of photosynthesis declined only slowly in infected tissue as compared with the rapid loss of photosynthetic capacity due to senescence in controls, so that the levels of fixation in infected tissue were eventually five or six times those of controls. Black et al. (1968), investigating the same host-parasite complex, used an infra-red CO_2 analyser to show conclusively

that at no time during infection did stimulation of photosynthesis occur (but merely maintenance of pre-existing after infection of the tissue); there was an overall drop in photosynthetic efficiency over the infection cycle. An interesting comment is made in their paper (Black et al., 1968) that some of the results of earlier experiments which indicated a stimulated photosynthesis in infected tissue may have been artifacts caused by the high (non-physiological) concentrations of CO_2 often used in the standard Warburg techniques to avoid rate-limiting deficits. A rather similar suggestion has been made by Edwards (1970): in powdery mildew infections of barley he found stimulation of photosynthesis in experiments conducted at 1.0% CO_2, but at physiological concentrations(0.04%) there was a biphasic inhibition of photosynthesis; the first phase of the inhibition came prior to sporulation of Erysiphe graminis hordei El.Marchal, when the infection court was rapidly accumulating metabolites (as elaborated on p.17), and the second phase when the fungus was sporulating heavily, chlorosis of the tissue was considerable, respiration was high and the flow of carbon from the host to the pathogen was proceeding at a very rapid rate.

Stimulation of photosynthesis by infection is not necessarily limited to infected organs. Livne (1964) found stimulation of the non-infected leaves of rusted plants of safflower, wheat and especially bean. Moreover, infected leaves themselves may not respond uniformly to infection: the findings of Shaw and Samborski (1956) of three zones with different photosynthetic rates around mildew infections of barley and rust infections of wheat are described above, and Wang (1961) found higher CO_2 uptake by the healthy regions of leaves lightly infected with rust than by diseased regions, though he made no comparisons between the photosynthetic rates of the healthy regions and those of healthy leaves.

The effects of infection on photosynthesis at the enzymic level have been little investigated. Scott and Smillie (1968) examined the capacity of infected leaves for partial reactions of photosynthesis such as the Hill reaction and the photoreduction of $NADP^+$ and found that on a fresh weight basis these decreased during the later stages of infection, though it can be calculated from their data that on a chlorophyll basis the rates of these reactions appear to have remained at least constant during the experimental period. Wynn (1963) showed that photosynthetic phosphorylation by chloroplasts isolated from rust-infected oats remained constant during the infection process on the bases of chlorophyll content of the isolated chloroplasts, though the overall photosynthetic rates of infected leaves decreased. Since changes in photosynthesis with infection do not parallel changes in chlorophyll content, it can be assumed that infection affects both the 'light' and 'dark' reactions of photosynthesis. Some enzymes of carboxylating systems were assayed in maize leaves infected with Helminthosporium carbonum Ullstrup by Malca et al. (1964): a stimulation of one or both enzymes involved in the conversion of ribose-5-phosphate to ribulose 1,5-diphosphate occurred soon after the fungus penetrated the leaves and before visual symptoms were evident, but this initial stimulation was followed by a rapid decline in activity; and RuDP carboxylase activity remained constant until the onset of symptoms, after which it declined. Similar enzyme changes were observed in mildewed barley (Malca et al., 1965) though the inhibition of activity of phosphoriboisomerase and phosphoribulokinase which occurred later in infection could be eliminated by washing the mildew off the leaves.

It would appear that the photosynthetic apparatus per se need not be functioning in host tissue for infection and fungal development to

occur in some diseases: mildew will develop on excised etiolated leaves floated on sucrose solutions in the dark (Trelease and Trelease, 1929); rust pustules will form on albino corn (Mains, 1926) and barley (Wu, 1952) if supplied with exogenous glucose; and Grainger (1947) observed the mildew Podosphaera oxycantha (DC.)de Bary growing on chlorophyll-less leaves of hawthorn. However this is not true of all diseases: Cutter (1951) found that only hypersensitive flecks developed on albino corn seedlings when invaded by a rust to which the corn variety was generally susceptible, even though glucose was supplied as a 2% solution to the root systems, and these flecks appeared only in the light; and he obtained pycnial rust lesions on variegated geranium leaves on the green but on neither the white nor virescent areas of the leaves. Cohen and Rotem (1970), using light deprivation and photosynthetic inhibitors, also showed a causal relationship between photosynthesis and parasite sporulation: when photosynthesis was inactivated in bean and potato respectively, U.phaseoli and Phytophthora infestans (Mont.)de Bary did not sporulate.

Though carbohydrate levels may be limiting, then, some parasites may require substrates other than carbohydrates for their development: Cutter (1951) suggested a phosphorylated photosynthetic intermediate could be the required substrate. Other workers have varied light and dark periods to determine the illumination necessary for fungal growth, and it would appear that illumination during the very early stages of infection generally has little if any favourable effect upon the establishment of the parasite in light-grown leaves (Sempio 1938, 1942), though further development may be delayed in continued darkness (Mains, 1917; Trelease and Trelease, 1929; Forward, 1932); however later in the infection process a certain minimum daily illumination may be necessary (Forward, 1932) and this may

be temperature dependent (Pratt, 1944). All these observations would suggest that the replenishment of some photosynthetically-produced nutrient, exhausted by the host-parasite complex, is necessary for infection to proceed.

Infection, then, may enhance the photosynthetic rates of infected plants, and such enhancement may be advantageous to the fungal parasite since photosynthetic intermediates as well as products such as carbohydrates are necessary for its development. Any enhancement is, however, short-lived, and though the photosynthetic efficiency may be maintained longer than the overall photosynthetic rate, there is eventual breakdown of the photosynthetic mechanism in all infected organs. Photosynthetic rates and chlorophyll levels are usually much reduced by the time the parasite is sporulating, when the abnormal carbon demands of the host-parasite complex may be maximal.

B. EFFECTS ON NON-PHOTOSYNTHETIC CARBOXYLATION.

In 1956, Shaw and Samborski reported the fixation of $^{14}CO_2$ in the dark by sunflower leaves ten days after infection with P.helianthi. Their autoradiographs of these leaves showed accumulation of the products of non-photosynthetic fixation of carbon around the infection sites, and Shaw (1961) later suggested enhanced β-carboxylation in the regions of the host leaves influenced by the fungus. Shortly after this, Daly and Krupka (1962) examined the organic acid content of leaves of rusted wheat and showed the malic, succinic and citric acid levels all to be greater than those of healthy leaves, a possible indication of an increased contribution by β-carboxylation to the total carbon pool (Walker, 1962). However such increases were not shown in other host-parasite complexes e.g. rusted

safflower (Bolar, 1962); and Malca and Zscheile (1963) recorded a 50% reduction in the malic acid content of corn seedlings inoculated with H.carbonum when these were compared with healthy controls. They also fed $^{14}CO_2$ to the corn-H.carbonum complex (Malca and Zscheile, 1963) and found that non-photosynthetic carboxylation was lower than in healthy tissue. Livne and Daly (1962) compared fixation of $^{14}CO_2$ in the dark in healthy and rusted wheat, safflower and bean, and found that diseased tissue became progressively less capable of such carboxylation from the time of appearance of flecks until the onset of sporulation of the parasite. The distribution of ^{14}C appeared to be similar in the organic acid fractions from both healthy and rusted tissues, but illumination prior to darkening for the experimental fixation period caused greater enhancement of fixation in diseased than in healthy tissue, suggesting to the authors (Livne and Daly, 1962) that some substrate of β-carboxylation, possibly PEP, which was exhausted more rapidly in diseased tissue (cf. Cutter's suggestion in 1951 of the requirement by rusts for a phosphorylated intermediate of host photosynthesis) was synthesised more rapidly in diseased tissue in the light. In later experiments, Livne and Daly (1966) did show some increase in fixation in the dark (as compared with the levels of controls) in rusted wheat and bean leaves after sporulation of the fungus, but only when the experiments were conducted immediately after a dark period; they felt that this was probably due to differences in the availability of CO_2 by diffusion through the epidermis in healthy and infected leaves: whereas after a dark period the stomata of healthy leaves would be closed, in infected tissue diffusion could occur through the ruptures in the epidermis caused by pustule formation. The parasite seemed to play little part in the increased β-carboxylation, because the latter was regulated by normal host functions

such as diurnal rhythms, and they concluded (Livne and Daly, 1966) that non-photosynthetic fixation of carbon was probably not of quantitative importance in the accumulation of carbon in rusted tissue.

Other research has indicated a more positive contribution of carbon from non-photosynthetic processes in host-parasite complexes. Zaki and Mirocha (1964) found much-enhanced (3.6- to 4.5-fold) fixation of $^{14}CO_2$ in the dark in rusted bean, with most of the fixed ^{14}C being localised around the infection sites. They did not attempt a quantitative estimate of the contribution of β-carboxylation to total carboxylation, but hypothesised (Zaki and Mirocha, 1965) that it could contribute significantly to the nutrition of the host-parasite complex, and perhaps to the substrates required for the synthesis of the storage lipids in the uredospores, since the dry weight of infected tissue increased even though photosynthesis declined and respiration rates increased. Support for their hypothesis comes from the work of Thrower (1965) who found a 25% increase in dry weight of detached clover leaflets in the dark. Furthermore, the results of Malca and Zscheile (1963) described above, where fixation in the dark by corn-H.carbonum complexes appeared to be lower than that of controls, have been contradicted by Kuo and Scheffer (1970), who found that infection by H.carbonum or applications of the toxins of H.carbonum or H.victoriae Meehan & Murphy increased the capacity of susceptible tissues for carboxylation in the dark.

Rick and Mirocha (1968) examined the activity of one of the enzymes of β-carboxylation, the malic enzyme (Ochoa, 1955), in extracts of rusted bean leaves and bean and oat stem-rust uredospores. They found a two- to five-fold increase in specific activity over healthy controls in infected tissue after the onset of sporulation of the parasite, and an active

malic enzyme in the uredospores of both rust fungi. Staples and Weinstein (1959) had earlier suggested an active PEP carboxylase (Bandurski and Greiner, 1953) in uredospores, but Rick and Mirocha (1968) were unable to demonstrate this enzyme, and suggested that the ^{14}C-labelling patterns of the products of fixation in uredospores reported by Staples and Weinstein (1959) were not incompatible with an active malic enzyme. They (Rick and Mirocha, 1968) concluded that the increases in fixation in the dark in the rusted tissue might be explained largely on the basis of the malic enzyme activity in the uredospores of the rust as these were formed; but there was some contribution from host metabolism since very high pustule density resulted in lower fixation of CO_2 (Rick and Mirocha, 1965), presumably because of the excessive disruption of host metabolism (cf. the effects of the intensity of infection on photosynthesis described on p.7).

As well as the malic enzyme, PEP carboxylase is active in β-carboxylation in healthy plants (Walker, 1962) and it has been assayed in extracts of some infected tissues. Malca et al. (1964) found that it had a lower specific activity in corn plants infected with H.carbonum than in healthy controls, but concluded that the reduced fixation in the dark that they had shown earlier (Malca and Zscheile, 1963), though perhaps due in part to the decrease in activity of PEP carboxylase, was more probably the result of the substrate PEP becoming limiting through disruption of the photosynthetic pathway. In contrast, Pao (1970) found a three- to four-fold increase in PEP carboxylase activity over healthy controls in rusted wheat leaves, though she hypothesised that the increase in this enzyme indicated a shift in photosynthetic carbon fixation in infected wheat from the Calvin cycle to the C_4-dicarboxylic acid pathway (Hatch and Slack, 1966) rather than increased non-photosynthetic carbon fixation.

C. EFFECTS ON TRANSLOCATION.

Translocation patterns have been shown to be altered in many plants after infection with both obligate and facultative parasites. Such an alteration is in part a general response to injury (Shaw et al., 1954; Shaw and Samborski, 1956; Shaw and Colotelo, 1961) and the accumulation of metabolites around infections of facultative parasites such as Helminthosporium spp. on Bromus L. and wheat (Shaw et al., 1954; Shaw and Samborski, 1956) seems to be little more specific a response. However obligate parasites have long been known to create powerful 'sinks' for metabolites in infected leaves and in some instances from other parts of infected plants. Gottlieb and Garner (1944) using rusted wheat provided the first clear demonstration of the extent to which accumulation can occur. They substituted $KH_2{}^{32}PO_4$ for the non-radioactive salt in Hoagland's solution supplied to healthy and infected plants, and found ^{32}P not only to be taken up in slightly greater quantities by infected plants, but also to accumulate around the infection sites. They then examined the ^{32}P-content of uredospores and found the specific activity to be less than that of infected or healthy tissue, and concluded from this that accumulation was largely in the host tissue, though the possibility of accumulation in the fungal mycelium also existed. Later work has indicated that there may be accumulation, to a greater or lesser extent, in both host and parasite. Accumulation continued to occur at infection sites in the host tissue after heat was used to selectively kill the pathogen in rusted bean (Yarwood and Jacobsen, 1955) and rusted wheat (Pozsar and Kiraly, 1967) and after powdery mildew was removed from infected barley leaves by rubbing the epidermis or inhibited with sulphur dust (Shaw and Samborski, 1956); and Bushnell and Allen (1962a) induced accumulation of

starch (and the formation of green islands) in barley leaves by the local application of aqueous extracts of both powdery mildew and rust spores.

Accumulation around infection sites depends on active metabolism of the host, as Shaw and Samborski (1956) demonstrated by feeding substrates to rusted wheat leaves either under anaerobic conditions or concomitant with the metabolic inhibitors sodium azide and 2,4 D; but, since in their experiments accumulation was maximal when the fungus was sporulating vigorously, at least some accumulation was no doubt occurring in the parasite (Shaw, 1961). Further evidence of the accumulation in the parasite came from the experiments on rusted wheat by von Sydow and Durbin (1962), where uredospores and hyphae acquired more of certain ^{14}C-labelled metabolites fed to the rusted leaves than did the host tissue; the results of Mount and Ellingboe (1970), who found that Erysiphe graminis DC. f.sp. tritici Em. Marchal took up ^{32}P and ^{35}S when these isotopes were fed to infected susceptible wheat during primary infection; and the work of Edwards and Allen (1966), who showed rapid transfer of ^{14}C fixed photosynthetically by barley to the parasitising powdery mildew organism during its sporulation. Reisener et al. (1970), in a dual labelling experiment on rusted wheat, found that rust both withdrew ^{14}C-alanine from the host and synthesised it internally from ^{3}H-glucose fed to the host.

As would be expected if the parasite exerts a specific influence on the host, there is some evidence that the metabolites of the parasite can be transferred to the host. Ehrlich and Ehrlich (1970) labelled uredospores with ^{14}C and showed using microautoradiography that ^{14}C could be detected in host cells of wheat after infection, and the ^{14}C was not localised in the haustorial encapsulation. Sivak and Shaw (1970) failed

to show a similar phenomenon after the infection of potato leaves with ^{3}H-labelled conidia of P.infestans, but felt that this was the result of the techniques employed rather than the absence of transfer of metabolites from parasite to host.

Translocation patterns may be disrupted throughout a plant by the infection of some leaves. The usual sinks for the photosynthate of older leaves are the young growing apices, but accumulation at the sites of infection of some of the older leaves of rust-infected bean plants may occur at the expense of the apices (Livne and Daly, 1966). However Edwards (1971) found that powdery mildew infections of barley were less powerful sinks than those of rusted bean described above: he spot-infected primary leaves of barley at their mid-points and found that only photosynthate from the distal portions of the leaves accumulated around the infection sites; moreover, little of the photosynthate produced in healthy parts of the plants entered the mildewed leaves. Infected older leaves may retain normally-exported photosynthate: Livne and Daly (1966) found that the export of photosynthetically fixed ^{14}C of unifoliate leaves of bean was reduced from 50% to 2% after infection by U.phaseoli; and Zaki and Durbin (1965) found the products of $^{14}CO_2$-fixation which accumulated around the infection sites in rusted bean leaves were not translocated to other organs.

D. EFFECTS ON NITROGEN METABOLISM.

Generally, the total nitrogen and protein content of the host-parasite complex is greater than that of healthy tissue. Shaw and Colotelo (1961) demonstrated increases in total nitrogen after rust-infection in wheat, accompanied by up to 2.5-fold increases in dry weight. Similar dry weight increases have been shown in many other studies

e.g. those by Yarwood and Cohen (1951) and Thrower (1965), the latter showing small increases even in the dark. In rusted beans (Pozsar and Kiraly, 1966) protein synthesis in the diseased parts, as indicated by the incorporation of cysteine labelled with ^{35}S into the protein fraction *in vivo*, is considerably higher at the infection centres than in the uninfected parts of the leaves. Staples and Ledbetter (1958) demonstrated that at least some of the protein increase in infected tissue is due to synthesis by the pathogen rather than the host: when they fed ^{3}H-labelled glycine to rusted bean leaves in the later stages of infection, the radioactive amino acid was incorporated mainly into the fungal mycelium and uredospores. However, increased protein synthesis by the host has been shown conclusively in some complexes, e.g. the green islands of rusted bean (Kiraly *et al.*, 1966); and Bhattacharya and Shaw (1967) showed incorporation of ^{3}H-labelled cytidine, uridine and leucine into host-cell nuclei in infected areas of rusted wheat as well as into the fungus, with increased protein synthesis in the host beginning very early in the development of rust-infection. In some complexes, there may be shifts in the protein synthesis of the host and possibly that of the fungus: Staples and Stahmann (1963, 1964) demonstrated using polyacrylamide gel electrophoresis that the number of isozymes of acid and alkaline phosphatase, succinate dehydrogenase and malate dehydrogenase as well as peroxidase activity increased in bean leaves after infection with *U.phaseoli*. The increased protein metabolism after infection of susceptible hosts appears to be correlated with increases in ribonucleic acid (Person, 1960; Quick and Shaw, 1964; Whitney *et al.*, 1962).

E. EFFECTS ON RESPIRATION.

The effects of leaf parasite on host respiration are the best documented of all the metabolic changes in such infected plants. Numerous

researchers have described the rises in respiration rate which seem invariably to accompany infection by both obligate and facultative parasites (e.g. Millerd and Scott, 1962; Shaw, 1963; Yarwood, 1967) as well as being a general reaction to injury (Yarwood, 1953). The increases in respiration generally begin when the first visual symptoms of disease appear, rise to a maximum as the parasite sporulates, and then decline, though there may be a second peak in the repiration if secondary infection occurs (Allen, 1942). Different host-parasite complexes have different respiratory patterns through the infection cycle, with the time of onset of the rise in respiration, the rate at which respiration increases, and the extent to which maximal respiration rates exceed the levels of controls all being to some extent characteristic, though influenced by environmental conditions (Millerd and Scott, 1956; Samborski and Shaw, 1956). Respiratory increases have been demonstrated on the bases of oxygen consumption (e.g. Bushnell and Allen 1962b; Collins and Scheffer, 1958; Samborski and Shaw, 1956; Scott and Smillie, 1966), carbon dioxide evolution (e.g. Allen, 1942), and the production of heat (Yarwood, 1953), and the respiration rates of host-parasite complexes may reach up to ten times those of healthy controls.

The respiratory levels of host-parasite complexes comprise the respiratory rates of both host and parasite, and the contribution of the parasite may be larger than expected since the relative rates of respiration of small organisms (the pathogens) are greater than those of larger organisms (the hosts) (James, 1953). Various methods have been used to estimate the relative contributions of host and parasite: the parasite has been selectively killed <u>in situ</u> by chemicals such as sulphur (Pratt, 1938; Yarwood, 1934) or by heat (Yarwood, 1953); it has been removed from infected leaves mechanically (Allen and Goddard, 1938; Allen, 1953); and host

respiration rates have been measured outside the areas of infected leaves occupied by the parasite (Samborski and Shaw, 1956). The findings in such experiments have lead to the general conclusion that the contribution of parasite respiration to the observed respiratory increases is small compared with that of the host under the influence of infection. Respiration in host-parasite complexes has usually been measured in the dark; since the increased respiration is largely host-mediated, it may be pertinent to re-assess the respiration of host-parasite complexes in the light period in view of the current hypothesis (Beevers, 1971) that 'classical' respiration is suppressed in the light and replaced by photorespiration.

Various hypotheses have been proposed to account for the increases in respiration in infected tissue. Bushnell and Allen (1962b) postulated that host respiration is stimulated by substances diffusing from the infection site since respiration at the centre of rust pustules (Samborski and Shaw, 1956) and mildew colonies (Bushnell and Allen, 1962b) is higher than that at the periphery. However, in general, the relative levels of ADP and inorganic phosphate to ATP, these being the pacemakers of respiration, have been considered to be more important than the influence of substrates, co-factors and inhibitors. Attempts have been made to measure directly the relative levels of organic and inorganic phosphate in infected as compared with healthy tissues: Mukherjee and Shaw (1962) found total phosphate and the ratio of inorganic to organic phosphate to increase in rust-infected wheat leaves, and Heitefuss and Fuchs (1961, 1963), using the same host-parasite complex, found that incorporation of ^{32}P fed as inorganic phosphate into the organic phosphates peaked with respiration. The synthetic processes stimulated in diseased plants, which lead to increased amounts of protein, nucleic acids and carbohydrates in infected

tissue, are all energy-requiring processes, and these, by accelerating ATP breakdown, might lead to increases in the rates of respiration. Daly and Sayre (1957) showed increased respiration rates accompanying pathologically stimulated cellular growth in safflower hypocotyls infected with Puccinia carthami Cda., and Yarwood and Cohen (1951) demonstrated that a rust-induced hypertrophy of cells in bean leaves was also paralleled by an increase in respiration. Correlations between increased respiration and increased ribonucleic acid synthesis in mildewed barley and rust-infected wheat have been shown by Millerd and Scott (1963) and Quick and Shaw (1964) respectively.

Another possible explanation for the respiratory rise in infected tissue is an uncoupling of phosphorylation from oxidation. This suggestion was initially made by Allen (1953) and there is certainly a degree of uncoupling in some infections. Farkas and Kiraly (1955) found that 2,4 D increased oxygen consumption in healthy but not rusted wheat, and Krupka (1959) showed a similar insensitivity to 2,4 D in oat tissue infected with H.victoriae or treated with its toxin victorin. However Shaw and Samborski (1957) showed some stimulation of respiration by chemical uncouplers in rusted wheat and mildewed barley, and complete uncoupling would not be consistent with the very evident synthetic activity and the energy-dependent accumulation of metabolites in infected tissue, as has been pointed out by Allen (1953), Daly and Sayre (1957) and Shaw and Samborski (1957). A partial uncoupling of respiration and phosphorylation is conceivable and might serve to explain some of the later degenerative changes in the susceptible reaction (Shaw, 1963); and there is some indication that the high rate of respiration in infected plants is at least in part wasteful: rust-infected wheat requires more oxygen per unit of ribonucleic acid

acid synthesised in the protoplasm than does healthy tissue (Shaw, 1963) and mitochondria isolated from victorin-treated plants exhibit little respiratory control and lower ratios of ADP to oxygen uptake than those from untreated plants (Wheeler and Hanchey, 1966). The abolition of the Pasteur effect in infected tissue (Daly et al., 1961) nas been cited as evidence of the uncoupling of oxidative phosphorylation; however any mechanism by which ADP levels are raised in the cell will raise respiratory rates and tend to abolish the significance of the Pasteur effect as an indication of respiratory efficiency (Sempio, 1950).

An altered balance of existing respiratory pathways and the initiation of new pathways have both been proposed to account for the enhanced respiration and characteristic metabolic effects of infection. There is some evidence for increased activity of the pentose phosphate pathway of glucose breakdown in diseased plants, based primarily on the measurements of the relative amounts of $^{14}CO_2$ respired from glucose-1-^{14}C and glucose-6-^{14}C, on the principle that in the Embden-Meyerhof-Parnas pathway CO_2 is produced equally from carbons 1 and 6, whereas in the pentose phosphate pathway CO_2 comes only from carbon 1. The C_6/C_1 ratio has been found to be lowered from 0.6 to 0.2 when safflower hypocotyls become heavily rust-infected (Daly et al., 1957) and to be lower in rusted wheat and mildewed barley than in healthy controls (Shaw and Samborski, 1957). Since the pentose phosphate pathway plays an important role in fungal respiration (e.g. Shu and Ledingham, 1956) the lowered ratios could accomodate the contribution of the fungus to the total respiration as well as a shift in the host metabolism. However, as Katz and Wood (1960) have pointed out, such measurements do not take into account the total metabolism of glucose in the tissue: aspects such as the recycling of hexose monophosphates and the randomisation of the carbon atoms in the pentose phosphate pathway;

the utilisation of glucose-6-phosphate as a substrate in pathways other than the respiratory pathways; and the incomplete CO_2 yields from the triose phosphates of both pathways cannot be accounted for. Increases in the activity of some of the enzymes of the pentose phosphate pathway have been demonstrated in rust-infected tissue (Kiraly and Farkas, 1962; Scott and Smillie, 1962) though this again is not concrete evidence of a shift in metabolism since increases in some of the enzymes of glycolysis have also been demonstrated (Lunderstadt et al., 1962; Scott and Smillie, 1962). However, since the pentose phosphate pathway has not been shown to be linked directly with oxidative phosphorylation, an increased participation of the pathway in the metabolic breakdown of glucose could be responsible for some of the uncoupling phenomena observed.

Some of the increased respiration in diseased tissue could be brought about by the activation of new oxidase pathways. The main terminal oxidase in both healthy and infected tissue appears to be cytochrome oxidase (Daly and Jensen, 1961), though after rust infection, ascorbic acid is oxidised by wheat-leaf tissue at rates which parallel the increase in oxygen uptake (Kiraly and Farkas, 1957b; Oaks, 1958). Polyphenol oxidase has been shown to be activated or induced in some diseased tissues (Farkas and Kiraly, 1962) and an electron transport system involving glutathione in infected tissue has been proposed by Mapson and Goddard (1951); but it is likely that some of the oxidase changes accompany tissue disintegration rather than representing a positive shift in metabolism in response to infection. It has been suggested (Kaul and Shaw, 1960) that the high levels of reduced auxin, ascorbic acid (Farkas and Kiraly, 1962) and glutathione (Sahai and Shaw, 1961) in susceptible tissue after infection may contribute to a high reducing potential in the host cells which creates a favourable environment for the growth and development of the parasite.

F. THE RESISTANT REACTION.

Resistance of plantsto pathogenic fungi is the rule rather than the exception: few of the many species of fungi which occur in the natural environment of a particular species of plant will be able to parasitise it. Any one or any combination of the physical and biochemical attributes of the plant and the fungus can result in the resistance of the plant to attack by the parasite. The resistance to be discussed here is specifically that encountered when a fungal leaf parasite can penetrate but not parasitise a host because of the incompatible hypersensitive reaction of the host. The results of genetic studies such as those of Flor (1941; 1942; 1946; 1947) suggest that there may be an interlocking of metabolic systems or mutual adjustment of metabolic rates in the susceptible reaction which is absent in the resistant reaction. Sempio (1950) emphasised the importance of the relative rates of photosynthesis, respiration and glycolysis in determining the establishment of a state of metabolic receptivity or resistance of the host tissue to fungal attack: his definition of metabolic resistance included the formation of substances injurious to the pathogen such as phenols within the host cell, but he postulated that these were often the manifestations of defence resulting from metabolic changes rather than defence mechanisms in themselves.

Various aspects of metabolism have been examined with the aim of demonstrating a relationship with resistance. Shaw and Colotelo (1961) found a relationship between resistance and nitrogen metabolism in rusted wheat leaves: in contrast to the changes in susceptible wheat varieties after infection, the resistant hypersensitive reaction was characterised by decreases in total, soluble and protein nitrogen. Increases in the

size and the subseqent collapse of the nuclei of infected cells occurred in both resistant and susceptible hosts, but the responses were very much more rapid in the resistant reaction and the ribonucleic acid levels did not rise in resistant host cells (cf. nitrogen metabolism in the susceptible reaction, p.19) and Rohringer and Heitefuss (1961) found in the same complex that the incorporation of ^{32}P into the RNA fraction was unaltered. Further indication of the importance of protein metabolism in the resistant reaction comes from observations of detached leaves: senescence and protein breakdown occur in such leaves, but the application of kinetin or benzimidazole to the detached leaves delays these effects; and, significantly, wheat leaves normally resistant to rust lose their resistance when detached and floated on water (Samborski et al., 1958), but when kinetin or benzimidazole are applied to such leaves, resistance is maintained. From such results Wang (1959) postulated a participation of specific proteins in disease resistance; and relationships have been found between the antigenic substances of parasites and their respective hosts. Doubly et al. (1960) prepared antisera of the globular protein fractions from the uredospores of each of four races of flax rust and from flax plants, and found antigens of the globular proteins of virulent races of flax rust present in extracts from flax plants susceptible to those races and absent in those from resistant plants; and Fedotova (1940) found a similar relationship in wheat leaf-rusts and their hosts. As well as affecting proteins, kinetin or benzimidazole applications to detached leaves result in the maintenance of the levels of total pyridine nucleotides in the leaves, and enhance the conversion of NAD to NADP in the tissues (Mishra and Waygood, 1968); and it is of interest that a relationship has been shown to exist between infection and the NAD(P) levels in the tissues: these rose initially upon infection of both susceptible and resistant wheat varieties with rust

(Rohringer, 1964), then reverted to the levels of controls in the resistant reaction while continuing to rise in the susceptible reaction.

From such considerations as the efficiency and rates of protein synthesis and breakdown upon infection (Samborski and Shaw, 1956) it might be supposed that changes in respiration upon infection would be of importance in determining the success or failure of fungi in parasitising different hosts. Respiration has not always been shown to be affected in the resistant reaction, e.g. there was no response to infection with H.carbonum in the respiration of resistant corn plants (Kuo and Scheffer, 1970). However, Scott and Smillie (1966) found increases in activity of the enzymes of glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle in barley resistant to powdery mildew after infection, and Millerd and Scott (1956) and Sempio and Barbieri (1964), investigating similar complexes, found the rise in respiration following infection to be more rapid, with the maxima lower, in resistant than in susceptible hosts. Samborski and Shaw (1956) found a similar relationship between the respiration changes and resistance and susceptibility to rust of different wheat varieties. They compared respiratory changes after infection in three varieties of wheat showing varying degrees of resistance to rust, and found that the more susceptible the host, the greater the duration and extent of the respiratory increase, though respiration increased at the same rate per unit area in all three varieties. Millerd and Scott (1956) postulated that the respiratory rise resulted from an uncoupling of oxidative phosphorylation, leading to cell collapse in resistant varieties; and the results of Shaw and Samborski (1957) also indicated uncoupling: in the later stages of infection in rusted and mildewed tissue, 2,4 D had little effect on oxygen consumption, the Pasteur

effect was abolished, and cell collapse subsequently occurred. Shaw and Samborski (1957) hypothesised that whereas only partial uncoupling occurred in the susceptible reaction, in resistant host cell oxidative phosphorylation was completely uncoupled. Shaw and Hawkins (1958) suggested that a higher rate of oxidation in the resistant than in the susceptible reaction might reduce the concentration of a metabolite essential to successful colonisation of the host by a particular parasite below a threshold level so that resistance resulted; the oxidation of normal cellular components to fungitoxic products could also be accomplished (Shaw, 1963). Support for these suggestions came from the results of Farkas and Kiraly (1962) who found decreases in ascorbic acid in rusted resistant plants; and Kaul and Shaw (1969), who showed changes in redox potential to a more strongly oxidising condition in the resistant reaction, accompanied by cell death.

The accumulation of metabolites around the sites of infection which frequently accompanies increased respiration in the susceptible reaction of plants to infection (p.17) has been observed to some extent in resistant reactions. Mizukami (1952) found starch accumulation around the lesions caused by Helminthosporium oryzae van Breda on resistant rice seedlings; and Shaw and Samborski (1956) showed that the amount of accumulation around rust and mildew lesions on different varieties of wheat and barley respectively was related to the degree of resistance of the varieties: the more resistant the variety, the less accumulation occurred, so that in the 'immune' reaction of barley to E.graminis hordei there was no accumulation.

There are few reports of the effects of infection on the fixation of carbon in resistant hosts. Scott and Smillie (1966) found photosynthesis and chlorophyll content in barley leaves resistant to powdery mildew

unaffected by infection. However Haspelova-Horvatovicova (1971) found some decrease in chlorophyll in a different resistant variety of barley infected with mildew, accompanied by decreases in carotenoids. An unusual relationship between pigments and resistance has been documented in oats by Harder et al. (1971), though the authors do not speculate as to the nature of the resistance: seedlings possessing gene pg-11 are susceptible to stem rust and have near-normal pigment levels, but with increasing age the pigment content decreases more rapidly than in plants without the gene, the ratio of chlorophyll to carotenoids remaining unchanged at all stages of plant development, and the plants become moderately resistanct to stem rust. Hassebrauk (1940) obtained some indication that photosynthesis or photosynthate is needed for the expression of resistance to rust in wheat: darkness increased the susceptibility to disease of some moderately resistant plants; and Allen (1954) reported that the susceptibility of certain hosts to invasion by parasites increased when the plants were incubated in the dark during the first few days after inoculation.

Non-photosynthetic fixation of CO_2 in the resistant reaction has been the subject of even less investigation than photosynthesis. Kuo and Scheffer (1970) found that slight increases occurred early in the infection of corn plants resistant to H.carbonum, whereas Malca and Zscheile (1963), upon examination of the same complex, found slight reductions in the fixation of CO_2 in the dark, accompanied by slightly reduced malic acid content. In the latter work, isocitric acid was shown to increase upon infection in both resistant and susceptible corn plants, and the authors postulated that this might be due to a decrease in activity of isocitric dehydrogenase or the enzymes of the glyoxalate cycle, presumably in the resistant as well as the susceptible reaction.

SECTION I: GENERAL

MATERIALS AND METHODS.

A. THE PLANT MATERIAL.

Certified seed of Parkland barley C.I.10001 was used, and seed of the Ethiopian variety C.I.5791 and the hybrid C.D.A. B.T.201 (C.I.5791 x Parkland^2) were supplied by Drs. D. R. Metcalf and W. C. McDonald of the Canada Department of Agriculture, Winnipeg.

Plants were grown in a greenhouse with a 16-hour light period; thermostat settings were 22^{o}C in the light period and 20^{o}C in the dark period, but afternoon temperatures frequently exceeded 32^{o}C. The soil mixture consisted of two parts sterilized soil to one part peat and one part sand. Plants for experiments on the fixation of CO_2 were grown in four inch plastic pots with six to eight plants per pot: these were usually thinned to five plants per pot before use.

B. THE STANDARD INOCULUM.

A culture of H.teres (C.D.A. acc. no. 102) was obtained from Dr. W. C. McDonald, C.D.A., Winnipeg. The fungus was grown on V8 vegetable juice agar comprising 10% V8 juice and 3% agar in distilled water, and subcultured by monoconidial transfers. Cultures for inoculum were grown on 50 or 100 ml agar slopes, in 125 and 250 ml Ehrlenmeyer flasks respectively, in a growth chamber at 21^{o}C with a 16-hour light period: the cultures were used as a source of inoculum when between two and three weeks old. H.teres is a very variable organism: colonies range from heavily pigmented, heavily sporing types to sparsely sporing types with little pigmentation, and patches and overgrowths of aberrant mycelium are common. According to McDonald (1967), virulence is linked with pigment production and conidial formation, and mutant colonies and

mixed cultures are obtained more often through subculturing by mass transfers of conidia and mycelium than by single spore transfers. Only heavily conidial, darkly pigmented cultures were used as an inoculum source.

The standard inoculum was prepared according to the method of Keeling (1966): a few ml of sterilized water containing one drop of Tween 20 per ml as wetting agent were poured onto the surface of the slope culture; the surface of the slope was gently scraped with a wire loop to loosen the spores and the resulting suspension strained through six layers of cheesecloth; and the culture surface was then washed several times with sterile distilled water containing one drop of Tween 20 per 100 ml, the filtrates being bulked as the inoculum. This contained conidia and small hyphal fragments in concentrations varying from ca. 6,000 - 10,000 units per ml. A haemocytometer estimate of spore density was difficult to obtain because of the large spore size; therefore a standard volume of water to a standard surface area of culture (25 ml per 50 ml slope, 50 ml per 100 ml slope) was used in preparing the inoculum: this gave reproducible results and will be referred to throughout as the 'standard inoculum'.

C. THE INOCULATION PROCEDURE.

Seven-day-old plants were inoculated in the greenhouse: at this time the first leaf was almost fully expanded and the second leaf had not yet emerged. Routinely they were sprayed with the standard inoculum using a hand atomiser (Maru Hachi Dahlia Sprayer) until the leaves were evenly wetted; control plants were sprayed with distilled water containing one drop of Tween 20 per 100 ml. In some experiments the first leaves were point-inoculated as follows: the distal portions (about one third of the

length) of the leaves were painted with the standard inoculum using a small sable brush; controls were painted with water containing one drop of Tween 20 per 100 ml.

After inoculation, the plants were incubated at 100% humidity for 24 hours. The plant containers were stood on wet gravel in the greenhouse and covered with a 58 x 30 x 16 inch hood made out of a softwood frame overlaid with polyethylene; a humidifier of five litres capacity spraying water at ambient air temperature was included under each hood and the hoods were draped with black cloth for the 24-hour period.

D. THE ASSAY SCHEDULE.

In early experiments, the progress of disease after inoculation was followed by macroscopic and microscopic examination at 12 and 24 hours after inoculation, and every 24 hours thereafter for nine days. Photographs of whole leaves were made at each examination using Kodachrome II film, and portions of leaves were treated with hot lactophenol-cotton blue solution (White and Baker, 1954) to clear the tissue and stain the fungal mycelium and then examined microscopically. The experimental conditions were constant, and thus from these records the typical appearance and state of infection of leaves of Parkland and C.I.5791 barley at regular intervals after inoculation was determined, and a standard infection cycle for these experimental conditions established to which all subsequent infections could be referred.

Early studies of enzyme activity in extracts of infected and control leaves of Parkland (involving assays of PEP carboxylase and RuDP carboxylase) were made at the time of inoculation and at 12 hours, 24 hours and every 24 hours thereafter for seven days (until sporulation of the fungus of susceptible leaves). Based on these results (pp.**61-71**) later enzyme assays

and the investigations of the early products of carbon fixation in the light in whole leaves were carried out at 24 hours (post-penetration stage), three days (early lesion development), and five days (spreading lesions present in the susceptible reaction, discrete necrotic spots in the resistant reaction). Second leaves from infected and control plants were assayed for enzyme activities at four days after inoculation of the first leaves, when first large enough to be harvested, and at six or seven days, when the fungus was sporulating on the first leaves in the susceptible reaction but had not spread from the discrete necrotic areas in the resistant reaction. Total fixation of CO_2 *in vivo* in the light and dark was estimated daily for seven days. Experiments in which the fixation and translocation of carbon in intact plants throughout the diurnal cycle were examined were carried out at four days (young spreading lesions with much chlorosis in the susceptible reaction, lesions reaching their maximal dimensions in the resistant reaction) and six days after inoculation.

E. DETERMINATION OF THE EXTENT OF FUNGAL SPREAD IN THE LEAF.

The first leaves of several seven-day-old plants were inoculated at their tips with the standard inoculum using a syringe to produce a drop of inoculum of standard size on the surfaces of the leaves. Lesions were allowed to develop fully (until sporulation of the fungus). Some leaves were then surface-sterilized with 10% hypochlorite solution; pieces of tissue were cut from the leaves at 0.5 cm intervals proximally from the lesions and transferred to petri plates af 10% V8 juice agar where they were incubated for the detection of viable units of *H.teres* within the tissue. Other leaves were treated with hot lactophenol-cotton blue solution (White and Baker, 1954) and examined microscopically for the extent of hyphal growth outside the lesions.

F. MEASUREMENT OF RADIOACTIVITY.

Radioactivity in all experiments using ^{14}C was assayed by liquid scintillation. The methods used in different experiments for the preparation of samples of various types before assay (water-soluble products; leaf residues; leaf-discs; radioactive areas from thin-layer chromatograms) have been described with the experiments to which they are applicable. The standard liquid fluor consisted of 100 gm naphthalene, 7 gm PPO (2,5-diphenyloxazole) and 0.3 gm POPOP (1,4-bis-2(4-methyl-5-phenyl-oxazolyl)bezene) in one litre of 1,4 dioxane. 10.0 ml of scintillation fluid were added to the sample in a standard 25 ml scintillation vial, and radioactivity was read in a Picker Nuclear Liquimat 220, using the external standard method for the determination of counting efficiency. Blank samples were routinely included for background corrections. Highly coloured samples were divided into fractions and these assayed separately, the d.p.m. in each being summed, so that the colour quenching was sufficiently low to be accurately corrected.

G. PROTEIN MEASUREMENT.

Protein was determined using the Folin-phenol reagent according to the method of Lowry et al. (1951). Protein was routinely measured prior to and after the purification procedures employed in the preparation of the various enzyme fraction from crude extracts of plant material.

H. CHLOROPHYLL ESTIMATION.

Chlorophyll was estimated by the rapid spectrophotometric method of Arnon (1949). Leaf material was ground with sand in a mortar with 80% acetone, filtered with suction through Celite filter aid (Baker, Phillipsburg, N.J.) and the residue washed with 80% acetone to a known volume. For estimates of the chlorophyll content of leaves from which enzyme extractions were made, the leaf material consisted of one gram of

representative leaf material extracted in 50 ml 80% acetone. For estimates of the chlorophyll content of leaves in which the fixation of CO_2 was examined, four or eight leaf-discs (depending on the sample size in the particular experiment) of seven millimetres diameter were cut with a cork borer from a representative leaf of each treatment and extracted in 10.0 ml 80% acetone. Optical density (A) was read in a Beckman SP500 spectrophotometer at 645 and 663 mu, and the total chlorophyll (C) determined according to the formula:-

$$C = 20.2\ A_{645} + 8.02\ A_{663} \text{ mg / litre}$$

SECTION I.

RESULTS.

THE STANDARD INFECTION CYCLE: THE SUSCEPTIBLE REACTION.

At 12 hours after inoculation of Parkland barley seedlings, microscopic examination of the surface of a typical first leaf showed that most of the fungal spores present had germinated: appressoria had been formed by about 50% of the germ tubes and the first infection pegs could be seen, especially in the areas of the stomata, though there was as yet no visible evidence of infection. By 24 hours after inoculation, the points of penetration of the leaf surface by the fungus were evident to the naked eye as pin-point lesions (Plate 1); microscopic examination showed penetration of the epidermis by the germ tubes of virtually all spores, and hyphae were visible within the mesophyll cells.

By 48 hours after inoculation, there were discrete necrotic spots on the leaves (Plate 2) and in areas where a number of fungal penetration points were in close proximity the lesions were coalescing; hyphae could be seen ramifying within and around the necrotic tissue, and there were narrow chlorotic halos around the lesions. By three days after inoculation the lesions were spreading so that the necrosis had begun to form the typical net pattern over the surface of the leaf (Plate 3); the chlorosis surrounding the lesions was more severe, and hyphae could be seen in and around the chlorotic and necrotic areas. The disease symptoms were intensified at four days after inoculation; the necrotic areas were enlarging and much of the leaf was now chlorotic (Plate 4).

By five days, hyphae could be seen within the tissue of virtually all parts of the leaf, and necrosis was more widespread (Plate 5); and at six days, the cells in the heavily necrotic areas of the lamina had

Plate 1: The susceptible reaction to infection of Parkland: one day after inoculation with H.teres.

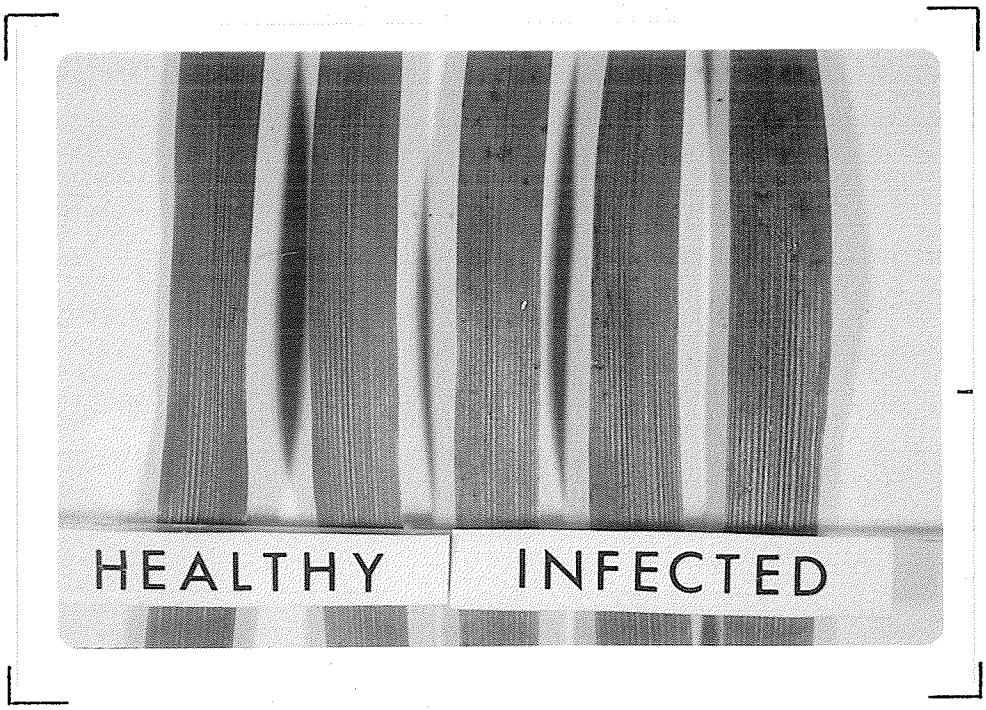


Plate 2: The susceptible reaction to infection of Parkland: two days after inoculation with H.teres.

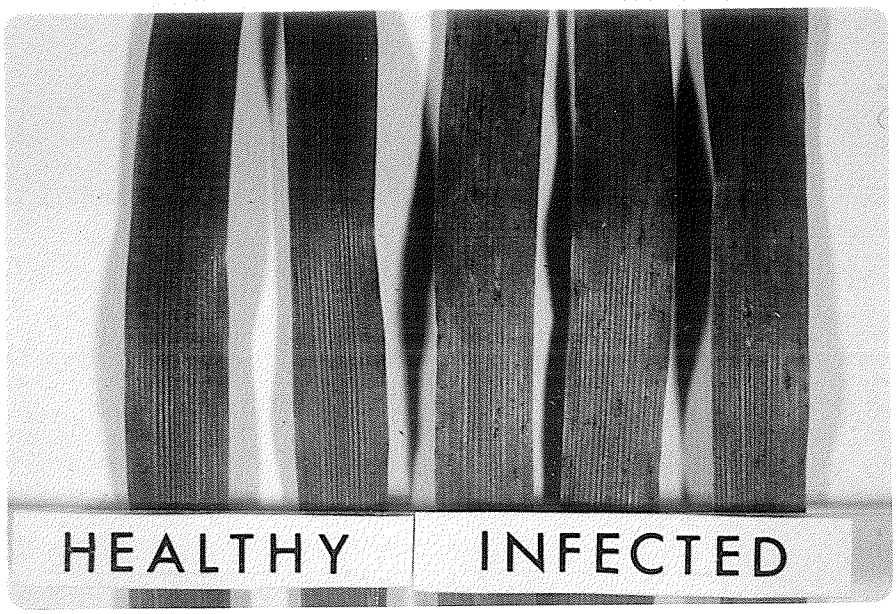


Plate 3: The susceptible reaction to infection of Parkland: three days after inoculation with H.teres.

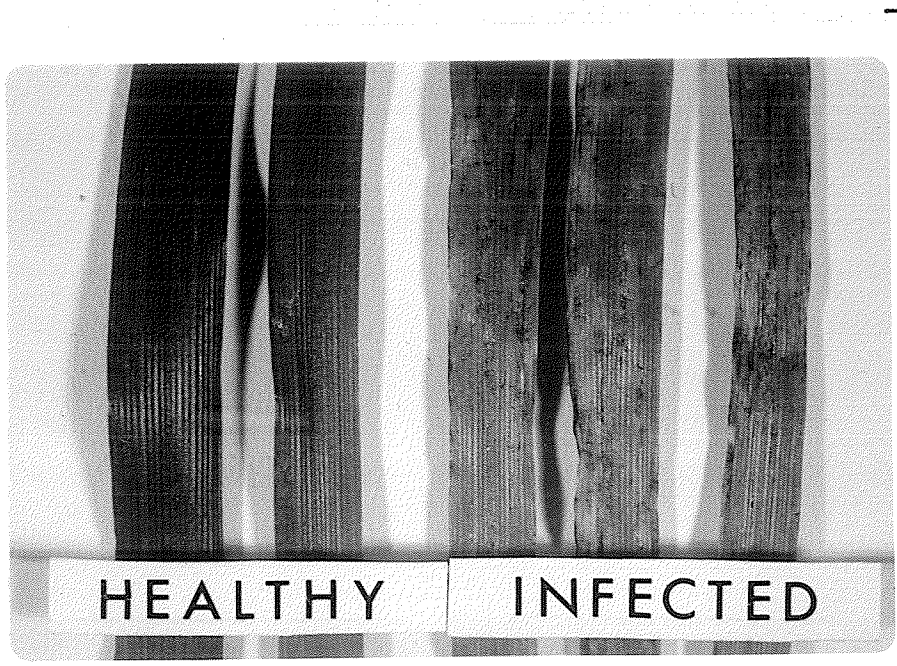


Plate 4: The susceptible reaction to infection of Parkland: four days after inoculation with H.teres.

collapsed and the leaf appeared desiccated and twisted (Plate 6),while conidiophores could be seen emerging from the collapsed cells. By seven days, further collapse of the leaf had occurred (Plate 7) and conidiophores were evident over much of both surfaces, with conidia being formed in patches. The dense hyphal network within the tissue was beginning to spread from the mesophyll over the surfaces of the leaf at eight days after inoculation, and conidia were being shed in large numbers from the conidiophores; the leaves were largely collapsed, and some of the newly-shed conidia were germinating on the leaf surface.

Infection of the first leaves affected to some extent the development of the second leaves. The second leaves emerged just after inoculation, when the plants were eight days old, and their early growth and development in infected plants was slower than in healthy plants. However, their growth apparently accelerated after they has achieved a certain surface area, so that the daily increment was similar to that of the second leaves of control plants.

THE STANDARD INFECTION CYCLE: THE RESISTANT REACTION.

At 12 hours after inoculation, most fungal spores had germinated on the surface of a typical first leaf of C.I.5791 barley, and by 24 hours, appressoria had been formed by most germ tubes and penetration of the leaf surface was occurring. However there was no visual evidence of infection until 48 hours after inoculation (Plate 8), when small discrete lesions were evident and hyphae were visible in the mesophyll tissue around the pentration points. Chlorosis was very limited in extent and increased only until some three days after inoculation (Plate 9); widespread chlorosis never occurred in resistant leaves. The necrotic spots increased in area until about four days after inoculation (Plate 10), after which time no further development of symptoms occurred. Although in areas where lesions

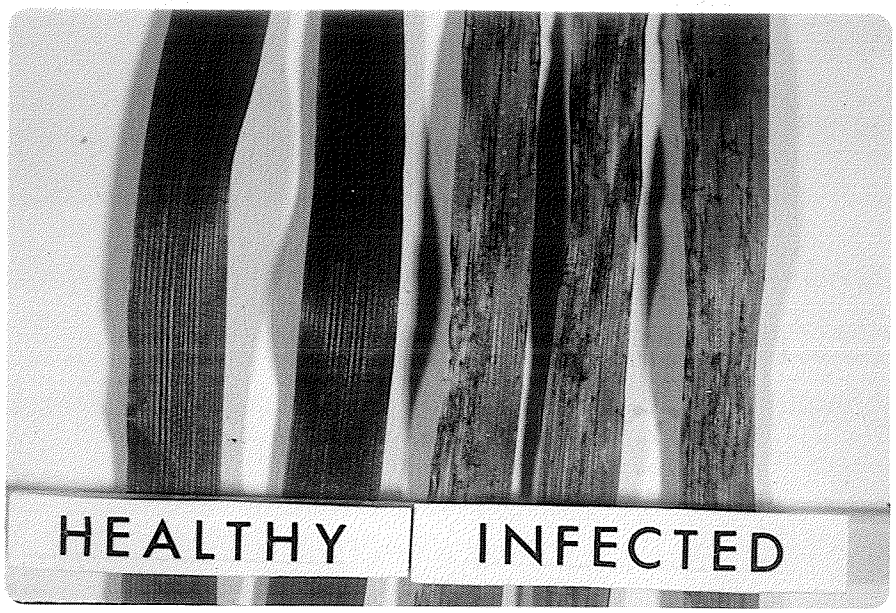


Plate 5: The susceptible reaction to infection of Parkland: five days after inoculation with H.teres.

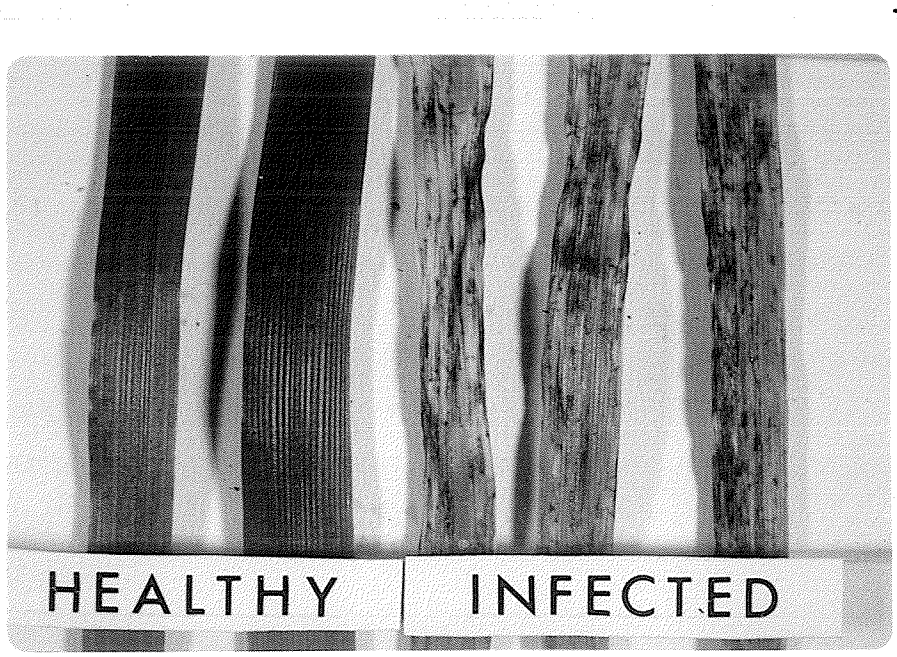


Plate 6: The susceptible reaction to infection of Parkland: six days after inoculation with H.teres.

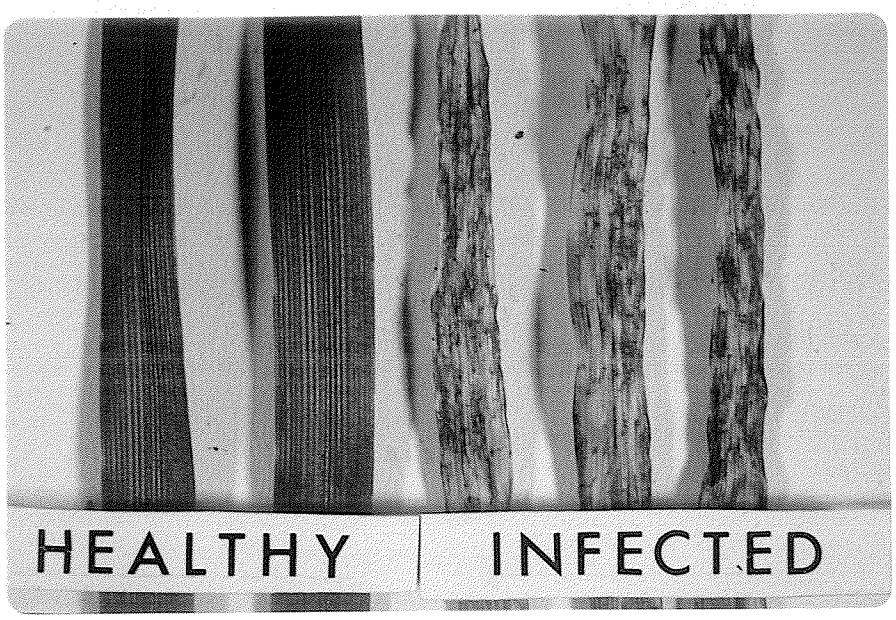


Plate 7: The susceptible reaction to infection of Parkland: seven days after inoculation with H.teres.

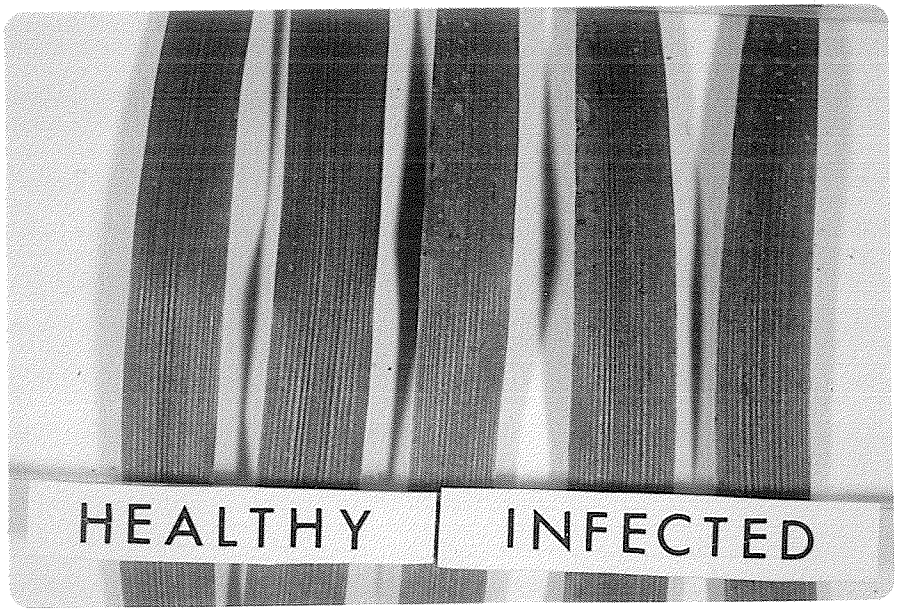


Plate 8: The resistant reaction to infection of C.I.5791: two days after inoculation with H.teres.

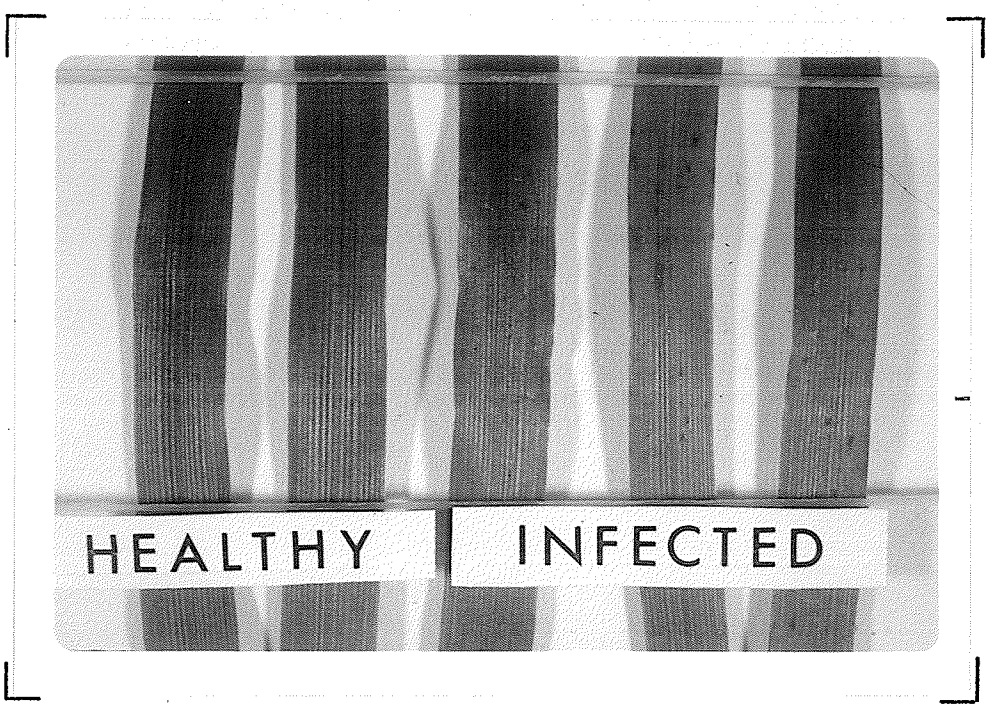


Plate 9: The resistant reaction to infection of C.I.5791: three days after inoculation with H.teres.

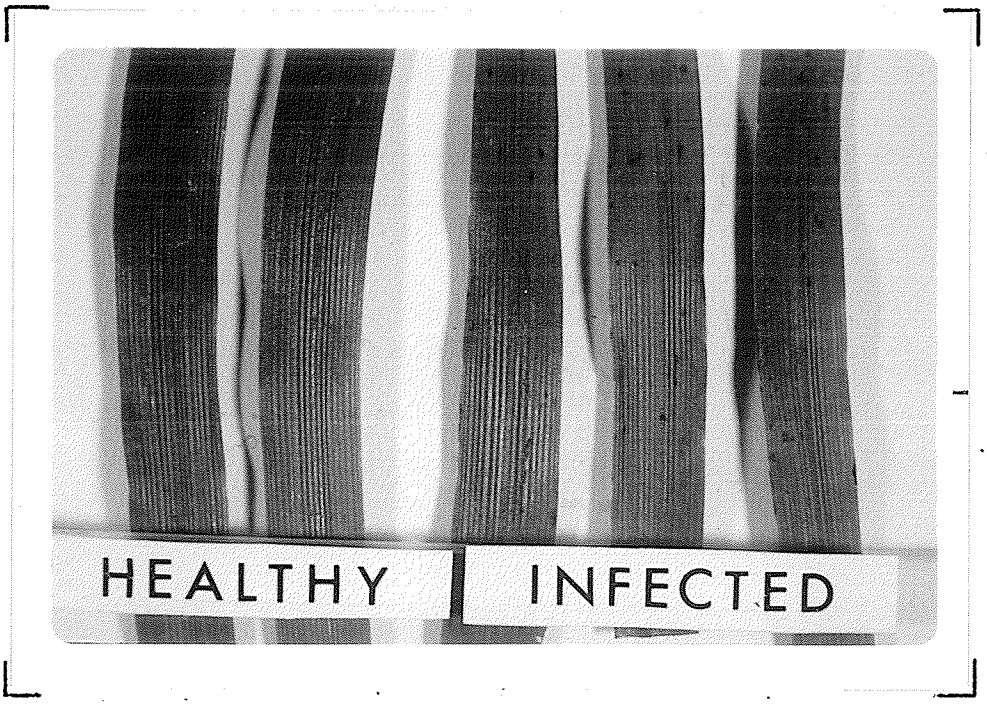


Plate 10: The resistant reaction to infection of C.I.5791: four days after inoculation with H.teres.

were in close proximity some coalescence of lesions occurred, the spreading necrotic areas typical of the susceptible reaction never developed. From four days after inoculation onwards, infected leaves had much the same appearance both macroscopically and microscopically each day: the fungal hyphae did not ramify through the tissue around the lesion but remained localised within the necrotic areas and there was in effect no further development of the disease. In experiments where leaves were point-inoculated (p.32), the density of lesions sometimes resulted in heavy necrosis and collapse of the heavily infected areas, but sporulation of the fungus was not observed. Infection of the first leaves of C.I.5791 apparently had little if any effect on the development of the second leaves, which proceeded similarly in infected and healthy plants.

THE EXTENT OF FUNGAL SPREAD OUTSIDE THE LESIONS IN PARKLAND.

Even when the fungus was sporulating in the inoculated areas of spot-infected leaves, hyphae were visible in the tissue (after clearing and staining) only within ca. 0.5 cm of the proximal end of the visible lesion. When spot-infected leaves were cut into 0.5 cm sections proximally from the visible lesion and incubated, viable units of H.teres were shown in ca. 60% of the first 0.5 cm sections and ca. 10% of the second 0.5 cm sections, but in the experimental period none were detected more than 1.0 cm from the proximal end of the lesion. From these results it was concluded that fungal spread in the leaves was quite rapidly followed by the appearance of visual symptoms of disease, and that leaf tissue more than 1.0 cm distant from the visible lesion was very unlikely to contain fungal mycelium. Point-inoculated leaves could thus be divided into invaded and uninvaded portions, and enzyme activities in these infected leaves be assessed within and outside the vicinity of the pathogen.

SECTION I.

DISCUSSION.

Some general observations on the nature of the resistance of barley to H.teres can be made at this point.

Keeling (1966) observed no differences between the number of spores germinating on or penetrating the surfaces of barley varieties susceptible and resistant to H.teres, even though he detected an inhibitor which reduced the growth of germ tubes in surface water of resistant varieties. He concluded that resistance was the result of the inability of the fungus to spread within the tissue of resistant varieties, due both to a constitutive resistance factor and the formation of a fungal inhibitor as a consequence of host-parasite interaction. The observations made in this study of the infection process support his findings in that germination, appressoria formation and the penetration by infection pegs proceeded similarly in Parkland and C.I.5791 barleys, but in resistant tissue the fungus was unable to spread from the necrotic areas surrounding the initial point of penetration. It is of interest that Keeling (1966) found that the growth rate of hyphae in resistant tissue was related to the degree of resistance of the host: growth was more greatly retarded in more resistant varieties, with the size of the lesions resulting from infection being correlated with the extent of fungal growth.

In the present study, though the macroscopic evidence of infection (small necrotic spots) became apparent more slowly in resistant leaves, similar numbers of lesions were formed on the leaves of the susceptible and the resistant varieties, as in the infection of susceptible and resistant varieties of barley by H.sativum Pam., King and Bakke (Mumford, 1966); though Keeling (1966) found fewer numbers of lesions on barley varieties

resistant to H.teres, he found this to be due to the smaller number of penetrations resulting in lesions in resistant hosts. Resistance of barley to net blotch, then, does not appear to be expressed either by a chemical inhibition of the germination of the spores of the pathogen on the surface of the resistant host, or by an inability of the fungus to penetrate the leaf surfaces of more resistant barley varieties, this latter mechanism being important in the resistance of barley to disease such as leaf blotch incited by Rhynchosporium secalis (Oudem.) J. J. Davis (Fowler and Owens, 1971).

Keeling (1966) observed but sparse sporulation of H.teres on his moderately resistant barley varieties and little or no sporulation on his highly resistant varieties; he found a close correlation between the extent of development of the pathogen and its ability to sporulate. The reaction of C.I.5791 to infection as observed in the present study was sufficiently extreme that no sporulation of H.teres was observed, even with fairly heavy inocula: the development of the pathogen was, then, either completely halted or merely inadequate for the production of spores.

SECTION II: ENZYME ANALYSES.

MATERIALS AND METHODS.

A. PREPARATION OF FUNGAL EXTRACTS.

1. Vegetative material: The vegetative mycelium was grown in a clarified liquid V8 juice medium (if the medium was prepared using unclarified juice, cellular debris acted as nuclei for the development of the fungus and colonies were of irregular size). 20% V8 juice in distilled water was centrifuged at 13,000 g for ten minutes; the supernatant was filtered through one layer of filter paper in a Buchner funnel, and 100 ml aliqots of the filtrate were placed in Ehrlenmeyer flasks. After sterilization, 1.0 ml of the standard inoculum (p.32) was added to each flask and the flasks were incubated on a rotary shaker at room temperature (ca. 25°C) for 48 hours: by this time small discrete colonies lacking in pigmentation had formed. Medium and fungus were decanted into 250 ml centrifuge buckets and centrifuged at 5,000 g for five minutes. The fungal mycelium formed a gelatinous precipitate and this was washed several times with 5 mM K_2HPO_4 buffer, pH 7.5, to remove adhering medium, centrifuging between washings at 5,000 g. The mycelium was frozen in liquid nitrogen and lyophilized for 48 hours, then stored in a desiccator at 5°C until use. One Ehrlenmeyer flask as prepared above yielded 100 - 150 mg dry weight of mycelium.

2. Sporulating material: Petri plates containing 10% V8 juice agar were heavily streaked with the standard inoculum using a bent glass rod. They were incubated for a week before the spores were collected at 5°C by a modification of the method of Harris (1972). Plates were individually lowered into liquid nitrogen with tongs; the agar was frozen slowly and the liquid nitrogen then allowed to flood over the surface. The plate was removed from the liquid nitrogen and placed in a shallow styrofoam dish,

and the surface of the fungal colony scraped with a spatula under liquid nitrogen: the spores became detached and were rinsed into a container and collected under liquid nitrogen. They were then lyophilised for 48 hours and stored desiccated at $5^{o}C$ until use. Each plate prepared as above yielded 3 - 5 mg dry weight af fungal material, largely spores.

3. Extraction: Two methods of extraction were used, and the extracts from both assayed for enzyme activity in case either method had resulted in loss of activity. In the first method, the Bronwill 'MSK' homogenizer made by Quigley-Rochester Inc., New York, was used. 500 mg of lyophilised material were placed in a 75 ml homogenization flask with 500 mg 0.45 - 0.50 mm diameter glass beads and 15 ml 0.05 M K_2HPO_4 buffer containing 0.01 M EDTA, pH 7.5. The material was homogenized with cooling for three two-minute periods, being allowed to warm up for 20 seconds between periods to prevent freezing. The supernatant was then decanted and centrifuged at 15,000 g for ten minutes in the cold. The glass beads were washed twice with buffer and centrifuged as above and the three supernatants combined and centrifuged at 20,000 g for 15 minutes. In order to concentrate the protein in solution, the supernatant was brought to 65% ammonium sulphate saturation in the cold with stirring. The resulting suspension was centrifuged at 15,000 g for ten minutes and the precipitate resuspended in the original buffer at one fifth the original volume, stirring overnight. Complete resuspension of the floccular precipitated protein of the vegetative material was never obtained, but that from the sporulating material was readily resuspended. The solutions were assayed immediately.

The second method of extraction was used only for the lyophilised vegetative material, the lyophilised spores being too powdery to be handled in the following way. The fungal material was ground dry in a mortar with a pestle and a little sand at $5^{o}C$. A small volume of buffer of composition

similar to that used in the first method was added and the somewhat gelatinous suspension centrifuged at 15,000 g for 15 minutes to clarify before immediate assay.

4. Assay: The fungal extracts were assayed for the activities of PEP carboxylase and the malic enzyme as described for the leaf extracts in the following sections.

B. PREPARATION AND ASSAY OF ENZYMES FROM LEAF EXTRACTS.

1. Extraction and assay of RuDP carboxylase, E.C.4.1.1.f.

Extraction was carried out by a modification of the method of Wilson and McCalla (1968). Leaves were harvested with scissors, washed in glass-distilled water and gently shaken dry in cheescloth; they were then chilled, and all extraction procedures were carried out at 5°C. The leaves were cut up finely into a mortar and ground with pestle and acid-washed sand in three times their own weight of a buffer containing 10 mM K_2HPO_4, 0.1 mM EDTA and 5 mM β-mercaptoethanol, pH 7.6. The grindate was filtered through six layers of cheesecloth and centrifuged at 15,000 g for 15 minutes. The supernatant was brought to 55% saturation with crystalline ammonium sulphate, and the resulting suspension allowed to stand overnight, after which it was centrifuged at 13,000 g for 30 minutes. The precipitate was resuspended in a second buffer containing 0.5 mM K_2HPO_4, 0.1 mM EDTA and 5 mM β-mercaptoethanol, of weight equivalent to twice the original weight of leaves extracted. The resulting solution was centrifuged at 147,000 g for 40 minuted to clarify, and the supernatant was then brought to 55% saturation with crystalline ammonium sulphate and stored as a suspension at 5°C.

Before assay, the protein suspension was centrifuged at 13,000 g for ten minutes and the resulting precipitate resuspended in a weight of the second buffer described above equivalent to the original weight of leaves.

The enzyme was assayed in a water bath maintained at 30°C; the assay system had a total volume of 0.5 ml and was constituted as follows: EDTA, 0.03 umoles; $MgCl_2$, 5 umoles; glutathione, 3 umoles; $NaHCO_3$, 25 umoles, $NaH^{14}CO_3$, 2 uCi; RuDP, 0.35 umoles; and enzyme preparation, 0.1 ml, containing ca. 50 ug protein; all in 0.1 M Tris-HCl buffer, pH 7.8. The reference reaction mixture contained all components except RuDP. The reaction was started by the addition of the enzyme preparation: it was found to be linear up to about 15 minutes and this was adopted as the standard incubation time. The reaction was stopped by the addition of 1.0 ml of 2 N HCl, from which mixture two 0.5 ml samples were taken for analysis and dried in scintillation vials in a stream of air over steam. Water-soluble components were redissolved in 0.2 ml water before the addition of scintillation fluid for the assay of the radioactive products of the reaction (p.35).

2. Extraction and assay of PEP carboxylase, E.C.4.1.1.31

The extraction procedure was a modification of the method of Waygood et al. (1969). Leaves were harvested, washed and chilled as in the extraction of RuDP carboxylase, and extracted at 5°C. They were cut up finely and ground with pestle and acid-washed sand in a mortar; twice their own weight of 50 mM K_2HPO_4 buffer, pH 7.6, was added. The grindate was filtered through six layers of cheescloth and centrifuged at 20,000 g for 20 minutes. The supernatant was brought to 25% ammonium sulphate saturation, using the crystalline salt with stirring; and the resulting suspension was then centrifuged at 15,000 g for 20 minutes, the precipitate being discarded. The supernatant was brought to 55% saturation with ammonium sulphate as before and centrifuged at 15,000 g for ten minutes. The precipitate was resuspended in a weight of the original buffer equivalent to the original weight of leaves, and re-precipitated at 50%

ammonium sulphate saturation. After centrifuging again at 15,000 g for ten minutes, protein was resuspended in 5 mM Tris-HCl buffer, pH 7.6, in the ratio of 1.0 ml of buffer to 6 - 7 gm original weight of leaves. The extracts were stored deep-frozen.

The enzyme was assayed by coupling the reaction with malic dehydrogenase and measuring the conversion of NADH to NAD (Cooper et al., 1968) according to the following reactions:-

$$PEP + CO_2 + H_2O \longrightarrow OAA + H_3PO_4$$

$$OAA + NADH \longrightarrow Malate + NAD$$

The conversion of NADH to NAD was followed by reading the decrease in optical density at 340 mu at room temperature (ca. 25°C) in cells with a light path of 1.0 cm in a Beckman SP500 spectrophotometer. The volume of the assay system was 3.0 ml and comprised the following: PEP, 12 umoles; $MgCl_2$, 48 umoles; $NaHCO_3$ 30 umoles; NADH, 0.78 umoles; malic dehydrogenase, one international unit; and the enzyme preparation, 0.1 ml, containing 400 - 700 ug protein; all in 0.05 M Tris-HCl buffer, pH 7.6. The reference cell contained all components except NADH. The reaction was initiated by the addition of PEP, and optical density was read every 15 seconds for three minutes, during which time the change in O.D. with time was linear.

3. Extraction and assay of the malic enzyme, E.C.1.1.1.40.

The methods used were a modification of those of Rick and Mirocha (1968) with reference to those of Ochoa (1955). Leaves were harvested, washed and chilled as for the extraction of RuDP carboxylase, and extracted at 5°C. They were cut up finely into a mortar and ground with pestle and acid-washed sand in three times their own weight of a buffer consisting of 20 mM Tris-HCl, 0.1 mM K_2HPO_4 and 0.1 mM EDTA, pH 7.4: the grindate

was filtered through six layers of cheesecloth and centrifuged at 12,000 g for ten minutes. The supernatant was adjusted to pH 6.2 with 1.0 N acetic acid, and allowed to stand in the cold for 30 minutes. It was then centrifuged at 20,000 g for ten minutes. The supernatant was brought to 35% saturation with crystalline ammonium sulphate with stirring, and the precipitate removed by centrifugation at 15,000 g for ten minutes. Saturation of the supernatant was increased to 60% by further addition of ammonium sulphate, and the suspension centrifuged. The supernatant was discarded and the precipitate re-dissolved in a weight of the original buffer equivalent to 0.2-0.3 the original weight of leaves. The enzyme solution was dialysed overnight against 0.1 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM K_2HPO_4 and 0.1 mM EDTA. The enzyme was assayed immediately: activity was found to be greatly reduced upon standing or freezing and thawing.

The enzyme was assayed in the direction of pyruvate formation according to the following reaction:-

$$\text{L-malate} + \text{NADP}^+ \xrightarrow{Mn^{++}} \text{Pyruvate} + CO_2 + \text{NADPH}$$

The production of NADPH (at room temperature, ca. 25°C) was followed in cells with a light path of 1.0 cm in a Beckman SP500 spectrophotometer at 340 mu. The volume of the assay system was 3.0 ml and comprised the following: malic acid, 10 umoles; NADP, 2 umoles; $MnCl_2$, 3 umoles; and the enzyme preparation 0.1 ml, containing ca. 1.0 mg protein; all in 20 mM Tris-HCl buffer, pH 7.4, with 0.1 mM K_2HPO_4 and 0.1 mM EDTA included in the buffer. The substrate was prepared by dissolving crystalline L-malic acid in distilled water and adjusting the solution to pH 7.4 with 1.0 N KOH. The reference cell contained all components except NADP. The reaction was initiated by the addition of malic acid, and optical density

was read every 15 seconds for three minutes, during which time the increase in O.D. was linear.

It was thought unnecessary to confirm the ability of the malic enzyme to carboxylate pyruvate, as this reaction has been demonstrated without difficulty for the malic enzyme from other sources, e.g. Mirocha and Rick (1967). The equilibrium constant favours malate formation (Harary et al., 1953) especially if the intracellular carbon dioxide is high (Walker, 1960), as it may well be in infected tissues (see Discussion, p.115) although the suggestion has been made (Walker, 1962; Ting and Dugger, 1965) that under certain conditions the malic enzyme may function in the utilization of malate.

4. Preparation of extracts from point-inoculated leaves: After the development of lesions, spot-infected leaves (p.32) and healthy controls were harvested with scissors, washed in glass-distilled water and gently shaken dry in cheesecloth. They were then divided into a distal portion which bore the lesion (the lamina of each leaf being cut with scissors about 1.0 cm below the visible lesion, p.44), and a basal portion which bore no external evidence of fungal infection; control leaves were cut into distal and basal portions of equivalent size. The distal portions of the leaves were bulked and chilled, and extracted and assayed according to the various procedures as the healthy and infected 'tip extracts'; the basal portions were treated similarly as the healthy and infected 'base extracts'.

SECTION II.

RESULTS.

A. BASES FOR THE EXPRESSION OF ENZYME ACTIVITY.

The expression as well as the examination of enzyme activity in extracts of the tissue of host-parasite complexes poses special problems. The results of biochemical investigations of carbon-fixing enzymes in normal tissues and chloroplasts are usually expressed on the bases of chlorophyll e.g. Hatch et al. (1969), Bjorkman and Gauhl (1969), or protein e.g. Kleinkopf et al. (1970), Waygood et al. (1969); total photosynthesis has been expressed on the bases of chlorophyll e.g. Slack and Hatch (1967), dry weight e.g. Hatch and Slack (1966), fresh weight e.g. Bjorkman and Gauhl (1969), and leaf area e.g. Hatch et al. (1969), Atkins and Canvin (1971). The results of experiments in which enzyme activities in infected plants have been compared with those of healthy controls have been expressed on many bases e.g. fresh weight (Scott and Smillie, 1963, 1966); dry weight (Malca and Zscheile, 1963); unit area (Zaki and Mirocha, 1965); per aliquot of the supernatant resulting from a standard extraction procedure (Malca et al., 1964) and per mg protein (Rick and Mirocha, 1968): yet many if not all of these parameters will have been affected by the presence of the parasite and the results obtained are not, therefore, necessarily comparable with the results of experiments on normal tissues.

Some authors have commented on the importance of the parameters in expressing enzyme and metabolic activities in tissues consisting of host and pathogen. Allen (1942) was one of the first to detail the very different results obtained when changes in photosynthesis upon infection were expressed on different bases. He compared the results of his

experiments on photosynthesis expressed on a fresh weight basis with the same results expressed on a chlorophyll basis and found meaningful differences between them: photosynthetic efficiency (per unit chlorophyll) was maintained after the decline in photosynthesis per unit fresh weight had begun; and photosynthetic efficiency then declined more slowly than total photosynthesis. Livne (1964), in his work on photosynthesis in rust-affected plants, noted differences of 7 - 9% in the comparative rates of photosynthesis in diseased and healthy plants when expressed on the bases of fresh weight and leaf area, since the weight per unit area in infected tissue was 10 - 15% greater than that of healthy tissue; however, he chose to use fresh weight as the basis for the expression of his data. Malca et al. (1964), whose data on enzyme changes in corn infected with H.carbonum were expressed ultimately on fresh weight, made dry weight determinations to assess whether the increasing desiccation of infected tissues with progressing infection would affect the validity of their data (they concluded that it was sufficiently slight to be disregarded). Sempio (1950) found the changes in dry weight per unit fresh weight in the infection of wheat by Oidium moniloides Sacc. to be sufficiently great to be routinely included in the results of his metabolic experiments. Rick and Mirocha (1968) were able to use protein as a basis for the expression of the results of their experiments on the comparative malic enzyme activity in healthy and rusted bean tissue because, even though there were increases with infection in the 35 - 60% ammonium sulphate fraction used as a source of the enzyme, they were quite small.

It was apparent from the literature that the available parameters should be examined through the infection cycle in a particular host-parasite complex before any one was chosen for the expression of the

results of metabolic studies in that complex. Accordingly, the possible parameters were examined through the infection cycle in the barley-H.teres complex under study. All the results in the following section represent the averages of at least three experiments.

Table 1: Changes in the water content through the infection cycle of infected first leaves of Parkland and C.I.5791 as compared with healthy controls.

days after inoculation	dry weight in grams / grams fresh weight the susceptible reaction			the resistant reaction		
	H	I	I/H	H	I	I/H
0	0.0617	0.0630	1.02	0.0630	0.0612	0.97
1	0.0622	0.0618	0.99	0.0623	0.0602	0.96
2	0.0659	0.0624	0.95			
3	0.0666	0.0632	0.95	0.0741	0.0703	0.94
4	0.0650	0.0735	1.13			
5	0.0645	0.0868	1.35	0.0742	0.0689	0.93
6	0.0650	0.0817	1.26			
7	0.0588	0.0742	1.26	0.0734	0.0740	1.01

From Table 1, it is evident that, in the susceptible reaction to infection, the dry weight per unit fresh weight of infected tissue increases as there begins to be a substantial amount of mycelium developed in the leaf tissue. Over the first three days of infection, the water content of infected tissue is slightly greater than that of healthy tissue, so that dry weight per unit fresh weight is slightly lower in infected tissue; increasing desiccation then ensues in the susceptible reaction which is not counterbalanced by the contribution to the fresh weight of

the fungal mycelium as it spreads through the leaf, and dry weight per unit fresh weight increases. There are slight but consistent changes in the water content of the resistant host-parasite complex, as evidenced by a decrease in dry weight per unit fresh weight: the water content increases slightly upon infection and then returns to the levels of controls.

The dry weight per unit area of the susceptible host-parasite complex increases as infection progresses (Table 2), as has been shown in the susceptible reaction in rusted bean (Zaki and Mirocha, 1965), rusted clover (Thrower, 1965) and rusted wheat (Samborski and Shaw, 1956). The increases in the dry weight will be due at least in part to the developing fungal mycelium, but probably reflect changes in the host tissue also, such as the accumulation of metabolites around the lesions which Shaw and Samborski (1956) showed in Helminthosporium infections of wheat and Bromus. The changes in the resistant complex are small compared with those in the susceptible complex, as would be expected since mycelial development is limited; however a slight increase in dry weight was observed, in contrast to the resistant reaction of wheat to rust (Samborski and Shaw, 1956) where there are marked losses in dry weight and total nitrogen in very heavily infected leaves.

The chlorophyll content of infected susceptible leaves falls increasingly below the levels of controls as infection progresses when measured as milligrams of chlorophyll per gram fresh weight (Table 3). Since chlorophyll is calculated on a fresh weight basis, and the fresh weight of infected tissue falls as infection progresses, the data only show the apparent loss of chlorophyll from infected tissue: the real loss may be substantially greater. In the resistant host there may be a slight decline in chlorophyll upon infection, but after the formation of the lesions no further loss occurs. Haspelova-Horvatovicova (1971)

Table 2: Changes in fresh and dry weight (on an area basis) of infected first leaves of Parkland and C.I.5791 as compared with healthy controls.

days after inoculation	weight in grams / dm^2											
	fresh weight						dry weight					
	susceptible			resistant			susceptible			resistant		
	H	I	I/H	H	I	I/H	H	I	I/H	H	I	I/H
3	1.414	1.440	1.02	1.656	1.672	1.01	0.157	0.162	1.03	0.186	0.189	1.02
5	1.603	1.164	0.73	1.784	1.687	0.95	0.152	0.170	1.12	0.169	0.185	1.10
7	1.855	0.982	0.53	1.797	1.685	0.94	0.147	0.193	1.31	0.153	0.178	1.16

Table 3: Changes in chlorophyll content (on a fresh weight basis) through the infection cycle in infected first leaves of Parkland and C.I.5791 as compared with healthy controls.

days after inoculation	mg chlorophyll / grams fresh weight					
	the susceptible reaction			the resistant reaction		
	H	I	I/H	H	I	I/H
0	1.09	1.02	0.94	1.21	1.17	0.97
12 hrs	0.96	0.98	1.02			
1	1.20	1.18	0.98	1.26	1.28	1.02
2	1.26	1.19	0.94			
3	1.42	1.17	0.82	1.38	1.27	0.92
4	1.48	1.28	0.86			
5	1.59	1.22	0.78	1.32	1.20	0.91
6	1.60	1.06	0.66			
7	1.35	0.78	0.58	1.42	1.38	0.97

showed a similar decline in chlorophyll in the resistant reaction of barley to mildew, though in a different mildew-resistant variety Scott and Smillie (1966) could find no change in chlorophyll upon infection.

The protein content of crude extracts of infected susceptible tissue was found to be somewhat greater than that of healthy controls on the bases of fresh weight, dry weight and area, as in the rust-wheat complexes examined by Shaw and Colotelo (1961) and Bhattacharya and Shaw (1966) and the rust-bean complex of Pozsar et al. (1966); but after the ammonium sulphate fractionation used in the extraction procedures of all enzymes assayed, the protein contents of the various samples were found to be similar to healthy controls, in contrast to the results of Rick and Mirocha (1968), who found changes in the 35 - 60% ammonium sulphate

fraction in extracts of corn infected with H.carbonum.

Because of the significant effects of infection of barley with H.teres on fresh weight and chlorophyll bases in the susceptible reaction, and the indirect relationships these parameters and dry weight measurements bear to the enzyme extracts as assayed, enzyme activities have not been routinely expresses on these bases: a protein basis has generally been used and activities computed as umoles of CO_2 consumed or evolved per minute per milligram protein. The results of RuDP carboxylase studies have been expressed on a chlorophyll basis since this enzyme is of primary importance in light-mediated carbon fixation (Bassham and Calvin, 1957) and strong correlations between RuDP carboxylase activity and photosynthesis have been documented in Calvin cycle plants (Neales et al., 1971). The activities of the enzymes which fix CO_2 non-photosynthetically (non-autotrophically) bear at most an indirect relationship to the light reaction in that substrates and co-factors may come from photosynthesis (e.g. Tamas et al., 1970) and have therefore been expressed only on a protein basis.

B. THE RESULTS OF THE ENZYME ASSAYS.

The activities of RuDP carboxylase, PEP carboxylase and the malic enzyme were examined in infected plants of Parkland and C.I.5791 barley and healthy controls daily through the infection cycle (seven days after inoculation). To investigate the extent to which the effects of the fungal infection were localised, the activity of the enzymes was examined in uninvaded tissue from infected plants at selected times after inoculation, in in extracts from point-inoculated leaves of susceptible and resistant plants and the uninoculated second leaves of infected plants. Assays for PEP carboxylase and the malic enzyme were also carried out on extracts from

vegetative and sporulating material of H.teres, since these enzymes are thought to fix CO_2 in other fungal parasites (Staples and Weinstein, 1959; Rick and Mirocha, 1968).

1a. RUDP CARBOXYLASE: THE SUSCEPTIBLE REACTION.

First leaves: The activity of RuDP carboxylase invariably declined with developing infection, the decline becoming more apparent after the appearance of symptoms, as in corn infected with H.carbonum (Malca et al.,1964, 1965). The rate and magnitude of the decline were related to the rate of development and the severity of the infection. Activities as low as 36% of healthy controls were recorded in some experiments seven days after inoculation. Figs. 1A and B show, respectively, the results of a typical experiment where infection followed the standard cycle (p.37), and those of an experiment where infection developed slowly owing to environmental factors such as low light and temperature. In both infections, the activity of RuDP carboxylase had begun to decline by the second day after inoculation. However, when the results of the typical experiment shown in Fig. 1A were expressed on a chlorophyll basis (Fig. 2), little change in RuDP carboxylase was noted until the sixth day after inoculation: the loss of enzyme activity apparently paralleled the loss of chlorophyll until this time. Fig. 3 shows the ratios of RuDP carboxylase activity in extracts of infected as compared with healthy leaves computed from the experimental results shown in Figs. 1A and 2.

Point-inoculated leaves: Spot-infected leaves were assayed at three and seven days after inoculation (to ascertain whether uninvaded portions of infected leaves exhibited changes in RuDP carboxylase activity similar to those in invaded portions), and a typical series of results is recorded in Table 4. The activity of RuDP carboxylase was found to be more greatly

FIG. 1: Changes in activity of RuDP carboxylase (on a protein basis) in extracts of infected first leaves of Parkland and healthy controls in a standard and a slowly developing infection.

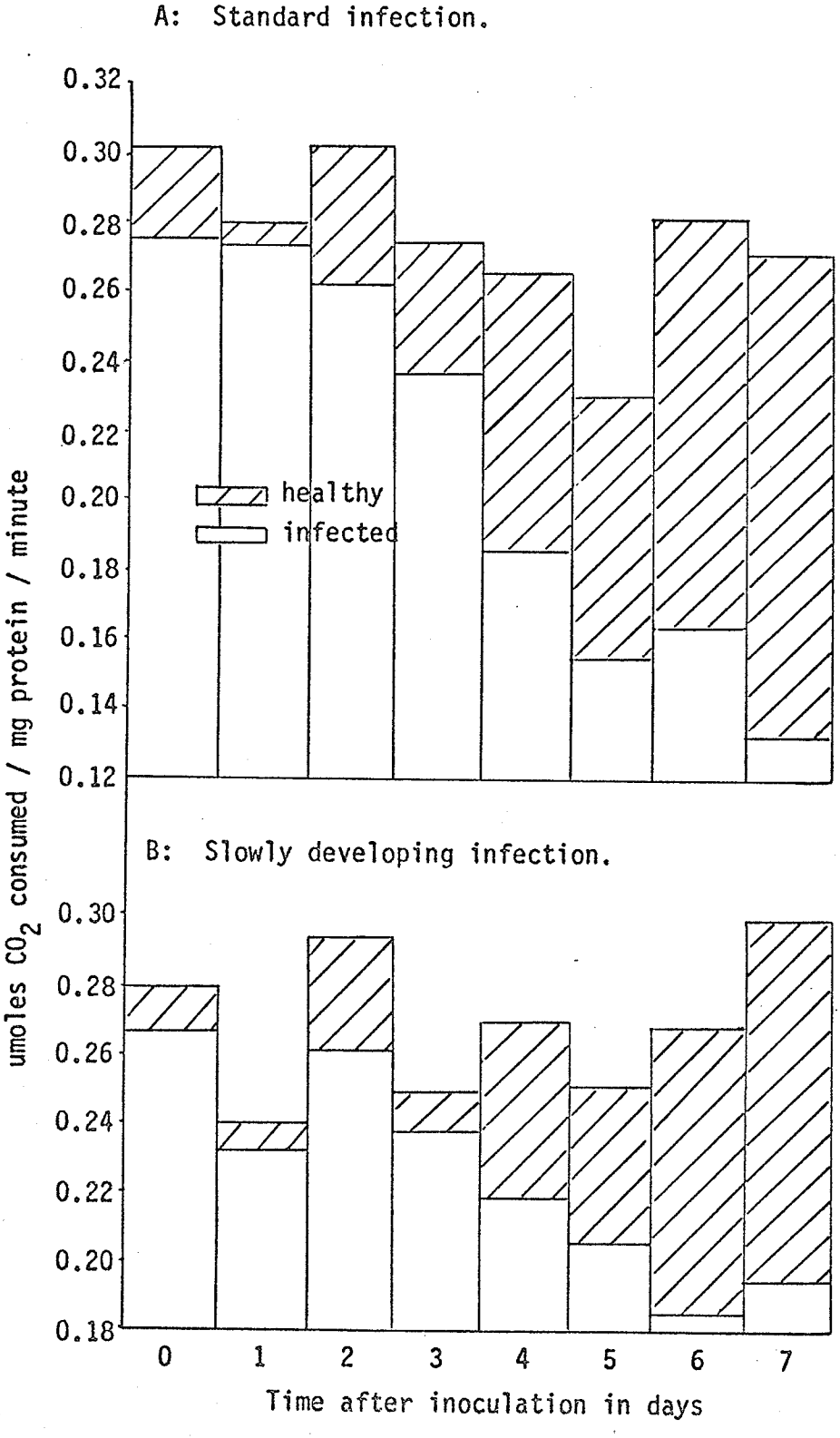


FIG. 2: Changes in activity of RuDP carboxylase (on a chlorophyll basis) in extracts of infected first leaves of Parkland and healthy controls through the standard infection cycle (cf. Fig. 1A).

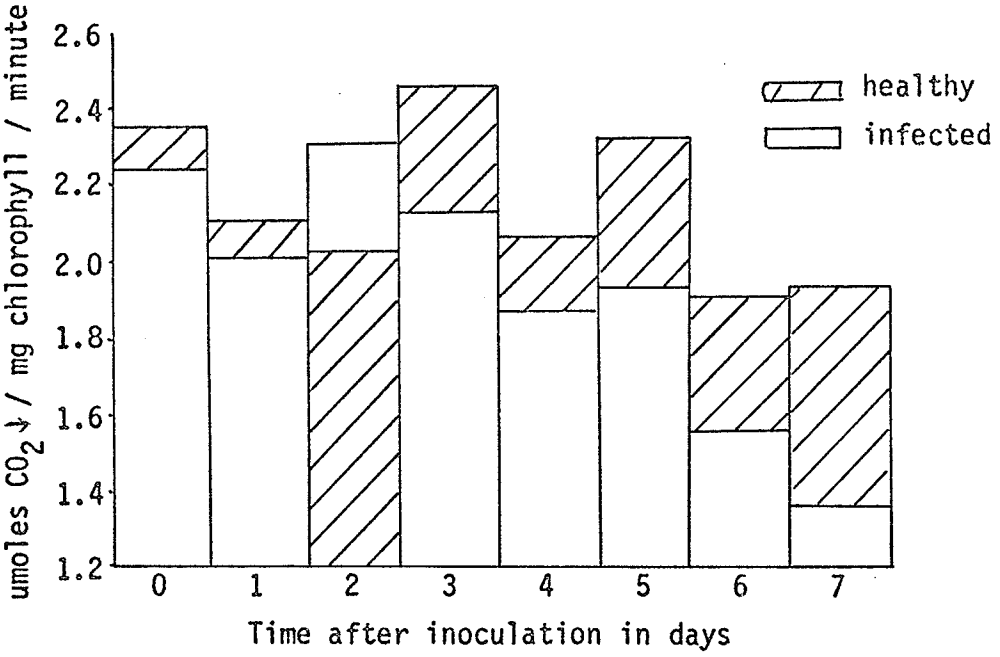
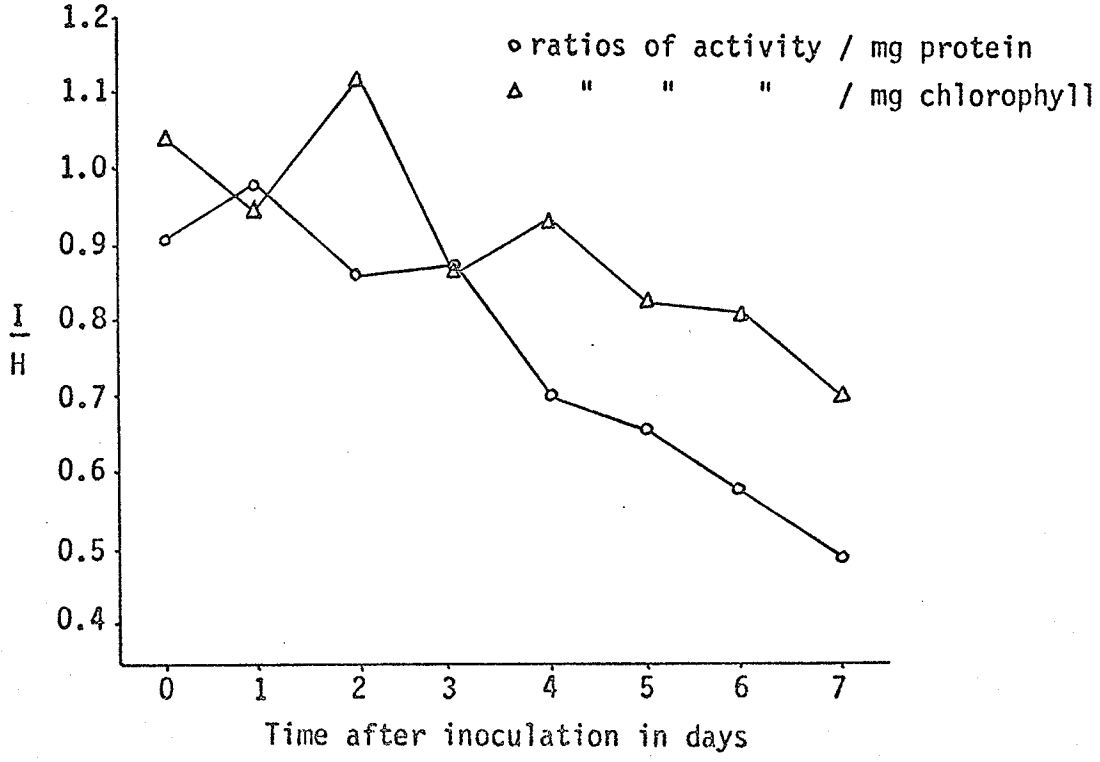


FIG. 3: Ratios of RuDP carboxylase on the bases of protein and chlorophyll through the standard infection cycle in extracts of the infected first leaves of Parkland as compared with healthy controls (from Figs. 1A and 2).



reduced in the vicinity of the fungus than outside it, but there was a reduction as compared with healthy controls throughout the infected leaf.

Table 4: Changes in activity of RuDP carboxylase through the infection cycle in extracts of the basal and distal portions of first leaves of Parkland infected at the tip as compared with healthy controls.

days after inoculation	umoles CO_2 ↓ / mg protein / min					
	Healthy		Infected		I/H	
	base	tip	base	tip	base	tip
3	0.284	0.271	0.221	0.165	0.78	0.61
7	0.198	0.240	0.123	0.108	0.62	0.45

Second leaves: Second leaves were assayed at three and seven days after inoculation of the first leaves. Results were variable: in most experiments, there was no significant difference between the activities of RuDP carboxylase from infected and healthy plants, but, as shown in Table 5, both increases and decreases in the enzyme's activity over controls were recorded in extracts from infected plants.

1b. RUDP CARBOXYLASE: THE RESISTANT REACTION.

First leaves: Table 6 shows the activity of RuDP carboxylase over the standard infection cycle in a typical experiment. On the third day after infection, activity per milligram protein was significantly reduced by infection, but by the fifth day its activity was again comparable to that of controls. On a chlorophyll basis, as in the susceptible reaction, the activity of RuDP carboxylase in extracts from infected resistant leaves

Table 5: Changes in the activity of RuDP carboxylase through the infection cycle in extracts of the uninoculated second leaves of infected plants of Parkland as compared with healthy controls in three experiments, A, B and C.

days after inoculation	expt. no.	umoles CO_2 ↓ / mg protein / min		
		H	I	I/H
3	A	0.328	0.372	1.13
	B	0.279	0.267	0.97
	C	0.223	0.238	1.07
7	A	0.281	0.265	0.94
	B	0.218	0.171	0.78
	C	0.219	0.285	1.30

was apparently proportional to chlorophyll content of the leaves. The ratios of RuDP carboxylase on a protein basis through the infection cycle in extracts of leaves of C.I.5791 as compared with healthy controls are shown with the equivalent ratios from the susceptible reaction (from the results shown in Fig. 1A) in Fig. 5A.

Table 6: Changes in the activity of RuDP carboxylase (on the bases of protein and chlorophyll) through the infection cycle in extracts of first leaves of C.I.5791 as compared with healthy controls.

days after inoculation	umoles CO_2 ↓ / minute					
	per mg protein			per mg chlorophyll		
	H	I	I/H	H	I	I/H
1	0.391	0.402	1.03	2.15	2.00	0.93
3	0.367	0.283	0.77	1.62	1.54	0.95
5	0.367	0.327	0.89	1.78	1.78	1.00
7	0.404	0.420	1.03	1.59	1.67	1.05

Point-inoculated and second leaves: No differences were observed between the activities of RuDP carboxylase in extracts of the bases of leaves of C.I.5791 whose tips were inoculated and those from healthy controls, nor in extracts from the second leaves of plants whose first leaves were infected and those from healthy controls: hence no tabular presentation of results is included.

2a. PEP CARBOXYLASE: THE SUSCEPTIBLE REACTION.

First leaves: In the standard infection, on a protein basis, the specific activity of PEP carboxylase increased through the infection cycle so that at the onset of sporulation of the parasite, it was more than double that of healthy controls (Fig. 4A). The rate of development of the disease regulated the rate at which the increase occurred, as was shown in experiments in which the disease developed slowly owing to environmental factors such as low light and low temperature (Fig. 4B). In an experiment where infection was light and coalescing lesions were just beginning to form at seven days after inoculation, little change in the specific activity of PEP carboxylase was recorded in this time (Fig. 4C). Fig. 5B shows the ratios of PEP carboxylase activity in the extracts of infected leaves as compared with healthy controls, derived from the results of the three experiments shown in Figs. 4A - C.

Point-inoculated leaves: Spot-infected leaves were assayed at three and five days after inoculation, and the results of a typical experiment are recorded in Table 7. At three days after inoculation, an increase in the specific activity of PEP carboxylase as compared with healthy controls was observed in extracts of the basal (uninvaded) portions of the leaves, the increase being as great as that in the distal portions where lesions were forming. At seven days after inoculation, the basal portions of

FIG. 4: Changes in activity of PEP carboxylase in extracts of infected first leaves of Parkland and healthy controls in a standard, a slowly developing and a light infection.

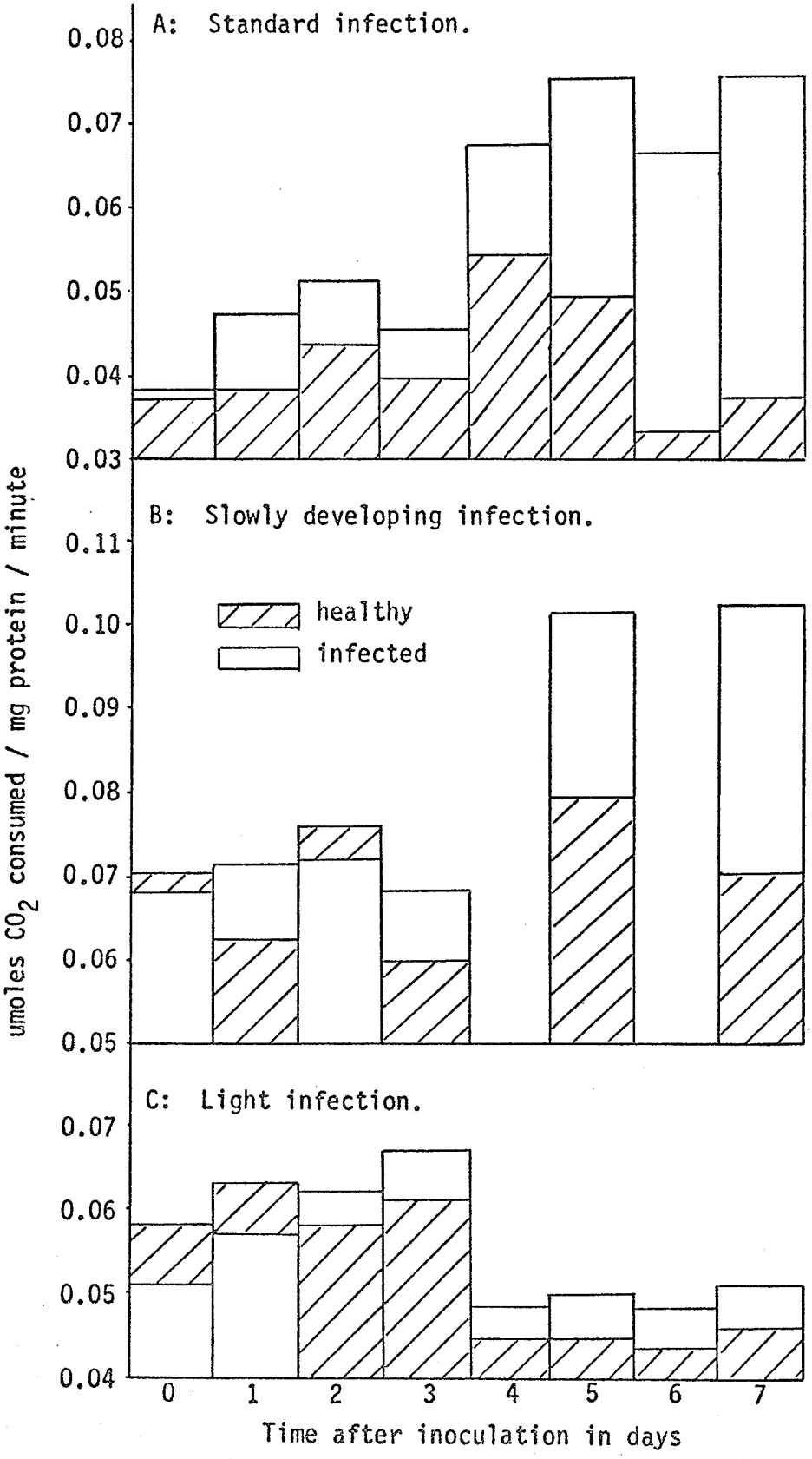
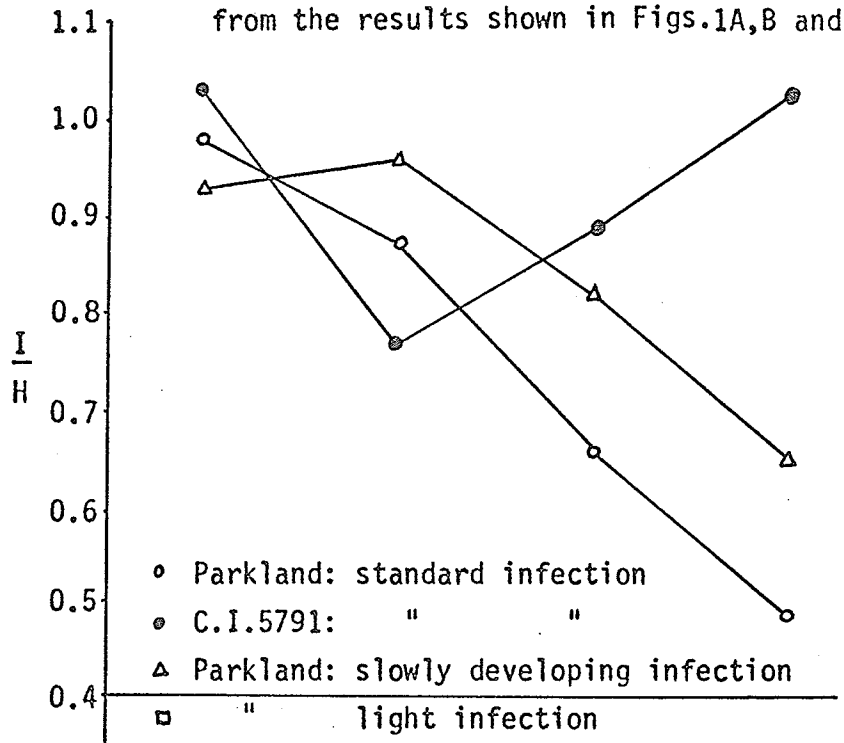


FIG. 5: Ratios of activity of RuDP and PEP carboxylases on a protein basis in extracts of the infected first leaves of Parkland and C.I.5791 as compared with healthy controls in standard, slowly developing and light infections.

A: RuDP carboxylase:
from the results shown in Figs.1A,B and Table 6.



B: PEP carboxylase:
from the results shown in Figs.4A-C and Table 10.

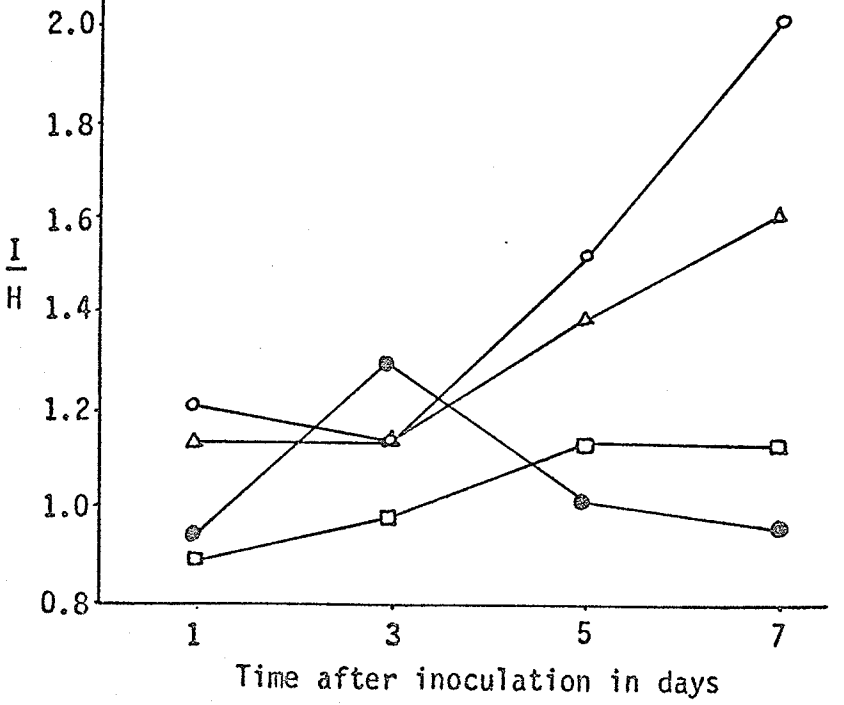


Table 7: Changes in the activity of PEP carboxylase through the infection cycle in extracts of the basal and distal portions of first leaves of Parkland infected at the tip as compared with healthy controls.

days after inoculation	umoles $CO_2 \downarrow$ / mg protein / min					
	Healthy		Infected		I/H	
	base	tip	base	tip	base	tip
3	0.034	0.027	0.043	0.034	1.28	1.24
7	0.043	0.040	0.060	0.048	1.40	1.19

the leaves showed a greater increase in PEP carboxylase activity over healthy controls than the heavily infected tips, perhaps due to the extensive damage caused by the fungus: Malca et al. (1964) have shown such an effect in the H.carbonum infection of corn.

Second leaves: The uninoculated second leaves were assayed for PEP carboxylase activity at three and seven days after inoculation of the first leaves. The specific activity of the enzymes in extracts from both healthy and diseased plants was lower than that in the first leaves; no consistent differences were found between the extracts from healthy and infected plants, but higher activities in those from infected plants were observed in some experiments as shown in Table 8: Experiment A was a typical experiment and Experiment B was one where differences between the extracts of infected and control leaves was observed. The variability of results was not apparently correlated with the rate of development or intensity of the infection.

Table 8: Changes in the activity of PEP carboxylase through the infection cycle in extracts of the uninoculated second leaves of infected plants of Parkland as compared with healthy controls in a typical experiment A and another experiment B.

days after inoculation	Expt. no.	umoles CO_2 ↓ / mg protein / min		
		H	I	I/H
3	A	0.030	0.027	0.90
	B	0.022	0.025	1.14
7	A	0.016	0.018	1.10
	B	0.024	0.038	1.60

2b. PEP CARBOXYLASE: THE RESISTANT REACTION.

First leaves: Table 9 shows the activity of PEP carboxylase in extracts of first leaves of C.I.5791 through the standard infection cycle as compared with healthy controls.

Table 9: Changes in the activity of PEP carboxylase (on a protein basis) through the infection cycle in extracts of first leaves of C.I.5791 as compared with healthy controls.

days after inoculation	umoles CO_2 ↓ / mg protein / min		
	H	I	I/H
1	0.057	0.054	0.95
3	0.052	0.068	1.31
5	0.046	0.047	1.02
7	0.060	0.058	0.96

At three days after inoculation, the extracts from infected leaves showed an increase over healthy controls in PEP carboxylase activity which often slightly exceeded that found in the susceptible reaction (Fig. 4A); however, by five days, the enzyme activity in these extracts was again similar to controls. In Fig. 5B, the ratios of PEP carboxylase activity on a protein basis through the infection cycle in extracts of leaves of C.I.5791 as compared with healthy controls are shown with the equivalent ratios from the susceptible reaction (from the experimental results shown in Figs. 4A - C).

Point-inoculated and second leaves: Slight increases in the activity of PEP carboxylase (up to 125% those of controls) were observed in extracts of the bases of leaves of C.I.5791 whose tips were infected, but no such differences were evident in extracts from the second leaves of plants whose first leaves were infected and those from healthy controls. Because the changes upon infection were slight, no tabular presentation of results is included.

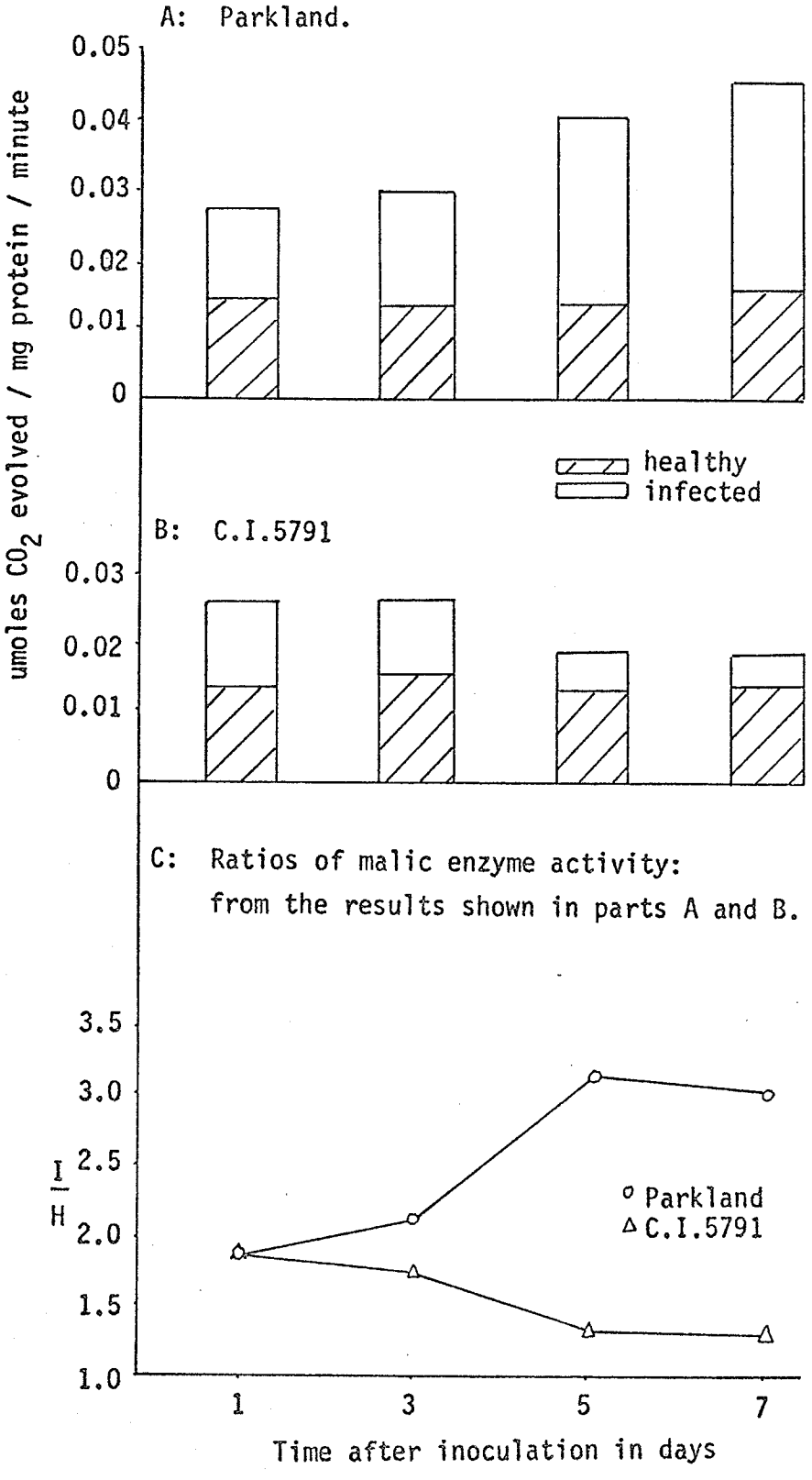
Assay of fungal extracts: Samples containing up to 1.0 mg of fungal protein could be assayed by the standard spectrophotometric method. No PEP carboxylase could be detected in the extracts obtained by homogenization or grinding from either the vegetative or the sporulating mycelium.

3a. THE MALIC ENZYME: THE SUSCEPTIBLE REACTION.

First leaves: In extracts of the infected first leaves of Parkland, the specific activity of the malic enzyme as compared with controls rose rapidly upon infection: substantial increases could be detected as early as 24 hours after inoculation, and at seven days the activity was three to four times that in healthy controls (Fig. 6A).

Point-inoculated leaves: In spot-infected leaves, substantially greater

FIG. 6: Changes in the activity of the malic enzyme on a protein basis through the standard infection cycle in extracts of the infected first leaves of Parkland and C.I.5791 as compared with healthy controls.



increases in the specific activity of the malic enzyme (over healthy controls) were found in extracts from the invaded distal portions of the leaves than those from the uninvaded basal portions at both three and five days after inoculation (Table 10), suggesting that some of the increase in infected tissue might be due to an active malic enzyme in the fungus itself.

Table 10: Changes in the activity of the malic enzyme (on a protein basis) through the infection cycle in extracts of the basal and distal portions of first leaves of Parkland inoculated at the tip, as compared with healthy controls.

days after inoculation	umoles $CO_2 \downarrow$ / mg protein / min					
	Healthy		Infected		I/H	
	base	tip	base	tip	base	tip
1	0.013	0.010	0.014	0.023	1.09	2.30
3	0.010	0.010	0.016	0.029	1.62	2.94
5	0.014	0.012	0.019	0.021	1.36	1.75

However, at least some of the increase in infected tissue could be attributed to a host response to infection since some increase was noted in uninvaded portions of infected leaves; and when the leaf tissue was heavily infected and damaged, as was the tip by the fifth day after inoculation, the malic enzyme activity was lower than in less damaged tissue (Day 3, tip). A similar relationship between the increase in the fixation of carbon in the dark by rusted bean leaves, probably due to an increase in the malic enzyme activity, and the pustule density, indicating the amount of damage to the tissue, has been shown by Rick and Mirocha (1965, 1968).

Second leaves: The specific activity of the malic enzyme in extracts of the second leaves of plants of which the first leaves were infected was no greater than that of healthy controls; in some experiments, a slight decrease in activity (to 80% of control values) was observed. Because the changes upon infection were slight, no tabular presentation of results is included.

3b. THE MALIC ENZYME: THE RESISTANT REACTION.

First leaves: As in extracts from susceptible plants, substantial increases in activity of the malic enzyme over healthy controls could be shown in extracts from the first leaves of C.I.5791 within 24 hours after inoculation; however, as infection progressed, the differences between the extracts from infected and healthy plants diminished so that at seven days after inoculation they were comparable (Fig. 6B). In Fig. 6C, the ratios of malic enzyme activity on a protein basis through the standard infection cycle in extracts of infected as compared with control leaves of C.I.5791 are shown with equivalent ratios from the susceptible reaction (from the experimental results shown in Fig. 6A).

Point-inoculated leaves: Extracts from point-inoculated leaves of C.I.5791 showed that the stimulation of malic enzyme activity evident in spray-inoculated leaves could not be detected outside the vicinity of the fungus: the basal extracts had a similar activity to healthy controls (Table 11). The relatively high activity of the malic enzyme in the tip extract at five days after inoculation, where the increase was greater than that in spray-inoculated leaves, could have been related to the high lesion density: the lesions coalesced in a manner superficially similar to the susceptible reaction, though sporulation of the pathogen in these areas was never observed; consequently a greater influence may have been exerted on the

Table 11: Changes in the activity of the malic enzyme through the infection cycle in extracts of the basal and distal portions of first leaves of C.I.5791 inoculated at the tip as compared with healthy controls.

days after inoculation	umoles CO_2 ↑ / mg protein / min					
	Healthy		Infected		I/H	
	base	tip	base	tip	base	tip
1	0.014	0.010	0.012	0.019	0.88	1.97
3	0.013	0.013	0.010	0.015	0.98	1.50
5	0.014	0.014	0.015	0.026	1.06	1.81

host tissue by the parasite than in the standard infection, with the greater amount of mycelium also possibly contributing an active malic enzyme to the total assay.

Second leaves: Since the specific activity of the malic enzyme in extracts of the second leaves of plants of which the first leaves were infected was not significantly different from healthy controls, no tabular presentation of results is included.

Assay of fungal extracts: Malic enzyme activity was found in extracts from both vegetative and sporulating H.teres mycelium made both by homogenization and grinding. Activity was low compared with that in leaf extracts and assay samples contained up to 2.5 mg of protein. The specific activity in extracts from sporulating mycelium was higher than that in extracts from vegetative mycelium: Table 12 shows a comparison of the activities with those found in leaves. The results obtained from the extracts made by homogenization and grinding were not significantly different.

Table 12: The specific activity of the malic enzyme from various sources.

source of enzyme		umoles CO_2 evolved / mg protein / min x 10^2
H.teres :	vegetative mycelium	0.069
H.teres :	sporulating mycelium	0.091
Parkland:	healthy first leaves	1.451
Parkland:	seven-day infected first leaves	4.530

SECTION II.

DISCUSSION.

All the carboxylases examined in Parkland and C.I.5791 were affected by infection; but they did not necessarily remain constant through the experimental period even in healthy control leaves. Under the standard experimental conditions of light and temperature, the specific activity per milligram protein of RuDP carboxylase in healthy leaves of Parkland declined; and this effect was much exaggerated by infection (Fig. 1A), possibly indicating a senescence-like effect induced by infection, as metabolic studies of other diseases have suggested (p.23). When enzyme activity in healthy and infected plants of Parkland was expressed per milligram chlorophyll, a downward trend was again apparent though less well defined, perhaps because the chlorophyll measurements were not made directly on the enzyme extracts as were the protein measurements (p.35). Chlorophyll was measured per gram fresh weight, and by five to seven days after inoculation, the fresh weight of infected tissue had dropped 25 - 45% below the levels of controls (Tables 1 and 2); the actual chlorophyll loss of infected tissue may therefore have been as much as 25 - 45% greater than that measured per gram fresh weight. RuDP carboxylase activity based on actual units of chlorophyll in infected tissue would therefore have remained more or less constant throughout the experimental period instead of dropping off as shown in Fig. 3. In the experiment carried out under low light and temperature (Fig. 1B), no decline in the specific activity of RuDP carboxylase in healthy leaves was shown: it remained constant or even showed a slight upward trend over the seven-day experimental period. It is conceivable that the first leaves of plants in this experiment may have been

in a different phase of their development from those grown under the standard environmental conditions, such that the difference in the relative levels of the various metabolic processes may have rendered these plants less susceptible to infection, as proposed by Sempio (1950) and Shaw and Samborski (1956).

The effects of infection on the specific activity of RuDP carboxylase appeared to be most strongly exerted in the immediate vicinity of the fungus, and were significant only after the initial penetration stages of parasitism, when the pathogen had developed considerably in the host tissue. This might be consistent with a direct effect of pathogenesis on the chloroplasts due to localised disturbances of metabolism, since RuDP carboxylase is a chloroplast enzyme; though this effect could not be merely physical disruption by the presence of the pathogen since the specific activity of RuDP carboxylase was affected outside the invaded areas of infected leaves (Table 4). However, the deleterious effects of infection may have been exerted on the chloroplast as a whole rather than on RuDP carboxylase specifically, since the loss of chlorophyll and the drop in specific activity of RuDP carboxylase apparently occurred at much the same rate (see above).

No consistent effect on the uninvaded second leaves of infected plants was noted; infection of the first leaves seemed to exert a general disruptive effect on the metabolism of the plant rather than a specific effect, such that the development of the second leaves (and possibly their carbon-fixing mechanisms, since the specific activity of RuDP carboxylase was initially lower in the second leaves) was initially inhibited. This could have been the result of a reduced supply of substrates, co-factors or growth factors normally provided by the first leaves but partially retained because of infection (see Table 24) and perhaps also synthesised

at a reduced rate in the first leaves because of their reduced photosynthetic capacity or efficiency. However, when the second leaves had achieved a certain minimum size and were presumably largely self-sufficient, development began to occur as rapidly as in healthy plants (see p.40).

In resistant plants, infection initially resulted in some loss of specific activity of RuDP carboxylase, but the loss was not permanent (Table 6). Since recovery occurred and the disease symptoms were relatively localised, no effect on RuDP carboxylase being evident outside the vicinity of the fungus (p.61), it seemed improbable that the decline in specific activity was caused by destruction of the chloroplasts but more probably was due to interference in enzyme or chloroplast function by some product of the host-parasite interaction, perhaps that which affected enzyme function in the basal portions of tip-inoculated leaves of Parkland. However, the effect was not intensified because only limited fungal development occurred in resistant tissue. When the changes in the activity of RuDP carboxylase in the resistant reaction were expressed per milligram chlorophyll, no change in activity was apparent (Table 6), perhaps further indicating the effect of infection on chloroplasts as a whole. The metabolism of plants of C.I.5791 was not sufficiently affected upon infection for there to be any effect on the development of the second leaves.

The specific activity of PEP carboxylase in healthy leaves of Parkland appeared to rise slightly through the experimental period and then decline (Fig. 4A and C); the peak of activity was slightly later under conditions of low light and temperature (Fig. 4B). Though PEP carboxylase may be particulate-bound in the cell, it is almost certainly not associated with the chloroplasts (Ting, 1971) and might therefore

be expected to be unaffected by the deleterious effects of infection on the chloroplasts; and indeed, in all the infection cycles examined, the specific activity of the enzyme increased until sporulation of the fungus commenced. Even though the peak of activity coincided with that of fungal growth in the leaves, there was apparently little if any contribution by the fungus of an active PEP carboxylase per se, since none was evident in extracts of the fungus. Moreover, after sporulation of the fungus had begun and extensive damage to the host tissue had occurred (as in the tips of tip-inoculated leaves at seven days after inoculation, Table 7), the overall specific activity of PEP carboxylase declined.

The effects of infection upon the specific activity of PEP carboxylase occurred more rapidly after inoculation than those upon RuDP carboxylase (Fig. 5), and they appeared to be more widespread, since the effects in the uninvaded portions of point-inoculated leaves (Table 7) were as great as those in the invaded portions, or even greater after extensive damage to the host tissue had occurred; and a stimulation of the specific activity was sometimes observed in the second leaves of infected plants.

In resistant leaves, during the phase of active growth of the fungus, a stimulation of the specific activity of PEP carboxylase occurred (Table 9); the stimulation was maintained only during active growth and the enzyme activity returned to the levels of controls after the inhibition of fungal development in the resistant tissue. However, further evidence of the ready diffusibility of the stimulating principle suggested above comes from point-inoculated resistant leaves, where slight stimulation was observed in the basal parts despite the limited fungal growth in the tips (p. 71).

Malic enzyme had relatively low specific activity in extracts of healthy leaves, and this acivity was relatively constant throughout the experimental period. The stimulating effects of infection on the malic enzyme occurred very rapidly: by 24 hours after inoculation its activity was almost doubled, and the peak in activity occurred before sporulation of the pathogen. Though an active malic enzyme was present in the fungus (Table 12), it was of relatively low specific activity (at least *in vitro*); and the amount of fungal mycelium in the leaves was still quite small when a substantial increase in the specific activity of the malic enzyme had occurred. It seems probable, then, that the contribution of enzyme of enzyme components by the fungus was relatively small, though such a contribution could conceivably considerably enhance the overall activity of the host-parasite complex (see General Discussion). The stimulation of activity within the vicinity of the fungus was greater than outside it (except after extensive damage to the host tissue had occurred); and no stimulation of activity was apparent in the second leaves of infected plants, the slightly reduced activity in such leaves probably being due to a general disruption of the metabolism of the infected plant rather than to a direct effect on the malic enzyme of the fungus *per se*.

In the resistant reaction to infection, the effects on the malic enzyme were again rapid: the peak in specific activity was reached at 24 hours after inoculation (rather than at three days, as in PEP carboxylase). Since the growth of the fungus in the resistant tissue was not arrested until *ca.* three days after inoculation, the increase in the specific activity was apparently not related to the extent of fungal development in the host and may therefore have been largely a response of the host tissue in the immediate vicinity of the fungus to infection, since the effects were localised (none was evident outside the invaded

areas in point-inoculated leaves) and the resistant reaction to infection was not as great as the susceptible reaction, though the possibility cannot be excluded of a contribution of some enzyme component from the fungus while it was actively metabolising in the resistant tissue.

Though the specific activity of the malic enzyme increased three to four-fold in extracts from infected tissue, the enzyme was still not capable of fixing as much CO_2 per minute per milligram protein as the PEP carboxylase extracted from healthy tissues; and the highest specific activities recorded for PEP carboxylase in extracts of infected material were only ca. one third those usually recorded for RuDP carboxylase from healthy tissues, though they were but little lower than those of RuDP carboxylase in extracts of heavily infected material.

SECTION III: THE FIXATION OF CO_2 IN VIVO

MATERIALS AND METHODS.

Several series of experiments were carried out in which intact barley plants in small pots (p.31) were exposed in a perspex chamber to air containing a small amout of $^{14}CO_2$. The environmental conditions were varied and the responses of the plants to the various conditions followed by studying their uptake of $^{14}CO_2$ or the movement of ^{14}C within the plants.

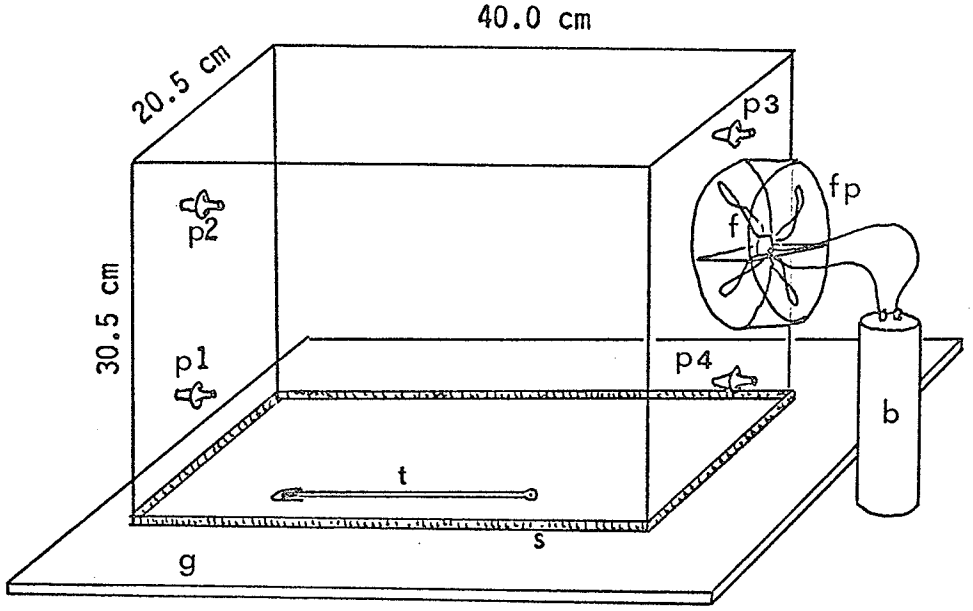
A. APPARATUS AND GENERAL METHODS.

The exposure chamber: The chamber had internal dimensions of 35 x 40 x 20.5 cm as shown in Fig. 7, and its total volume (including fan pocket) was 19440 cc. It was constructed as a hood with an open underside so that it could be placed over plants, water containers, etc. arranged on the glass base sheet. The underside was edged with a dense foam rubber strip, so that there was airtight contact with the glass base sheet: this form of seal allowed easy access to the experimental material and other apparatus in the chamber. The atmosphere of the chamber was circulated constantly during experiments by a small battery-driven fan screwed to a ledge in the drum-shaped perspex pocket in the side of the chamber. During exposure of plants to $^{14}CO_2$, all chamber ports were sealed with plastic clay.

Equilibration of plant material in the chamber: In experiments on the fixation of CO_2 in the light other than those involving a 16-hour light period, plants were allowed to equilibrate in the chamber at the experimental light intensity (q.v.) for 20 - 30 minutes before the release of $^{14}CO_2$. Humidified air was passed into the chamber through a hose attached to port 1 (Fig. 7); port 3 was left unsealed to serve as the air outlet.

Release of $^{14}CO_2$ into the chamber atmosphere: The air hose was first

FIG. 7: Plexiglass chamber used in the exposure of barley seedlings to $^{14}CO_2$ in air.



b	battery
f	fan with small motor
fp	fan pocket
g	glass base plate
p1 - 4	polyethylene ports 1 - 4
t	thermometer
s	foam rubber strip

disconnected and port 3 sealed. The required amount of $NaH^{14}CO_3$ (q.v.) was pipetted into a small petri dish and placed in the chamber under port 1. A few drops of 2.0 N HCl were introduced into the $NaH^{14}CO_3$ solution through port 1 using a long-tipped pasteur pipette (Plate 11) to generate $^{14}CO_2$ and a short burst of air was then applied to port 1 to disperse the $^{14}CO_2$ through the chamber. Port 1 was then sealed and the chamber rendered airtight.

Experimental conditions in the light: All experiments were conducted at 25°C unless otherwise specified in the detailed experimental descriptions. In the experiments of 15 minutes' duration, the average light intensity received by leaves in the chamber was 900 f.c., provided by an incandescent lamp; in longer experiments, because of the difficulty in maintaining a constant temperature inside the chamber at this light intensity since the lamp had a heating effect, the light intensity was reduced to 600 - 700 f.c. Though these light intensities are lower than those often used in experiments on the fixation of CO_2, they were considered to be adequate since the light in the greenhouse in which the experimental material was grown often did not exceed these intensities and growth and infection of the plants proceeded readily under such greenhouse conditions; and in the experiments with barley seedlings conducted by Edwards (1970) there was little increase in the fixation of CO_2 with increasing light intensity above 600 f.c. Two 50 ml beakers of water were included in the chamber to maintain humidity; when necessary for the maintenance of a temperature of 25°C, the beakers were filled with ice cubes, the ice being replaced when the chamber was flushed in long-term experiments (q.v.).

Experimental conditions in the dark: All experiments were conducted at room temperature (ca. 25°C) unless otherwise described. The exposure chamber was darkened by covering with a heavy black cloth before the

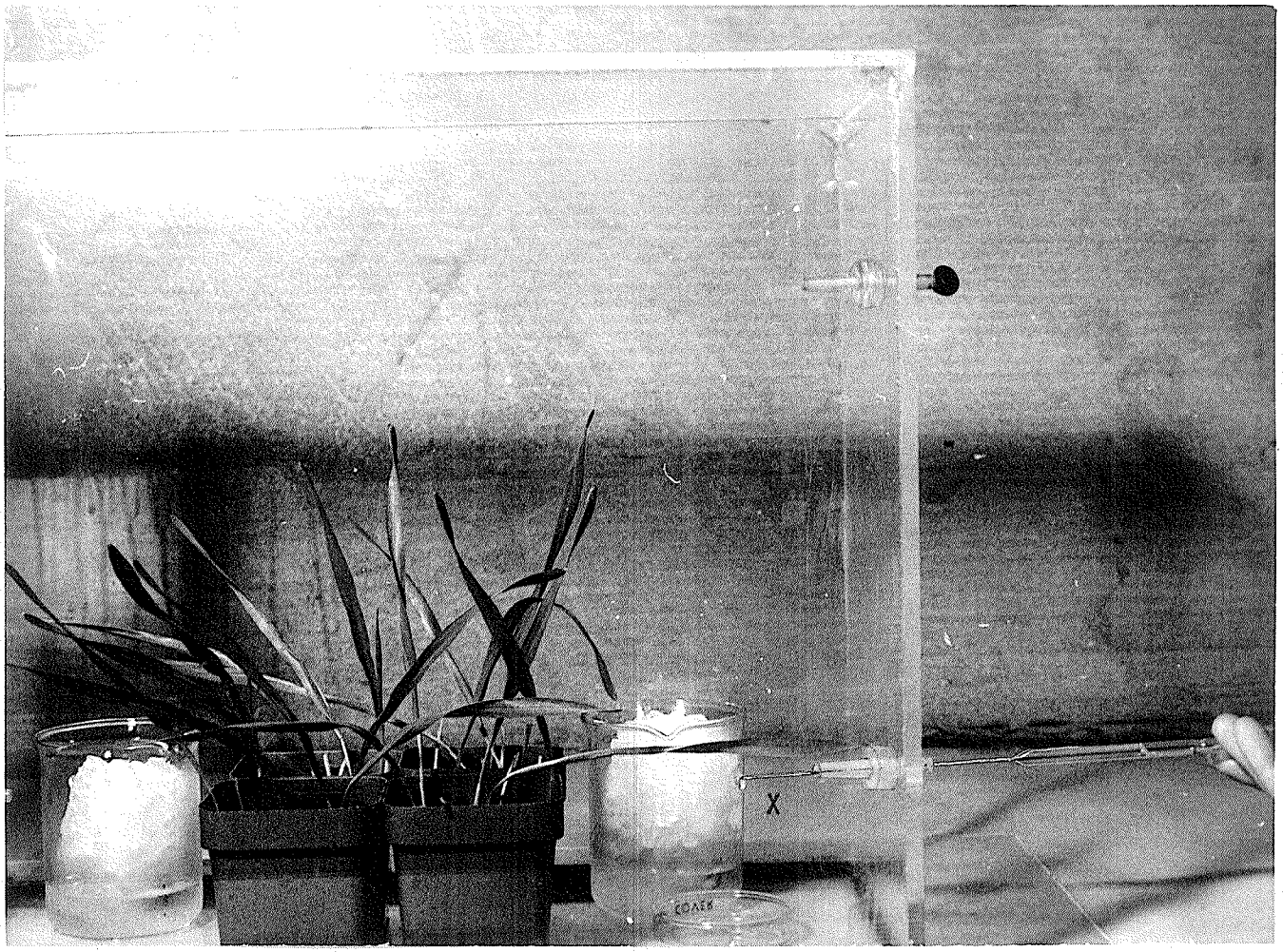


PLATE 11: The generation of $^{14}CO_2$ from $NaH^{14}CO_3$ in the exposure chamber by the introduction (at point X) of hydrochloric acid.

release of $^{14}CO_2$ into the chamber atmosphere, and remained covered for the duration of the experiment.

Maintenance of CO_2 levels in the fixation chamber during long-term experiments: In experiments of eight, 16 and 24 hours' duration, with typically 20 barley seedlings at the two-leaf stage present in the chamber, the chamber atmosphere would be expected to become depleted of CO_2 with consequent limiting of CO_2 fixation; to avoid this, the chamber was flushed at intervals with air and re-charged with $^{14}CO_2$. The flushing procedure was as follows: ports 1 and 3 were unsealed and the air-hose attached to port 1; humidified air was passed through the chamber for three minutes, after which port 3 was sealed. The small petri dish used to contain the $NaH^{14}CO_3$ from which $^{14}CO_2$ was generated was removed from the chamber and washed, and the required amount of $NaH^{14}CO_3$ was again pipetted into it before it was replaced in the chamber. 2.0 N HCl was pipetted into the dish as at zero time; a short burst of air dispersed the $^{14}CO_2$, and port 1 was again sealed to close the system.

To establish the periods in light and dark after which CO_2 levels might become limiting in the closed system, experiments were carried out under the standard conditions of light (600 - 700 f.c. for long-term experiments) and temperature (25°C) with four pots in the chamber each containing five seedlings of Parkland or C.I.5791 at the two-leaf stage. The plants were allowed to fix $^{14}CO_2$ in air (in the proportions used in the long-term experiments) for periods of one, one and a half and two hours, in three separate experiments; after each experiment samples of eight leaf discs from each of five first leaves taken at random from the plants in the chamber were cut and assayed for radioactivity as described later in this section. From these experiments it was deduced (p.101) that two-hour periods between flushings would be adequate in the

light, and, since fixation proceeds very much more slowly in the dark, that four-hour intervals would be adequate in the dark; these intervals have been used in the long-term experiments unless otherwise described. The sampling procedure: Leaf discs of diameter 7.0 mm were cut from the first, and in some experiments, the second, leaves of plants to be sampled. In experimental Series 1, one sample of four discs was cut from each of five of the first leaves of each treatment (Parkland, infected and control; C.I.5791, infected and control). In Series 2 - 4 , two groups of four discs were cut from each first leaf, and one or two groups of four from each second leaf, depending on its size. Each group of four discs was rapidly transferred to a scintillation vial containing hot 2.0 N HCl and boiled to disintegration (three to five minutes): Bieleski (1964) advised the use of acid for rapid halting of metabolism. Each vial was then dried in a stream of cold air and, when dry, 0.25 ml of water was added, and the residue resuspended by scraping with a blunt dissecting needle. The radioactivity was measured by a modification of the method of Gordon and Wolfe (1960): 10.0 ml of scintillation fluid containing ca. 2.5% w/w thixotropic gel powder (Cabosil, Canlab) were added to each vial and the vial shaken so that a fine homogeneous gel was formed; the radioactivity was then measured as previously described (p.35). Where two samples of four leaf discs were cut from one leaf, the radioactivity in both was summed for a measurement of carbon fixation in that leaf.

The chlorophyll content of four or eight leaf discs (as appropriate to the experiment) equivalent to those cut for each treatment was assayed to obtain an estimate of the chlorophyll content of the sample discs as described on p.36.

B. THE EXPERIMENTAL SERIES.

Series 1: The fixation of CO_2 by infected plants and healthy controls in light and dark was followed through the standard seven-day infection cycle in Parkland and C.I.5791 and a third variety, B.T.201 (p.4). B.T.201 was included because members of the population exhibited reactions to H.teres ranging from those which were superficially similar to C.I.5791 in that small necrotic lesions resulted from infection, to those where lesions similar to a susceptible reaction were formed upon infection, the parasite sporulating on some plants; the aim of the experiments was to determine whether a similar variation existed in the metabolic reaction to infection.

In both light and dark, the $^{14}CO_2$ from 5 uCi of $NaH^{14}CO_3$ of specific activity 60 uCi per umole was generated in the fixation chamber at the beginning of the fixation period. Plants were allowed to fix CO_2 at a light intensity of 900 f.c. for 15 minutes at ca.25°C; fixation experiments in the dark at 25°C were of one hour's duration.

Series 2: The effects on carbon fixation in the light and dark of damage to leaves other than that caused by fungal infection were investigated. Some of the metabolic effects of fungal infection seem to be part of a general response to injury rather than a specific response to the parasite (Allen, 1959): among these effects are accumulation of metabolites (Shaw et al., 1954; Shaw and Colotelo, 1961); increased respiration (Yarwood, 1953; Uritani, 1971); changes in proteins (Shaw and Colotelo, 1961) and the activities of some enzymes, including RNase (Bagi and Farkas, 1967).

Damage to seven-day-old leaves of Parkland was investigated. To create small necrotic lesions, three dressmaker's steel pins were blunted and pressed close together through a piece of cork; they were heated in a

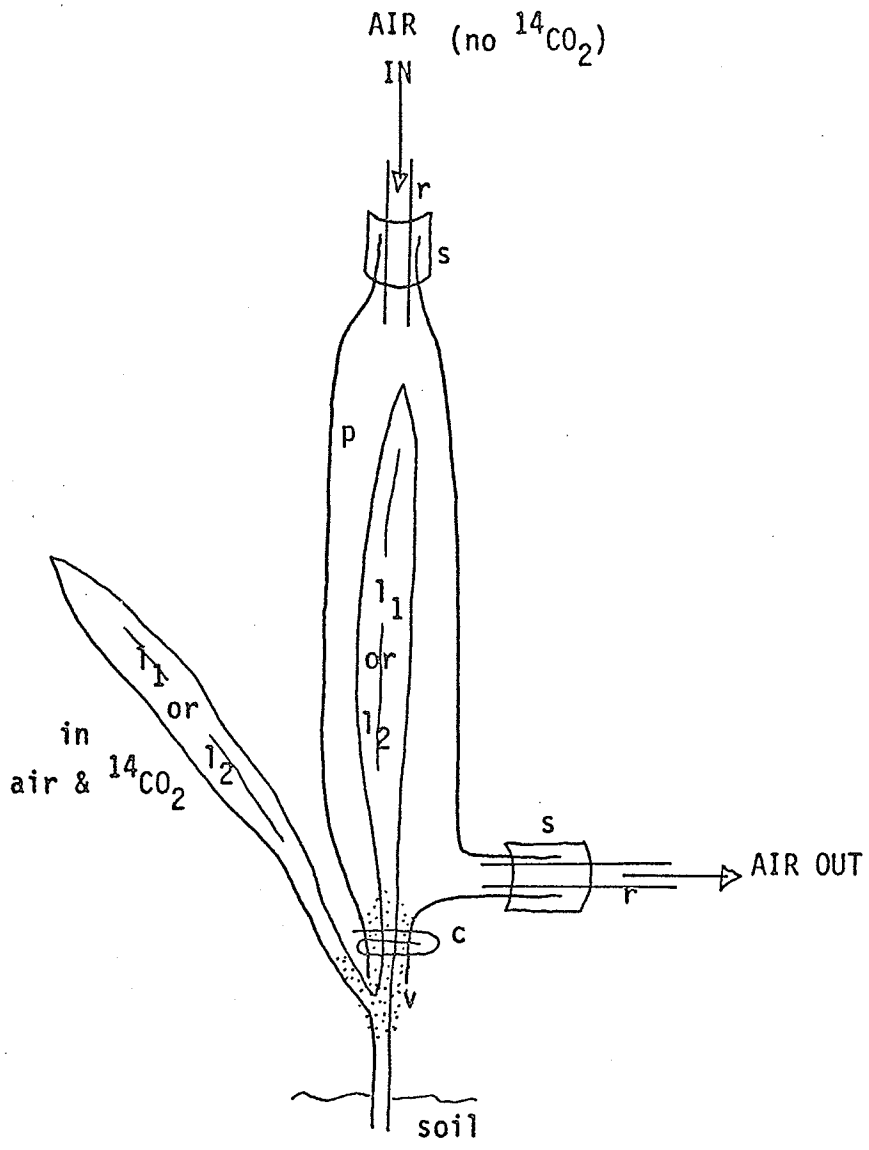
flame until red-hot and lightly touched in a regular pattern along the upper surfaces of the leaves, being reheated as necessary. Striations were also made on some leaves by drawing the heated pins along the leaves. The leaf cells touched by the needles were probably immediately killed but necrotic areas did not reach their maximal development until ca. 36 hours after damaging: at this time the leaves were used for experimentation. To create chlorotic areas, an ammonium sulphate solution was used: to 20 ml of a 50% solution of ammonium hydroxide a drop of Tween 20 was added; cotton wool was wrapped around the tip of an inoculating needle and soaked in the solution, and this was touched at approximately 1.0 cm intervals along the upper surfaces of the leaves, re-wetting as necessary to give small discrete drops on the leaf surface. The leaves were used for experimentation after two days, when bleached areas corresponding in size to the original drops of solution were well-developed on the leaves, often surrounded by necrotic zones. The leaves with chlorotic and necrotic areas were allowed to fix CO_2 in light and dark under conditions similar to those described in experimental Series 1.

Series 3: At four and six days after inoculation (p.34), infected plants and healthy controls of Parkland and C.I.5791 were allowed to fix CO_2 for 24-hour periods consisting, as in the greenhouse conditions under which they were grown, of 16 hours' light and eight hours' dark. At the end of the 24-hour period, first and second leaves were sampled and an indication of the net gain of carbon by the different parts of the plant after one diurnal cycle obtained. The exposure chamber was flushed every two hours in the light and every four hours in the dark, and the $^{14}CO_2$ from 5 uCi of $NaH^{14}CO_3$ of specific activity 60 uCi per umole was released after each flushing. Experiments of 16 and eight hours' duration respectively, using the same methods, were carried out to measure, separately,

the net carbon accumulation in the light period and the dark period. Fixation of carbon in the dark was also examined in the middle of the light period of the diurnal cycle. Diurnal fluctuations in the organic acid content of corn and wheat and bean seedlings have been mentioned by Malca and Zscheile (1963) and Daly and Livne (1966), who worked on the H.carbonum and rust infections of these hosts respectively, as being of possible importance when comparing the fixation by healthy and diseased tissue in the dark. In that such diurnal fluctuations of organic acids in host-parasite complexes might indicate fluctuations in the enzymes of β-carboxylation, it seemed reasonable to compare fixation in the dark in the H.teres-barley complexes under study with the fixation in healthy plants at different times in the diurnal cycle. Fixation of CO_2 in an eight-hour dark period arranged to fall in the middle of the usual 16-hour light period of the diurnal cycle was compared with fixation in the usual eight-hour dark period of the diurnal cycle: plants were placed in the darkened exposure chamber at four hours after the commencement of the light period. In the eight-hour experiments at both times in the diurnal cycle, the chamber was flushed every two hours (rather than every four as in the 24-hour experiments) to avoid the possibility of the CO_2 concentrations becoming rate-limiting.

Series 4: Translocation patterns in infected plants of Parkland and C.I.5791 as compared with healthy controls were followed in experiments of 24 hours' duration comprising a 16-hour light period and an eight-hour dark period. The first and the second leaves of the experimental plants were isolated from one another; both were allowed air and 16 hours' light, but $^{14}CO_2$ was fed to only one of the leaves in any one experiment. For the isolation of one of the seedling leaves from the other, small polyethylene bags as shown in Fig. 8 were made using the heat-sealing device

FIG. 8: The atmospheric isolation of the first and second leaves of barley seedlings from one another (experimental Series 4).



c	paper-clip closing bag around leaf base
l_1 or l_2	first or second leaf of barley seedling.
p	clear polyethylene bag
r	rubber tubing
s	scotchtape holding tubing to bag
v	vaseline to improve seal

manufactured by Laboratory Apparatus Co., Cleveland, Ohio. The first or the second leaves of four plants in each of four pots (Parkland, infected and control, and C.I.5791, infected and control) were connected up to air input and output manifolds, which were themselves connected to chamber ports 2 and 4 respectively (Fig. 7), as shown in Plates 12 and 13. A stream of humidified air was passed into the bags at their upper ends and out near their bases continuously during the 24-hour experiments: the leaves in the bags were thus exposed to air without $^{14}CO_2$. $^{14}CO_2$ from 5 uCi of $NaH^{14}CO_3$ of specific activity 60 uCi per umole was released in the chamber by the usual method, and the chamber flushed every two hours in the light and every four hours in the dark, using chamber ports 1 and 3, with the $^{14}CO_2$ being replenished after each flushing: thus the leaves outside the bags were exposed to air containing $^{14}CO_2$. After the 24-hour experimental period, both leaves were assayed for radioactivity using the method described above.

PLATE 12A.

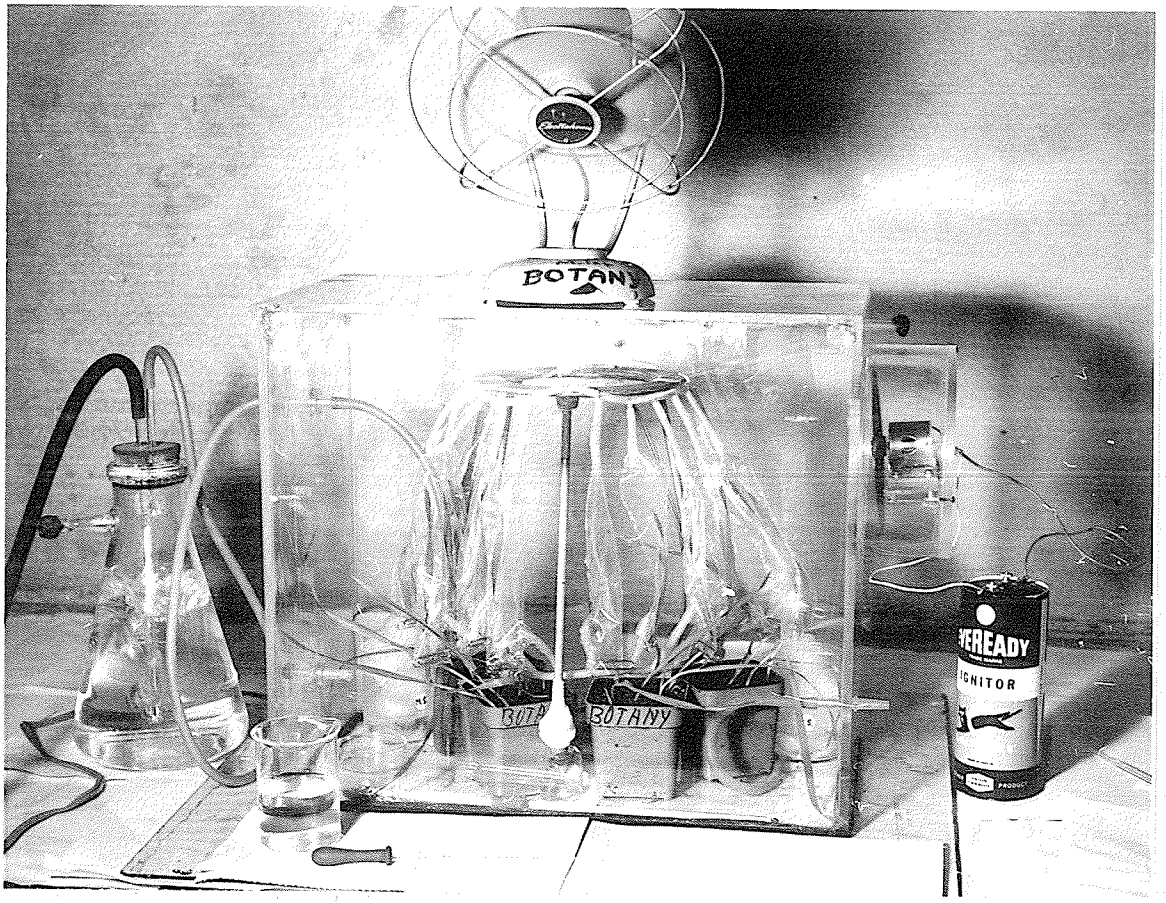


PLATE 12A: Translocation experiments (Series 4): apparatus for feeding ($^{12}CO_2$ + $^{14}CO_2$) to one leaf and $^{12}CO_2$ to the other leaf of barley seedlings at the two-leaf stage.

PLATE 12B.

PLATE 12B: Translocation experiments (Series 4): apparatus for the atmospheric isolation of one leaf from the other within the exposure chamber (cf. Fig. 8).

SECTION III.

RESULTS.

SERIES 1.

The fixation of CO_2 by infected plants of three barley varieties and appropriate controls was followed through the infection cycle. Unless otherwise defined, the results given in the tables represent the the average of those obtained in three different inoculation experiments, with five samples of four leaf discs being averaged for each treatment in each experiment. The results have been computed on the bases of area and chlorophyll as umoles CO_2 fixed / dm^2 / minute and umoles CO_2 fixed / mg chlorophyll / minute. Individual values are necessarily only approximate since it has been assumed in calculation that the CO_2 levels remained constant in the exposure chamber during the fixation period; however, it was felt that the results would be more meaningful if expressed in standard units rather than d.p.m., etc., and, since experimental conditions remained constant, comparisons of the results of different treatments can more readily be made.

1a. CO_2 FIXATION IN THE LIGHT: the susceptible reaction.

Table 13A shows the gradual decline in the fixation of CO_2 per unit area in infected plants as compared with healthy controls from the third day after inoculation until, at seven days, when the fungus was sporulating, it was about 50%; a similar reduction was found by Livne (1964) in rusted bean, wheat and safflower upon sporulation of the parasite. No stimulation of fixation in the light was apparent in infected leaves even in the early stages of infection. Photosynthetic efficiency in infected leaves, in terms of fixation of CO_2 per unit chlorophyll, was maintained at levels near those of controls until the fifth day after

Table 13: Fixation of CO_2 in the light on the bases of area and chlorophyll through the standard infection cycle in the infected first leaves of Parkland and C.I.5791 as compared with healthy controls.

A: The susceptible reaction.

days after inoculation	umoles CO_2 fixed / minute per dm^2			per mg chlorophyll		
	H	I	I/H	H	I	I/H
0	0.724	0.666	0.92	45.25	48.10	1.06
1	1.045	0.909	0.87	69.71	70.43	1.01
2	1.097	0.998	0.91	57.75	58.32	1.01
3	0.925	0.734	0.80	62.79	66.11	1.05
4	0.994	0.775	0.78	48.11	44.25	0.92
5	1.150	0.655	0.57	53.86	49.00	0.91
6	0.783	0.431	0.55	51.46	42.71	0.83
7	1.077	0.517	0.48	43.25	34.61	0.80

B: The resistant reaction.

days after inoculation	umoles CO_2 fixed / minute per dm^2			per mg chlorophyll		
	H	I	I/H	H	I	I/H
0	1.047	1.005	0.96	47.61	48.07	1.01
1	1.258	1.120	0.89	62.93	61.68	0.98
2	1.169	0.935	0.80	58.43	55.50	0.95
3	1.051	0.842	0.80	50.04	50.00	1.00
4	1.341	1.019	0.76	63.89	56.86	0.89
5	1.074	0.918	0.84	48.79	49.29	1.01
6	1.025	0.933	0.91	46.57	52.61	1.13
7	1.169	1.204	1.03	58.43	60.18	1.03

inoculation, after which it began to decline; such maintenance of efficiency while the overall fixation dropped has been shown in powdery mildew infections of wheat (Allen, 1942) and barley (Scott and Smillie, 1966).

1b. CO_2 FIXATION IN THE LIGHT: the resistant reaction.

The typical resistant reaction is shown in Table 13B: per unit area total fixation was initially depressed by infection, but by six to seven days after inoculation, levels were again comparable to those of controls. In contrast, Scott and Smillie (1966) could show no effect upon total fixation in the resistant reaction of barley to powdery mildew infection. Fixation in infected plants per unit chlorophyll appeared to be little affected by infection.

In Fig. 9A, the resistant reaction of C.I.5791 to H.teres is compared with the susceptible reaction of Parkland, the ratios of fixation on an area basis in infected plants of both varieties as compared with healthy controls from Tables 13A and B being presented together.

Table 14 shows the fixation of CO_2 in infected plants of B.T.201 as compared with healthy controls in two representative experiments, A and B. The ratios clearly demonstrate the variable resistance to infection of these plants under the experimental conditions employed: they ranged from those which resembled Parkland, with photosynthesis being progressively reduced by advancing infection, to those which resembled C.I.5791, with the photosynthetic rate being depressed below the levels of the healthy controls early in the infection, but later recovering. Since the samples taken in experiments were random and each sample came from a different plant, all types of reaction to infection are represented in the data in Table 14 and no particular pattern of response emerges from the data.

FIG. 9: Ratios of fixation of CO_2 in the light and the dark on an area basis in the infected first leaves of Parkland and C.I.5791 as compared with healthy controls.

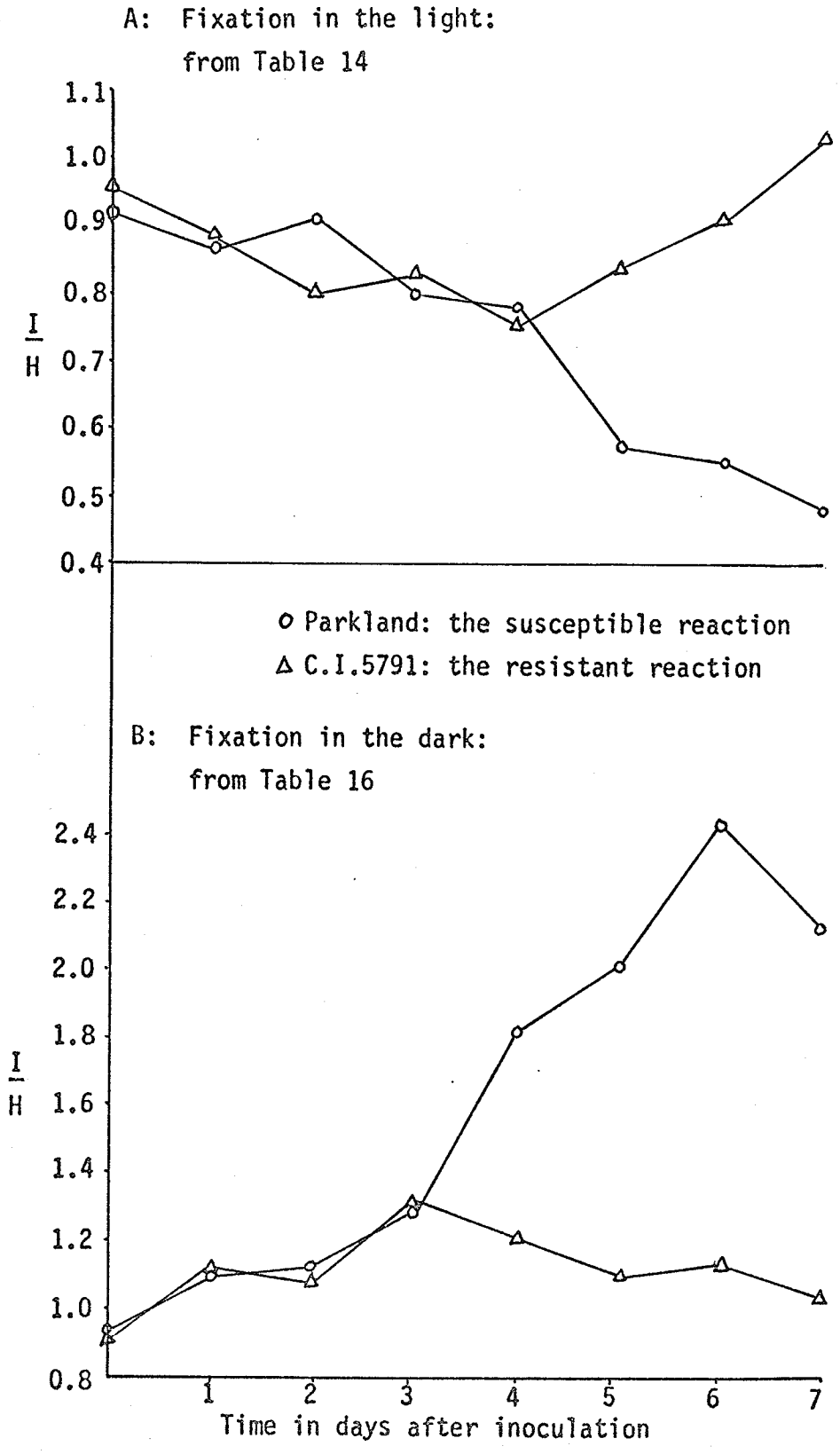


Table 14: The ratios of CO_2 fixation expressed as umoles CO_2 fixed / minute / dm^2 in light and dark through the infection cycle in the infected first leaves of B.T.201 as compared with healthy controls in two experiments, A and B.

days after inoculation	Ratios I/H			
	Light		Dark	
	Expt A	Expt B	Expt A	Expt B
0	0.95	1.05	1.09	0.97
1	1.08	0.92	1.14	0.89
2	1.04	0.78	1.00	1.16
3	0.80	1.08	1.32	1.48
4	0.91	0.80	1.40	1.20
5	0.61	0.95	1.09	1.39
6	0.84	1.03	0.92	1.89
7	0.76	0.68	1.28	1.62

2. CO_2 FIXATION IN THE DARK: the susceptible and resistant reactions.

Table 15 shows the fixation of CO_2 per minute on an area basis in infected and control plants of Parkland and C.I.5791. In susceptible plants, fixation of CO_2 in the dark increased as the disease developed, reaching a maximum just before sporulation of the pathogen; in resistant plants, a slight increase was observed early in infection, but after the third day after inoculation fixation of CO_2 dropped to the levels of healthy controls. The resistant reaction resembled that shown by Kuo and Scheffer (1970) in corn plants resistant to H.carbonum upon infection. The ratios from Table 15 are shown graphically in Fig. 9B, as a comparison of the effects on fixation in the dark in susceptible and resistant plants.

The ratios of fixation in infected plants of B.T.201 as compared with healthy controls are shown in Table 14, which represents the results

of two typical experiments, A and B. As in the results of the experiments on the fixation of carbon in the light also shown in Table 14, the variability in the reactions of these plants to infection under the experimental conditions employed is clearly demonstrated. The increases in fixation in the dark exhibited by infected plants as compared with healthy controls were on average greater than those of plants of C.I.5791 which showed complete resistance to H.teres, but not as great as those of plants of Parkland: the plants of B.T.201 exhibited a range of reactions to H.teres between complete resistance and complete susceptibility.

Table 15: Fixation of CO_2 in the dark on an area basis through the standard infection cycle in the infected first leaves of Parkland and C.I.5791 as compared with healthy controls.

days after inoculation	umoles CO_2 fixed / minute / $dm^2 \times 10^3$					
	Parkland			C.I.5791		
	H	I	I/H	H	I	I/H
0	4.63	4.26	0.92	4.66	4.23	0.91
1	4.83	5.30	1.10	5.43	6.02	1.11
2	3.95	4.47	1.13	5.21	5.63	1.08
3	4.24	5.51	1.30	4.05	5.35	1.32
4	3.52	6.38	1.82	4.90	5.88	1.20
5	4.41	8.87	2.01	6.49	7.08	1.09
6	5.64	13.76	2.44	6.36	7.18	1.13
7	4.25	9.06	2.13	3.95	4.07	1.03

SERIES 2.

Seedlings of Parkland with chlorotic or necrotic areas on the first

leaves as illustrated in Plates 13 and 14 were allowed to fix $^{14}CO_2$ in air in the light and the dark. The experiments were repeated three times with five samples of eight leaf discs being averaged for each treatment in each experiment, and the results presented represent the averages of the three: they were computed as in experimental Series 1.

a. CO_2 FIXATION IN THE LIGHT: damaged leaves.

Table 16 shows the fixation in leaves with chlorotic and necrotic areas as compared with controls, on the bases of leaf area and chlorophyll content.

Table 16: Fixation of CO_2 in the light on the bases of area and chlorophyll in mechanically or chemically damaged first leaves of Parkland as compared with untreated controls.

sample treatment	mg c'phyll per sample (8 lf. discs)	umoles CO_2 fixed / min dm^2	umoles CO_2 fixed / min mg c'phyll	Ratios damaged/untreated dm^2	Ratios damaged/untreated mg c'phyll
untreated	0.031	2.12	2.13	-	-
chlorotic	0.022	1.67	2.38	0.79	1.11
necrotic	0.026	1.80	2.15	0.85	1.01

Both bleaching and mechanical damage reduced CO_2 fixation; bleaching caused the greater reduction, and also the greater loss of chlorophyll: the depression of fixation appeared to be closely related to the extent of chlorophyll loss. Photosynthesis in the chlorotic leaves appeared from the results to be slightly more efficient (per unit chlorophyll) than that in the untreated leaves; however, when, in a control experiment, only the unbleached areas of the chlorotic leaves were sampled, similar fixation rates to those of untreated leaves were found.

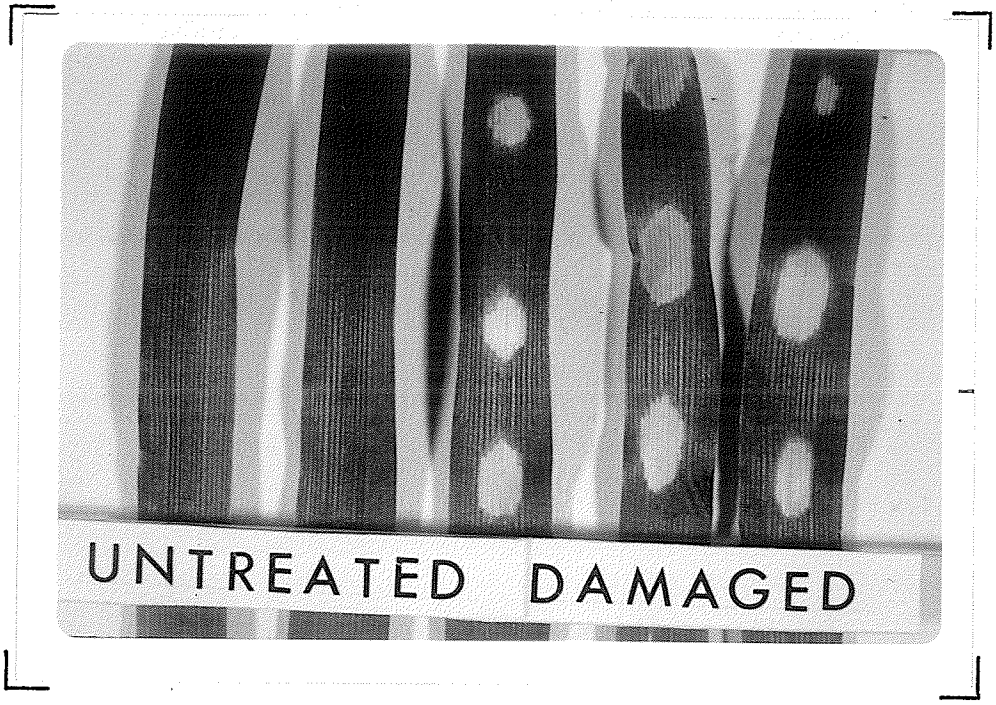


Plate 13: 'Chlorotic' leaves of Parkland at two days after treatment with ammonium hydroxide solution.

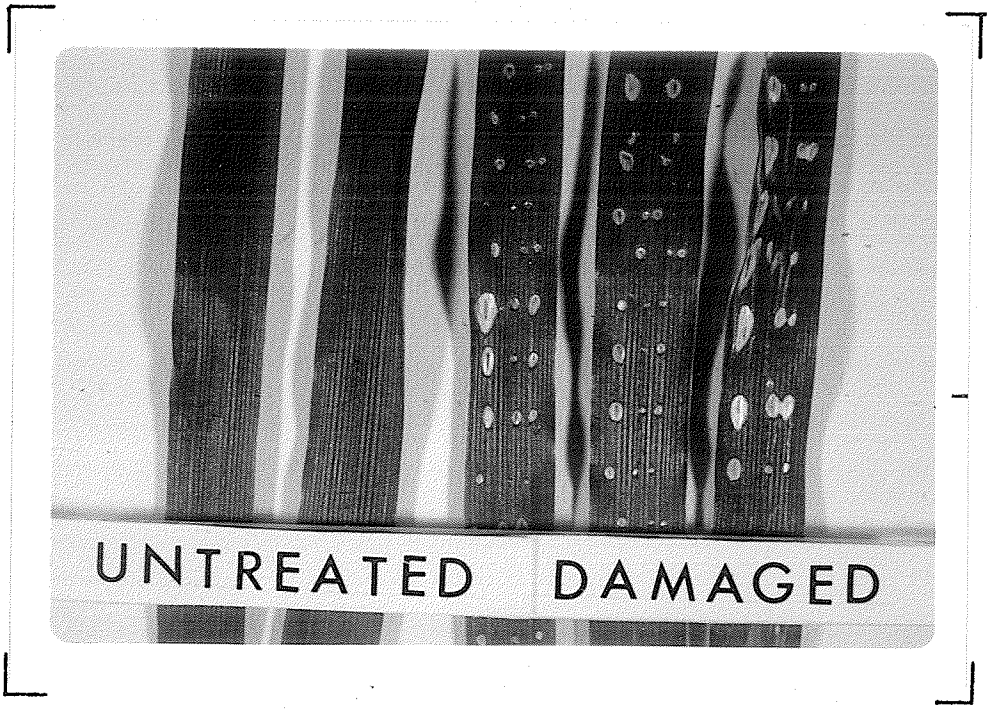


Plate 14: 'Necrotic' leaves of Parkland at 36 hours after treatment with heated steel pins.

b. CO_2 FIXATION IN THE DARK: damaged leaves.

Higher CO_2 fixation in the dark resulted from both mechanical damage and bleaching, as shown in Table 17.

Table 17: Fixation of CO_2 in the dark on an area basis in mechanically or chemically damaged first leaves of Parkland as compared with untreated controls.

sample treatment	umoles CO_2 fixed / min / dm^2 x 10^3	Ratios $\frac{\text{damaged}}{\text{untreated}}$
untreated	6.43	-
chlorotic	8.10	1.26
necrotic	8.68	1.35

SERIES 3.

In these experiments the fixation of CO_2 in infected and control plants of Parkland and C.I.5791 was followed through the 24-hour diurnal cycle.

A. The interval between flushings of the exposure chamber.

Table 18: Fixation of CO_2 in the light on an area basis by first leaves of Parkland, the plants being held in the closed exposure chamber to establish the period after which CO_2 became limiting.

fixation time in hours	umoles carbon present / dm^2 after fixation period
1.0	34.30
1.5	51.08
2.0	65.36

The results shown in Table 18, computed as described below, indicate that fixation in the light was proceeding more or less linearly up to two hours after the closing of the system under the standard experimental conditions, and therefore the chamber was flushed every two hours in the light and every four hours in the dark as described (p.86).

B. The expression of results of long-term experiments (eight, 16 and 24 hours).

The results of the analyses of ^{14}C in leaves exposed to $^{14}CO_2$ for long periods of time represent the $^{14}CO_2$ fixed *in situ* by the enzymes of photosynthetic and non-photosynthetic carboxylation and the ^{14}C transported from elsewhere in the plant to those areas of the leaves which were sampled for radioactivity. Unrepresented in the results will be the ^{14}C lost by respiration *in situ*, and by the translocation of the products of carboxylation *in situ* to other parts of the plant. To indicate that the results of the ^{14}C analyses represent the sum of several metabolic processes, they have been expressed as umoles of the carbon fixed during the experimental period present in the leaves after the experimental period. The computation of results in these units was as follows. Allowing for the volume occupied by beakers and pots (Plate 11), the volume of air in the fixation chamber was *ca.* 18840 cc. Assuming that the concentration of CO_2 in the air in the chamber was 0.03% at the start of each experiment and the temperature was maintained at 25°C, the chamber contained approximately 230 umoles of CO_2 at zero time. To this, 5 uCi of $^{14}CO_2$ (0.083 umoles, of negligibly small volume) were added, with radioactivity of $\propto$ d.p.m. Assuming that $^{12}CO_2$ and $^{14}CO_2$ were fixed equally readily by the tissues, the proportion of the $^{14}CO_2$ supplied which was fixed by any tissue sample in the experimental period could be taken to represent the proportion of the total CO_2 ($^{12}CO_2$ + $^{14}CO_2$) available in the chamber fixed by that sample: then one umole CO_2 fixed would be represented

by $\propto$/ 230 d.p.m. in the tissue sample. In 24 hours, the chamber was flushed every two hours in the light and every four hours in the dark; thus the plants in the chamber were exposed to ca. (10 x 230) umoles of $^{12}CO_2$ + $^{14}CO_2$, represented by 10$\propto$ d.p.m. In eight- and 16-hour experiments, when the chamber was flushed every two hours, the plants were exposed to (4 x 230) and (8 x 230) umoles of $^{12}CO_2$ + $^{14}CO_2$ respectively, represented by 4$\propto$ d.p.m. and 8$\propto$ d.p.m. Using these conventions, the proportion of the CO_2 available to the plants over the experimental period which was incorporated into, and remained in, a sample at the end of the experimental period could be estimated. If y d.p.m. were present in a sample upon analysis, then y (230 /$\propto$) d.p.m. were taken to represent the umoles of carbon fixed by the plant during the experimental period which were present in the sample at the end of the period.

C. THE RESULTS OF LONG-TERM EXPERIMENTS.

1a. NET ACCUMULATION OF CARBON AFTER 24 HOURS: the susceptible reaction.

First leaves: As shown in Table 19, there was little reduction in the net accumulation of the carbon fixed in the experimental period in infected as compared with healthy leaves after the 24-hour diurnal cycle at four days after inoculation, though the leaves showed extensive streaking and chlorosis by this time (p.37); by six days, however, when conidiophores were forming in at least the more heavily infected areas of the inoculated leaves, the reduction in the net carbon accumulation was greater. A control experiment was conducted at 30°C (all other experimental conditions remaining constant) to determine whether temperature had any effect on the relative accumulation of carbon in infected and healthy leaves: samples from both showed slightly higher carbon levels, probably indicating higher rates of fixation, but the ratios at four and six days after inoculation remained the same as at 25°C.

Table 19: Net accumulation of the products of CO_2 fixation in the 24-hour diurnal cycle on an area basis in the inoculated first leaves and the uninoculated second leaves of infected plants of Parkland and C.I.5791 as compared with healthy controls.

leaf number	days after inoculation	umoles carbon present / dm^2 after 24 hours susceptible			resistant		
		H	I	I/H	H	I	I/H
1	4	56.23	50.04	0.89	60.36	64.21	1.06
	6	48.59	37.90	0.78	57.89	62.52	1.08
2	4	80.29	68.25	0.85	90.08	97.29	1.08
	6	122.16	123.38	1.01	130.72	133.33	1.02

Second leaves: Table 19 includes the results of the investigation of net carbon accumulation in the second leaves. At four days after inoculation of the first leaf, when the second leaves of infected plants were usually smaller than those of healthy controls (p.40), the net accumulation in the second leaf of the carbon fixed by the plant in the 24-hour diurnal cycle per unit area was lower in infected than healthy plants, indicating therefore considerably lower net accumulation by the second leaf as a whole. By six days, however, the second leaves of infected and healthy plants showed similar net accumulation: the extensive infection developed on the first leaves appeared to have little effect on the second leaves. Livne (1964) obtained somewhat similar results in wheat seedlings of which the first leaves were infected with rust: he found little effect of infection on the photosynthesis of the second leaves.

1b. NET ACCUMULATION OF CARBON AFTER 24 HOURS: the resistant reaction.

First and second leaves: Infection appeared to have little effect on the net accumulation of carbon fixed in the previous 24 hours (Table 19) in the

first (inoculated) or the second (uninoculated) leaves of infected plants of C.I.5791 as compared with healthy controls.

2a. NET ACCUMULATION OF CARBON AFTER 16 HOURS' LIGHT: the susceptible reaction.

First leaves: The results of the experiments of 16 hours' duration in the light are shown in Table 20.

Table 20: Net accumulation of the products of CO_2 fixation in the 16-hour light period of the diurnal cycle on an area basis in the inoculated first leaves and the uninoculated second leaves of infected plants of Parkland and C.I.5791 as compared with healthy controls.

leaf number	days after inoculation	umoles carbon present / dm^2 after 24 hours					
		susceptible			resistant		
		H	I	I/H	H	I	I/H
1	4	103.31	89.88	0.87	118.90	108.20	0.91
	6	110.06	82.55	0.75	124.93	127.43	1.02
2	4	170.30	151.57	0.89	183.68	172.66	0.94
	6	190.11	174.90	0.92	190.74	186.93	0.98

The ratios of carbon accumulation after 16 hours' light in infected and healthy first leaves are very similar to those of 24 hours' accumulation, Table 19. At four days after inoculation, the carbon fixed in the 16-hour light period was slightly lower, and at six days, considerably lower, than that in healthy plants. At six days, the values of samples from infected plants were sometimes as low as 0.64 of healthy controls, though usually greater than 0.70 as shown (Table 20) by the average of three experiments.

After 16 hours' light, samples from both healthy and infected first leaves of Parkland contained higher radioactivity, and therefore more of

the carbon fixed by the respective plants in the light period, than equivalent samples after the 24-hour diurnal cycle.(Table 19), indicating net loss of carbon in the eight-hour dark period of the diurnal cycle: however, the losses from infected plants and those from healthy controls in the eight-hour dark period appeared to be much the same.

Second leaves: At four days after inoculation of the first leaves, the net gain of carbon by the second leaves after 16 hours' light was a little lower than that in healthy controls; however, by six days, samples from infected plants gave similar results to those from healthy controls (Table 20).

2b. NET ACCUMULATION OF CARBON AFTER 16 HOURS' LIGHT: the resistant reaction

First and second leaves: Infection did not significantly affect the net accumulation of the products of 16 hours' fixation in the light in the first (inoculated) and second (uninoculated) leaves of C.I.5791 (Table 20). It can be seen from a comparison of Tables 19 and 20 that, as in infected and control plants of Parkland, there was a net loss of carbon from plants of C.I.5791 in the eight-hour dark period of the diurnal cycle, of similar magnitude in infected and control plants.

3a. NET ACCUMULATION OF CARBON IN EIGHT HOURS' DARK: the susceptible reaction.

First leaves: Table 21 shows the average results of three experiments. At four days after inoculation, the net accumulation of the products of carbon fixation in the dark by infected leaves of Parkland was considerably higher than that of healthy leaves, and the differences were even more pronounced at six days after inoculation. The ratios of net carbon gain (I / H) from the two series of experiments at the different times in the diurnal cycle were fairly similar at both four and six days after inoculation, though on both days they were slightly higher in experiments 'D' conducted during the usual dark period of the diurnal cycle than in the experiments

Table 21: Net accumulation of the products of CO_2 fixation in eight-hour dark periods, 'D' coincident with the dark period and 'L' falling in the middle of the light period of the diurnal cycle, on an area basis in the inoculated first leaves and the uninoculated second leaves of Parkland as compared with healthy controls.

days after inoculation	time of dark period	umoles carbon present / dm^2 after eight hours					
		first leaves			second leaves		
		H	I	I/H	H	I	I/H
4	D	0.269	0.917	3.41	0.431	0.366	0.85
	L	0.344	1.118	3.25	0.415	0.324	0.78
6	D	0.233	0.983	4.22	0.467	0.406	0.87
	L	0.300	1.170	3.90	0.468	0.421	0.90

'L' conducted in the middle of the light period. It was apparent from the results that diurnal fluctuations in metabolism occurred in both healthy and infected leaves: during the usual dark period of the diurnal cycle 'D', both healthy and infected leaves showed a smaller net accumulation of the carbon fixed during the experiment than was shown in the dark period which fell in the middle of the light period of the diurnal cycle, 'L'. The diurnal fluctuations were more marked in infected than in healthy leaves; at four days after inoculation, the difference in the net carbon gain at the two experimental times was 0.075 umoles for healthy plants and 0.201 umoles for infected plants; at six days, the differences were 0.067 and 0.187 umoles respectively.

Second leaves: Table 21 shows the average results of three experiments and at both four and six days after inoculation, the net carbon accumulation of the products of fixation in the dark by the second leaves of

infected plants was lower than in healthy plants at both times 'D' and 'L' in the diurnal cycle, but the difference was never more than 25% (per unit area) even at four days after inoculation when the effect was more pronounced. The diurnal fluctuations in enzyme activity shown in the first leaves could not be demonstrated in the second leaves of either healthy or infected plants; however, the second leaves of healthy plants showed greater net accumulation of carbon than did the first leaves at both times in the diurnal cycle, while in infected plants the first leaves consistently showed the greater accumulation.

3b. NET ACCUMULATION OF CARBON IN EIGHT HOURS' DARK: the resistant reaction.

First leaves: The average results of three experiments are shown in Table 22.

Table 22: Net accumulation of the products of CO_2 fixation in eight-hour dark periods, 'D' coincident with the dark period and 'L' falling in the middle of the light period of the diurnal cycle, on an area basis in the inoculated first leaves and the uninoculated second leaves of C.I.5791 as compared with healthy controls.

days after inoculation	time of dark period	umoles carbon present / dm^2 after eight hours					
		first leaves			second leaves		
		H	I	I/H	H	I	I/H
4	D	0.285	0.465	1.63	0.468	0.473	1.01
	L	0.321	0.446	1.39	0.400	0.376	0.94
6	D	0.264	0.279	1.06	0.390	0.359	0.92
	L	0.314	0.363	1.16	0.428	0.462	1.08

At four days after inoculation, the net accumulation of the products of carbon fixation in the dark by infected leaves of C.I.5791 was significantly

greater than that of healthy leaves, though at six days there was little difference between the samples. At four days, the ratios of net carbon gain (I / H) from the experiments conducted in the usual dark period 'D' were higher than those from the experiments 'L' conducted in the usual light period. As in Parkland, diurnal fluctuations in metabolism were evident in some extracts of C.I.5791: both healthy and infected leaves at six days after inoculation showed lower net gain of carbon in experiments in the usual dark period 'D' than in experiments which fell in the light period 'L'. Infected leaves at four days after inoculation showed little difference in net accumulation in the two series of experiments.

Second leaves: Infection of the first leaves had little effect upon the net accumulation of the products of carbon fixation in the dark in experiments at either time in the diurnal cycle, as shown in Table 22. Second leaves of healthy plants of C.I.5791 and those of infected plants at six days after inoculation showed greater net carbon gain than the first leaves of these plants; carbon accumulation was very similar or somewhat lower in the second leaves of infected plants at four days after inoculation than in the first leaves of these plants.

SERIES 4.

$^{14}CO_2$ in air was fed to either the inoculated first leaves or the uninoculated second leaves of infected plants of Parkland and C.I.5791 and healthy controls. After 24 hours, both first and second leaves were assayed for radioactivity. The results have been computed as umoles carbon present after 24 hours per dm^2 as described for the experiments of 24 hours duration in Series 3 (p.102), assuming the CO_2 levels in the chamber to be constant between flushings of the chamber.

1a. TRANSLOCATION FROM THE FIRST TO THE SECOND LEAF: the susceptible reaction.

The results of a typical experiment are given in Table 23. In healthy plants, the radioactivity found in the second leaves per dm^2 ranged from 0.15 to 0.44 that found in the first leaves, but was usually ca. 0.35, as shown in the typical experiment. It must be emphasised that this does not indicate the fraction of the total CO_2 fixed in the first leaves translocated to the second, since the results are expressed per dm^2 and first and second leaves did not have the same surface areas; moreover, individual plants differed in the size of the second leaf over the experimental period, and the second leaves of infected plants were usually smaller than those of healthy plants of equivalent age.

Table 23: Net accumulation of the products of CO_2 fixation in the 24-hour diurnal cycle on an area basis in the inoculated first leaves and the uninoculated second leaves of infected plants of Parkland and C.I.5791 as compared with healthy controls when $^{14}CO_2$ was fed only to the first leaves.

days after inoculation	sample	umoles carbon present / dm^2 after 24 hours susceptible 1st leaf	susceptible 2nd leaf	susceptible 2/1	resistant 1st leaf	resistant 2nd leaf	resistant 2/1
4	H	43.45	16.51	0.38	40.35	14.17	0.35
	I	40.55	7.30	0.18	39.10	11.73	0.30
6	H	37.45	11.15	0.33	34.75	13.20	0.38
	I	31.00	2.45	0.08	36.80	14.68	0.40

In diseased plants, translocation from the first to the second leaf was much reduced so that at six days after inoculation there was apparently little movement of the products of carbon fixation in the 24-hour feeding

period out of the diseased leaf into the young second leaf. Livne and Daly (1966), working with bean plants, found similarly large reductions in the translocation of the products of fixation in the light from the older to the younger leaves when the older leaves were infected with rust: whereas the older leaves normally exported 50% of their fixation products to younger leaves, after the infection of the older leaves, export was negligible.

1b. TRANSLOCATION FROM THE FIRST TO THE SECOND LEAF: the resistant reaction.

The results of a typical experiment are given in Table 23. Infection apparently had little effect upon translocation from the first to the second leaf at either four or six days after inoculation.

2a. TRANSLOCATION FROM THE SECOND TO THE FIRST LEAF: the susceptible reaction.

The results of a typical experiment are shown in Table 24.

Table 24: Net accumulation of the products of CO_2 fixation in the 24-hour diurnal cycle on an area basis in the inoculated first leaves and the uninoculated second leaves of infected plants of Parkland and C.I.5791 as compared with healthy controls when $^{14}CO_2$ was fed only to the second leaves.

days after inoculation	sample	umoles carbon present / dm^2 after 24 hours susceptible			resistant		
		2nd leaf	1st leaf	2/1	2nd leaf	1st leaf	2/1
4	H	86.95	1.95	0.022	102.70	2.05	0.020
	I	67.70	1.55	0.023	90.00	1.55	0.017
6	H	158.70	4.30	0.027	172.29	2.05	0.012
	I	143.45	4.60	0.032	152.50	2.75	0.018

There was very little movement of the products of carbon fixation in the 24-hour experiment from second to first leaves in either healthy or infected seedlings of Parkland. Never more than 5% of the radioactivity present in the second leaves (per unit area) was recovered in the first leaves, and infection of the first leaves did not create a 'sink' for the products of fixation in the younger leaves. These results are in contrast to those of Livne and Daly (1966) who found that there was little movement from younger to older leaves in healthy bean plants, but that rust-infection of the older leaves greatly increased this movement.

2b. TRANSLOCATION FROM THE SECOND TO THE FIRST LEAF: the resistant reaction.

The results of a typical experiment are shown in Table 24. As in Parkland, there was very little movement of the products of the carbon fixation in the 24-hour experiment from second to first leaves in either healthy or infected seedlings.

SECTION III.

DISCUSSION.

There are obvious parallels between the effects of infection on total fixation in the light as observed in the experimental Series 1 (Table 13) and those on the specific activity of RuDP carboxylase observed *in vitro* (Fig. 1). In neither was infection at any time stimulatory (cf. other infections discussed on p.7); in the susceptible reaction, fixation in the light and the specific activity of RuDP carboxylase over the experimental period declined gradually to *ca.* 50% of the levels of controls, probably indicating the predominance of RuDP carboxylase-mediated carboxylation in the light in both healthy and infected tissue (as would be expected from the relative activities of RuDP carboxylase, PEP carboxylase and the malic enzyme *in vitro*); and in the resistant reaction both total fixation and the specific activity of RuDP carboxylase fell up to three or four days after inoculation and then reverted to the levels of controls. These parallels are consistent with the strong correlations between RuDP carboxylase activity and photosynthesis which have been documented in normal C_3 plants (Neales *et al.*, 1971).

Total fixation was probably estimated more reliably than the specific activity of RuDP carboxylase on a chlorophyll basis, since the leaf-disc samples upon which the chlorophyll estimates for the *in vivo* experiments were made did not suffer from the disadvantages of the one-gram samples of leaf material used in the *in vitro* experiments (Section II, Discussion). However, a slight overestimate of the chlorophyll content of infected tissue may have resulted from the shrinking of such

tissue with resulting inaccuracies in the leaf-disc samples: the data (Table 13) indicate that the efficiency of fixation was maintained until at least the fifth day after inoculation, while the activity of RuDP carboxylase on a chlorophyll basis may have been maintained longer. It was impossible to deduce from these experiments whether the eventual drop in efficiency of fixation in the light *in vivo* was due to induced defects of the light-harvesting mechanism or to disruption of the enzyme steps in carboxylation, but it would appear that part of the photosynthetic mechanism other than the initial RuDP carboxylase-mediated step was first deleteriously affected by infection.

The increases in CO_2 fixation in the dark upon infection may indicate that the increases noted *in vitro* in the specific activities of PEP carboxylase and the malic enzyme are functional *in vivo*. Non-photosynthetic fixation usually reached levels *ca*. 250% those of controls; this increase was more than that usually shown in the specific activity of PEP carboxylase (*ca*. 200%, Fig. 4) and less than that usually shown in the specific activity of the malic enzyme (*ca*. 300%, Fig. 6). However, the specific activities of these enzymes as measured *in vitro* were very different: that of PEP carboxylase was at least five-fold that of the malic enzyme.

The *in vivo* fixation per minute in the dark measured in these experiments (Table 15) was only *ca*. 0.5% that of fixation per minute in the light (Table 13), whereas, *in vitro*, the relative activities of PEP carboxylase and the malic enzyme were *ca*. 20% and 4% respectively that of RuDP carboxylase. While the experiments in light and dark *in vivo* are not stricly comparable because of their different durations (15 and 60 minutes respectively), it would nevertheless appear that the *in vitro* studies do not necessarily give a realistic estimate of *in vivo* activity,

where the availability of substrates, co-factors etc. may be of greater significance than specific activity; but it is also conceivable that β-carboxylation levels measured in the dark do not give a reliable indication of the capacity of the plants for β-carboxylation when substrates such as PEP and pyruvate and reduced co-factors such as NAD(P)H are more readily available, i.e., in the light. It is even conceivable that the CO_2 of photorespiration would be readily available for re-fixation in vivo by the non-photosynthetic reactions, especially that mediated by PEP carboxylase, as has been proposed to account for the apparent lack of photorespiration in C_4 plants (Osmond, 1971); and the enhanced respiration in the host-parasite complex could also generate an increased supply of the metabolites necessary for β-carboxylation in infected tissue. Since the overall fixation in the dark in infected tissue peaked before sporulation of the parasite (as did malic enzyme activity), it is likely that maximal enhancement from infection-induced changes in metabolism could occur only until the tissue was so disrupted by the presence of the parasite that efficient metabolism was impossible (pp.69 & 80).

Total fixation in the dark through the infection cycle in C.I.5791 paralleled the changes in the β-carboxylation enzymes in vitro in that after an initial increase in fixation which lasted until about three days after inoculation and was correlated with the formation of pin-point lesions, fixation reverted to the levels of controls, apparently corresponding with the cessation of fungal growth. The stimulation of metabolism may, therefore, have been a function of active interaction between host and parasite.

The changes in fixation in the light and the dark in B.T.201 (Table 17) were closely correlated with the degree of development of disease

symtoms in the infected plants. The results of the experiments in the light with B.T.201 support the concept of a relationship between loss of chlorophyll and chloroplast disruption in diseased tissue and the decreases in fixation as infection progressed; the more extensively the tissues were damaged, the greater the decline in the fixation of CO_2 in the light. The increases in CO_2 fixation in the dark would be consistent both with a greater response of the host to more widespread pathogenesis, and a greater stimulation of either the β-carboxylation enzymes (at the operational or synthetic level) by some metabolic product of either the fungus per se as it develops in the infected tissue or by some product of its interaction with the host.

That the changes in CO_2 fixation in the light and dark which have been documented upon infection of barley with H.teres are in part a general response of the host to injury is apparent from the results of experimental Series 2, when a loss of capacity for CO_2 fixation in the light and a slightly enhanced CO_2 fixation in the dark were observed in bleached and mechanically injured leaves (Tables 16 and 17). The extent to which CO_2 fixation in the light was reduced by injury was directly correlated with the amount of chlorophyll loss, and probably the result of direct damage to the chloroplasts, since no effect was produced in undamaged areas of treated leaves. The effects of infection upon carbon fixation in the light would seem, therefore, at least in the susceptible reaction, to be more specific than those of damage, in that they were exerted outside the immediate vicinity of the fungus; and fixation in the light was substantially decreased before widespread tissue death occurred. The effects of infection on fixation in the light in the resistant reaction were probably more comparable to those of damage, in that discrete areas of tissue were rapidly killed, and a temporary loss of capacity for the

fixation of CO_2 resulted, which was more or less proportional to the loss of chlorophyll; however, the tissue damage by infection was not sufficiently widespread to cause a permanent reduction in the photosynthetic capacity of infected leaves.

The responses of leaves to damage as measured by their fixation in the dark was a little different from the response in the light in that the 'necrotic' leaves, of which a smaller surface area was damaged than in bleached leaves, showed a greater response than the 'chlorotic' leaves. This would seem to indicate that the response was in the living tissue at the periphery of the damaged areas rather than in the damaged areas themselves, the effect thus being greater in leaves with numerous small lesions than in leaves with few large lesions. The stimulation caused by infection of susceptible leaves with H.teres cannot, then, be accounted for merely as a general response to damage, since not only is the effect more widespread in the leaves, being exerted in uninvaded tissue, but it is greater in leaves with larger lesions and more extensive damage than it is in leaves with numerous small lesions, and the stimulation increases until extensive tissue death occurs. It could, however, be the result of a more specific host response to the special stress of pathogenesis and is not necessarily mediated by the pathogen per se.

In 24-hour experiments, with 16 hours' illumination, the net accumulation of the products of CO_2 fixation in the infected first leaves of Parkland was more similar to that in healthy leaves at both four and six days after inoculation (Table 19), than might be expected from the relative rates of fixation of CO_2 in the light estimated in 15-minute experiments (Table 13) or from the effects of infection on the main photosynthetic enzyme, RuDP carboxylase (Fig. 1). The similarity in the net accumulation is especially note-worthy since the rates of respiration of

infected tissues are greater than those of healthy tissues (p.20), at least in the dark (the condition under which respiration *in vivo* in host-parasite complexes is usually measured); although, since the significance of photorespiration has been realised and the suggestion made (Beevers, 1971) that normal (i.e.'classical') respiration may be largely repressed in green plants in the light, it may be pertinent to reasses the overall differences in respiration rates in parasitised and healthy tissue in the light period of the 24-hour diurnal cycle. If, however, there is in fact a net increase in the respiration of infected tissue over the 24-hour diurnal cycle, then at least some of the respiratory substrate might be expected to come from carbon fixed in that period, with consequently reduced net accumulation of carbon in the infected tissue.

Since the relative rates of fixation in healthy and infected leaves in the 16-hour light period (Table 20) were very similar to those after the 24-hour period, the unexpectedly high net accumulation in infected tissue after 24 hours was obviously not due to the enhanced fixation in the dark period indicated by the increased activity of the β-carboxylation enzymes (Table 21) and the one-hour experiments in the dark (Table 15). Indeed, though fixation in the dark period in infected first leaves was three to four times that in healthy leaves (Table 21), the net accumulation of products was lower after 24 hours than after 16 hours light in both infected and healthy first leaves, so that there was net loss of carbon in the dark period of the diurnal cycle. Since this loss was no greater in infected than in healthy tissue, the substrates for the increased respiration which can be assumed in infected tissue in the dark must have come either from some source other than that of carbon fixed by the leaves

in the preceding 16-hour light period, or else from carbon fixed in the preceding light period normally exported by healthy leaves but retained by infected leaves (Table 23). The amount of carbon lost from the first leaves during the dark period, as estimated from the differences in net accumulation after 16 hours and 24 hours (Tables 19 and 20), was very much greater than net accumulation in the dark (Table 21); while the gross carbon fixation in the dark might have been considerably higher than the net carbon accumulation, it seems probable that most of the loss in the dark period was of the carbon fixed in the preceding light period.

Another possible explanation for the unexpected similarity in net accumulation in infected and healthy first leaves could be an increased non-photosynthetic fixation of CO_2 in the light in the infected material. That this could not be due to differences in the diurnal fluctuations of the β-carboxylation enzymes in infected and healthy tissue was shown (Table 21). However, in the light, the increased availability of substrates and co-factors for β-carboxylation might result in substantially higher activity than that indicated by the experiments in the dark (Tables 15 and 21). That the β-carboxylation enzymes have a higher capacity for the fixation of CO_2 than that shown in the dark *in vivo* (Tables 15 and 21) when substrates and co-factors are non-limiting was shown *in vitro*: PEP carboxylase and the malic enzyme fixed 20% and 4% respectively of the CO_2 fixed by RuDP carboxylase per minute per milligram protein, whereas *in vivo* (as discussed on p.114) fixation in the dark was only 0.5% that in the light. However, neither the results of the *in vitro* enzyme experiments nor the one-hour *in vivo* experiments (Table 15) can fully account for the three- to four-fold increase in net accumulation in infected as compared with healthy tissue in the dark; it seems probable that this increase was due in part to the retention of some products of carboxylation in the infected first leaf which would be removed by translocation in the healthy leaf (Table 24).

A direct relationship between the induced increase in respiration and that in β-carboxylation in infected tissue is conceivable. The increased respiration would generate intracellular CO_2 and reduced co-factors which could perhaps be utilised by the non-photosynthetic carboxylation enzymes: this might offset the expected substrate depletion by the increased respiration. If the process of photorespiration, by which more than 25% of the carbon fixed in photosynthesis in healthy leaves may be subsequently evolved (Atkins et al., 1971), were disrupted in infected tissue, less carbon would be lost by this route. Such a disruption might be suggested by the decrease of glycolic acid oxidase, an enzyme currently thought to be of major importance in photorespiration (Beevers, 1971), in some infected tissues (Kiraly and Farkas, 1957a; Sanwal and Waygood, 1963). Not only would greater accumulation of the products of photosynthesis result from an inhibition of photorespiration, but also intermediates normally turned over by this route, such as sugar diphosphates (Bassham and Kirk, 1964; Gibbs, 1971) or serine (Atkins et al., 1971), might become available as precursors of the substrates of the enzymes of β-carboxylation or even contribute directly to the nutrition of the parasite.

Another possible source of carbon for the infected first leaves is translocation from uninfected parts of the plant. It is clear from the results of the experiments in Series 4 that the infected first leaves did not act as 'sinks' for the products of 24 hours' fixation in the second leaves (Table 24), unlike certain obligate parasite infections as discussed on p.17. However, export from the infected leaves was decreased by ca. 50% at four days, and ca. 75% by six days after inoculation (Table 23). From Table 23, it can be deduced that the net accumulation in the

the infected first leaf at six days after inoculation would have been only ca. 21 umoles per unit area if the levels of export of the controls had been maintained, resulting in an I / H ratio for net accumulation in the first leaves in these experiments of 0.57 rather than the value of 0.83 actually obtained. Thus, even though the calculations are necessarily only approximate (p.110), it is highly probable that much of the unexpectedly high net accumulation of carbon in infected first leaves in comparison with controls over the 24-hour diurnal cycle is the result of reduced export of carbon.

In all the various series of experiments in vivo and in vitro, the results obtained with second leaves were variable, probably owing to variation in the stage of development of the second leaves of individual plants, with this variability being exacerbated by infection throughout the experimental period. However, some general conclusions can be drawn. Despite the usually lower activity of RuDP carboxylase in second than in first leaves of all plants (p.64), the net accumulation in the second leaves of all plants was greater than that in first leaves (Tables 19 and 20), probably as a result of the accumulation of translocated carbon from the first leaves. At four days after inoculation, in the 24-hour, 16-hour and eight-hour experiments (Tables 19, 20 and 21), infection of the first leaves of Parkland resulted in reduced accumulation in the second leaves, with translocation to the second leaves also reduced (Table 23); accumulation in the second leaves at six days after inoculation was more similar to healthy controls, even though translocation from the first leaves was further reduced (Table 23). It seems possible that the second leaves are more dependent on the first leaves as a source of carbon in the very early stages of their development than in the later stages; when less carbon is forthcoming from the first leaves as a result

of infection, development of the second leaves and perhaps their systems of carbon fixation are retarded but not prevented (p.78) and a stage is reached, though later than in healthy plants, after which the leaves are largely self-sufficient. It is even conceivable that debilitation of the first leaves and reduced amounts of carbon coming from them might result in a compensatory increased capacity for fixation in the second leaves once a certain degree of maturity was achieved: Neales et al. (1971) have shown that partial defoliation of bean plants leads to increased photosynthesis and delayed senescence in the remaining leaves, and infection of first leaves with consequent reduced translocation of products to the rest of the plant could perhaps exert a similar effect. It is evident that the products of H.teres infection do not directly affect the second leaves: fixation in the light was not increasingly reduced by the developing infection of the first leaves, nor was fixation in the dark stimulated. No evidence was shown in vivo of the enhanced activity of PEP carboxylase sometimes shown in vitro in the second leaves (Table 8), indicating perhaps that the effect on PEP carboxylase was, as with RuDP carboxylase and the malic enzyme (Section II, Discussion), the result of a general disturbance of plant metabolism due to infection rather than specifically the result of infection.

After long-term experiments (Table 19), the net accumulation of carbon in first leaves of C.I.5791 was less affected by infection than might have been expected from the results of the in vitro examination of the carboxylation enzymes (Table 13) and the short-term experiments (Table 15). From the latter experiments, it might have been predicted that resistant plants would show effects of infection somewhat similar to the susceptible reaction at four days after inoculation, but that these effects would have largely disappeared at six days after inoculation.

Though after 24 hours' metabolism in air containing $^{14}CO_2$, net accumulation in both first and second leaves of infected plants was very similar to that in healthy plants, results similar to those predicted were obtained in the 16-hour experiments in the light (Table 20), where net accumulation in both first and second leaves of infected plants was somewhat lower, and in the eight-hour experiments in the dark (Table 22), where net accumulation in the first leaves of infected plants was significantly higher, than in healthy controls. It is conceivable that over the 24-hour diurnal cycle (Table 19), the various effects of infection were such that there was little net effect on carbon accumulation: export by the infected first leaves was perhaps sufficiently reduced (Table 23) to compensate for the loss of photosynthetic capacity (Tables 6, 13 and 20); and a positive contribution of carbon by β-carboxylation processes as proposed for the susceptible reaction is also conceivable. The lack of, or slight, net effect in the resistant reaction could be merely a result of resistance, but could conceivably contribute towards resistance, the balance of the various effects on metabolism being such that the effects of fungal infection were negated.

The resistant reaction appeared to be limited to the first (inoculated) leaves; not only was the net accumulation of carbon in the second leaves in light and dark unaffected by infection of the first leaves, but there was apparently no increase in translocation from the second to the first leaves after infection, which might have indicated a contribution to the first leaves of some metabolite or growth factor whose increased concentration in infected tissue was part of the resistant reaction.

SECTION IV: PATH OF CARBON IN FIXATION IN THE LIGHT.

The infected first leaves of Parkland and C.I.5791 at one, three and five days after inoculation, and healthy controls, were exposed to $^{14}CO_2$ in air for ca. two seconds (experimental Series 1) and sixty seconds (experimental Series 2). The water-soluble compounds were then extracted from the leaves and fractionated using ion-exchange resins and thin-layer electrophoresis; the radioactive products were further analysed using chromatographic techniques.

For comparative purposes, a study was made of the early products of carbon fixation in rusted barley. Parkland was resistant, and C.I.5791 susceptible, to P.graminis tritici. Infected first leaves of both varieties at seven, and 14 or 16 days after inoculation, and healthy controls, were exposed to $^{14}CO_2$ in air for ca. two seconds, followed by the extraction and analysis of products by the methods outlined for leaves infected with H.teres.

MATERIALS AND METHODS.

SERIES 1: ca. two seconds' exposure to $^{14}CO_2$.

A flat-bottomed Pyrex test-tube of 1.7 cm internal diameter and 11.9 cm length was used for the exposure of single leaves to $^{14}CO_2$ in air. It was clamped vertically in a fume hood at room temperature (ca. 25°C) with incandescent lamp beams directed onto it to give an internal light intensity of ca. 900 f.c., the air in the fume hood being circulated by a fan to maintain a constant temperature. Immediately before their individual exposure to $^{14}CO_2$, leaves were cut with their bases under water; cotton thread was passed through the base of each leaf.

In early experiments, a few drops of 2.0 N HCl were pipetted into the bottom of the Pyrex tube, and the leaf was then freely suspended by

the cotton thread inside the tube from a clamp positioned above the tube (Plate 15A). 2 uCi of $NaH^{14}CO_3$ were pipetted into the tube, and after two seconds, boiling 80% methanol was poured into the tube over the lamina of the leaf until the tube was full in order to stop metabolism. Using a similar method, Hess and Tolbert (1966) found that metabolism in whole tobacco leaves was halted ca. two seconds after the addition of the methanol. The contents of the tube was then poured into a 100 ml reflux condenser flask and further extracted (p.129). This will be referred to as the 'hot methanol method'.

In later experiments, for the reasons discussed on pp.145-151, a second method for short-term exposure of leaves to $^{14}CO_2$ was developed. In this method, to be termed the 'liquid nitrogen method', the leaf was not suspended in the exposure tube; first, 2 uCi of $NaH^{14}CO_3$ were pipetted into the tube and the $^{14}CO_2$ generated with a few drops of 2.0 N HCl, and then the leaf, suspended from the cotton thread, was dipped manually into the tube for ca. two seconds before being plunged into liquid nitrogen (Plate 15B), where it was held until further extraction in the cold (p.129).

Experiments with rusted barley: Standard methods were used for the growth of the plant material (p.31), and spores for inoculation were obtained from Dr. G. F. Green, C.D.A., Winnipeg. As in H.teres infections, plants were inoculated at seven days after planting, when the first leaves were fully expanded. Spores of P.graminis tritici were spread over the dry first leaves of Parkland and C.I.5791 with a small sable-hair brush; control seedlings were brushed without spores. Plants were then sprayed with water containing one drop of Tween 20 per 100 ml and kept in a humid chamber in the greenhouse (p.32) for 48 hours. Experiments with C.I.5791 (susceptible to the rust) were conducted at seven and 14 days, and those with Parkland

PLATES 15A and B: The exposure of single leaves to $^{14}CO_2$ in air for ca. two seconds:
A: 'hot methanol method' B: 'liquid nitrogen method'

(resistant to the rust) at seven and 16 days, after inoculation: at seven days, the leaves of both varieties bore chlorotic spots , and uredosori were beginning to form, the earliest uredospores being evident; at 14 days in C.I.5791, large coalescing lesions were present and uredosori were well developed and vigorously producing uredospores (reaction type 3); and at 16 days in Parkland, discrete lesions were present, with small uredosori formed at the centres of the chlorotic areas (reaction type 1 to 1+). Leaves were exposed to $^{14}CO_2$ in air for ca. two seconds as in the experiments with H.teres where leaves were dipped manually into the exposure tube, and extracted by the 'liquid nitrogen method'.

SERIES 2: sixty seconds' exposure to $^{14}CO_2$.

Infected first leaves were cut with their bases under water from four seedlings of Parkland or C.I.5791, and cotton thread was passed with a needle through the bases of the four leaves. The upper laminae were blotted dry, and the leaves were placed in a shallow dish containing water, with a similar group of four leaves of healthy control seedlings, in the exposure chamber described on p.83, as shown in Plate 16. Beakers of water were included in the chamber and the leaves were allowed to equilibrate under the experimental conditions of light intensity 900 f.c. and temperature 25^{o}C as described on p.85 for 20 to 30 minutes. After the equilibration period, 20 uCi of $NaH^{14}CO_3$ were pipetted into a small petri dish which was then placed in the chamber under port 1 (Fig. 7). At zero time, a few drops of 2.0 N HCl were pipetted into the petri dish through port 1, as in Plate 11, and the leaves were allowed to fix $^{14}CO_2$ in air for 60 seconds. The two groups of four leaves were then quickly removed from the chamber and plunged into liquid nitrogen where they were held until further extraction in the cold.

PLATE 16: Sixty seconds' exposure of excised first leaves (in two groups of four) to $^{14}CO_2$ in air in the exposure chamber.

A. EXTRACTION OF PLANT MATERIALS: 'hot methanol method'.

Leaves which had been exposed to $^{14}CO_2$ in air for ca. two seconds were individually refluxed in the 80% methanol in which they had been killed for ten to 15 minutes. The leaf residues were further refluxed for ten minutes in each of three solutions: 80% methanol, 40% methanol (Wang, 1960) and distilled water, to flush the tissues of all water-soluble compounds. Each residue was then ground with sand in a mortar and transferred to a scintillation vial in 0.25 ml water; scintillation fluid and thixotropic gel powder were added (p.87) and the radioactivity was measured. The methanol and water extracts were pooled and then fractionated with chloroform to remove chlorophylls, carotenoids and lipids. The methanol and chloroform fractions were each washed twice with chloroform and 80% methanol respectively, and both fractions were then dried in a stream of cold air. The chloroform fraction was redissolved in 1.0 ml chloroform, and 0.1 ml of this was added to 10.0 ml scintillation fluid and assayed for radioactivity. The dried methanol-water extract was stored at -10°C for further analysis.

B. EXTRACTION OF PLANT MATERIALS: 'liquid nitrogen method'.

Leaves held under liquid nitrogen after exposure to $^{14}CO_2$ were extracted by a modification of the method of Bieleski and Turner (1966). Each leaf was ground under liquid nitrogen with sand in a mortar and the resulting suspension poured into a 25 ml glass vial. When the liquid nitrogen had evaporated, a few millilitres of a solution of methanol: water: chloroform v/v 12 : 5 : 3 (MCW) at -10°C were added. The sample could be stored in this form at -10°C until required. Upon further extraction, the suspension was shaken for one minute, then centrifuged at 15,000 g for ten minutes. The residue was washed twice with MCW, the three MCW supernatants being pooled, and the residue was then assayed for radioactivity using

thixotropic gel powder as before (p.87). Chloroform was added to the pooled MCW extract in the ratio 1 : 12 and water in the ratio 1 : 8 (v/v) and the two phase mixture separated using a pasteur pipette. The chloroform fraction was washed twice with a few millilitres of 80% methanol, which were then added to the methanol-water extract; and the methanol-water fraction was washed twice with a few millilitres of chloroform, which were then added to the chloroform extract. The fractions were dried in a stream of cold air. The chloroform fraction was assayed for radioactivity as before (p.129) and the dry methanol-water extract stored at -10^{o}C for further analysis.

C. FRACTIONATION OF METHANOL-WATER EXTRACTS (from both extraction methods).

Water-soluble products were separated into their basic, acidic and neutral fractions by the use of two ion-exchange resins, essentially by the method of Wang (1960), and the acidic fraction further separated using thin-layer electrophoresis. Before and after every fractionation step, samples of the extracts were taken and their radioactivity assessed by liquid scintillation (p.35).

Preparation of resins: The resins used were Dowex 50 W-X8 (H^{+}; 200 - 400 mesh) and Dowex AG 1-X10 (Cl^{-}; 200 - 400 mesh) supplied by Bio-Rad Laboratories, Richmond, California. Both were normally prepared in bulk and stored in distilled water at 3^{o}C. They were first treated to remove large and fine particles according to the procedure of Atkins and Canvin (1971): this allowed a more closely-packed column with regular internal flow. About 100 gm of each resin was suspended in three volumes distilled water, stirred vigorously and immediately decanted into a second container: this was repeated three times to remove heavier particles. The suspension was allowed to stand until most of the resin beads had settled, and then the upper suspension of fines was decanted and discarded: this was repeated

four or five times. Dowex 50 W-X8 (H^+) was then treated several times with 6.0 N HCl to ensure that it was charged with H^+ ions, and then subsequently washed several times with water. Each resin was slurried into a glass burette of 1.0 cm internal diameter and 12 - 13 cm length which had been plugged with glass wool, to give columns of height 8.0 cm. Columns of Dowex 50 W-X8 (H^+) (Column 1) were washed until neutral and were then ready for use. Columns of Dowex AG 1-X10 (Cl^-) (Column 2) were treated with 1.0 M sodium formate until the effluent was free of Cl^- ions (as tested with acidified silver nitrate) to convert the resin to the formate form: these were then rinsed with one to two bed-volumes of 1.0 N formic acid and washed with water until neutral.

Column fractionation: A similar procedure was followed for all extracts. The extract was dissolved in 1.0 ml distilled water and loaded onto Column 1; acidic and neutral substances were not adsorbed and were washed through the column with 50 ml water. The basic substances, essentially amino acids, remained on the column and were eluted with 25 ml 1.0 N HCl followed by 15 ml 6 N HCl and 15 ml water. Both fractions were dried in a stream of cold air; the basic fraction was redissolved and re-evaporated to get rid of any traces of HCl.

The dried effluent from the first column was dissolved in 1.0 ml water and loaded onto Column 2. Neutral substances, mainly sugars, were washed through with 50 ml distilled water; acidic substances, essentially organic acids and phosphate esters, were retained on the column and eluted with 25 ml 1.0 N formic acid followed by 15 ml 6.0 N formic acid and 15 ml water. Both fractions were then dried as before.

At least 85% of the initial radioactivity in samples fractionated as above was recovered in the three resulting fractions: hence no corrections for loss of radioactivity were considered necessary in assessing the activity residing in each fraction.

Separation of organic acids and phosphate esters: An attempt was made to adapt the column purification method for phosphate esters described by Bieleski and Young (1963) to the separation of organic acids and phosphate esters. Cellex P (Bio-Rad Laboratories) was prepared in the H^+ form by their method and the combined eluate from Column 2 washed through it. Phosphate esters passed through the column while organic acids were retained, and these were then eluted using 6.0 N HCl followed by 2.0 N HCl and finally water. The organic acid fraction could be adequately chromatographed, but good separation of the phosphate ester fraction could not be obtained with the effluent: it was therefore passed through a column of Cellex D (Bio-Rad Laboratories) in the HCO_3^- form, prepared as described by Bieleski and Young (1963) for further purification. Good elution from the latter column was achieved with 15% ammonium bicarbonate, but this salt could not be completely removed from the eluate with the apparatus available and it interfered greatly with subsequent chromatography.

A more satisfactory method for the separation of organic acids and phosphate esters was developed which involved thin-layer electrophoresis. In their work on the fractionation of plant extracts by thin-layer electrophoresis, Cook and Bieleski (1969) described a method for the separation of amino acids from total extracts as follows: the total extract was spotted on a mixed cellulose-silica gel layer plate in a band; the plate was sprayed with a buffer consisting of 17 ml 90% formic acid and 57 ml acetic acid per litre, pH 2.0, and electrophoresis was carried out at 1000 V, 25 mA at 15^0C for 25 minutes. The amino acids moved towards the cathode, and the authors observed that phosphate esters were lost to the anode compartment, while organic acids and sugars remained at or near the origin, slight shifts being due to electrosmosis and buffer flow. It

seemed from their work that, if the fraction containing phosphate esters were spotted near the cathode, the organic acids should remain near the cathode in electrophoresis while the phosphate esters ran towards the anode. Mixtures of known organic acids and phosphate esters were spotted on M & N cellulose plates, and subjected to electrophoresis using equipment as described on p.137 for various lengths of time using the buffer and voltage systems as described above. When the sample was streaked 5.0 cm from the edge of the plate at the cathode and the current run for 45 minutes, good separation of organic acids and phosphate esters was obtained (Fig. 10), though some drifting of the origin occurred: the organic acid band diffused up to 2.0 cm in the direction of the anode. The method was subsequently used for the radioactive samples, and after electrophoresis, the plates were autoradiographed for a few days to ascertain the position of the origin (Plate 17). To obtain the organic acid fraction, the origin was scraped off as a roll of thin-layer adsorbent with a portion of razor blade and transferred to a small glass column plugged with glass wool, the column being tapped gently to settle the contents during loading; the adsorbed organic acids were then eluted with aqueous 10% isopropanol and the eluate dried. For the phosphate ester fraction, the area of the plate between the origin and the anode was scraped, eluted and dried as above. Total recovery of radioactivity in organic acids and phosphate esters after separation by this method was always greater than 90% of the activity of the combined extract before spotting.

D: CHROMATOGRAPHIC AND ELECTROPHORETIC METHODS.

Preparation of plates: Prior to use, 20 x 20 cm thin-layer plates were soaked in dichromate-H_2SO_4 cleaning solution, scrubbed with scouring cleanser, and rinsed in distilled water. The standard layer powders were

FIG. 10.

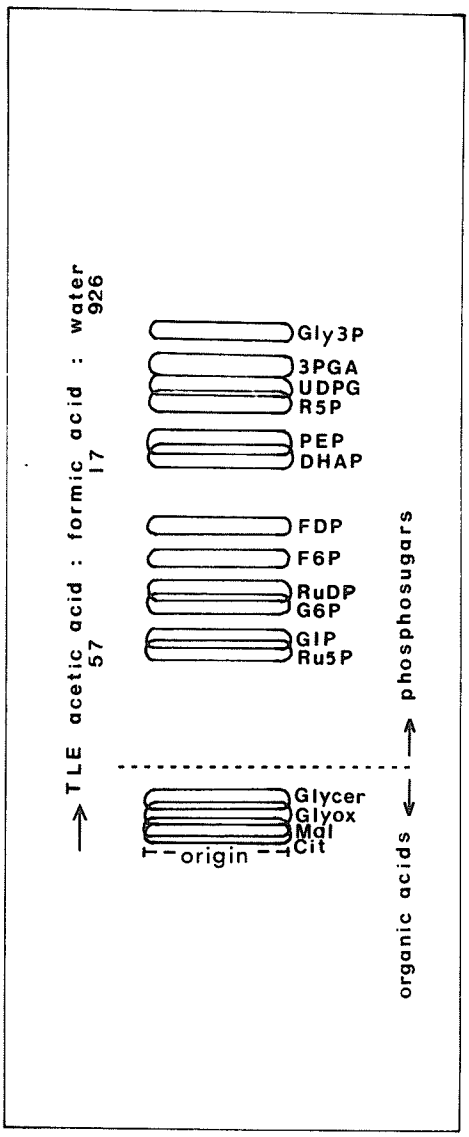


FIG. 10: Diagram of a typical separation by TLE of a mixture of authentic samples of organic acids and phosphate esters.

PLATE 17.

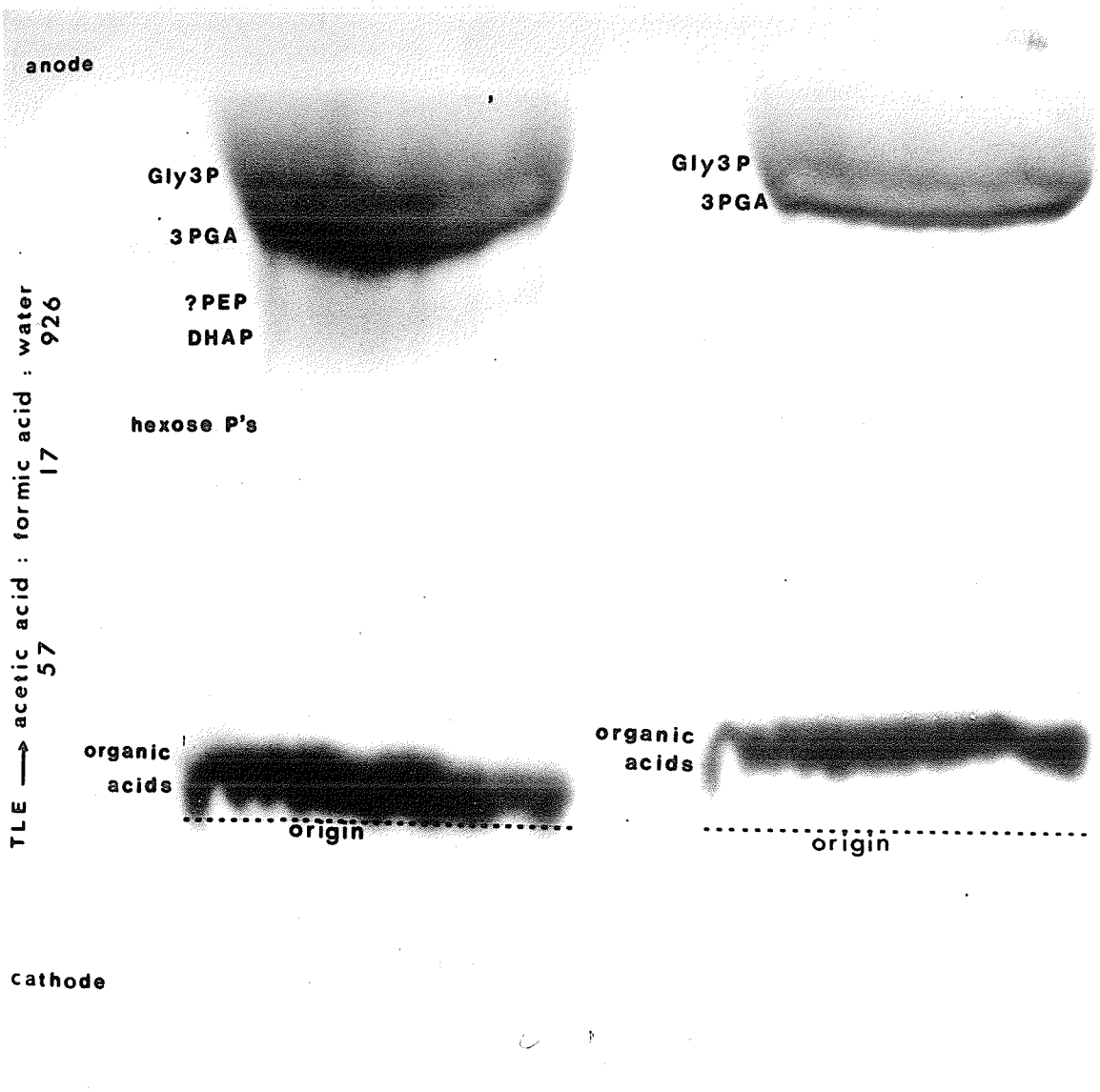


PLATE 17: Autoradiograph of a typical separation by TLE of the organic acid and phosphate ester fractions from extracts of first leaves of Parkland and C.I.5791 after exposure to $^{14}CO_2$.

Cellulose M & N 300 without binder (Macherey, Nagel & Co., Duren, Germany) and Silica Gel G (Merck, from Kensington Scientific Corp., California): different mixtures of these were used for the separation of each group of compounds. The powder mixtures were homogenized in 90 - 100 ml water prior to spreading with Desaga (Heidelberg) spreading equipment. Layers were always 250 mu thick. Extracts were routinely spotted in a 2.5 cm band, 2.5 cm from the edges of the cellulose in the bottom left-hand corner of the plate. Individual compounds separated as bands in chromatography or electrophoresis in the first dimension; the bands were converted into spots by elution with distilled water before chromatography in the second dimension (Cook and Bielseki, 1969). Amino acid plates were eluted through the entire layer so that the spots were formed at the left side of the original band and the layer was washed. All other plates were eluted from the left side so that spots were formed at the right side of the original band.

Analysis of standards: The two-dimensional thin-layer electrophoretic and chromatographic procedures used in the analysis of each fraction, of which the descriptions follow, were standardised using samples of known compounds. The samples were run individually in each dimension separately, and thus values for R_{Asp} for the amino acids, R_{Gluc} for the sugars, R_{Mal} for the organic acids and R_{Pi} for the phosphate esters in both directions were obtained. These values were relatively constant, though there was minor variation in the R_F values. The chromatographic separations of authentic samples of the four groups of compounds are shown in Figs. 11 - 14. For maximal separation, the solvent front was allowed to rise 16 - 17 cm above the origin in each dimension before chromatography was interrupted; the times given for chromatography in each solvent in the following sections are therefore approximate.

When extracts from experimental Series I and II were analysed, relatively small aliquots of each fraction were used in chromatography because better resolution of the radioactive products of $^{14}CO_2$ fixation was obtained when the total concentration of compounds (radioactive and non-radioactive) was low.

Amino acid fraction: The method used was a modification of that of Bieleski and Turner (1966). Amino acids were separated on a mixed cellulose-silica gel layer: the spreading mixture contained 12.5 gm cellulose powder and 5.0 gm silica gel powder in 100 ml water. Aliquots of amino acid fractions containing ca. 5,000 d.p.m. were spotted on plates in bands as described above. The amino acids were separated in the first dimension by electrophoresis, using equipment supplied by Savant, New York, at 1000 V, 25 mA at 15°C for 25 minutes, in a buffer containing 17 ml 90% formic acid and 57 ml acetic acid per litre, pH 2.0. Wicks were made of 20 x 8 cm strips of Miracloth, with the edge adpressed to the layer enclosed in a pieces of dialysis-tubing slit and folded over the Miracloth; the wick unit was presoaked in buffer before use. The amino acids moved as discrete bands towards the cathode. The plates were chromatographed twice in the second dimension, first for ca. one hour in methyl ethyl ketone : pyridine : water : acetic acid 70 : 15 : 15 : 2 v/v to separate threonine and glutamic acid; and then for ca. four hours in n-propanol : water : n-propyl acetate : acetic acid : pyridine 120 : 60 : 20 : 4 : 1 v/v for the separation of the amino acids.

Sugar fraction: The method used was derived from that of Cook and Bieleski (1969). The cellulose powder from which the layer used in the separation of sugars was made was washed prior to spreading according to the method of Redgewell and Bieleski (1967) as follows: 33 gm cellulose powder were slurried in 175 ml methanol and poured into a Buchner funnel over two layers

of Whatman No. 1 filter paper and filtered; the filtrate was recycled through the funnel twice so that the 'fines' were retained, and further washes (6 x 50 ml methanol; 6 x 50 ml n-propanol; 3 x 50 ml 1% acetic acid; 4 x 50 ml water) were followed by a final wash in methanol to dehydrate the cellulose, which was then dried at 30°C. The spreading mixture contained 15 gm of the washed cellulose and 90 ml water, and the plates were aged one week after spreading to ensure layer stability. Aliquots of sugar fractions containing ca. 5,000 d.p.m. were spotted on the plates, and chromatographed in the first direction for ca. three hours in n-propanol : water : n-propyl acetate : acetic acid : pyridine 120 : 60 : 20 : 4 : 1 v/v. They were then chromatographed in the second dimension for ca. two and a half hours in n-butanol : acetic acid : water 12 : 5 : 3 v/v.

Phosphate ester fraction: The method used was that of Waring and Ziporin (1964). The spreading mixture for phosphate ester separation consisted of 15 gm cellulose powder in 100 ml water, and the plates were activated before use at 105°C for two hours. Aliquots of phosphate ester fractions containing ca. 8,000 - 10,000 d.p.m. were spotted, and the plates chromatographed for ca. seven hours in the first dimension in the lower phase from a separated mixture of 60 ml tert-amyl alcohol, 30 ml water and 2.0 gm p-toluenesulphonic acid. Chromatography in the second dimension took place for ca. four hours in isobutyric acid : ammonium hydroxide : water 66 : 33 : 1 v/v.

Organic acid fraction: The method was derived from that of Ahmed (1968). The spreading mixture for the layer consisted of 15 gm cellulose in 100 ml water. Aliquots of the organic acid fraction containing ca. 5,000 d.p.m. were spotted on the plates, which were then chromatographed in the first dimension for ca. four hours in a solvent consisting of n-propanol :

ammonium hydroxide : water 6 : 3 : 1 v/v, and for ca. one and a half hours in the second dimension in propyl acetate : formic acid : water 11 : 5 : 3 v/v.

E. DETECTION OF COMPOUNDS BY SPRAY PROCEDURES.

Amino acids: A ninhydrin solution consisting of 0.2% ninhydrin in acetone was used. After spraying, the plates were heated at 105°C for ca. five minutes to develop the colour.

Sugars: Aniline hydrogen phthalate was used. 930 mg aniline and 1.6 gm phthalic acid were dissolved in 100 ml water-saturated n-butanol. The layer was dried after spraying and heated at 105°C for five minutes to develop the colour.

Organic acids: Aniline xylose was used. 2.0 gm xylose were dissolved in 20 ml ethanol, and 2.0 gm aniline were dissolved in 20 ml water. The solutions were combined and the volume made to 100 ml with n-butanol. After spraying, the layer was dried and heated at 110°C for five minutes to develop the colour.

Phosphate esters: Three spray reagents were used consecutively according to the method of Waring and Ziporin (1964). The first consisted of 0.2 M m-phenylenediamine dihydrochloride in 76% ethanol. After spraying with this reagent, the layer was heated for five minutes at 110°C and viewed under ultra-violet light. The second reagent consisted of a mixture of 5.0 ml 60% perchloric acid, 10.0 ml 1.0 N HCl, 25 ml 4% ammonium molybdate and 60 ml water. The layer was dried after spraying and heated at 110°C for ten minutes. The third reagent was applied while the layer was still warm, and consisted of 10% stannous chloride in concentrated hydrochloric acid, which had been freshly diluted 200-fold with 0.5 M sulphuric acid. The colour and ultra-violet absorption characteristics of the various compounds in the three spray reagents aided in their identification.

F. DETECTION OF RADIOACTIVE COMPOUNDS AND MEASUREMENT OF THEIR RADIOACTIVITY.

Radioactive compounds on chromatographs were located by autoradiography, for which Kodak No-Screen Medical X-Ray film was used. Radioactivity in individual compounds was low because small aliquots of fractions were chromatographed in order to keep the total concentration of radioactive and non-radioactive compounds in each aliquot sufficiently low for good resolution to be obtained in chromatography, and chromatographs therefore required up to twelve weeks' exposure to X-ray film for adequate visualisation of compounds. After exposure, the radioactive areas were removed from the chromatograms for identification or measurement of radioactivity: the outlines of the areas were traced from the X-ray film onto the layer with a fine needle, and the layer powder was then scraped from these areas with a piece of razor blade. If the identity of a particular compound was known, the layer powder was transferred to a scintillation vial; 0.25 ml of water was added to dissolve the water-soluble compounds, then 10.0 ml of scintillation fluid, and the mixture was shaken and assayed for radioactivity as described (p.35). When the identity of a compound was to be confirmed, the compound was eluted from the layer powder with 10% isopropanol, dried in a stream of cold air, resuspended in water and spotted on a fresh thin-layer plate with known compounds; the plate was chromatographed in two dimensions by the standard methods, re-exposed to X-ray film, and later sprayed for the relative positions of the unknown radioactive compounds and the known compounds.

FIG. 11

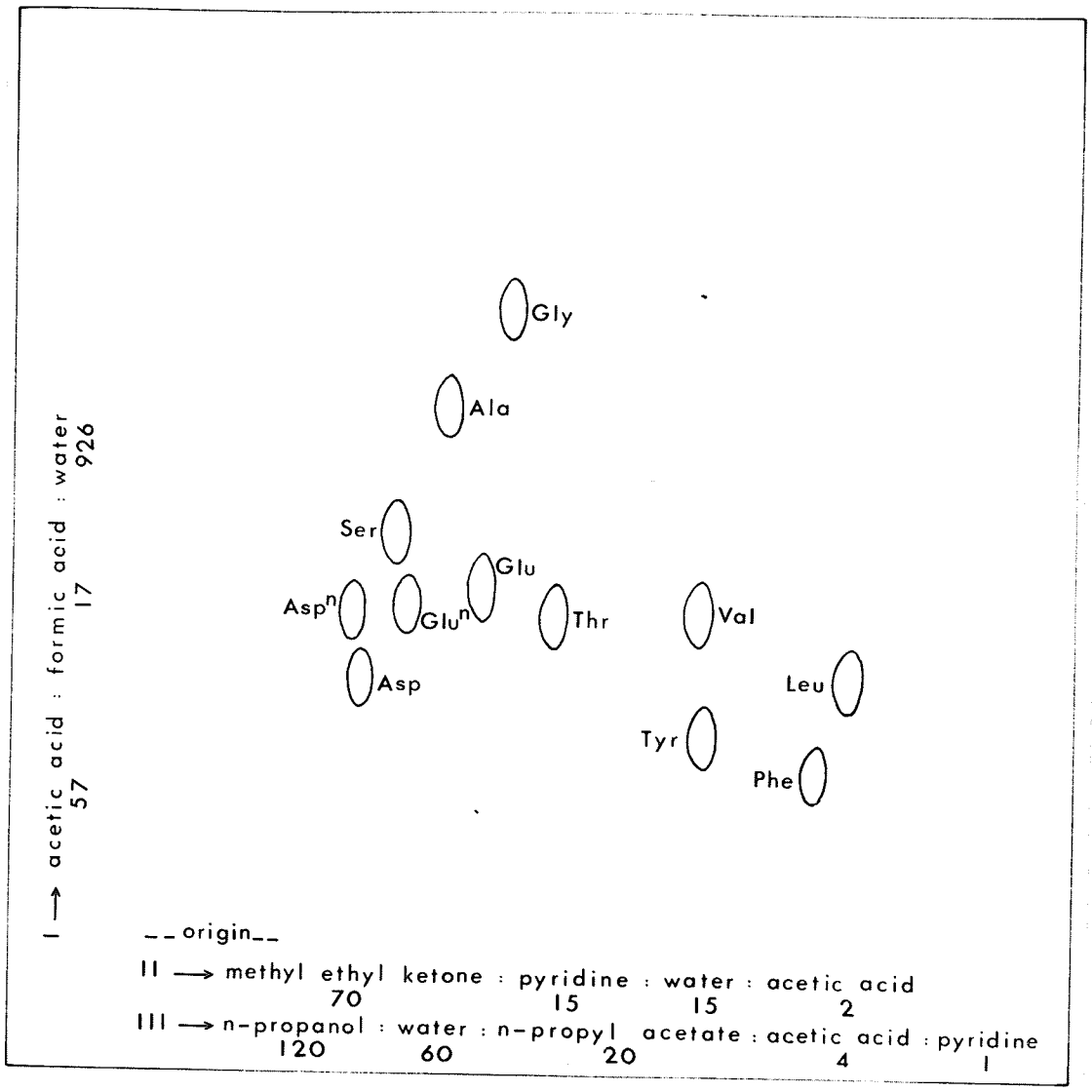


FIG. 11: Diagram of a typical analysis by TLE and TLC of a mixture of authentic samples of amino acids.

FIG. 12.

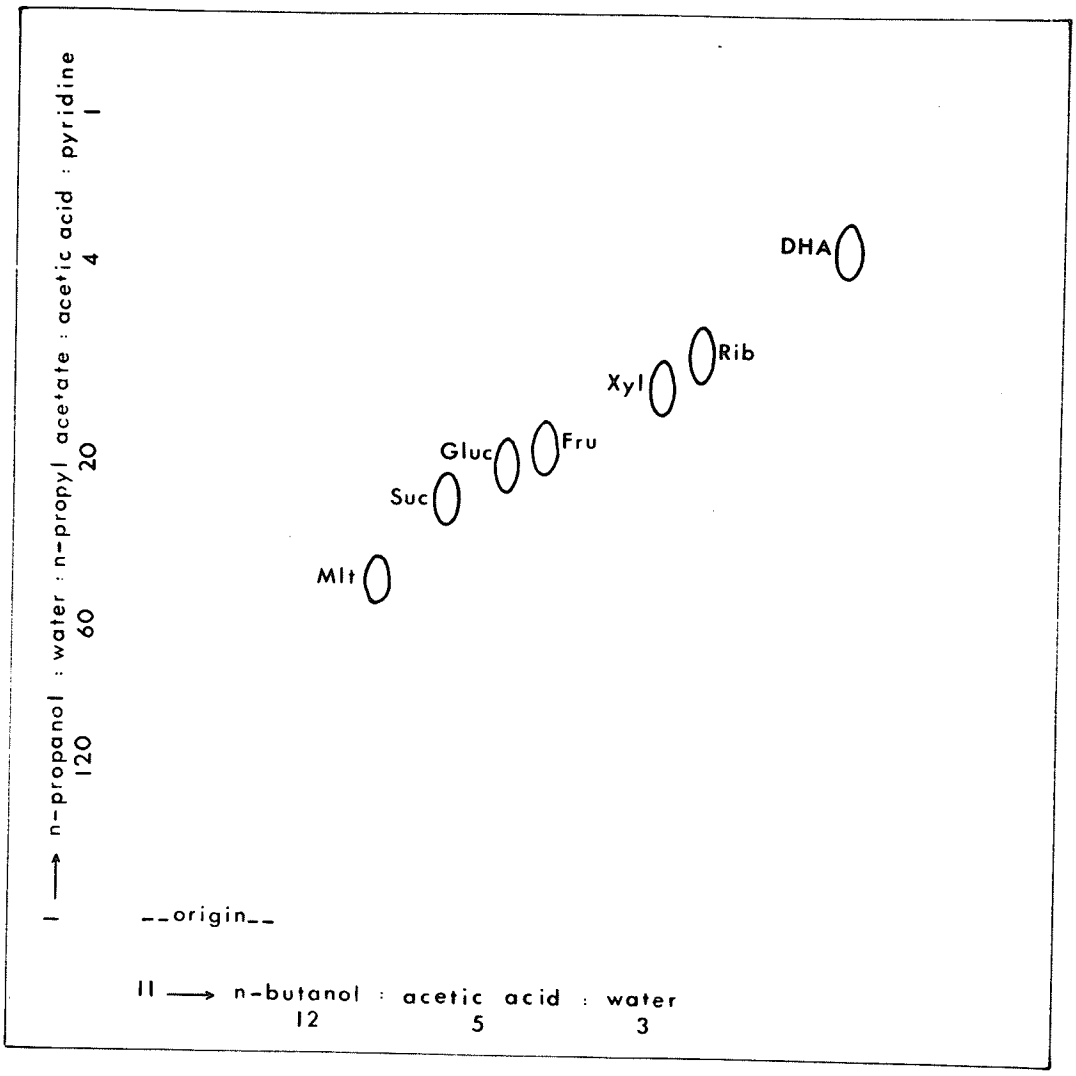


FIG. 12: Diagram of a typical analysis by TLC of a mixture of authentic samples of sugars.

FIG. 13.

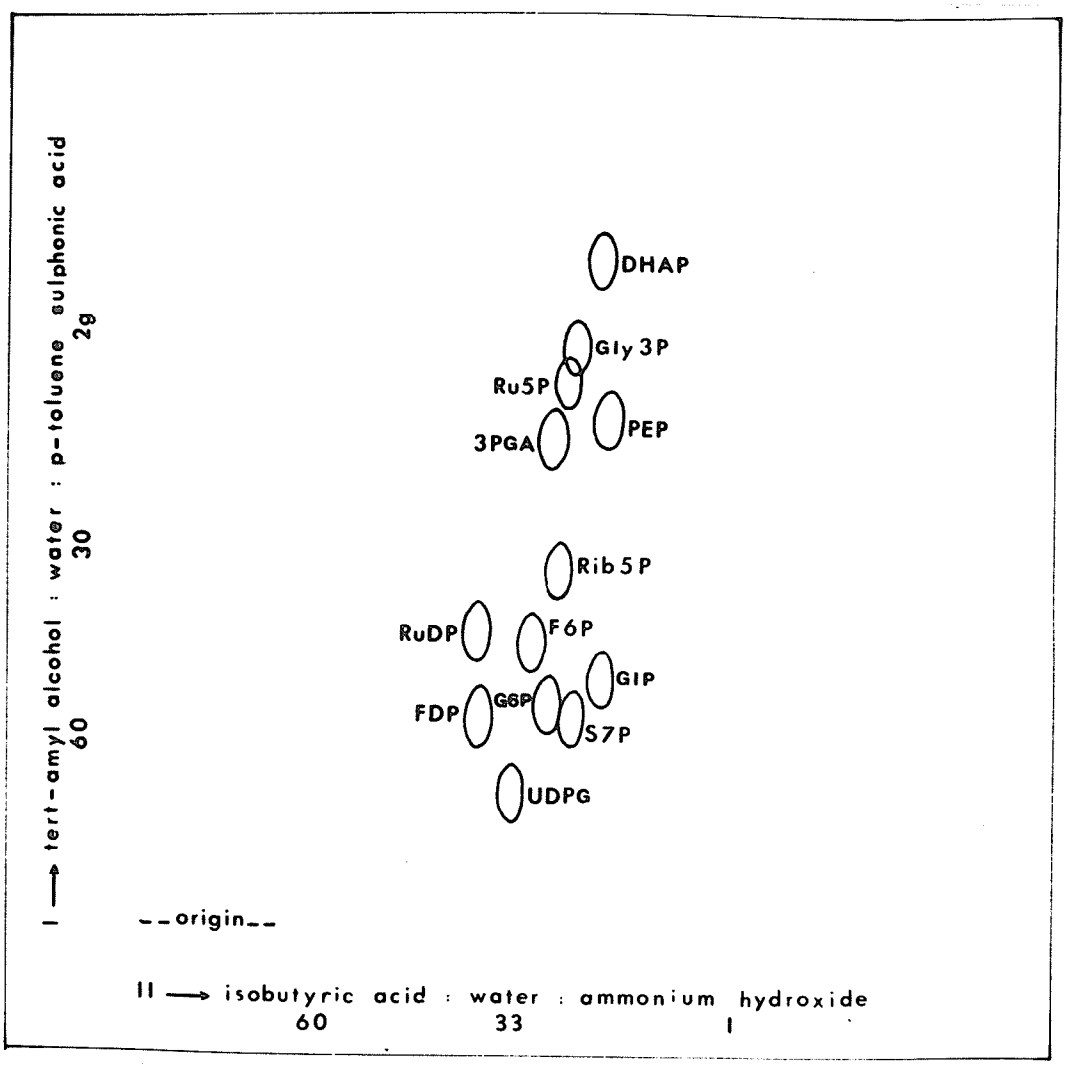


FIG. 13: Diagram of a typical analysis by TLC of a mixture of authentic samples of phosphate esters.

FIG. 14.

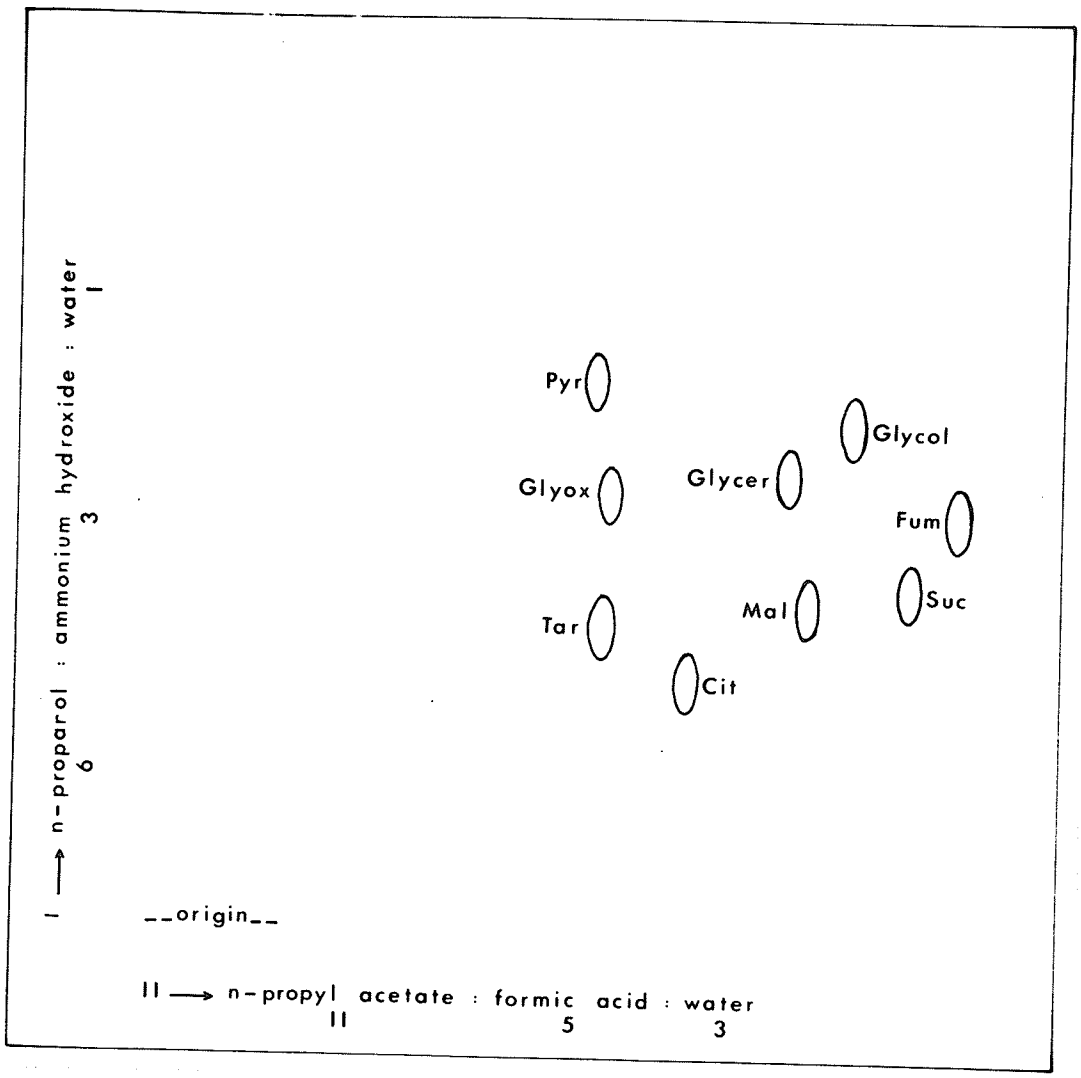


FIG. 14: Diagram of a typical analysis by TLC of a mixture of authentic samples of organic acids.

SECTION IV

RESULTS.

A. THE ANALYSIS OF STANDARDS.

Figs. 11 - 14 indicate the results of the analyses of mixtures of authentic samples of amino acids, sugars, organic acids and phosphate esters respectively by the two-dimensional thin-layer electrophoretic and chromatographic methods used in this work. The relative positions of the various compounds in each group are similar but not identical with published results: of the amino acids, differences from those described by Cook and Bieleski (1963) are especially noticeable in the position of glutamic acid relative to the positions of aspartic acid and threonine; and of the phosphates esters, the position of PEP relative to 3PGA is quite different from that described by Waring and Ziporin (1964). The separations of authentic compounds obtained in this work were used as the basis for identification of the radioactive products of $^{14}CO_2$ fixation experiments.

B. SERIES 1: ca. TWO SECONDS' EXPOSURE TO $^{14}CO_2$.

1a. 'HOT METHANOL METHOD': the susceptible reaction.

The distribution of radioactivity between the various fractions of extracts of infected leaves of Parkland after exposure to $^{14}CO_2$ for ca. two seconds at one, three and five days after inoculation as compared with healthy controls is shown in Table 25A. These results represent the average of three experiments, but the distribution of radioactivity in different experiments was very variable. However, the sum of the radioactivity in the organic acid and phosphate ester fractions was fairly constant (59 - 70% in the experiments shown) and there appeared to be an inverse relationship between the two, with most of the radioactivity in the phosphate ester

Table 25: Percentage distribution of ^{14}C in the various fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after ca. two seconds' exposure to $^{14}CO_2$: 'hot methanol method'.

A: The susceptible reaction.

days after inoculation	% of the total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
1	34	39	31	28	26	25	9	8
3	50	56	14	14	31	25	3	5
5	28	31	31	34	34	25	7	10

B: The resistant reaction.

days after inoculation	% of the total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
1	37	38	33	34	25	20	5	8
3	51	45	22	28	22	23	5	4
5	33	29	35	38	23	27	9	6

fraction being resident in 3PGA and most of that in the organic acid fraction being found in glyceric acid. The reciprocity of these two compounds seemed to indicate phosphatase activity during the extraction procedure (Mortimer, 1961). Though hot ethanol and methanol have commonly been used for extracting the radioactive products of $^{14}CO_2$ fixation from plant tissue, it seemed likely that, in whole barley leaves under the experimental conditions employed in these experiments, the inactivation of enzymes was insufficiently rapid to prevent phosphatase activity during extraction, in contrast to the results of Tolbert and Gailey (1955), who, using a very similar method, found all metabolism in whole tobacco leaves to be halted in two seconds. The work of Benson (1955) showed that plant phosphatases might be inactivated only after a few minutes in boiling 80% ethanol, and Mortimer (1961) commented on the possibility of a stimulation of phosphatase activity during the moments of initial contact between plant tissue and hot alcohol such that dephosphorylation of 3PGA could occur before the phosphatases were destroyed, though when he (Mortimer, 1961) tested the dephosphorylation of ^{14}C-3PGA either formed by photosynthesis in $^{14}CO_2$ by whole leaves (including barley) or added to leaf homogenates <u>in vitro</u> under a variety of conditions favouring enzyme activity, he found but little dephosphorylation. However, in the present experiments, further indications of phosphatase activity during extraction were obtained from the results of the analyses of the sugar fractions of the same experiments: a typical autoradiograph of one of these analyses is shown in Plate 18. Radioactive hexoses and pentoses are present in unphosphorylated form, which would not be expected in experiments of this duration (Benson and Calvin, 1951). Moreover, dephosphorylation of phosphosugars could account for the relatively low proportion of the radioactivity recovered in the

PLATE 18.

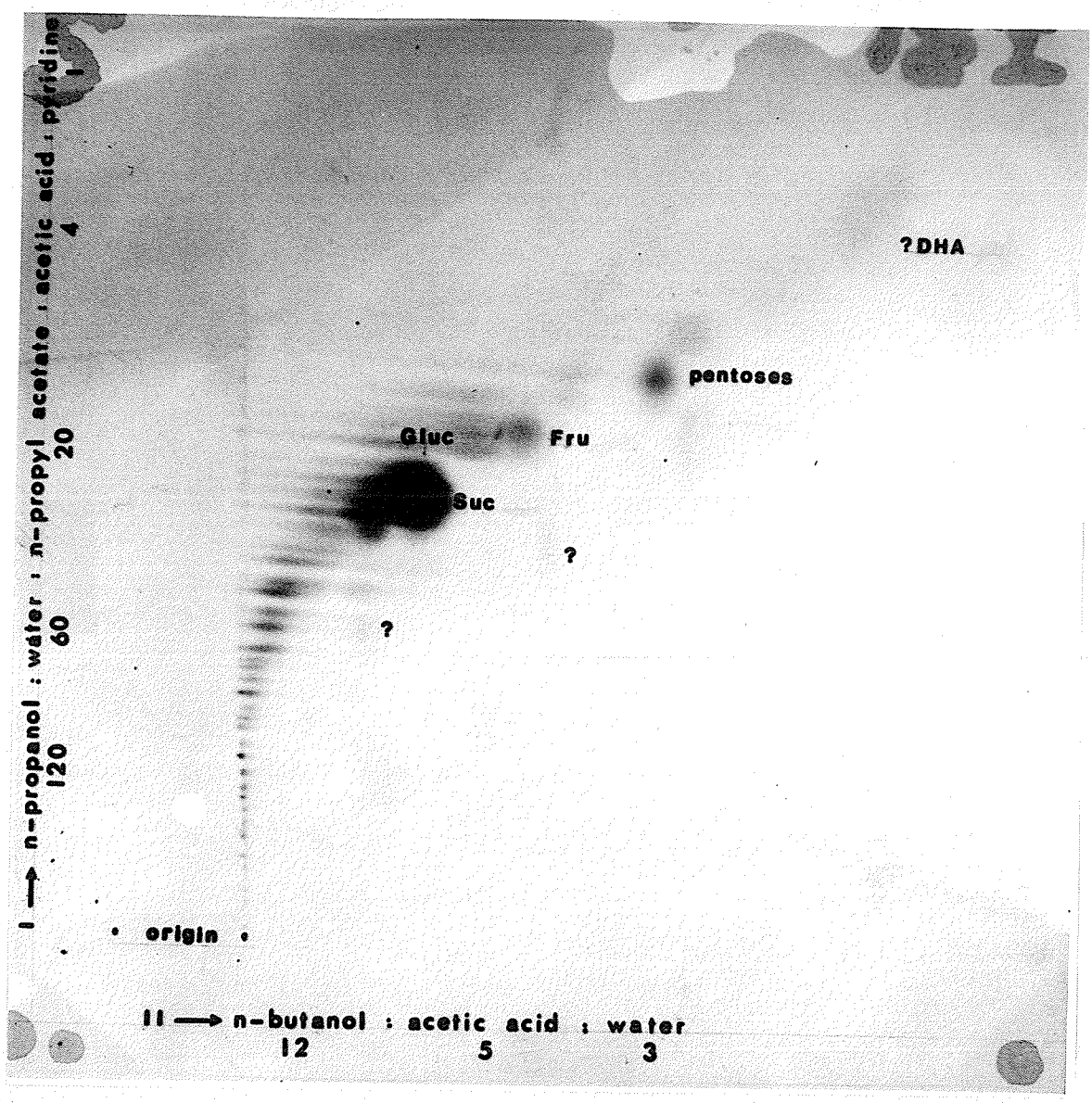


PLATE 18: Autoradiograph of a typical analysis by TLC of the sugar fraction of extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' exposure to $^{14}CO_2$: 'hot methanol method'.

phosphate ester fraction, and the correspondingly high proportion in the sugar fraction compared with the results of similar experiments published by other workers (e.g. Benson and Calvin, 1951; Mortimer, 1961).

It seems possible from the results in Table 25 that enzymes other than phosphatases had continued to function after the subjection of the leaves to the boiling 80% methanol: the recovery of up to 10% of the total radioactivity in amino acids is higher than might be expected in $^{14}CO_2$ fixation experiments of this duration. Plate 19 shows a typical autoradiograph of an amino acid fraction in these experiments: glutamine especially contains a higher concentration of radioactivity than would be expected since Bassham and Kirk (1964) showed that glutamic acid, from which glutamine is probably formed (Calvin and Bassham, 1962; Van der Meulen and Bassham, 1959; Smith et al., 1961) is not a product of a primary carboxylation and is usually labelled relatively late (after 30 seconds) in photosynthesis in $^{14}CO_2$ because of the many pools of intermediates between the products of the initial carboxylation and this compound; moreover, Benson and Calvin (1951) found neither glutamine nor glutamic acid labelled after 30 seconds' photosynthesis in $^{14}CO_2$ in barley. Small amounts of an amino acid tentatively identified as asparagine were also labelled (Plate 19) and, if correctly identified, this would also represent a secondary amino acid (being formed from aspartic acid) and hence be unexpected in a short-term experiment.

1b.'HOT METHANOL METHOD': the resistant reaction.

The average results of three experiments are shown in Table 25B. Comparison with Table 25A shows that variability similar to that apparent in experiments with Parkland existed in the results of the experiments with C.I.5791, and again there were indications of phosphatase activity: there was an inverse relationship between the radioactivity of the phosphate

PLATE 19.

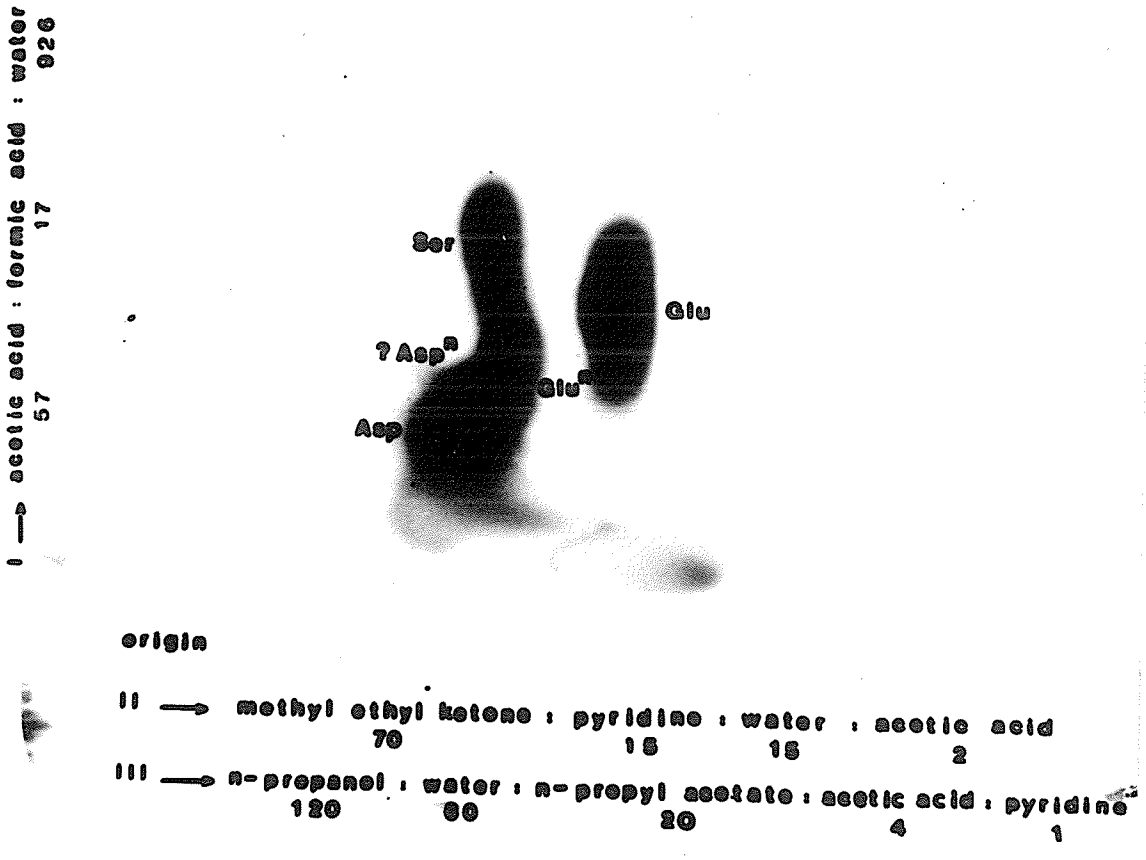


PLATE 19: Autoradiograph of a typical analysis by TLE and TLC of the amino acid fraction from extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' exposure to $^{14}CO_2$: 'hot methanol method'.

ester and organic acid fractions, primarily between 3PGA and glyceric acid, the sum of the activities in the two fractions being 67 - 73%. Radioactive unphosphorylated hexoses and pentoses were present, and activity in the amino acid fraction was up to 9% of the total recovered in the extracts.

Because of the probability of there being a time-lag in the inactivation of some of the enzymes in whole barley leaves after the addition of hot 80% methanol, leaves in later experiments were plunged into liquid nitrogen after exposure to $^{14}CO_2$ (p.125) as advocated by Bieleski (1964) in his discussion of the problem of halting metabolism.

2. 'LIQUID NITROGEN METHOD'.

Table 26 shows the distribution of radioactivity between the residue, chloroform and alcohol-water fractions in samples processed by the 'liquid nitrogen method'.

Table 26: Percentage distribution of ^{14}C between the various fractions of extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' and 60 seconds' exposure to $^{14}CO_2$ and processing by the 'liquid nitrogen method'.

fraction	percentage of total ^{14}C recovered in extract	
	ca. 2 seconds' exposure	60 seconds' exposure
residue	1.2 - 2.9	3.8 - 5.6
chloroform	2.5 - 3.9	9.4 - 13.2
methanol-water	93.6 - 96.3	81.3 - 85.6

The percentage of the total radioactivity recovered in the alcohol-water fraction by this method exceeded that of the 'hot methanol method'

where a maximum of 85% of the total radioactivity was recovered in the alcohol-water fraction. Little of the ^{14}C taken up by the leaf in experiments of ca. two seconds was retained in the insoluble and chloroform fractions of the total extract, and a similar distribution of radioactivity was found in extracts from infected and control leaves of Parkland and C.I.5791.

2a. 'LIQUID NITROGEN METHOD': the susceptible reaction.

The distribution of radioactive products in the various fractions of extracts of leaves of Parkland after ca. two seconds' fixation in the light is given in Table 27A: each figure represents the average of those obtained in separate analyses of extracts of three leaves from three different inoculation experiments. A very much higher level of radioactivity was present in the phosphate ester fraction, with correspondingly lower concentrations in the other fractions, than in the results of the 'hot methanol method' (cf. Tables 25 and 27). The high concentration of ^{14}C in the phosphate esters is consistent with the results of other short-term experiments with Calvin cycle plants (Bassham and Calvin, 1957).

The results obtained with extracts of infected and control leaves of Parkland and C.I.5791 at three and five days after inoculation were very similar, indicating that the pathways by which carbon entered metabolism in infected leaves were similar to those in healthy leaves. However, small but consistent differences could be observed in the gross analyses at three days after inoculation, the percentages of the total radioactivity in the sugar fraction of extracts of infected leaves was always slightly lower than that in extracts from healthy controls, and the difference was more pronounced at five days; and the percentages of the total radioactivity in the amino acid and organic acid fractions from infected leaves, though always low, were slightly higher at both three and five days after inoculation

Table 27: Percentage distribution of ^{14}C in the various fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after ca. two seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

A: The susceptible reaction.

days after inoculation	% of total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
3	74.4	77.1	0.7	1.2	23.8	19.8	1.1	1.9
5	73.0	74.9	1.5	2.9	23.3	18.0	2.2	4.2

B: The resistant reaction.

days after inoculation	% of total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
3	74.1	75.2	0.8	1.6	24.3	21.1	0.8	2.0
5	70.9	71.5	1.7	1.4	25.4	23.9	2.0	3.2

than those from healthy controls. Somewhat similar effects of infection have been noted in other diseased tissues: Livne (1964) found reductions in the ^{14}C fixed in 30 minutes recovered in the sugar fractions of rust-infected bean leaves as compared with healthy controls; and Daly and Krupka (1962) reported a rise in the organic acid content of wheat leaves after infection with rust. Similar relationships between the percentages of the total radioactivity recovered in the various fractions of infected leaves of Parkland as compared with healthy controls can be seen in the results of the 'hot methanol' extraction method as shown in Table 25A.

2b. 'LIQUID NITROGEN METHOD': the resistant reaction.

The distribution of radioactive products in the various fractions of extracts of leaves of C.I.5791 after ca. two seconds' fixation of $^{14}CO_2$ in the light is given in Table 27B, with each figure representing the average of those obtained in separate analyses of extracts of three leaves from different inoculation experiments. 70 - 75% of the total radioactivity recovered in all extracts was located in the phosphate ester fraction, and the results indicate similar carbon fixation patterns in infected and healthy leaves of C.I.5791. However, slight but consistent differences in the distribution of radioactivity in the various fractions existed between extracts of infected and control leaves at both three and five days after inoculation, the effects being more pronounced at three days: a slightly lower percentage of the total radioactivity was recovered in the sugar fraction, and a proportionally greater percentage in the amino acid fraction and the organic acid fraction, in extracts of infected leaves. These effects are reminiscent of those of infection in the susceptible reaction (Table 27A) and may therefore be of some significance.

2c. 'LIQUID NITROGEN METHOD': analysis of fractions.

This section is divided into four parts, each dealing with the

analyses of one of the four groups of radioactive products of ca. two seconds' fixation of $^{14}CO_2$, viz. phosphate esters, organic acids, sugars and organic acids, in infected and control leaves of Parkland and C.I.5791. The results for the various treatments are described together to facilitate comparisons between them. The extracts from two leaves of each treatment, obtained from different inoculation experiments, were fractionated and analysed chromatographically, and the results presented are the averages of the two experiments.

Phosphate ester fraction: A typical autoradiograph obtained in the analysis of phosphate ester fractions in these experiments is shown in Plate 20. Table 28 shows the percentages of the total radioactivity recovered in the various components of the phosphate ester fractions of extracts of infected and control leaves of Parkland and C.I.5791 barley respectively at three and five days after inoculation.

Table 28: Percentage distribution of ^{14}C in the components of the phosphate ester fraction of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after ca. two seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
3PGA	81	69	75	79	78	68	69	71
Gly 3 P	11	19	17	10	11	10	21	23
DHAP &PEP	3	2	2	4	5	4	3	4
FDP	5	10	6	7	6	10	7	3

PLATE 20.

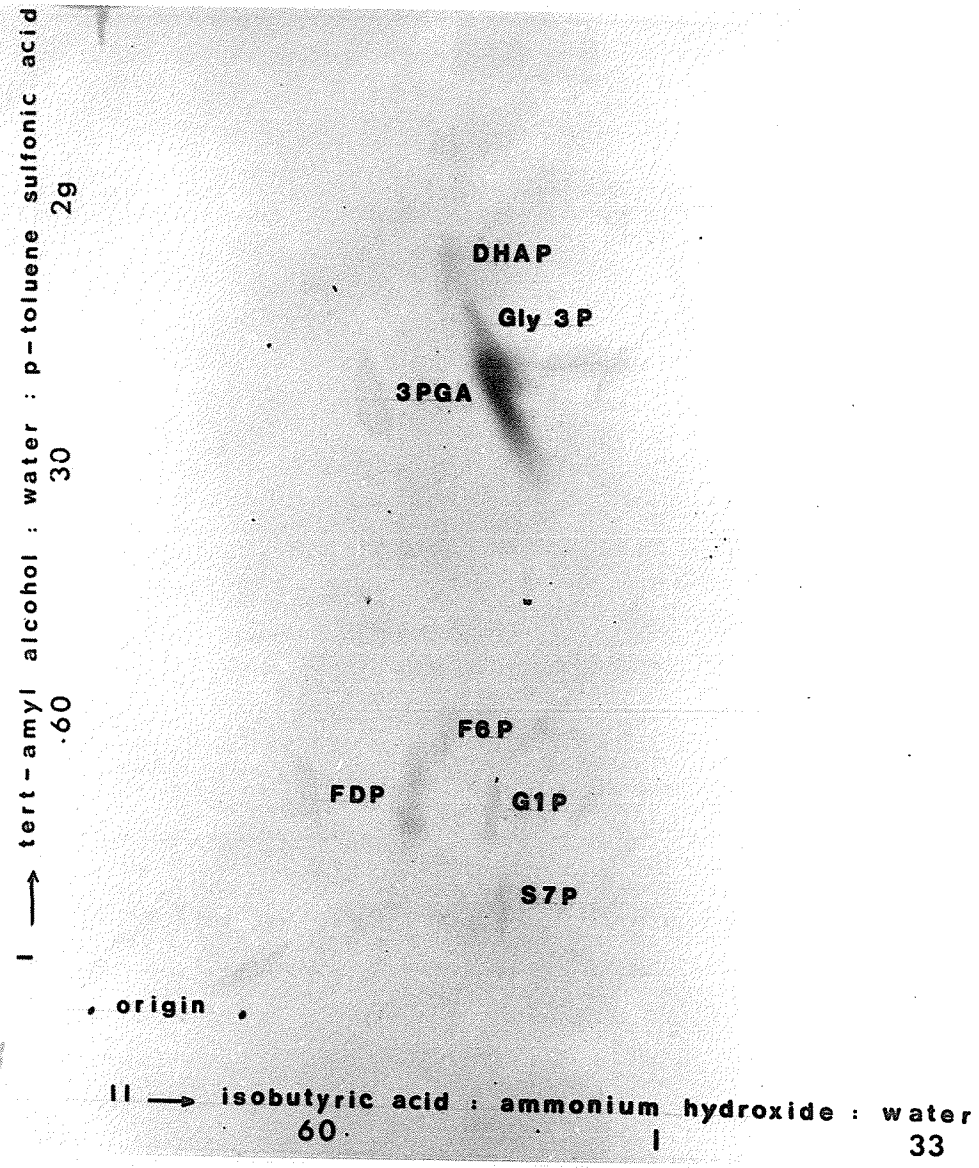


PLATE 20: Autoradiograph of a typical analysis by TLC of the phosphate ester fraction from extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

3PGA and 2PGA could not be separated by the chromatographic method used and hence the percentage radioactivity shown for 3PGA will include any which may be resident in 2PGA. Similarly, the percentage radioactivity in Gly 3 P may include that resident in ribulose-5-phosphate. Radioactivity in the hexose phosphates was largely confined to fructose 1,6-diphosphate. Because the percentages of the total radioactivity recovered in DHAP and PEP were low, these compounds were analysed together.

No consistent differences were observed between the analyses from infected and control leaves of either Parkland or C.I.5791. In all fractions, radioactivity was concentrated in the triose phosphates. Since these compounds are formed in the first steps of the Calvin cycle (Bassham and Calvin, 1957), this pathway of carbon fixation must predominate in infected as well as healthy leaves of Parkland and C.I.5791. Little radioactivity was recovered in the pentose and hexose phosphates; since these compounds occur later in the Calvin cycle, this would seem to indicate a rapid halting of metabolism in these experiments. The varying percentages of the total activity resident in 3PGA may reflect slight differences in the time taken for metabolism to be halted in different leaves as well as slight differences in the rate of movement of ^{14}C out of the 3PGA pool.

Organic acid fraction: A typical autoradiograph obtained in the analyses of organic acid fractions in these experiments is shown in Plate 21. Table 29 shows the percentages of the total radioactivity recovered in the components of the organic acid fraction of infected and control leaves of Parkland and C.I.5791 at three and five days after inoculation.

No consistent differences were observed between the extracts of infected and control leaves of Parkland or C.I.5791, though accurate

PLATE 21.

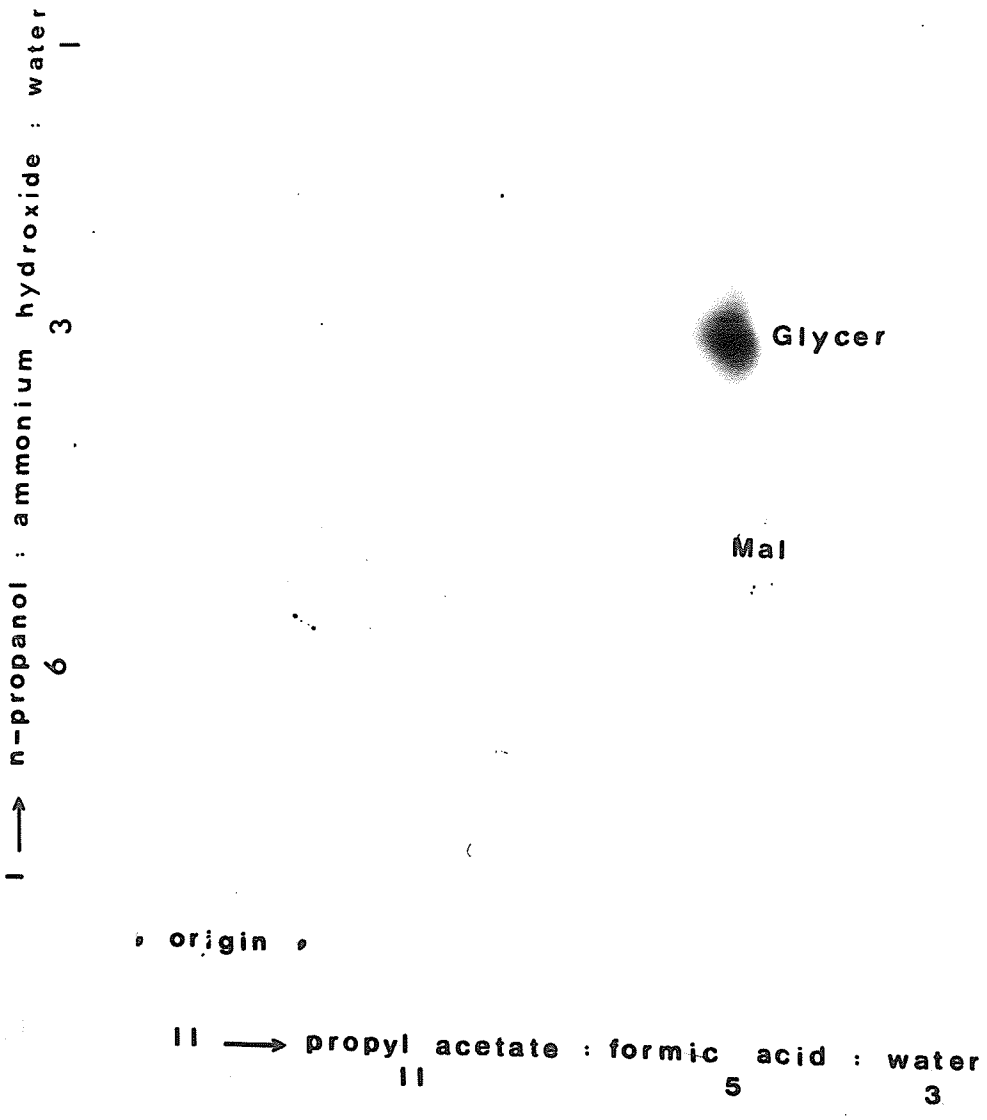


PLATE 21: Autoradiograph of a typical analysis by TLC of the organic acid fraction from extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

Table 29: Percentage distribution of ^{14}C in the components of the organic acid fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after ca. two seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
Glycerate	78	72	75	68	67	72	80	76
Malate	22	28	25	32	33	28	20	24

estimates of the ^{14}C resident in individual compounds were difficult to obtain since radioactivity was low. Only radioactive glyceric and malic acids were recovered in detectable quantities, and glyceric acid always contained a greater proportion of the radioactivity then malic acid. The formation of ^{14}C-malic acid after short periods of fixation of $^{14}CO_2$ *in vivo* has been described by Smith *et al.* (1961) as evidence of the carboxylation of PEP; and Benson and Calvin (1951) found radioactive malic acid to be common to extracts of barley leaves which had been fixing $^{14}CO_2$ in the light and the dark. The pathway by which ^{14}C-glyceric acid would be formed in experiments of this nature is a little less clear. However, it regularly occurs in the radioactive products of short-term fixation of $^{14}CO_2$ in the light, and in greater concentrations than ^{14}C-malic acid: Benson and Calvin (1951) found substantial quantities in extracts of barley after 30 seconds, with lesser amounts of ^{14}C-malic acid; and Mortimer (1961) found 8% of the total ^{14}C incorporated into the water-soluble components of barley leaves after 60 seconds to be resident in glyceric acid, while malic acid contained only trace amounts. Various

hypotheses have been put forward to account for the ^{14}C-glyceric acid: Mortimer (1960) proposed, from the results of iodoacetate inhibition studies, that it was a by-product resulting from the over-production of the primary fixation and reduction products, its immediate precursor being glyceraldehyde-3-phosphate; and Hess and Tolbert (1966) interpreted the results of their carboxyl-group labelling experiments as indicating that glycerate may be formed from the hydrolysis of 3PGA *in vivo* (prior to extraction).

Sugar fraction: A typical autoradiograph obtained in these experiments is shown in Plate 22. Table 30 shows the percentages of the total radioactivity in the components of the sugar fractions of extracts of infected and control leaves of Parkland and C.I.5791 at three and five days after inoculation.

Table 30: Percentage distribution of ^{14}C in the components of the sugar fraction of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after *ca.* two seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
Sucrose	74	68	75	74	79	77	78	71
Fructose	26	32	25	26	21	23	22	29

There were no consistent differences in the distribution of radioactivity in the extracts. Sucrose contained most of the radioactivity in this fraction, this being a usual end-product of all pathways of CO_2 fixation

PLATE 22.

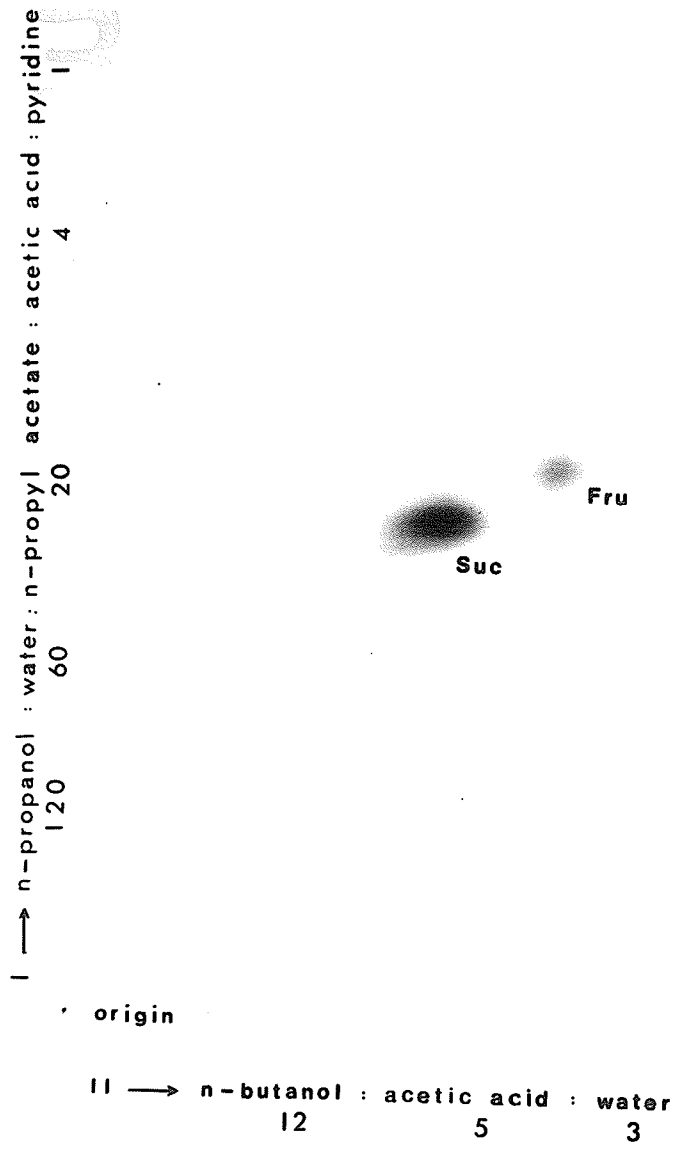


PLATE 22: Autoradiograph of a typical analysis by TLC of the sugar fraction from extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

(Norris et al., 1955; Hatch and Slack, 1970). The only unphosphorylated monosaccharide containing ^{14}C was fructose; the reasons for its appearance are unclear, since phosphatase activity in extraction was apparently minimal (cf. Tables 25 and 27), and the formation of sucrose in green plants proceeds largely by the pathway (Leloir, 1964):-

$$\text{fructose-6-P} \xrightarrow{\text{UDP-glucose}} \text{sucrose-6-P} \longrightarrow \text{sucrose}$$

No unphosphorylated hexoses were reported after 30 seconds' fixation in $^{14}CO_2$ in barley by Benson and Calvin (1951). However, Mortimer and Wylan (1962) found traces of free glucose and fructose labelled with ^{14}C after 30 seconds' fixation of $^{14}CO_2$ by sugar beet leaves; and Norris et al. (1955) found small amounts of free ^{14}C-fructose in extracts of barley leaves after five minutes' exposure to $^{14}CO_2$.

Amino acid fraction: A typical autoradiograph obtained in the analyses of amino acids in these experiments is shown in Plate 23. Table 31 shows the percentages of the total radioactivity recovered in the components of the amino acid fractions of infected and control leaves of Parkland and C.I.5791 at three and five days after inoculation.

Table 31: Percentage distribution of ^{14}C in the components of the amino acid fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after ca. two seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
Asp	39	38	47	50	38	47	40	43
Ser	46	38	40	40	37	35	38	32
Glu	15	24	13	10	24	18	22	25

PLATE 23.

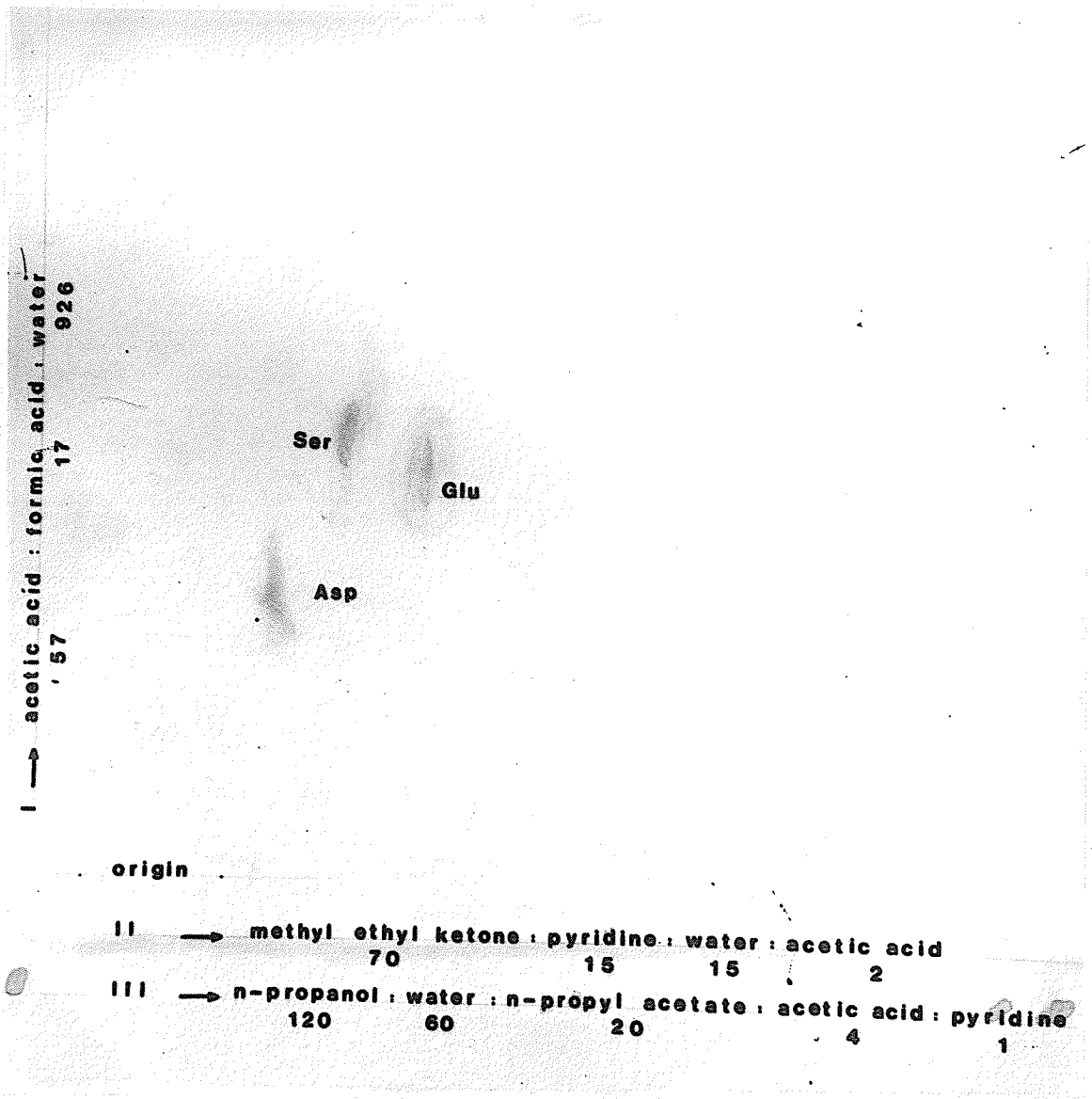


PLATE 23: Autoradiograph of a typical analysis by TLE and TLC of the amino acid fraction from extracts of first leaves of Parkland and C.I.5791 after ca. two seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

The total amino acid fraction contained little radioactivity, but radioactive aspartic acid, serine and glutamic acid were found in all extracts; because of the very low total activity, little significance can be attached to differences in activity of the various amino acids between extracts. It is of interest that radioactive glycine and alanine, if present in the extracts, were of insufficient concentration to be detected: these amino acids have frequently been found amongst the labelled products of short-term fixation of $^{14}CO_2$ e.g. Benson and Calvin (1951) found glycine to be the most highly radioactive amino acid in barley after 30 seconds' fixation of $^{14}CO_2$ (with serine and aspartic acids also substantially labelled), and Towers and Mortimer (1956) found that glycine, serine and alanine were labelled after 30 seconds' exposure of barley to $^{14}CO_2$ in their experiments. The differences between the results of the present experiments and those of other workers might be attributable to experimental factors such as the light intensities used in the fixation period or the conditions under which the experimental material was grown.

3. EXPERIMENTS WITH RUST-INFECTED BARLEY.

In all experiments with rust-infected barley and healthy controls, the 'liquid nitrogen method' was used for halting metabolism and the extraction of the plant material after ca. two seconds' exposure to $^{14}CO_2$. All experimental results presented in the following sections represent those obtained in the individual analyses of extracts of two leaves from different inoculation experiments.

3a. RUST-INFECTION: the susceptible reaction.

The distribution of radioactivity in the various fraction of extracts of infected and control leaves is shown in Table 32A. Comparison with Table 27A (the susceptible reaction in Parkland infected with H.teres) shows

Table 32: Percentage distribution of ^{14}C in the various fractions of extracts of rust-infected first leaves of C.I.5791 and Parkland and healthy controls after ca. two seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

A: The susceptible reaction (C.I.5791).

days after inoculation	% of total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
7	76.2	80.3	1.8	2.0	19.7	15.7	2.3	1.9
14	68.4	79.1	1.3	3.2	28.3	15.1	2.0	2.6

B: The resistant reaction (Parkland).

days after inoculation	% of total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
7	77.5	73.2	2.9	3.4	18.7	22.1	0.9	1.3
16	70.2	73.4	2.5	3.2	25.4	20.5	1.9	2.9

marked similarities between the results of the two series of experiments. At neither the time of appearance of the first lesions nor at the time of sporulation of the parasite was the distribution of ^{14}C in the products of fixation of $^{14}CO_2$ substantially different from that in healthy controls, indicating that the pathways by which carbon enters metabolism in infected leaves are similar to those in healthy leaves. However, as in the extracts from leaves exhibiting the susceptible reaction to H.teres infection (Table 27A) small but consistent differences were noted: the proportion of the radioactivity recovered in the sugar fraction of infected leaves was lower than that of healthy controls at seven days after inoculation; and the percentage radioactivity recovered in the amino acid and organic acid fractions was slightly higher in the extracts from infected leaves than in those from healthy controls at 14 days after inoculation.

3b. RUST-INFECTION: the resistant reaction.

The distribution of radioactivity in the various fractions of extracts of infected and control leaves of Parkland is given in Table 32B. The results obtained were very similar to those of the susceptible reaction (Table 32A). However, at 16 days after inoculation, the proportion of radioactivity recovered in the sugar fraction of extracts of infected leaves was lower than that of healthy controls; and there were consistently slightly higher proportions of the total radioactivity in the organic acid and amino acid fractions from extracts of infected leaves than in those from healthy controls. These results are similar to those obtained in the resistant reaction to H.teres (Table 27B).

3c. RUST-INFECTION: analysis of fractions:

The analysis of the distribution of ^{14}C in the individual components of the various fractions of rust-infected and control leaves gave very similar results to those of extracts of barley leaves infected with H.teres

and controls. Photographs of autoradiographs and detailed analyses of the components of each of the four fractions as designated on p.155 will therefore not be presented; however, any differences in the results of the two series of experiments with the different infections have been included, and comparisons drawn between extracts of leaves of C.I.5791 and Parkland infected with rust and those of healthy controls.

Phosphate ester fraction: The distribution of radioactivity between the four main radioactive components of the phosphate ester fractions (which are outlined on p.157) was similar to those shown in Table 28 for the H.teres experiments. The proportion of the radioactivity in FDP and the other hexose phosphates was slightly higher (4 - 20% rather than the 3 - 14% shown in Table 28) at the expense of the triose phosphate fractions, possibly indicating a somewhat longer time for the inactivation of metabolism to be accomplished in this series of experiments, since in the typical Calvin cycle of carboxylation (Bassham and Calvin, 1957) newly fixed carbon moves from the triose to the hexose phosphates.

Organic acid fraction: The organic acid fractions of extracts of barley leaves of all treatments at seven days after inoculation showed a similar distribution of radioactivity to that outlined in Table 29 for the experiments with H.teres. However, at 14 and 16 days after inoculation, small amounts of radioactive glycolic and glyoxylic acids were present in the extracts, their appearance not apparently correlated with infection nor with susceptibitlity or resistance to P.graminis tritici. Glycolic acid contained 2 - 12%, and glyoxylic acid 1 - 3%, of the total ^{14}C present in the fractions. These acids have frequently been shown in the products of short-term fixation experiments in barley (Schou et al., 1950; Benson and Calvin, 1951) and they appeared in this work in the analyses of the products of 60 seconds' fixation of $^{14}CO_2$ (p.174). In the extracts of leaves

exposed to $^{14}CO_2$ for ca. two seconds, their presence seems to be correlated only with the age of the leaves from which the extracts were made (21- and 23-day-old leaves rather than ten- and 15-day-old leaves as used in all other experiments), possibly indicating a more active production in these leaves, or perhaps due to some factor in experimentation connected with the age of the leaves, such as the time taken for the halting of metabolism.

Sugar fraction: In the sugar fractions of all extracts, 69 - 80% of the total radioactivity was located in sucrose, with the remainder resident in fructose as in Table 30. In some autoradiographs, traces of unphosphorylated pentoses were also evident (but apparently correlated neither with rust-infection nor with susceptibility or resistance to rust).

Amino acid fraction: The radioactivity recovered in the amino acids was distributed between aspartic acid, serine and glutamic acid in proportions similar to those outlined in Table 31. Small amounts of ^{14}C-alanine (2 - 6%) were recovered in the extracts of older leaves (infected and controls at 14 and 16 days after inoculation); however, its presence could not be correlated with infection, nor with susceptibility or resistance to P.graminis tritici.

C. SERIES 2: SIXTY SECONDS' EXPOSURE TO $^{14}CO_2$.

Table 26 (p.151) shows the distribution of radioactivity between the residue and the chloroform and alcohol-water fractions of extracts. After 60 seconds' fixation of $^{14}CO_2$, a greater proportion of the total radioactivity fixed was retained in the residue and chloroform fraction than after only ca. two seconds (Table 26). Total fixation in diseased leaves was lower than that in healthy leaves, but no consistent differences were observed between extracts from healthy or infected leaves of Parkland or C.I.5791, in contrast to the results of experiments by Livne (1964)

on rusted bean, in which greater incorporation of ^{14}C into the alcohol-insoluble fraction occurred in infected than in healthy plants.

1. The susceptible reaction.

The distribution of radioactivity in the various fractions of extracts of infected and control leaves at three and five days after inoculation is given in Table 33A: the figures represent the average of those obtained from the analyses of extracts of two leaves from different inoculation experiments. Significant amounts of ^{14}C were recovered in every fraction. In extracts of both healthy and infected leaves, the phophate ester fraction contained the highest proportion of the radioactivity, though this comprised only 39 - 42% of the total recovered as compared with 73 - 77% after ca. two seconds' fixation (Table 27A). Comparatively more radioactivity was located in the sugars after 60 seconds than after ca. two seconds (30 - 37% as compared with 18 - 24%) as would be expected if sucrose is one of the main products of carbon fixation in the light (Bassham and Calvin, 1957); the sugar fractions from extracts of infected leaves consistently contained a lower percentage of the total activity than those from healthy leaves, as after ca. two seconds' fixation. 15 - 21% of the total ^{14}C recovered had been incorporated into amino acids (which is very similar to the 17% found by Benson and Calvin, 1951, and the 22% found by Mortimer, 1961, in extracts of barley after 60 seconds' exposure to $^{14}CO_2$) as compared with only 1 - 4% after ca. two seconds' fixation; however, as in the latter experiments, extracts from infected leaves showed a slightly higher percentage than extracts from healthy leaves. As after ca. two seconds' fixation, the fraction containing the least radioactivity was the organic acids: extracts from healthy plants contained 6 - 8% of the total radioactivity in the organic acid fraction (which is very similar to the 8% found by both Benson and Calvin, 1951, and Mortimer, 1961, in extracts of barley after 60 seconds' exposure to

Table 33: Percentage distribution of ^{14}C in the various fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after sixty seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

A: The susceptible reaction.

days after inoculation	% of total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
3	40.8	38.7	9.9	13.1	34.8	30.4	14.5	17.8
5	40.7	39.9	6.2	10.0	37.9	30.3	17.0	19.8

B: The resistant reaction.

days after inoculation	% of total ^{14}C in extract recovered in each fraction							
	phosphate esters		organic acids		sugars		amino acids	
	H	I	H	I	H	I	H	I
3	46.6	44.4	9.2	12.0	29.8	27.2	14.4	16.4
5	42.3	43.2	10.1	12.5	34.3	32.7	13.3	11.8

$^{14}CO_2$) whereas extracts from infected plants contained 10 - 13% of the total activity in organic acids.

2. The resistant reaction.

The distribution of radioactivity in the various fractions of extracts of infected and control leaves of C.I.5791 at three and five days after inoculation is given in Table 33B, each figure representing the average of those obtained from the analyses of extracts of two leaves from different inoculation experiments. Significant amounts of ^{14}C were recovered in every fraction and the distribution of radioactivity between the various fractions was generally similar to that in Parkland (Table 33A). Slight differences were apparent between extracts from healthy and infected leaves at three days after inoculation: the percentage of the total radioactivity recovered in the sugar fraction was very slightly lower, and that recovered in the organic acid and amino acid fractions slightly higher, than in healthy controls; as previously discussed (p.154), these differences may be significant since they parallel the changes in the susceptible reaction (Table 33A), though they are of a different magnitude. At five days after inoculation, the distribution of radioactivity in extracts from healthy and infected leaves was much the same.

3. Analysis of fractions.

As in experimental Series 1 (p.155), the results of the analyses of the individual fractions of the various extracts from infected and control leaves of Parkland and C.I.5791 are dealt with together to facilitate comparisons between them. The extracts from two leaves of each treatment were fractionated and analysed chromatographically, and the results presented are the averages of the two experiments.

Phosphate ester fraction: A typical autoradiograph obtained in the analyses of phosphosugar fractions from extracts of healthy and infected leaves of Parkland and C.I.5791 is shown in Plate 24. Table 34 shows the percentages of the total radioactivity recovered in the various components of the phosphate ester fraction from Parkland and C.I.5791.

Table 34: Percentage distribution of ^{14}C in the components of the phosphate ester fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after 60 seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
3PGA	48	53	55	57	51	55	48	50
Gly 3 P	9	11	6	5	7	5	10	7
DHAP	5	3	6	3	7	3	4	2
PEP	6	8	5	4	5	7	8	6
Ru5P	4	2	5	4	4	3	5	6
Rib 5 P	4	-	3	6	2	3	3	4
F6P	2	4	2	2	4	2	4	-
FDP & hexose Ps	20	18	17	19	19	21	18	24
UDPG	1	2	1	-	1	1	-	1

The components as designated do not necessarily imply pure compounds: '3PGA' may include 2PGA; 'F6P' may include RuDP; and the figures for 'FDP' includes the radioacitivity resident in glucose-1-phosphate, glucose-6-phosphate, sedoheptulose-7-phosphate and possibly other compounds. In all fractions analysed, about half the ^{14}C recovered was resident in 3PGA, compared with 69 - 81% after ca. two seconds' fixation (Table 28); and the

PLATE 24.

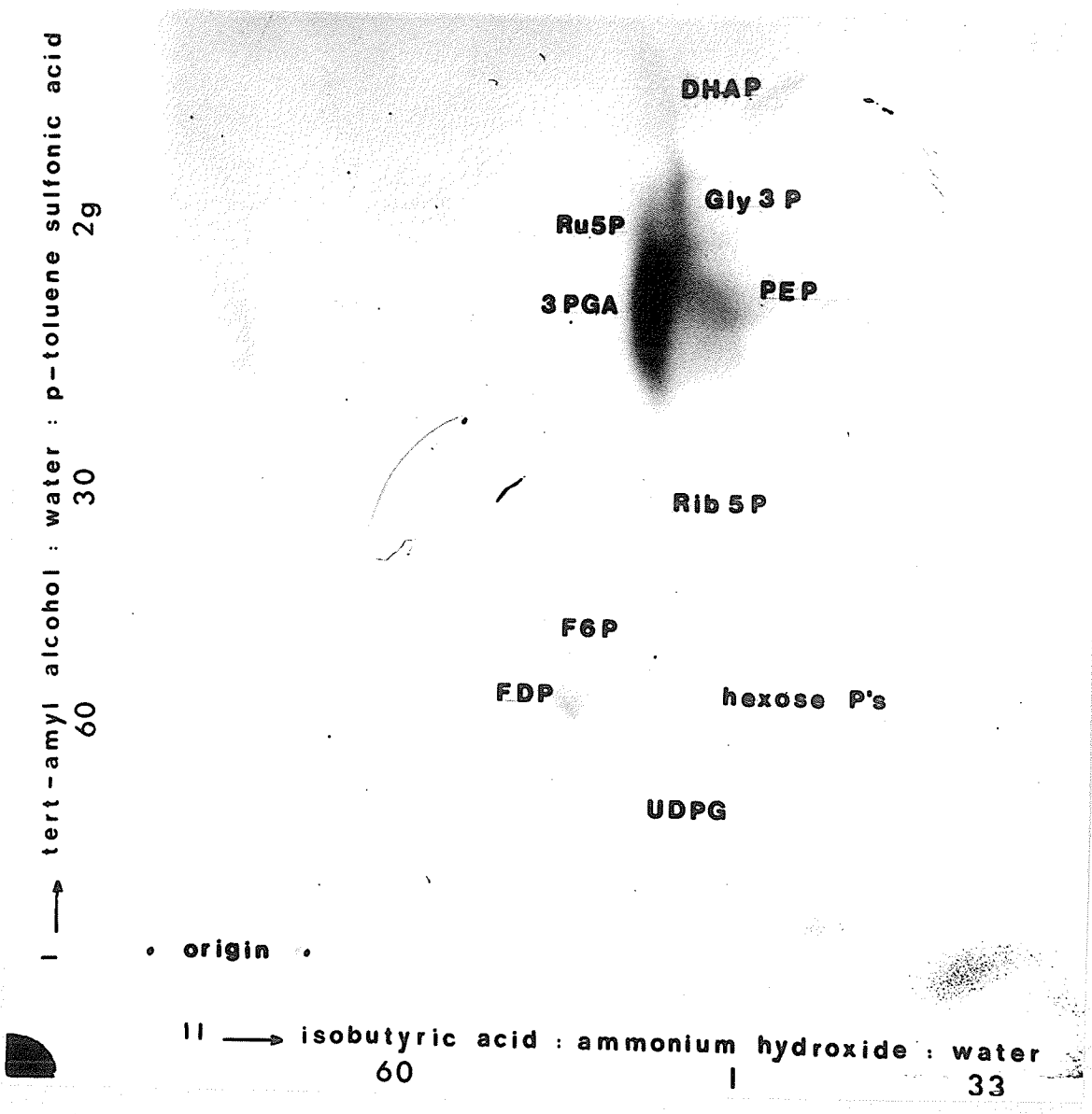


PLATE 24: Autoradiograph of a typical analysis by TLC of the phosphate ester fraction from extracts of first leaves of Parkland and C.I.5791 after 60 seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

combined triose phosphates contained up to 77% of the activity. Many more pools of individual phosphate esters were significantly labelled after 60 seconds' fixation of $^{14}CO_2$ than after ca. two seconds, as would be expected: the hexose phosphates and sedoheptulose contained 19 - 21% of the total radioactivity, and the pentose phosphates up to 10%. Only traces of ^{14}C-UDPG were found; this compound seems to be recovered in substantial quantities relatively rarely after short-term fixation of $^{14}CO_2$ e.g. Benson and Calvin (1951), Norris et al. (1955) and Mortimer (1960) reported none in extracts of barley after such experiments; one of the few reports of large amounts of ^{14}C-UDPG is that of Tolbert and Gailey (1955) who examined extracts of wheat after ten minutes' exposure to $^{14}CO_2$. No significant differences were noted between the phosphate ester fractions from infected and control leaves of Parkland or C.I.5791.

Organic acid fraction: A typical autoradiograph obtained in the analyses of organic acid fractions in these experiments is shown in Plate 25. Table 35 shows the percentages of the total radioactivity recovered in the various components of the organic acid fractions from extracts of infected and control leaves of Parkland and C.I.5791, at three and five days after inoculation. The relative proportions of ^{14}C in glyceric acid and malic acid were quite similar, with the equivalence being more marked in extracts from infected leaves. After ca. two seconds' fixation, the activity in glyceric acid was higher than that in malic acid: the glyceric acid pool was apparently both labelled and turned over more rapidly than the malic acid pool. Benson and Calvin (1951) found similar changes in the relative quantities of these acids between 30 and 60 seconds' fixation of $^{14}CO_2$ in barley, though they did not comment on the relationship. ^{14}C-glycolate was recovered in all organic acid fractions, though in

smaller relative quantities (8 - 16%) than those found by Benson and Calvin (1951) after 60 seconds' exposure of barley to $^{14}CO_2$, from whose published data it can be calculated that ca. 23% of the total activity recovered in the organic acids resided in this acid. The results of some researchers (e.g. Bidwell et al., 1970; Zelitch, 1971) indicate that glycolate may be part of major photosynthetic pathways in green plants; but, as in these experiments, the glycolate pool is usually labelled more slowly than the 3PGA pool (e.g. Hess and Tolbert, 1966) and an early proposition (Wilson and Calvin, 1955) that the glycolic acid is derived from an intermediate of the Calvin cycle has currently received some support (Gibbs, 1971). ^{14}C-glyoxylate was sometimes recovered in the organic acid fractions, though its appearance could not be correlated with infection, nor with susceptibility or resistance to H.teres; this acid may well be formed from glycolic acid by glycolic acid oxidase (an enzyme whose activity may be reduced by infection such as that of wheat leaves by rust, as shown by Kiraly and Farkas,1957a) in the initial reaction of photorespiration.

Table 35: Percentage distribution of ^{14}C in the components of the organic acid fraction of extracts of infected first leaves of Parkland and C.I.5791 and healthy controls after 60 seconds' fixation of $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
Glycer	50	44	48	40	49	49	55	47
Mal	40	42	39	40	35	44	33	40
Glycol	8	10	13	15	16	7	8	11
Glyox	2	4	-	5	-	-	4	2

PLATE 25.

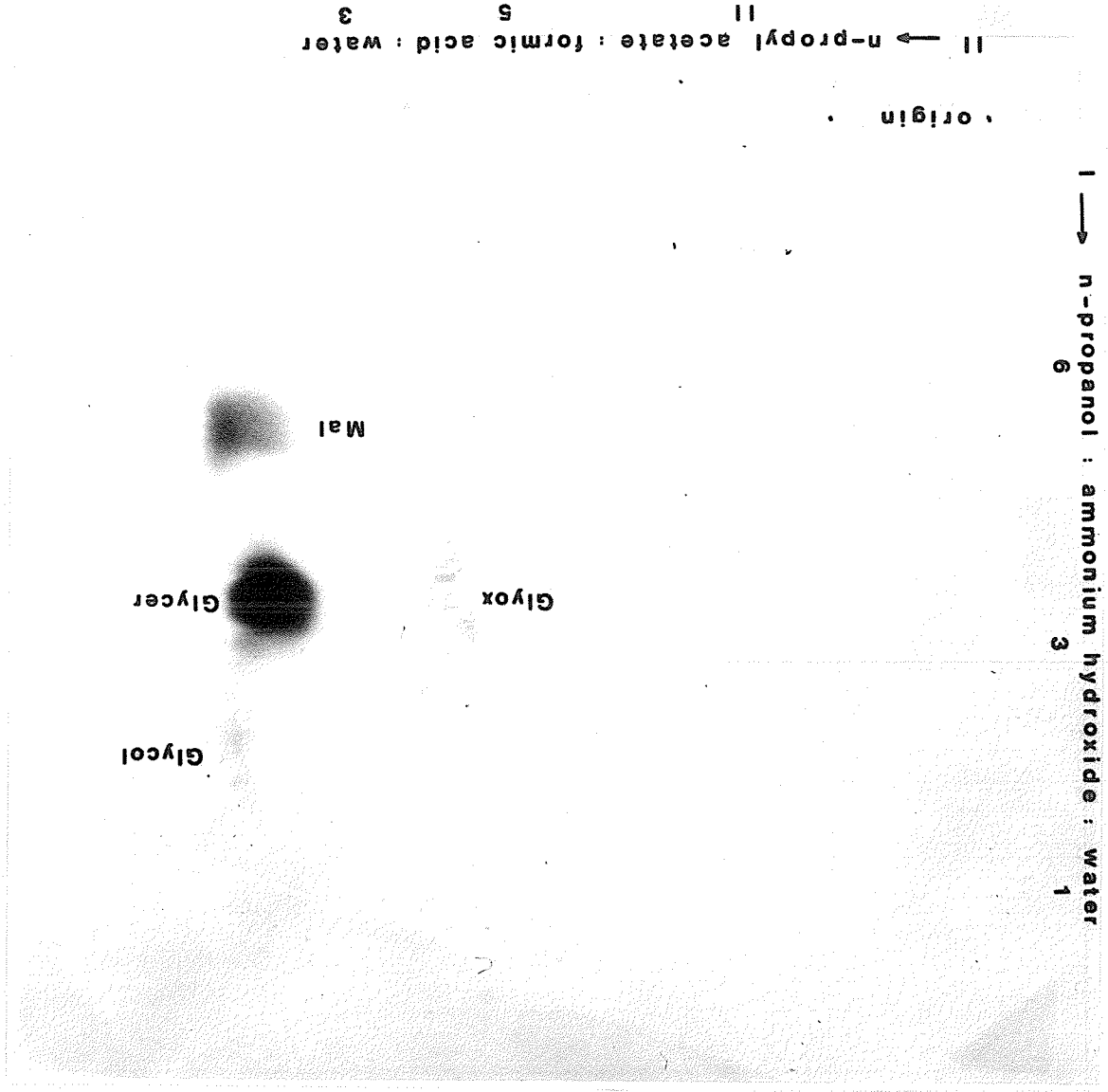


PLATE 25: Autoradiograph of a typical analysis by TLC of the organic acid fraction from extracts of first leaves of Parkland and C.I.5791 after 60 seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

Sugar fraction: A typical autoradiograph obtained in the analyses of sugar fractions in these experiments is shown in Plate 26. Table 36 shows the percentages of the total radioactivity in the components of the sugar fractions of infected and control leaves of Parkland and C.I.5791 at three and five days after inoculation.

Table 36: Percentage distribution of ^{14}C in the components of the sugar fractions of extracts of the infected first leaves of Parkland and C.I.5791 and healthy controls after 60 seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
Sucrose	82	85	88	87	86	90	87	83
Fructose	14	12	6	8	8	7	9	13
Glucose	4	3	4	4	6	3	2	3
pentoses	-	-	2	1	-	-	2	1

A similar pattern of distribution of activity was obtained in all extracts. A smaller proportion of the total radioactivity in the sugar fraction was recovered in fructose after 60 seconds' fixation than after ca. two seconds (Table 30); however, in terms of the percentage of the total radioactivity ofthe extract, the amounts of ^{14}C-fructose were very similar in the two series of experiments, 4 - 7% being present after ca. two seconds and 2 - 5% after 60 seconds: this may be an indication of its formation during extraction rather than in metabolism. Small amounts of labelled glucose were present in all fractions, and traces of ^{14}C-pentoses in some fractions, probably again the result of the extraction procedure.

PLATE 26.

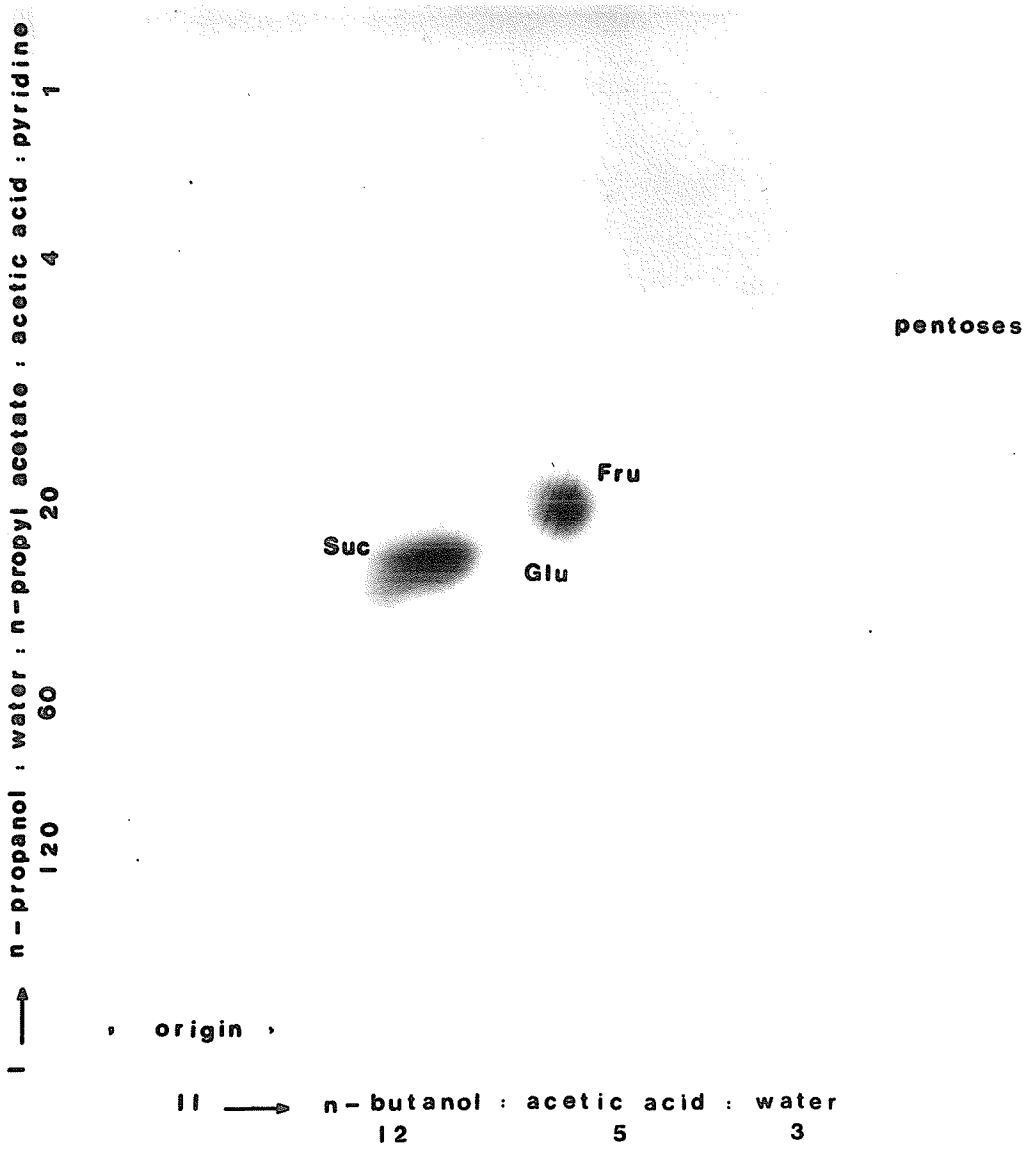


PLATE 26: Autoradiograph of a typical analysis by TLC of the sugar fraction from extracts of first leaves of Parkland and C.I.5791 after 60 seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

Amino acid fraction: A typical autoradiograph obtained in the analysis of amino acid fractions in these experiments is shown in Plate 27. Table 38 shows the percentages of the total radioactivity recovered in the components of the amino acid fractions of extracts of infected and control leaves of Parkland and C.I.5791 at three and five days after inoculation.

Table 38: Percentage distribution of ^{14}C in the components of the amino acid fractions of extracts of infected first leaves of Parkland and C.I.5791 and healthy controls after 60 seconds' exposure to $^{14}CO_2$.

radio-active products	% of total ^{14}C recovered in fraction							
	the susceptible reaction				the resistant reaction			
	3 days after inoculation		5 days after inoculation		3 days after inoculation		5 days after inoculation	
	H	I	H	I	H	I	H	I
Asp	42	54	40	51	35	49	42	47
Ser	45	34	50	38	53	39	48	43
Glu	5	3	4	2	5	4	3	4
Glu^n	8	9	6	9	7	8	7	6

As in the results of ca. two seconds' fixation (Table 31), most of the recovered radioactivity was resident in aspartic acid and serine, with neither glycine nor alanine being labelled in detectable quantities. Extracts from infected leaves of Parkland, and those from C.I.5791 at three days after inoculation, contained slightly higher proportions of ^{14}C-aspartic acid than ^{14}C-serine, whereas extracts from healthy controls and C.I.5791 at five days after inoculation showed rather more radioactivity in serine than in aspartic acid. Activity in glutamic acid was low in all extracts, probably indicating a rapid turnover of the glutamic acid pool

PLATE 27.

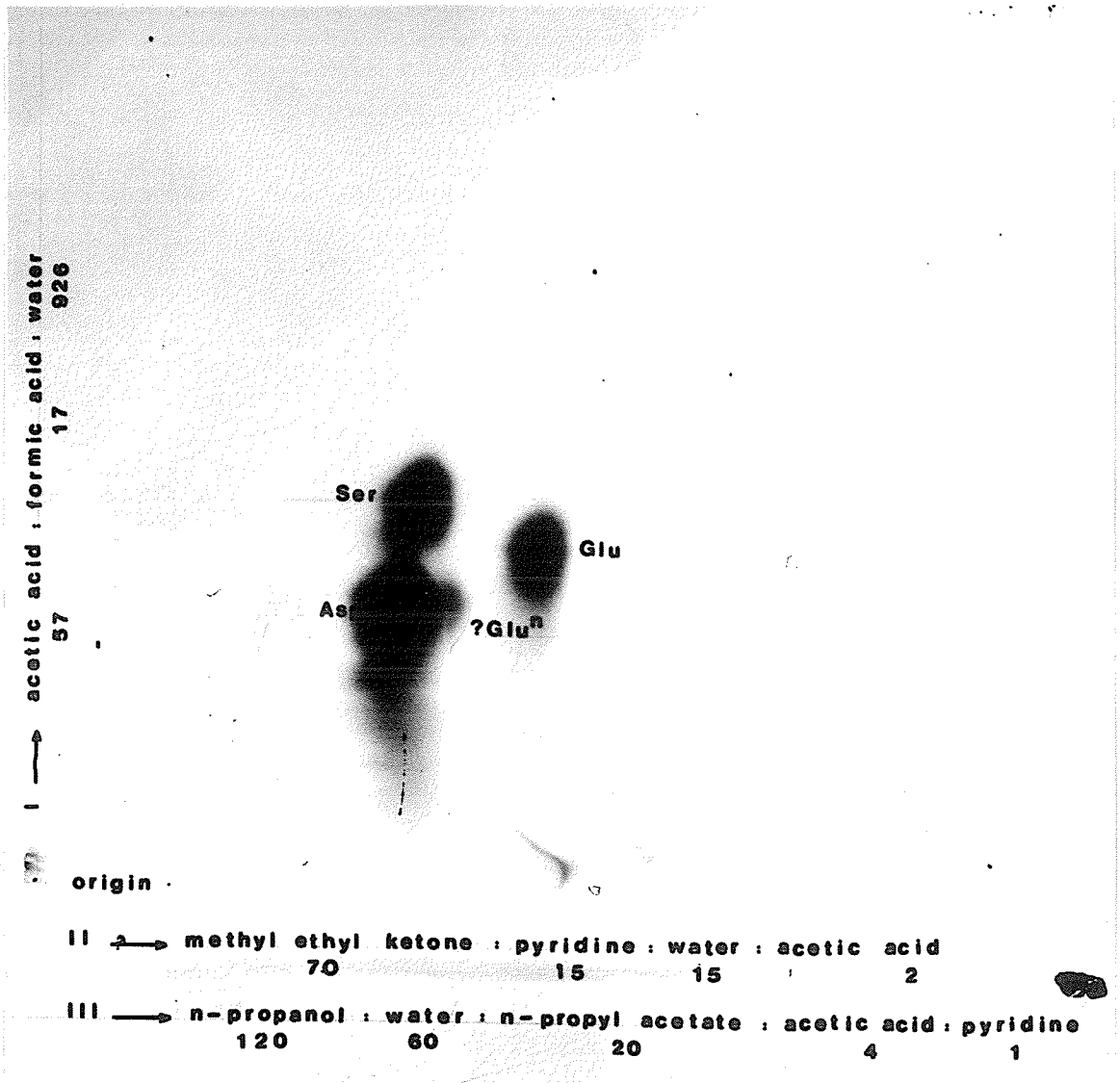


PLATE 27: Autoradiograph of a typical analysis by TLE and TLC of the amino acid fraction of extracts of first leaves of Parkland and C.I.5791 after 60 seconds' exposure to $^{14}CO_2$: 'liquid nitrogen method'.

since this amino acid was labelled after ca. two seconds' exposure to $^{14}CO_2$ (Table 31). The amino acid rather tentatively identified as glutamine (co-chromatography with authentic glutamine not always being obtained) contained a somewhat higher percentage of the total radio-activity than glutamic acid, which would be consistent with its formation from glutamic acid (Calvin and Bassham, 1962).

SECTION IV.

DISCUSSION.

In order to be detected by the methods employed in these experiments, a product of $^{14}CO_2$ fixation would have to contain a certain basic level of radioactivity. The inevitable loss in analysis in individual compounds will have a relatively greater effect on those compounds which contain little radioactivity than on those which have higher levels of ^{14}C: therefore a true representation of the comparative fixation of carbon by highly active and less active carboxylation systems will not necessarily be obtained from the results of such analyses. After sixty seconds' fixation of $^{14}CO_2$, a greater proportion of the products will be significantly labelled than after ca. two seconds; though some further metabolism of the radioactive products may have occurred in the longer experimental period, it should be of limited extent. Hence, though the data from the experiments of ca. two seconds' duration are useful in indicating the predominant compounds into which carbon dioxide is first incorporated in healthy and infected plants, and will show major changes in fixation pathways, the data from the experiments of sixty seconds' duration should give the better picture of carbon fixation as a whole, including indications of any minor changes in the early metabolism of carbon in infected as compared with healthy plants.

From the results of ca. two seconds' fixation of $^{14}CO_2$, it is apparent that there is no major alteration in the pathway by which carbon enters metabolism in Parkland or C.I.5791 barley leaves after infection with H.teres, thus confirming the observations in vitro of the relative activities of RuDP carboxylase, PEP carboxylase and the malic enzyme, and the results of the in vivo fixation of carbon in the light and dark

in healthy and infected leaves. The results of the isolated experiment with barley infected with P.graminis tritici indicate that rust-infection, like H.teres infection, does not cause any major shift in fixation pathway in either susceptible or resistant leaves.

From the results of the sixty-second experiments, an impression of the effects of infection on metabolism as a whole can be obtained. In the susceptible reaction, the percentage of the total radioactivity recovered in the phosphate esters was very similar in extracts from healthy and infected plants; thus, though less $^{14}CO_2$ was fixed by infected first leaves, it would appear upon first inspection that the relative contribution to the overall fixation by the Calvin cycle was not significantly altered, even though there were increasing reductions in the specific activity of RuDP carboxylase as infection progressed (Fig. 1). However, there were apparently disruptions along the route from the carboxylation step to the formation of sucrose, because an increasingly reduced proportion of the total radioactivity recovered was resident in the sugars after infection. This effect was probably due at least in part to a general slowing of metabolism upon infection, so that the labelled carbon remained in the individual phosphate ester pools for a longer period (such an effect might also result in a similarity in the proportion of the radioactivity recovered in healthy and infected material such as that observed in the phosphate esters when in fact a smaller proportion of the total $^{14}CO_2$ fixed was entering metabolism via the Calvin cycle in infected tissue); the Calvin cycle enzymes were apparently fairly uniformly affected since there was no evidence of a build-up of radioactivity in any specific phosphate ester pool. The slowing of sugar formation upon infection might also have been due to the siphoning of co-factors or even phosphorylated intermediates from the tissue by the fungal mycelium, as Cutter (1951)

proposed might occur in rusted tissue.

Both ^{14}C-labelled organic acids and amino acids showed slight increases in concentration in infected tissue. Glyceric acid has been cited as an overflow product of carbon fixation (p.160), and might therefore have been found ^{14}C-labelled in increasing amounts in tissue where tha Calvin cycle was in some way inhibited; but in fact there was no build-up of ^{14}C in this acid: indeed, the concentration of radioactive glyceric acid was somewhat decreased by infection. With reference to the total radioactivity recovered in each extract, malic, glycolic and glyoxylic acids all contained marginally more radioactivity in infected than in healthy tissue. It is not inconceivable that an increase in organic acids might result not only from an increased β-carboxylation but also from a build-up of the intermediates of photorespiration (see Discussion, Section III). A slightly greater proportion of acid products in infected cells could also create a more favourable environment for the growth of the fungal parasite.

In the amino acid fraction, aspartic acid contained significantly more radioactivity relative to the total ^{14}C recovered in the extracts after infection than in healthy controls. When the proportions of ^{14}C in serine are related to the radioactivity of the total extracts, it is apparent that the formation of serine was little changed by infection: the ratio of radioactivity I / H for serine at three and five days after inoculation is 6.0 / 6.5 and 8.5 / 7.5 respectively. Since serine is probably formed largely from 3PGA in the tissue, this is perhaps further evidence for the apparent similarity in the relative contribution of the Calvin cycle to total carboxylation in infected and healthy tissue. The small increase in radioactivity resident in the amino acid fraction, then, is largely located in aspartic acid. This acid is usually formed

from oxalacetic acid, and ^{14}C-oxalacetic acid probably comes largely from the products of β-carboxylation in short-term experiments: hence its presence could indicate an increased contribution of β-carboxylation to metabolism. It is conceivable that amino acids might be among the products of metabolism readily available to a parasitising fungal mycelium, and that enhanced levels of amino acids might be of value to the fungus in its development in the tissues.

It is of interest that some evidence of the effects of infection on overall metabolism deduced from the results of the sixty-second experiments are apparent in the results of the experiments of only ca. two seconds' duration.

If only ^{14}C-malic and ^{14}C-aspartic acids are assumed to come from β-carboxylation (Saltman et al., 1956; Kunitake et al., 1959) and all other ^{14}C-labelled products of sixty seconds' fixation of $^{14}CO_2$ directly or indirectly from the Calvin cycle, then at three days after inoculation ca. 15% of the total carbon comes from β-carboxylation in infected plants as compared with 10% in healthy plants; and at five days after inoculation, 16% comes from β-carboxylation in infected plants as compared with 9% in healthy plants. While these figures are gross approximations for both practical and theoretical reasons, they may perhaps indicate that the contribution of β-carboxylation to the total carbon fixed in the light is more significant than suggested by the short-term experiments in the light and the dark (Tables 13 and 15) where fixation in the dark was only ca.0.5% of that in the light, and nearer the relative carboxylation capacity of the enzymes as established in vitro, with a significant increase occurring in infected tissue.

The results of the sixty-second fixation experiments in infected resistant tissue tend to support the conclusions reached in earlier

experiments *in vitro* and *in vivo* that up until about three days after inoculation, the changes in metabolism in the resistant reaction to infection parallel those in the susceptible reaction, but after this time metabolism gradually reverts to the levels found in healthy tissue. The disruptions in metabolism were apparently not sufficient to significantly affect the incorporation of carbon into sugars at three days after inoculation and are therefore perhaps less severe than the effects of infection upon susceptible tissue at this time.

GENERAL DISCUSSION.

Since metabolism is in a dynamic state, shifts in one metabolic system lead to shifts in others. It would be tempting to interpret all the changes in metabolism observed in these experiments in Parkland infected with H.teres on the bases of changes in the relative levels of co-factors and substrates in infected tissue, particularly the levels of oxidised and reduced pyridine nucleotides and the balance of the various C_3 and C_4-dicarboxylic acids in the cells. Such changes could be pathogen-mediated; or host-mediated as a general response to stress or a more specific response to pathogenesis; or could be a function of the host-parasite interaction.

However, as well as changes in the overall rates of carboxylation in the light and the dark in vivo, there were changes at the specific level in the enzymes assayed; and hence the possibility of specific inhibition or stimulation of the various carboxylating enzymes must be considered. The enzymes as assayed in vitro were by no means purified: their activities as expressed per milligram protein changed only in relation to the total proteins precipitating in the various ammonium sulphate fractions. It is therefore quite conceivable that, rather than there being a specific activation or inhibition of the individual enzymes, the relative amounts of the functional enzymes in the tissues changed. This in itself would be an interesting effect of infection: while a decrease in functional RuDP carboxylase would seem almost certainly to be related to a general deleterious effect on the chloroplasts, it is perhaps significant that both the enzyes which increased in activity, PEP carboxylase and the malic enzyme, consist of multiple molecular forms, each form having a different solubility or location in the cell (Ting, 1971); if infection were to

bring about a change in the isoenzyme complement of these enzymes in the cell, an enhancement of their total activity could result. This hypothesis seems especially reasonable for the malic enzyme, of which both host and pathogen contain active molecules so that the enhanced activity could be a product of the host-parasite interaction; but should not be excluded for PEP carboxylase even though no enzyme was shown in extracts from the fungus in culture.

An increased activity of PEP carboxylase and the malic enzyme *in vivo* could be mediated by an increase in the soluble components of the enzymes; soluble protein has been shown to increase with infection in various tissues such as wheat when invaded by stem-rust (Shaw and Colotelo, 1961). Such soluble isoenzymes could mediate in the passage of carbon and co-factors between pools in the cell and enhance various metabolic processes of which β-carboxylation might be only a part.

It seems unlikely that there would be translocation of isoenzymes or enzyme components over more than small distances in the tissues, though diffusion of substrates etc. could presumably occur. Because the stimulatory effects of infection on the malic enzyme and more especially PEP carboxylase were exerted outside the immediate vicinity of the pathogen, specific stimulation of these enzymes by some product or products of the pathogen, the host or the host-parasite complex cannot be ruled out. Other species of *Helminthosporium*, including *H.maydis* Nis. & Miy., *H.sacchari* Butler, *H.carbonum* and *H.victoriae* are well-known for their ability to produce substances which enhance their infective capability; among the effects on susceptible tissues of the toxins of *H.carbonum* and *H.victoriae* are increases in the free amino acid content (as were shown in tissues infected with *H.teres*, p.169) as well as increases in the fixation of CO_2 in the dark (see summary by Yoder and Scheffer, 1972); *H.teres*

might well produce an analogous substance. The H.carbonum toxin does not act directly on the β-carboxylation system because, when added after extraction of the enzymes, there was no increase in activity (Kuo and Scheffer, 1970). H.carbonum and H.victoriae infections result in increases in the IAA content of the tissues, the H.carbonum infection (and application of the toxin) being accompanied by an increased growth and protein synthesis; if H.teres infection of susceptible barley were to produce a similar response, an effect at some point in enzyme synthesis, resulting in increased activity, could perhaps be envisaged. The fungi themselves might be capable of producing growth regulator substance: H.sativum in culture secretes terpenes with growth-regulating activity (van Andel and Fuchs, 1972). Such host-parasite interactions in the H.teres infection would not exclude the possibility of the formation of hybrid enzyme systems.

The deleterious effects of infection on the chloroplasts proposed earlier (p.78) to account for the loss of specific activity of RuDP carboxylase could similarly be due to toxic products of the fungus or host-parasite interaction, though the effects on the 'light' and 'dark' mechanisms in the chloroplasts would appear to be separate since the activity of RuDP carboxylase was affected in uninvaded tissue but chlorophyll levels were not (Table 4). The effects could conceivably be on the chloroplast membranes (see below), with RuDP carboxylase being affected before the integrity of the chlorophyll.

It seems probable that the primary lesion of the toxins of H.carbonum and H.victoriae is in the plasma membrane (Scheffer and Yoder, 1972): cell permeability is considerably affected and electrolyte leakage occurs, and Scheffer and Yoder (1972) proposed that other effects such as those on protein synthesis may be secondary. Keeling (1966) found some evidence

of a change in the permeability of cells of barley after infection with H.teres, perhaps a further indication of analogous effects in the different Helminthosporium diseases. Metabolites and enzymes are organised spatially within cells, so that an orderly sequence of metabolic events is maintained; there may be several pools of key intermediates within each cell (Ting, 1971). Once this organisation is disrupted, as it could be either by the presence or the products of a pathogen or by the host response to pathogenesis, general metabolic alterations may occur and certain stimulations result simply from the increased availability of reaction sites. The increases in respiration in infected tissue are probably in part of this nature, and it seems likely that the increases in β-carboxylation shown in this work similarly result from changes in the relative levels of co-factors, substrates etc. available to different enzyme systems, as proposed in the first paragraph of this Discussion; and indeed, the two could well be related, respiration producing the substrates and reduced co-factors for β-carboxylation. Moreover, a loss of capacity for photorespiration, as proposed on p.120, could result from the loss of integrity of the membrane systems of the microbodies, or even the membranes of the chloroplasts if there is close association between glycolate synthesis and photosynthetic electron transport (Gibbs, 1971).

Cell response to disorganisation usually leads to the death of the cell, and it is notable that the stimulation of fixation in the dark and the activities of the malic enzyme and PEP carboxylase only persisted until the onset of sporulation of H.teres, when tissue damage began to be severe. However, the stimulation of metabolism, though relatively short-lived, is probably advantageous to the pathogen, which is in a phase of very active anabolism: for example, the increased β-carboxylation reactions

of the host-parasite complex could act as anaplerotic sequences (Kornberg, 1966) for the replenishment of pools of acid intermediates depleted by the pathogen, as suggested by Mirocha (1972). Moreover, in terms of the total contribution of carbon to the tissue, β-carboxylation itself may be of greater significance than is sometimes proposed (e.g. Livne and Daly, 1966). There may be other advantages to the parasite of a more active β-carboxylation: Luke and Freeman (1965) have proposed that the increase in malic acid often shown in infected cells (and in H.teres infection of barley, p.169) could buffer the excessive amounts of cations leaking out of the vacuole and maintain a physiologically functional cell environment.

The alteration in translocation patterns of H.teres-infected plants may be in part a result of general disorganisation of the tissues, but might also be the result of the secretion in the host-parasite complex of specific substances such as growth factors as proposed earlier. Auxins and cytokinins have been shown to induce accumulation (Mothes and Engelbrecht, 1961; Pozsar and Kiraly, 1964), though attempts to isolate cytokinins from obligately parasitic micro-organisms or plants attacked by them have not so far been successful (Shaw and Srivastava, 1964). The retention of photosynthate by the infected first leaves may, per se, bring about reductions in the photosynthetic rate of the leaves by feed-back mechanisms as discussed by Durbin (1967), which are quite apart from the disruptions of photosynthesis induced more directly by infection. If the effects of H.teres infection on the leaves of more mature plants of Parkland are similar to those on the first leaves, then maturation and reproduction are no doubt seriously impaired by fairly limited infection.

From the results obtained in this work, it seems unlikely that the responses to infection of the carboxylation mechanisms or of translocation

in C.I.5791 are connected directly with the resistance of this barley variety to parasitism by H.teres, but rather are part of the response to infection. The metabolic effects observed in C.I.5791 were very similar to those in Parkland as disease symptoms developed in both varieties; but while fungal growth and development continued in Parkland, it was curtailed in C.I.5791 after the formation of pin-point lesions at three or four days after inoculation, and the metabolism of the resistant leaves then returned to normal. It would seem that the effects of infection on the aspects of the metabolism of Parkland and C.I.5791 examined are more or less proportional to the extent of development of the the pathogen. This, of course, gives no indication as to whether they are host-mediated as the host responds to the stress of infection; or a function of host-parasite interaction before the death of the infected cells and the related inhibition of the fungus; or mediated by some product of active metabolism of the fungus and therefore only persisting as long as the fungus can actively develop in the resistant tissue.

The host-parasite interaction in the susceptible and resistant complexes could be essentially similar, but curtailed in the resistant tissue by an earlier inhibition of fungal growth. The inhibition could, at least in part, be brought about by some of the very changes in host cells which are in their early stages stimulatory, such as cellular disorganisation, the differences between resistance and susceptibility merely being the speed at which the effects become deleterious. The results of Keeling (1966) working with H.teres infections of barley tend to support this hypothesis: he showed that the permeability changes in the cells following infection occurred more rapidly in resistant than in susceptible varieties. Kaul and Shaw (1960) have proposed that disturbances

between oxidative and reductive processes leading to excessive oxidation of polyphenol compounds and a breakdown of cellular structure account for the hypersensitive response of wheat to some races of stem rust, with a normal redox potential being maintained longer in susceptible tissues. Certainly, eventual breakdown of metabolism occurred in the susceptible reaction of Parkland to H.teres, when the stimulated β-carboxylations and enzyme activities began to decline; it is highly conceivable that such a breakdown occurred three or four days after infection in those tissues which were responsible for the early increases in carboxylation in the dark and the stimulated enzyme activities in C.I.5791.

Such an explanation of the changes in metabolism observed in the resistant reaction of C.I.5791 to infection with H.teres implies a constitutive resistance mechanism, without, of course, excluding the possibility of induced mechanisms; indeed, Keeling (1966) has proposed both constitutive resistance and induced phytoalexin-like products to account for the resistance of some barley varieties to infection with H.teres. There is evidence to suggest that in H.carbonum and H.victoriae infections, susceptibility and resistance result from the presence of a toxin receptor or sensitive site in the susceptible cell and the absence of such a site in the resistant cell (Scheffer and Yoder, 1972); but there is a metabolic response in resistant corn tissue to infection with H.carbonum since carboxylation in the dark rises upon infection, as Kuo and Scheffer (1970) demonstrated, though the origin of the metabolic response is not clear: it could conceivably be part of a general response of the tissues to disorganisation, as proposed above, and the metabolic response of C.I.5791 could be of a similar nature.

Until specific compounds which can bring about the metabolic changes observed in Parkland and C.I.5791 barley upon infection with H.teres are isolated from the pathogen, or from the host after its specific response to the stress of pathogenesis, or the host-parasite complex as a result of their interaction, it will be impossible to determine the origins of the metabolic effects. However, it seems very likely that both the changes in translocation patterns and the stimulation of the β-carboxylation mechanisms in Parkland barley, in the face of increasing tissue disorganisation and the concomitant loss of photosynthetic ability as the pathogen develops, make a very significant contribution to the reproductive capacity in situ of H.teres.

SUMMARY.

1. Some metabolic effects of H.teres infections of Parkland (susceptible) and C.I.5791 (resistant) were examined in vitro and in vivo. The first leaves of seedling plants were inoculated, either uniformly or at their tips: aspects of the metabolism of these and the young (uninoculated) second leaves were investigated.

2A. Over the seven-day infection cycle (from inoculation to sporulation) in susceptible first leaves, per mg protein: RuDP carboxylase activity fell to ca. 50% the levels of controls (remaining more or less constant per mg chlorophyll), while PEP carboxylase activity increased two-fold and that of the malic enzyme three- to four-fold. Similar though less extreme changes were detected in the uninvaded tissue of tip-inoculated leaves. The effects on the second leaves seemed to be indicative of a general disturbance of metabolism rather than being specifically the result of infection.

2B. In resistant first leaves at three days after inoculation, per mg protein: RuDP carboxylase activity had fallen by ca. 30% while PEP carboxylase activity had increased by ca. 30%; but by five days, the activities of these enzymes were again similar to controls. The malic enzyme activity had increased two-fold by 24 hours after inoculation, but then slowly declined to the levels of controls. Only the effects on PEP carboxylase were detectable in uninvaded tissue; metabolism in the second leaves was unaffected.

3. No activity of PEP carboxylase could be detected in fungal

extracts, but the malic enzyme was present and its activity was higher in sporulating than vegetative mycelium.

4A. Over the seven-day infection cycle, CO_2 fixation in the light per dm^2 in susceptible leaves fell to ca. 50% the levels of controls, though falling by only 20% per mg chlorophyll. Fixation in the dark per dm^2 increased ca. 2.5-fold, peaking just before sporulation of the fungus.

4B. In resistant leaves, fixation in the light had fallen by 25% while fixation in the dark had increased by ca. 30% (per dm^2) at three to four days after inoculation; but by five days, fixation in the light and the dark was again similar to controls.

4C. The effects of infection on CO_2 fixation over the infection cycle in a barley variety B.T.201, in which resistance to H.teres was variable, were examined in light and dark and found to be correlated with the apparent degree of resistance of individual plants.

5. Bleaching and mechanical damage of leaves of Parkland resulted in reductions in CO_2 fixation in the light correlated with the amount of chlorophyll loss, and a stimulation of ca. 30% in fixation in the dark.

6A. The net accumulation of carbon per dm^2 after a 24-hour diurnal cycle (16 hours' illumination) in infected susceptible leaves (I) was reduced (as compared with controls, H) by ca. 10% at four days, and 25% at six days, after inoculation. Net accumulation in the second leaves was reduced at four days, but was equivalent to controls at six days. The ratios I / H of net

accumulation per dm^2 after 16 hours' illumination only were very similar, though the net accumulation per dm^2 in all samples was higher after 16 than after 24 hours.

6B. The net carbon accumulation over 24 hours in infected resistant plants was unaffected in either first or second leaves at four or six days after inoculation. The ratios I / H after 16 hours' illumination only were very similar, except at four days after inoculation, when infection resulted in a 10% reduction in net accumulation by the first leaves. Net accumulation was again higher than after the 24-hour diurnal cycle.

7A. Net carbon accumulation in the eight-hour dark period in infected susceptible first leaves was 3.2 - 4.2-fold that in controls at both four and six days after inoculation. The slight diurnal fluctuations in net accumulation in the dark were similar in healthy and infected leaves. Net accumulation in second leaves was reduced 15 - 25% at four days, and 10 - 15% at six days.

7B. Net accumulation in the eight-hour dark period in infected resistant first leaves was 40 - 60% greater than controls at four days, but similar to controls at six days, after inoculation. There was little change in the second leaves.

8. Translocation from infected susceptible first leaves to the uninfected second leaves was decreased by ca. 50% at four days, and 75% at six days, after inoculation. Export by infected resistant leaves was slightly reduced at four days, but was similar to controls at six days. There was little movement of carbon from second to first leaves, and this was unaffected by infection.

9. Healthy and infected first leaves of susceptible and resistant plants were exposed to $^{14}CO_2$ in air for ca. two seconds at three and five days after inoculation: distribution of ^{14}C in the products indicated that in both healthy and infected plants, carbon entered metabolism predominantly via the Calvin cycle. Comparative experiments on Parkland and C.I.5791 infected with the obligate parasite Puccinia graminis tritici, to which the barley varieties were resistant and susceptible respectively, at seven and 14 or 16 days after inoculation similarly indicated no change in the main carboxylation pathway in barley upon infection.

10. $^{14}CO_2$ in air was fed to healthy and infected first leaves of susceptible and resistant plants for 60 seconds: the distribution of ^{14}C in the products showed that infection resulted in reduced sucrose formation, though there was no indication of a specific blockage in the Calvin cycle; and an increase in the synthesis of organic acids and amino acids seemed to be due at least in part to increased β-carboxylation in infected tissue.

11. The possible origins of the shifts in metabolism upon infection in Parkland and C.I.5791 are discussed and some suggestions made as to their significance in the development of the pathogen in susceptible tissue.

BIBLIOGRAPHY.

Ahmed, A. M. M. Veranderungen der $^{14}CO_2$ fixierung im entwicklungs-cyclus der Chlorella pyrenoidosa. Ph.D. Thesis, Universitat Tubingen, Tubingen, Germany.

Akai, S. and H. Tanaka. 1955. Change of the respiration and carbon-assimilation in leaves of rice plants infected by Cochliobolus miyabeanus. Forsch. Gebeit Pflanzenkrankenheit (Kyoto) 5: 95-104.

Allen, P. J. 1942. Changes in the metabolism of wheat leaves induced by infection with powdery mildew. Amer. J. Bot. 29: 425-435.

Allen, P. J. 1953. Toxins and tissue respiration. Phytopath. 43: 221-229.

Allen, P. J. 1954. Physiological aspects of fungus diseases of plants. Ann. Rev. Plant Physiol. 5: 225-248.

Allen, P. J. 1959. The physiology and biochemistry of defence. In Plant Pathology: An Advanced Treatise. Edited by J. G. Horsfall and A. E. Dimond. Academic Press, N.Y. pp. 435-467.

Allen, P. J. and D. R. Goddard. 1938. A respiratory study of powdery mildew of wheat. Amer. J. Bot. 25: 613-621.

Allen, R. F. 1926. A cytological study of Puccinia triticina physiologic form 11 on Little Club wheat. J. Agr. Research 33: 201-222.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol-oxidases in Beta vulgaris. Plant Physiol. 24: 1-15.

Atanasoff, D. 1920. Treatment of cereal seeds with dry heat. J. Agr. Research 18: 379-390.

Atkins, C. A. and D. T. Canvin. 1971. Photosynthesis and CO_2 evolution by leaf discs: gas exchange, extraction, and ion-exchange fractionation of ^{14}C-labelled photosynthetic products. Can. J. Bot. 49: 1225-1234.

Atkins, C. A., D. T. Canvin and H. Fock. 1971. Intermediary metabolism

in relation to carbon dioxide evolution in sunflower. In Photosynthesis and Photorespiration. Edited by M. D. Hatch, C. B. Osmond and R. O. Slatyer. Wiley-Interscience, N.Y. pp. 497-505.

Bagi, G. and G. L. Farkas. 1967. On the nature of increase in ribonuclease activity in mechanically damaged tobacco leaf tissue. Phytochem. 6: 161-169.

Bandurski, R. S. and C. M. Greiner. 1953. The enzymatic synthesis of oxaloacetate from phosphoryl enolpyruvate and carbon dioxide. J. Biol. Chem. 204: 781-786.

Bassham, J. A., A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson and M. Calvin. 1954. The path of carbon in photosynthesis XXI. The cyclic regeneration of the carbon dioxide acceptor. J. Amer. Chem. Soc. 76: 1760-1770.

Bassham, J. A. and M. Calvin. 1957. The Path of Carbon in Photosynthesis. Prentice-Hall, Inc., Englewood Cliffs, N.J.

Bassham, J. A. and M. Kirk. 1960. Dynamics of the photosynthesis of carbon compounds I. Carboxylation reactions. Biochim. Biophys. Acta 43: 447-464.

Bassham, J. A. and M. Kirk. 1964. Photosynthesis of amino acids. Biochim. Biophys. Acta 90: 553-562.

Beevers, H. 1971. Photorespiration: assessment. In Photosynthesis and Photorespiration. Edited by M. D. Hatch, C. B. Osmond and R. O. Slatyer. Wiley-Interscience, N.Y. pp. 541-543.

Benson, A. A. 1955. Phosphorylated sugars. In Modern Methods of Plant Analysis. Vol. 2. Edited by K. Paech and M. V. Tracey. Springer-Verlag, Berlin. pp. 113-144.

Benson, A. A. and Calvin, M. 1951. The path of carbon in photosynthesis VII. Respiration and photosynthesis. J. Exptl. Bot. 1: 63-68.

Bhattacharya, P. K. and M. Shaw. 1967. The physiology of host-parasite relations XVIII. Distribution of tritium-labelled cytidine, uridine and leucine in wheat leaves infected with the stem rust fungus. Can. J. Bot. 45: 555-563.

Bidwell, R., W. B. Levin and D. C. Shephard. 1970. Intermediates of photosynthesis in Acetabularia mediterranea chloroplasts. Plant Physiol. 45: 70-75.

Bieleski, R. L. 1964. The problem of halting enzyme activity when extracting plant tissues. Anal. Biochem. 9: 431-442.

Bieleski, R. L. and N. A. Turner. 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Anal. Biochem. 17: 278-293.

Bieleski, R. L. and R. E. Young. 1963. Extraction and separation of phosphate esters from plant tissues. Anal. Biochem. 6: 54-68.

Bjorkman, O. and E. Gauhl. 1969. Carboxydismutase activity in plants with and without β-carboxylation photosynthesis. Planta 88: 197-203.

Black, L. L., D. T. Gordon and P. H. Williams. 1968. Carbon dioxide exchange by radish tissue infected with Albugo candida measured with an infrared CO_2 analyser. Phytopath. 58: 173-178.

Bolar, M. L. 1962. Studies of tricarboxylic acid cycle intermediates in safflower infected with Puccinia carthami. Phytopath. 52: 4 (Abstr.)

Bushnell, W. R. and Allen, P. J. 1962a. Induction of disease symptoms in barley by powdery mildew. Plant Physiol. 37: 50-59.

Bushnell, W. R. and P. J. Allen. 1962b. Respiratory changes in barley leaves produced by single colonies of powdery mildew. Plant Physiol. 35: 751-758.

Calvin, M. and J. A. Bassham. 1962. The Photosynthesis of Carbon Compounds. Benjamin, N.Y.

Cohen, Y. and J. Rotem. 1970. The relatioship of sporulation to photosynthesis in some obligatory and facultative parasites. Phytopath. 60: 1600-1604.

Collins, R. P. and R. P. Scheffer. 1958. Respiratory responses and systemic effects in Fusarium-infected plants. Phytopath. 48: 349-355.

Cook, A. R. and R. L. Bieleski. 1969. Fractionation of plant extracts by thin-layer electrophoretic and chromatographic procedures. Anal. Biochem. 28: 428-435.

Cooper, T. G., T. T. Tchen, H. G. Wood and C. R. Benedict. 1968. The carboxylation of phosphoenolpyruvate I. The active species of 'CO_2' utilized by phosphoenolpyruvate carboxykinase, carboxytransphosphorylase and pyruvate carboxylase. J. Biol. Chem. 243: 3857-3863.

Cornu, M. 1881. See Allen, P. J. 1942.

Cutter, V. M. Jr. 1951. The isolation of plant rusts upon artificial media and some speculations on the metabolism of obligate plant parasites. Trans. N.Y. Acad. Sci. 14: 103-108.

Dabala, I. and I. Muteanu. 1969. Changes in the proportion of the main pigments of the barley leaves infected with Helminthosporium gramineum Rb. Phytopath. Z. 65: 373-378.

Daly, J. M., A. A. Bell and L. R. Krupka. 1961. Respiration changes during development of rust diseases. Phytopath. 51: 461-471.

Daly, J. M. and S. G. Jensen. 1961. Evidence for functional cytochrome oxidase in higher plant tissues infected with obligate parasites. Phytopath. 51: 759-765.

Daly, J. M. and L. R. Krupka. 1962. Effect of Puccinia graminis tritici on organic acid content of wheat leaves. Plant Physiol. 37: 277-282.

Daly, J. M. and A. Livne. 1966. Dark fixation of CO_2 by healthy and rust-affected leaves of wheat and bean. Phytopath. 56: 164-169.

Daly, J. M. and R. H. Sayre. 1957. Relations between growth and respiratory metabolism in safflower infected by Puccinia carthami. Phytopath. 47: 163-168.

Daly, J. M., R. H. Sayre and J. H. Pazur. 1957. The hexose monophosphate shunt as the major respiratory pathway during sporulation of rust of safflower. Plant Physiol. 32: 44-48.

Dickson, J. G. 1956. Diseases of Field Crops. McGraw-Hill, N.Y.

Doubly, J. A., H. H. Flor and C. O. Clagett. 1960. Relation of antigens of Melampsora lini and Linum usitatissimum to resistance and susceptibility. Science 131: 229-246.

Drechsler, C. 1934. Phytopathological and taxonomic aspects of Ophiobolus, Pyrenophora, Helminthosporium and a new genus, Cochliobolus. Phytopath. 24: 953-983.

Durbin, R. D. 1967. Obligate parasites: effect on the movement of solutes and water. In The Dynamic Role of Molecular Constituents in Plant-Parasite Interaction. Edited by C. J. Mirocha and I. Uritani. The American Phytopathological Society, Inc. St. Paul, Minn. pp. 80-99.

Edwards, H. H. 1970. Biphasic inhibition of photosynthesis in powdery mildewed barley. Plant Physiol. 45: 594-597.

Edwards, H. H. 1971. Translocation of carbon in powdery mildewed barley. Plant Physiol. 47: 324-328.

Edwards, H. H. and Allen, P. J. 1966. Distribution of the products of photosynthesis between powdery mildew and barley. Plant Physiol. 41: 683-688.

Ehrlich, H. G. and Ehrlich, M. A. 1962. Electron microscopy of the host-parasite relationship in stem rust of wheat. Amer. J. Bot. 50: 123-130.

Ehrlich, M. A. and H. G. Ehrlich. 1970. Electron microscope radioautography of ^{14}C transfer from rust uredospores to wheat host cells.

Phytopath. 60: 1850-1851.

Farkas, G. L. and Z. Kiraly. 1955. Studies on the respiration of wheat infected with stem rust and powdery mildew. Physiol. Plantarum 8: 877-887.

Farkas, G. L. and Kiraly, Z. 1962. Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopathol. Z. 44: 105-150.

Fedotova, T. I. 1940. Immunological character of various species and varieties of wheat in relation to wheat leaf rust (Puccinia triticina) Vestnik Zashch. Rast. 4: 123-130.

Flor, H. H. 1941. Inheritance of rust reaction in a cross between the flax varieties Buda and J. W. S. J. Agr. Research 63: 369-388.

Flor, H. H. 1942. Inheritance of pathogenicity in Melampsora lini. Phytopath. 32: 653-669.

Flor, H. H. 1946. Genetics of pathogenicity in Melampsora lini. J. Agr. Research 73: 335-357.

Flor, H. H. 1947. Inheritance of reaction to rust in flax. J. Agr. Research 74: 241-162.

Forward, D. F. 1932. The influence of altered host metabolism upon modification of the infection type with Puccinia graminis tritici p.f.21. Phytopath. 22: 493-555.

Fowler, A. M. and H. Owens. 1971. Studies on leaf blotch of barley (Rhynchosporium secalis). Trans. Br. mycol. Soc. 56: 137-152.

Gibbs, M. 1971. Biosynthesis of glycolic acid. In Phtosynthesis and Photorespiration. Edited by M. D. Hatch, C. B. Osmond and R. O. Slatyer. Wiley-Interscience, N.Y. pp. 433-441.

Gordon, C. F. and A. L. Wolfe. 1960. Liquid scintillation counting of aqueous samples. Anal. Chem 32: 574-575.

Gottlieb, D. and J. M. Garner. 1946. Rust and phosphorus distribution in wheat leaves. Phytopath. 36: 557-564.

Grainger, J. 1947. The ecology of Erysiphe graminis DC. Trans. Br. mycol. Soc. 31: 54-65.

Grecusnikov, A. I. 1936. The physiology of the inoculation period in rust infections. Compt. rend. acad. sci. U.R.S.S. N.S. 2: 245-247.

Harary, I., S. R. Karey and S. Ochoa. 1953. Biosynthesis of dicarboxylic acids by carbon dioxide fixation VII. Equilibrium of "malic" enzyme reaction. J. Biol. Chem. 203: 595-604.

Harder, D. E., J. W. Martens and R. I. H. McKenzie. 1971. Changes in chlorophyll and carotenoid content in oats associated with the expression of adult plant resistance to stem rust conferred by gene pg-11. Can. J. Bot. 49: 1783-1786.

Harding, H., P. H. Williams and S. S. McNabola. 1968. Chlorophyll changes, photosynthesis and ultrastructure of chloroplasts in Albugo candida induced 'green islands' on detached Brassica juncea cotyledons. Can. J. Bot. 46: 1229-1234.

Harris, J. L. 1972. A method for separating aerial hyphae from surface growth. Mycologia 64: 221-223.

Haspelova- Horvatovicova, A. 1971. Changes of leaf pigments in resistant barley infected with powdery mildew. Biologia (Bratislava) 26: 19-26.

Hassebrauk, K. 1940. Zur frage der Wirkung von Aussenfaktoren auf verschiedene Stadien von Weizenbraun-rostinfectionen. Phytopath. Z. 12: 490-508.

Hatch, M. D. and C. R. Slack. 1966. Photosynthesis by sugar-cane leaves. A new carboxylation reaction and the pathway of sugar formation. Biochem. J. 101: 103-111.

Hatch, M. D. and C. R. Slack. 1970. Phtosynthetic CO_2-fixation pathways.

Ann. Rev. Plant Physiol. 21: 141-162.

Hatch, M. D., C. R. Slack and T. A. Bull. 1969. Light-induced changes in the content of some enzymes of the C_4-dicarboxylic acid pathway of photosynthesis and its effects on other characters of photosynthesis. Phytochem. 8: 697-706.

Heitefuss, R. and W. H. Fuchs. 1961. Untersuchungen zum Phosphatstoffwechsel von Weizenkeimpflanzen nach Infektion mit Puccinia graminis tritici. Naturwissenschaften 48: 505-506.

Heitefuss, R. and W. H. Fuchs. 1963. Phosphatstoffwechsel und Sauerstoffaufnahme in Weizenkeimpflanzen nach Infektion mit Puccinia graminis tritici. Phytopathol Z. 46: 174-198.

Hess, J. L. and N. E. Tolbert. 1966. Glycolate, glycine, serine and glycerate formation during photosynthesis by tobacco leaves. J. Biol. Chem. 241: 5705-5711.

Iljin, W. S. 1923. Einfluss des Welkens auf die Atmung der Pflanzen. Flora 116: 379-404.

James, W. P. 1953. Plant Respiration. Clarendon Press, Oxford.

Katz, J. and H. G. Wood. 1960. The use of Glucose-C^{14} for the evaluation of the pathways of glucose metabolism. J. Biol. Chem. 235: 2165-2177.

Kaul, R. and M. Shaw. 1960. The physiology of host-parasite relations VI. Oxidation-reduction changes in wheat leaf sap caused by rust-infection. Can. J. Bot. 38: 399-407.

Keeling, B. L. 1966. Studies on the nature of barley resistance to Helminthosporium teres. Ph.D. Thesis, University of Minnesota, St. Paul-Minneapolis, Minnesota.

Kiraly, Z. and G. L. Farkas. 1957a. Decrease in glycolic acid oxidase activity of wheat leaves infected with Puccinia graminis var. tritici. Phytopath. 47: 277-278.

Kiraly, Z. and G. L. Farkas. 1957b. On the role of ascorbic oxidase in the parasitically increased respiration of wheat. Arch. Biochem. Biophys. 66: 474-485.

Kiraly, Z. and G. L. Farkas. 1962. Relation between phenol metabolism and stem rust resistance in wheat. Phytopath. 52: 756-664.

Kiraly, Z., B. I. Pozsar and M. El Hammady. 1966. Cytokinin acitivity in rust-infected plants: Juvenility and senescence in diseased leaf tissue. Acta Phytopathol. Hung. 1: 29-37.

Kleinkopf. G. E., R. C. Huffaker and A. Matheson. 1970. A simplified purification and some properties of ribulose 1,5-diphosphate carboxylase from barley. Plant Physiol. 46: 204-207.

Kornberg, H. L. 1966. Anaplerotic sequences and their role in metabolism. In Essays in Biochemistry. Edited by P. N. Campbell and C. D. Greville. Vol. 2. Academic Press, N.Y. pp. 1-32.

Kourssanov, A. L. 1928. De l'influence de l'Ustilago tritici sur les fonctions physiologique du froment. Rev. Gen. Bot. 40: 343-371.

Krupka, L. R. 1959. Metabolism of oats susceptible to Helminthosporium victoriae and victorin. Phytopath. 49: 587-594.

Kunitake, G., C. Stitt and P. Saltman. 1959. Dark fixation of CO_2 by tobacco leaves. Plant Physiol. 34: 123-127.

Kuo, M. S. and R. P. Scheffer. 1970. Comparative effects of host-specific toxins and Helminthosporium infections on respiration and carboxylation by host tissue. Phytopath. 60: 1391-1394.

Leloir, L. F. 1964. Nucleoside diphosphate sugars and saccharide synthesis. Biochem. J. 91: 1-8.

Livne, A. 1964. Photosynthesis in healthy and rust-infected plants. Plant Physiol. 39: 614-621.

Livne, A. and J. M. Daly. 1962. CO_2-fixation in healthy and rust-affected

tissues. Phytopath. 52: 18 (Abstr.)

Livne, A. and J. M. Daly. 1966. Translocation in healthy and rust-affected beans. Phytopath. 56: 170-175.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Luke, H. H. and Freeman, T. E. 1965. Effects of victorin on Krebs cycle intermediates of a susceptible oat variety. Phytopath. 55: 967-969.

Lunderstadt, J., R. Heitefuss and W. H. Fuchs. 1962. Aktivitat einiger Enzyme des Kohlehydratstoffwechsels aus Weizenkeimpflanzen nach Infektion mit Puccinia graminis tritici. Naturwissenschaften 17: 403-407.

Mains, E. B. 1917. The relation of some rusts to the physiology of their hosts. Amer. J. Bot. 4: 179-220.

Mains, E. B. 1926. Studies in rust resistance. J. Heredity 17: 313-325.

Malca, I., R. C. Huffaker and F. P. Zscheile. 1964. Stimulation and depression of cell-free carboxylating systems in relation to development of the Helminthosporium leaf spot disease of maize. Phytopath. 54: 663-669.

Malca, I., R. C. Huffaker and F. P. Zscheile. 1965. Changes in enzyme activity in relation to powdery mildew disease development in barley. Phytopath. 55: 442-446.

Malca, I. and F. P. Zscheile. 1963. Effect of the Helminthosporium leaf-spot disease on organic acid content of corn seedlings. Phytopath. 53: 341-343.

Mapson, L. W. and D. R. Goddard. 1951. The reduction of glutathione by plant tissues. Biochem. J. 49: 592-601.

McDonald, W. C. 1967. Variability and the inheritance of morphological mutants in Pyrenophora teres. Phytopath. 57: 747-755.

McDonald, W. C. and K. W. Buchannon. 1964. Barley yield reductions attributed to net blotch infection. Can. Plant Dis. Survey 44: 118-119.

Millerd, A. and K. J. Scott. 1956. Host-pathogen relations in powdery mildew of barley II. Changes in respiratory pattern. Aust. J. Biol. Sci. 9: 37-44.

Millerd, A. and K. J. Scott. 1962. Respiration in the diseased plant. Ann. Rev. Plant Physiol. 13: 559-574.

Millerd, A. and K. J. Scott. 1963. Host-pathogen relations in powdery mildew of barley III. Utilization of respiratory energy. Aust. J. Biol. Sci. 16: 775-783.

Mirocha, C. J. 1972. Phytotoxins and metabolism. In Phytotoxins in Plant Diseases. Edited by R. K. S. Wood, A. Ballio and A. Graniti. Academic Press, Lond. pp. 191-210.

Mirocha, C. J. and P. D. Rick. 1967. Carbon dioxide fixation in the dark as a nutritional factor in parasitism. In The Dynamic Role of Molecular Constituents in Plant-Parasite Interaction. Edited by C. J. Mirocha and I. Uritani. The American Phytopathological Society, Inc. pp. 121-141.

Mishra, D. and E. R. Waygood. 1968. Effect of benzimidazole and kinetin on the nicotinamide nucleotide content of senescing wheat leaves. Can. J. Biochem. 46: 167-178.

Mizukami, T. 1952. On the Helminthosporium disease resistance of rice seedlings treated with ether vapour (IV). Sci. Bull. Fac. Agr. Kyushu Univ. 12: 299-303.

Montemartini, L. 1904. Note di fisiopatologia vegetale. Atti. 1st Botan. Univ. Pavia (II) 9: 39-97.

Moore, R. T. and McAlear, J. H. 1961. Fine structure of Mycota 8. On the aecidial stage of Uromyces caladi. Phytopathol. Z. 42: 297-304.

Mortimer, D. C. 1960. Iodoacetate inhibition of photosynthetic carbon dioxide assimilation in sugar beet and soybean leaves. Can. J. Bot. 38: 623-634.

Mortimer, D. C. 1961. Some observations on the formation of glyceric acid during photosynthesis experiments. Can. J. Bot. 39: 1-5.

Mortimer, D. C. and C. B. Wylan. 1962. The incorporation of ^{14}C into cellulose and other polysaccharides of sugar beet during short-term photosynthesis in $^{14}CO_2$. Can. J. Bot. 40: 1-11.

Mothes, K. and L. Engelbrecht. 1961. Kinetin-induced directed transport of substances in excised leaves in the dark. Phytochem. 1: 58-62.

Mount, M. S. and A. H. Ellingboe. 1969. ^{32}P and ^{35}S transfer from susceptible wheat to Erysiphe graminis f. sp. tritici during primary infection. Phytopath. 59: 235.

Mukherjee, K. L. and M. Shaw. 1962. The physiology of host-parasite relations XI. The effect of stem rust on the phosphate fractions in wheat leaves. Can. J. Bot. 40: 975-985.

Mumford, D. I. 1966. Factors associated with resistance of barley to spot blotch. Phytopath. 56: 79-83.

Neales, T. F., K. J. Treharne and P. F. Wareing. 1971. A relationship between net photosynthesis, diffusive resistance and carboxylating enzymes in bean leaves. In Photosynthesis and Photorespiration. Edited by M. D. Hatch, C. B. Osmond and R. O. Slatyer. Wiley-Interscience, N.Y. pp. 89-96.

Norris, L. T., R. E. Norris and M. Calvin. 1955. A survey of the rates and products of short-term photosynthesis in plants of nine phyla. J. Exptl. Bot. 6: 64-74.

Oaks, A. 1958. A survey of the factors controlling the activity of the indole-acetic acid oxidase system in healthy and rust-infected wheat leaves. Ph.D. Thesis, University of Saskatchewan, Saskatoon.

Ochoa, S. 1955. "Malic" enzyme. In Methods in Enzymology. Vol. 1. Edited by S. P. Colowick and N. O. Kaplan. Academic Press, N.Y.

Omar, A. M., A. K. A. Selim and M. I. Kalifa. 1971. Inheritance of resistance to net blotch disease of barley in U.A.R. J. Bot. U.A.R. 13: 29-43.

Osmond, C. B. 1971. The absence of photorespiration in C_4 plants: real or apparent? In Photosynthesis and Photorespiration. Edited by M. D. Hatch, C. B. Osmond and R. O. Slatyer. Wiley-Interscience, N.Y. pp. 472-482.

Pao, L. 1970. Metabolism of nicotinamide nucleotides in wheat leaves infected with Puccinia graminis tritici. M.Sc. Thesis, University of Manitoba, Winnipeg, Manitoba.

Person, C. 1960. A preliminary note on the histochemical localization of DNA and RNA in rust-infected wheat leaves. Can. J. Genet. Cytol. 2: 103-104.

Peterson, R. F. 1956. Progress Report, Central Breeding Laboratory, Winnipeg, Manitoba.

Peyton, G. A. and C. C. Bowen. 1963. The host-parasite interaction of Peronospora manshurica on Glycine max. Amer. J. Bot. 50: 787-797.

Pon, D. S. 1949. Physiologic specialization and variation in Helminthosporium teres. Phytopath. 39: 18 (Abstr.).

Pozsar, B. and Z. Kiraly. 1964. Phloem transport in rust-infected plants and the cytokinin-directed long-distance movements of nutrients. Phytopathol. Z. 56: 297-309.

Pozsar, B. and Z. Kiraly. 1967. (unpublished) In The Biochemistry and

Physiology of Infectious Plant Diseases. Edited by R. N. Goodman, Z. Kiraly and M. Zaitlin. D. van Nostrand Co., Inc., Princeton, N.J. p. 298.

Pozsar, B., K. Kristev and Z. Kiraly. 1966. Rust resistance induced by amino acids: A decrease of the enhanced protein synthesis in rust-infected bean leaves. Acta Phytopathol. Hung. 1: 203-208.

Pratt, R. 1938. Respiration of wheat infected with powdery mildew. Science 88: 62.

Pratt, R. 1944. Influence of light on the infection of wheat by the powdery mildew, Erysiphe graminis tritici. Bull. Torrey Botan. Club 71: 134-143.

Quick, W. A. and M. Shaw. 1964. The physiology of host-parasite relations XIV. The effect of rust infection on the nucleic acid content of wheat leaves. Can. J. Bot. 42: 1531-1540.

Redgewell, R. J. and R. L. Bieleski. 1967. Washing of adsorbents for thin-layer chromatography. J. Chromatog. 30: 231-232.

Reed, H. S. and C. H. Crabhill. 1915. The cedar rust disease of apples caused by Gymnosporangium juniperi-virginianae. Virginia Agr. Exptl. Sta. Tech. Bull. 9: 1-106.

Reisener, H. S., E. Zeigler and A. Prinzing. 1970. On the metabolism of the mycelium of Puccinia graminis var. tritici on wheat leaves. Planta 92: 355-357.

Rick, P. D. and C. J. Mirocha. 1965. Effects of pustule density on dark fixation of CO_2 by bean leaves infected with Uromyces phaseoli. Phytopath. 55: 1073 (Abstr.).

Rick, P. D. and C. J. Mirocha. 1968. Fixation of CO_2 in the dark by the malic enzyme of bean and oat stem rust uredospores. Plant Physiol. 43: 201-207.

Rohringer, R. 1964. Drifts in pyridine nucleotide levels during germination of rust spores and in wheat leaves after inoculation with leaf rust. Z. Pflanzenkr. Pflanzenschutz. 71: 160-170.

Rohringer, R. and R. Heitefuss. 1961. Incorporation of ^{32}P into ribonucleic acid of rusted wheat leaves. Can. J. Bot. 39: 263-267.

Sacchardo, P. A. 1882. Fungi Veneti Novi V. Critici V. Mycologiae Venetae. Michelia 2: 528-648.

Sahai, B. I. and M. Shaw. 1961. The physiology of host-parasite relations VIII. Effects of rust infection on ascorbic acid and glutathione in wheat leaves. Can. J. Bot. 39: 1327-1336.

Saltman, P., G. Kunitake, H. Spolter and C. Stitt. 1956. The dark fixation of CO_2 by succulent leaves: the first products. Plant Physiol. 31: 464-468.

Samborski, D. J., F. R. Forsyth and C. Person. 1958. Metabolic changes in detached wheat leaves floated on benzimidazole and the effect of these changes on rust reaction. Can. J. Bot. 36: 591-601.

Samborski, D. J. and M. Shaw. 1956. The physiology of host-parasite relations II. The effect of Puccinia graminis tritici Eriks. and Henn. on the respiration of the first leaf of resistant and susceptible species of wheat. Can. J. Bot. 34: 601-619.

Sanwal, B. D. and E. R. Waygood. 1963. Glycolic acid oxidase and fusariose wilt of tomatoes. Can. J. Bot. 41: 55-63.

Scheffer, R. P. and O. C. Yoder. 1972. Host-specific toxins and selective toxicity. In Phytotoxins in Plant Diseases. Edited by R. K. S. Wood, A. Ballio and A. Graniti. Academic Press, Lond. pp. 251-272.

Schou, L., A. A. Benson, J. A. Bassham and M. Calvin. 1950. The path of carbon in photosynthesis XI. The role of glycolic acid. Physiol. Plantarum 3: 487-498.

Scott, K. J. and R. M. Smillie. 1962. Enzyme changes in barley leaves after infection with powdery mildew. Plant Physiol. 37 (suppl.); lvii.

Scott, K. J. and R. M. Smillie. 1963. Possible relationship between photosynthesis and the rise in respiration in diseased leaves. Nature 197: 1319-1320

Scott, K. J. and R. M. Smillie. 1966. Metabolic regulation in diseased leaves I. The respiratory rise in barley leaves infected with powdery mildew. Plant Physiol. 41: 289-297.

Sempio, C. 1938. Riv. patal. vegetale 28: 241-351 (see Allen, P.J. 1954).

Sempio, C. 1942. Riv. biol. (Rome) 34: 22-47 (see Allen, P. J. 1954).

Sempio, C. 1950. Metabolic resistance to plant diseases. Phytopath. 40: 799-819.

Sempio, C. and G. Barbieri. 1964. Attivita repiratoria e difesa in varieta di fagiolo variamente resistenti all'Uromyces appendiculatus. Phytopathol. Z. 50: 270-282.

Shaw, M. 1961. The physiology of host-parasite relations IX. Further observations on the accumulation of radioactive substances at rust infection. Can. J. Bot. 39: 1393-1407.

Shaw, M. 1963. The physiology of host-parasite relations of the rusts. Ann. Rev. Phytopathol. 1: 259-294.

Shaw, M., S. A. Brown and D. Rudd-Jones. 1954. Uptake of radioactive carbon and phosphorus by parasitized leaves. Nature 173: 768-769.

Shaw, M. and N. Colotelo. 1961. The physiology of host-parasite relations VII. The effect of stem rust on the nitrogen and amino acids in wheat leaves. Can. J. Bot. 39: 1351-1372.

Shaw, M. and A. R. Hawkins. 1958. The physiology of host-parasite relations V. A preliminary examination of the level of free endogenous indoleacetic acid in rusted and mildewed cereal leaves and their ability

to decarboxylate endogenously supplied radioactive indoleacetic acid. Can. J. Bot. 36: 1-16.

Shaw, M. and D. J. Samborski. 1956. The physiology of host-parasite relations I. The accumulation of radioactive substances at infections of facultative and obligate parasites including tobacco mosaic virus. Can. J. Bot. 34: 389-405.

Shaw, M. and D. J. Samborski. 1957. The physiology of host-parasite relations III. The pattern of respiration in rusted and mildewed cereal leaves. Can. J. Bot. 35: 389-407.

Shaw, M. and B. I. S. Srivastava. 1964. The physiology of host-parasite relations XIII. The effect of stem rust on purines in wheat leaves. Can. J. Bot. 42: 139-144.

Shoemaker, R. A. 1959. Nomenclature of Drechslera and Bipolaris, segregated from Helminthosporium. Can. J. Bot. 37: 879-887.

Shu, P. and G. A. Ledingham. 1956. Enzymes related to carbohydrate metabolism in uredospores of stem rust. Can. J. Microbiol. 2: 489-495.

Singh, S. 1956. Physiology and epidemiology of Helminthosporium teres. Ph.D. Thesis, University of Minnesota, St. Paul, Minnesota.

Singh, S. 1962. Biotic factors affecting barley net blotch (Helminthosporium teres) epidemiology. Indian Phytopath. 15: 195-202.

Sivak, B. and M. Shaw. 1970. Microautoradiography of potato leaves inoculated with ^{3}H-labelled conidia of Phytophthora infestans. Can. J. Bot. 48: 613-615.

Slack, C. R. and M. D. Hatch. 1967. Comparative studies on the activity of carboxylases and other enzymes in relation to the new pathway of photosynthetic CO_2 fixation in tropical grasses. Biochem. J. 103: 660-665.

Smith, D. C., J. A. Bassham and M. Kirk. 1961. Dynamics of the photosynthesis

of carbon compounds II. Amino acid synthesis. Biochim. Biophys. Acta 48: 299-313.

Staples, R. C. and M. C. Ledbetter. 1958. A study by microautoradiography of the distribution of tritium-labelled glycine in rusted pinto bean leaves. Contrib. Boyce Thompson Inst. 19: 349-354.

Staples, R. C. and M. A. Stahmann. 1963. Malate dehydrogenases in the rusted bean leaf. Science 140: 1320-1321.

Staples, R. C. and M. A. Stahmann. 1964. Changes in proteins and cereal enzymes in susceptible bean leaves after infection by the bean rust fungus. Phytopath. 54: 760-764.

Staples, R. C. and L. H. Weinstein. 1959. Dark carbon dioxide fixation by uredospores of rust fungi. Contrib. Boyce Thompson Inst. 20: 71-81.

Tamas, I. A., E. W. Yemm and R. G. S. Bidwell. 1970. The development of photosynthesis in dark-grown barley leaves upon illumination. Can. J. Bot. 48: 2313-2317.

Thrower, L. B. 1965. On the host-parasite relationship of Trifolium subterraneum and Uromyces trifolii. Phytopathol. Z. 52: 269-294.

Ting, I. P. 1971. Nonautotrophic CO_2 fixation and Crassulacean acid metabolism. In Photosynthesis and Photorespiration. Edited by M. D. Hatch, C. B. Osmond and R. O. Slatyer. Wiley-Interscience, N.Y. pp. 169-185.

Ting, I. P. and W. M. Dugger. 1965. Transhydrogenation in root tissue: mediation by carbon dioxide. Science 150: 1727-1728.

Tolbert, N. E. and F. B. Gailey. 1955. Carbon dioxide fixation by etiolated plants after exposure to white light. Plant Physiol. 30: 491-499.

Towers, G. H. N. and D. C. Mortimer. 1956. The role of keto acids in photosythetic carbon dioxide assimilation. Can. J. Biochem. Physiol. 34: 511-519.

Trelease, S. F. and H. M. Trelease. 1929. Susceptibility of wheat to mildew

as influenced by carbohydrate supply. Bull. Torrey Botan. Club 56: 65-92.

Uritani, I. 1971. Protein changes in diseased plants. Ann. Rev. Phytopathol. 9: 211-234.

van Andel, O. M. and A. Fuchs. 1972. Interference with plant growth regulation by microbial metabolites. In Phytotoxins and Plant Diseases. Edited by R. K. S. Wood, A. Ballio and A. Graniti. Academic Press, Lond. pp. 227-250.

van der Meulen, P. Y. F. and J. A. Bassham. 1959. Study of inhibition of azaserine and diazo-oxo-norleucine (DON) in the algae Scenedesmus and Chlorella. J. Amer. Chem Soc. 81: 2233-2241.

von Sydow, B. and R. D. Durbin. 1962. Distribution of C^{14}-containing metabolites in wheat leaves infected with stem rust. Phytopath. 52: 169-170.

Walker, D. A. 1960. Physiological studies on acid metabolism 7. Malic enzyme from Kalanchloe crenata: effects of carbon dioxide concentration. Biochem. J. 74: 216-223.

Walker, D. A. 1962. Pyruvate carboxylation and plant metabolism. Biol. Rev. 37: 215-256.

Wang, D. 1959. Effect of benzimidazole, dimethylbenzimidazole, glucose and metal ions on the development of Puccinia graminis tritici on detached leaves of Khapli wheat. Can. J. Bot. 37: 239-244.

Wang, D. 1960. An ion-exchange resin method for the fractionation of alcoholic plant extracts. Nature, 186: 326.

Wang, D. 1961. The nature of starch accumulation at the rust infection site in leaves of Pinto bean plants. Can. J. Bot. 39: 1595-1604.

Waring, P. P. and Z. Z. Ziporin. 1964. The separation of hexosephosphates and triosephosphates by thin-layer chromatoraphy. J. Chromatog.

15: 168-172.

Waygood, E. R., R. Mache and C. K. Tan. 1969. Carbon dioxide, the substrate for phosphoenolpyruvate carboxylase from leaves of maize. Can. J. Bot. 47: 1455-1458.

Wheeler, H. and P. J. Hanchey. 1966. Respiratory control: loss in mitochondria from diseased plants. Science 154: 1569-1571.

White, N. H. and E. P. Baker. 1954. Host-pathogen relations in powdery mildew of barley I. Histology of tissue reactions. Phytopath. 44: 657-662.

Whitney, H. S., M. Shaw and J. M. Naylor. 1962. The physiology of host-parasite relations XII. A cytophotometric study of the distribution of DNA and RNA in rust-infected leaves. Can. J. Bot. 40: 1533-1544.

Wilson, A. T. and M. Calvin. 1955. The photosynthetic cycle. CO_2-dependent transients. J. Amer. Chem. Soc. 77: 5948-5957.

Wilson, J. H. and D. R. McCalla. 1968. A simple method for the isolation of Fraction I protein of chloroplasts. Can. J. Biochem. 46: 441-444.

Wu, Y. S. 1952. Production of stem rust sori on albino barley. Phytopathol. 42: 177-178.

Wynn, W. K. Jr. 1963. Photosynthetic phosphorylation by chloroplasts isolated from rust-infected oats. Phytopath. 53: 1376-1377.

Yarwood, C. E. 1934. Effect of mildew and rust infection on dry weight and respiration of excised clover leaflets. J. Agr. Research 49: 549-558.

Yarwood, C. E. 1953. Heat of respiration of injured and diseased leaves. Phytopath. 43: 675-681.

Yarwood, C. E. 1967. Response to parasites. Ann. Rev. Plant Physiol. 18: 419-438.

Yarwood, C. E. and J. F. L. Childs. 1938. Some effects of rust infection on the dry weight of host tissue. Phytopath. 28: 723-733.

Yarwood, C. E. and M. Cohen. 1951. Hypertrophy from the uredial stage of bean rust. Botan. Gaz. 112: 294-300.

Yarwood, C. E. and L. Jacobsen. 1955. Accumulation of chemicals in diseased areas of leaves. Phytopath. 45: 43-48.

Zaki, A. I. and R. D. Durbin. 1965. The effect of bean rust on the translocation of photosynthetic products from diseased leaves. Phytopath. 55: 528-529.

Zaki, A. I. and C. J. Mirocha. 1964. Respiration, photosynthesis and dark fixation of $C^{14}O_2$ by healthy and rusted bean leaves. Phytopath. 54: 913 (Abstr.).

Zaki, A. I. and C. J. Mirocha. 1965. Carbon dioxide fixation by rust-infected bean plants in the dark. Phytopath. 55: 1303-1308.

Zelitch, I. 1971. Photosynthesis, Photorespiration and Plant productivity. Academic Press, N.Y.