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BIOCHEMICAL ASPECTS OF THE RESISTANCE
OF BEAN TO HALO BLIGHT DISEASE

by

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Ph.D. Thesis presented to the
University of London

September 1967

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ABSTRACT

The author assigned isolates of the causal organism of halo blight, Pseudomonas phaseolicola (Burk.) Dows. from various parts of the world, to two races using differential varieties of dwarf bean (Phaseolus vulgaris L.) Other workers have shown that race 1 is non-virulent on variety Red Mexican but infects all others and that race 2 infects all varieties.

Homogenates of healthy bean leaves resistant to race 1 of P. phaseolicola took up oxygen more rapidly than homogenates of healthy susceptible leaves. This confirmed earlier work by others. The substrate of oxidation was extracted, purified and identified as two galactolipids, mono- and di-galactosyl diglyceride. Other workers have shown and the author confirmed that these galactolipids were hydrolysed on homogenisation of leaves, releasing linolenic and linoleic acids. It was shown that these fatty acids then underwent lipoxidation.

The difference in oxygen uptake by homogenates of leaves resistant and susceptible to race 1 shown by other workers was found to be due to a high level of galactolipid hydrolase activity in resistant leaves.

Analysis of lipids in cell walls of P. phaseolicola showed that galactolipids were absent. Fatty acid composition of wall lipids of races 1 and 2 were dissimilar in content of one substance.

When race 1 was incubated with linolenic acid for four days it was found that respiration ceased.

It is suggested that resistance of Red Mexican to race 1 is due to the action of free linoleic and linolenic acids on the respiratory mechanism of the bacterial cell.

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INTRODUCTION

The causal organism of the halo blight disease of beans (Phaseolus vulgaris L.) was first isolated and described by Burkholder (1926), who named the organism Phytomonas medicaginis var phaseolicola. Dowson (1943) later renamed the organism Pseudomonas phaseolicola (Burk.)Dows.

Infection of plants normally commences with the leaves, on which the first symptoms are small water soaked spots which within a few days become dry and brown and may be surrounded by a ring of chlorotic tissue, the halo. As the organism becomes systemic leaves turn yellow, although many of them regain their green colour after a few weeks. Redening of petioles, pulvini and veins may occur. (Patel and Walker, 1963a). Leaves may wilt (Burkholder, 1926) and become brown and dry. Frequently plants die before flowering if infection occurs when plants are young, but when more mature plants become infected flowering may occur and diseased seed may be produced. The infected seed carries the pathogen throughout the winter and the infected plant which grows from this acts as the source of infection in a new bean crop. (Butler and Jones, 1949).

Within a crop the disease is spread by rain splash of the organism after it has been exuded from lesions or by direct contact of these lesions with healthy plants.

Under the conditions of humidity in the crop bacteria enter the plant through stomata (Butler and Jones, 1949). When artificially inoculated as will be described (page 18) and grown in a greenhouse, symptoms on susceptible plants are normally limited to water-soaking of the inoculation site about two to four days after inoculation followed by gradual necrosis of the area which usually becomes surrounded by a narrow chlorotic area (see photograph, figure 4). New trifoliate leaves show interveinal chlorosis when they emerge.

Within an infected leaf bacteria occupy the substomatal space and intercellular spaces. Omer (1966) states that bacteria do not enter the plant cells and that cells break down only when the lesion becomes necrotic. Omer also observed the population increase of bacteria in artificially infected resistant and susceptible leaves and found that the viable cell count of race 1 increased after infection in both resistant and susceptible leaves. However, in resistant leaves, the viable cell count ceased to increase 3-5 days after infection whereas in susceptible leaves the count continued to increase at a rapid rate until 5 days after infection and only decreased in number of viable cells between 5 and 7 days after infection. Following cessation of bacterial growth, in susceptible leaves, the number of viable cells remained constant but in resistant leaves gradually decreased until readings stopped 12 days after

infection.

The present study arose from a series of investigations at the University of Wisconsin by Professor J. C. Walker and his colleagues on resistance to halo blight disease. It should be pointed out that at present there is no understanding of the mechanism of varietal resistance in higher plants to bacterial infections. Studies on the halo blight disease should make important contributions to this general problem.

Walker and Patel (1964) using isolates of Pseudomonas phaseolicola from several parts of the world, showed that resistance to halo blight in variety Red Mexican U.I. No. 3, was inherited as a monogenic dominant character. Such isolates were assigned to race 1. One isolate caused a susceptible reaction on Red Mexican and was assigned to race 2.

A number of lines of dwarf bean, homozygous for resistance and susceptibility to race 1 of P. phaseolicola were developed by Deverall and Walker (1963) from a cross between Red Mexican and the susceptible variety Kinghorn Wax. These lines have been used by the author in this study and were previously used in a series of studies on physiological differences between resistant and susceptible lines before and after infection by Patel and Walker (1963), Deverall and Walker (1963) and Deverall and Daly (1964).

Patel and Walker (1963b) studied the content of free amino acids and amides in leaves of resistant and susceptible bean varieties. Before infection they found negligible differences between varieties, which they stated could not be responsible for resistance and susceptibility.

After infection susceptible plants showed an increase in ornithine, histidine, methionine, asparagine-glutamine, β -alanine and lysine in inoculated leaves, and even greater increases of these amino-acids in chlorotic leaves away from the site of infection, to which there had been limited movement of the pathogen. In resistant plants there was little difference between the amino acid content of healthy and inoculated plants.

The effect of halo blight on bean leaf hydrolases was studied by Rudolph and Stahmann (1966). Using polyacrylamide disc electrophoresis they found that after infection the major acid phosphatase of the host increased during early and decreased during later infection stages. An acid phosphatase of bacterial origin was found in infected leaves and they suggest that this takes part in the uptake of metabolites by the pathogen.

Although several esterase bands decreased after infection, one with activity towards α -naphthyl butyrate increased. Also the pathogen showed an esterase band with high activity towards α -naphthyl butyrate.

Their finding of greatest relevance to this thesis was that they were unable to relate the synthesis of new host isoenzymes to resistance and susceptibility.

Deverall and Walker (1963) and Deverall and Daly, (1964) showed that homogenates of healthy resistant leaves took up O_2 more rapidly than those of healthy susceptible leaves. This was checked on 24 different lines of bean, and under controlled conditions for plant growth. Deverall and Walker (1963) eliminated cytochrome oxidase and copper oxidases including polyphenol oxidase and ascorbic acid oxidase, as the enzymes mediating the endogenous oxidation. Deverall and Daly, (1964) showed that there was no difference in respiratory rates of leaf discs, between the resistant and susceptible lines and Deverall and Walker (1963) showed that homogenate respiration was not a major contributor to oxygen uptake.

Therefore the present work was done to find out what was being oxidised in the homogenates, and then to discover which component of the oxidation reaction differed between resistant and susceptible leaves. The ultimate aim would be to discover the relationship between this component and the mechanism of resistance.

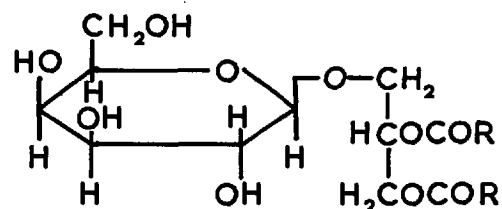
Rapid oxygen uptake by freshly prepared plant homogenates has been reported by Fritz and Beevers (1955) working with corn seedlings, and by Gamborg and Zalik (1958) with sunflower

seeds and seedlings, although the relevance of this work was not realized at the commencement of the present study. These workers found that unsaturated fatty acids were being oxidized by the action of lipoxidase.

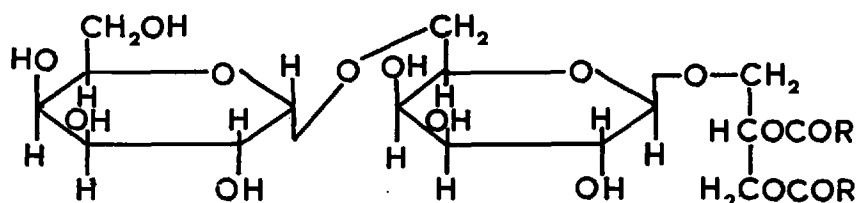
During the course of the work reported in this thesis, while attempts were being made to purify the then unidentified lipids which were releasing linolenic acid in homogenates, Sastry and Kates (1964a,b) published papers describing the purification of mono- and di-galactosyldiglycerides and the existence in bean leaves of enzymes capable of hydrolysing these lipids. They state that this hydrolysis occurs on homogenisation of leaves and results in the release of linolenic acid and small amounts of other fatty acids. Chromatographic properties and chemical constitution suggested similarity between these lipids and the substrate responsible for the stimulation of oxidation in bean leaf homogenates. Adoption of the chromatographic procedures of Sastry and Kates (1964a) confirmed this hypothesis and enabled identification and purification of the substrate to be achieved more rapidly than would otherwise have been possible.

The structures of mono- and di-galactosyl diglycerides are shown in figure 1.

It should be conceded that a weakness in the entire argument that the level of endogenous oxidation is associated with resistance to halo blight is the possibility that the



Monogalactosyl diglyceride (MGD)



Digalactosyl diglyceride (DGD)

R = FATTY ACID RADICALS present in the following proportions:-

	MGD	DGD
Linolenic acid	95.5%	93.2%
Linoleic acid	2.2%	1.3%
Palmitic acid	2.3%	4.5%
Stearic acid	trace	1.0%
Palmitoleic acid	trace	trace
Oleic acid	trace	trace

(after Sastry and Kates, 1964a.)

LINOLENIC ACID



LINOLEIC ACID

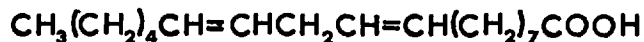


FIGURE 1. Structure and composition of mono- and di-galactosyl diglycerides.

difference between resistant and susceptible leaf homogenates in the rate of endogenous oxidation is based on a gene which is very closely linked to the gene conferring resistance. As no attempt has been made at genetic separation by workers in this field the most direct approach to the problem is that followed in this thesis.

MATERIALS AND METHODS

PLANT MATERIAL

Varieties of bean were obtained from Watkins and Simpson, Pound Hill, Crawley, Sussex, with the exception of the resistant variety, Red Mexican U.I. No. 3, Highgrade, Kinghorn Wax and progeny from a cross between the resistant, Red Mexican and the susceptible variety, Kinghorn Wax, which were sent by Professor J. C. Walker, University of Wisconsin, U.S.A.

PLANT CULTURE

Plants were grown in John Innes No. 1 potting compost in a greenhouse at 18° - 25°C and apart from available daylight, were illuminated with "Warm White" fluorescent tubes for 16 hr. per day. Unless otherwise stated, monofoliates were used in all experiments between 2 and 8 days after emergence.

SOURCE OF BACTERIAL ISOLATES

Isolates of Pseudomonas phaseolicola (Burk.) Dows were obtained from the National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Harpenden, Herts. (N.C.P.P.B.). The numbers and origins of cultures used are listed in Table 1. (see overleaf)

TABLE 1

Origin of cultures of Pseudomonas phaseolicola (Burk.)
Dows.

N.C.P.P.B. No.	Origin	
	Year	Country
380	1956	Sweden
604	1958	U.K.
605	1958	U.K.
1057	1961	Ethiopia
1098	1953-54	New Zealand
1103	1956	Hungary
1321	1961	U.K.
1341	1961	Tanganyika

CULTURE OF BACTERIA

Bacteria were grown on the following media:-

a) For storage of isolates in pure culture

Nutrient Agar pH. 7.4

Blood Base Agar (Difco)	20 g.
Bacteriological Peptone (Oxoid)	2.5 g.
Beef Extract (Oxoid)	1.5 g.
Agar (Davis Standard)	15.5 g.
Distilled water	1,000 ml.

After two days incubation on slopes in McCartney bottles at 22°C, cultures were stored at 4°C for 3 months before subculturing.

b) For growth of bacteria for inoculation of plants.

Nutrient - Dextrose

Beef extract (Oxoid)	3 g.
Bacteriological Peptone (Oxoid)	5 g.
Agar (Davis Standard)	15 g.
Dextrose	10 g.
Distilled Water	1,000 ml.

Agar was sloped in medical flats. Cultures were incubated at 22°C for 3 days before use.

c) For liquid culture of bacteria.

Dextrose	10 g.
Casein hydrolysate	4 g.
KH_2PO_4	1 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g.
Distilled water	1,000 ml.

130 ml. of medium were dispensed into 500 ml. conical flasks. After inoculation flasks were incubated for two days at 25°C on a shaker, rotating at approx. 200 r.p.m. with an excentric throw of 6 cm.

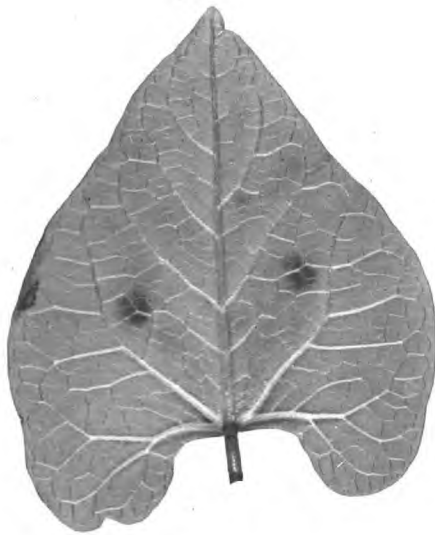
INOCULATION OF PLANTS

A number of different methods of inoculation were attempted as follows:-

- a) a metal plate bearing two to three dozen pins at 0.5 cm. intervals was dipped in a bacterial suspension and then pressed into a leaf. The plant was kept in a humid chamber for 48 hours.
- b) a mixture of carborundum and bacterial suspension were gently rubbed into leaves and plants kept in a humid chamber for 48 hours.
- c) under-surfaces of leaves were painted with a bacterial suspension and kept in a humid chamber for 48 hours.
- d) using a fine hypodermic needle a bacterial suspension was injected by syringe into the veins of leaves, the suspension also frequently passing into the surrounding leaf lamina.

All the above methods were not satisfactory. No infection was achieved by methods a, b and c. Method d was only of limited success as it was difficult to perform and frequently caused excessive damage to the leaf.

Finally a successful method was found and used for all experiments involving inoculation of bean leaves with bacteria. Bacteria were rinsed from the surface of nutrient-dextrose agar cultures, washed twice by centrifugation and resuspended in sterile water. Leaves 2 to 3 days old were inoculated by spraying



Lower surface



Upper surface

Figure 2. Water-soaking on recently inoculated leaf.

milky white bacterial suspensions through the undersides of leaves, by means of a spray pen (No. 2a from Colour Sprays Ltd., North Road, London, N.7.) attached to a compressed air line at 10 lb. p.s.i. The suspensions contained between 10^{10} and 10^6 bacteria per ml. It has been shown that the concentration does not influence the ability to create a resistant reaction on resistant plants as long as there are sufficient bacteria present to infect a susceptible plant. (Omer 1966).

Penetration of the bacteria occurs through the stomata into the substomatal spaces and it has been suggested that the suspension will also penetrate the cuticle of the undersurface and pass into the inter-cellular spaces (K. J. Dormer, personal communication).

The entry of the bacterial suspension can be observed as the spreading of water soaking outwards from the inoculation site (Fig. 2). This water soaking disappears within a few hours and should not be confused with water soaking which appears later being the onset of disease symptoms (Fig. 4).

OXYGEN UPTAKE MEASUREMENT

Oxygen uptake by healthy leaf homogenates was measured on a Warburg apparatus at 25°C, following the general procedure of Deverall and Daly (1964).

Preparation of the flasks was performed in a cold room at 4°C . and apparatus and leaves were chilled for at least 15 minutes before use. Monofoliolate leaves were removed from plants, weighed, wrapped in aluminium foil while chilling and placed in a mortar with volumes of $0.1\text{ M KH}_2\text{PO}_4$ in ml. five times the fresh weight of leaves in g. The phosphate buffer was pH. 3.6, having been acidified with HCl. The final pH. of the homogenates was 5.2. When the leaves had been homogenised to a slurry after about 1 min. the homogenate was filtered through 4 layers of muslin. Two ml. of filtered homogenate were added to each Warburg flask containing 1 ml. of $0.1\text{MKH}_2\text{PO}_4$, ph. 4.7. When desired, plant extracts or other addenda were suspended or dissolved in the phosphate buffer. Flasks also contained 0.2 ml. 10% KOH in the centre well, into which filter paper strips were placed before putting the flasks on the manometers and placing them in the constant temperature bath. No more than three min. was allowed to elapse between removal of flasks from the cold room and placing them in the constant temperature bath. A thermobarometer control containing 3 ml. $0.1\text{ M KH}_2\text{PO}_4$ was treated in the same way as the flasks containing homogenates. After 5 min. equilibration in the bath, zero readings were taken and the stopcocks closed. Readings were taken at intervals depending on the rate of oxygen uptake.

Attempts were made to suspend dried plant extracts and fatty acids by shaking with phosphate buffer and glass beads. As this proved unsuccessful ultrasonic vibrations were used to suspend the material. Although this removed dried extracts from the walls of tubes in which they had been dried, the extracts remained in immiscible lumps. Addition of a surface active agent enabled the dispersion of these by the ultrasonic vibrations.

The method thus devised enabled dried plant extracts or fatty acids to be suspended in 0.1 M KH_2PO_4 containing 0.1% vol./vol. Tween 80 (Honeywill-Atlas, Sorbitan mono-oleate, a surface active agent) with the aid of ultra-sonic vibrations supplied for 1 min. from a 60 watt M.S.E. ultra-sonic probe and power unit. Controls included the same amount of Tween 80.

EXTRACTION AND FRACTIONATION METHOD

A known fresh weight of about 40 g. of leaves were extracted for substrate of oxidation by maceration for 3 min. with a Kenwood liquidiser at maximum speed in an ethanol/water mixture (10 ml. per g. fresh weight tissue) adjusted so that the final ethanol concentration was 80%, allowing for a 90% water content of fresh leaves. After centrifugation of the extract at 130xg. for 10 min. to remove insoluble material, the supernatant was washed four times with equal volumes of petroleum spirit. The residue from centrifugation only retained a very slight green colouration indicating that nearly all cells had been broken.

The aqueous ethanol phase remaining after washing with petroleum spirit was reduced to a small volume (about 20 ml. mainly water) in vacuo in a rotary film evaporator at 35°C to reduce the possibility of oxidation or destruction by heat. It was not possible to measure the volume to which the ethanol extract was reduced due to its high viscosity.

One ml. of chloroform per 2 ml. of 80% ethanol used in substrate extraction of leaves, was added to the residue of the dried ethanol extract without shaking. The solution was washed x3 by dropwise addition to equal volumes of water to remove water soluble material from

the chloroform solution. It was essential to avoid shaking chloroform with water to prevent emulsion formation. The bulked water phase was extracted with chloroform to remove any remaining chloroform soluble material. This water washing procedure was facilitated by the incomplete removal of water when the 80% ethanol extract was dried in vacuo (above).

The combined chloroform solutions were dried in vacuo by rotary film evaporator at 35°C. to remove any traces of water or ethanol. The residue was taken up in 5 ml. of chloroform and put on a column of silica gel/ celite as used by Kates and Eberhardt (1957). The use of this column necessitates the above mentioned water washing of the chloroform solution of the extract and the drying in vacuo of this chloroform solution before dissolving the extract in chloroform because the omission of these steps causes clogging of the column by a hard "pan" on the surface of the adsorbent, greatly reducing the rate of solvent flow through the column.

The column is prepared by the mixing of 36 g. silica gel M.F.C. (B.D.H.) with 9g. Celite (Johns-Manville). The mixed adsorbent was heated for two hours at 110°C., allowed to cool in a desiccator and mixed to a slurry with chloroform. The slurry was poured into a chromatography column (2.5 cm. diam.) over $\frac{1}{2}$ in. depth of acid-washed

sand which covered a grade 2 porosity sintered glass filter. The column volume was approximately 80 ml.

Elution of the chloroform solution of the extract was with 10 column volumes (= 800 ml.) successively of chloroform, chloroform/methanol, 5/1 (v./v.), chloroform/methanol 1/1, (v./v.). Each of these 10 column volume fractions was dried in vacuo by rotary evaporator at 35°C, taken up in 10 ml. chloroform and stored under N₂ at -20°C.

For reasons which will be explained later (page 66) the investigation continued with the chloroform/methanol 5/1 fraction only. The 10 ml. chloroform solution of this fraction was dried in a stream of N₂ and the residue was taken up in 2 ml. diethyl ether. Phospholipids were precipitated by the addition of 40 ml. "Analar" acetone at 0°C and the precipitate removed by filtration through Whatman No. 1 paper.

The acetone solution of the phospholipid-free chloroform/methanol, 5/1 fraction was dried in a stream of N₂ and taken up in 5 ml. chloroform which was fractionated on a column of silica gel.

The column was prepared by heating 45 g. silica-gel (M.F.C.) overnight at 110°C. After cooling it was slurried in methanol and placed in a water jacketed 2.5 cm. diameter column above ½ in. acid-washed sand

over a grade 2 porosity sintered glass filter. The water jacket was kept at 2°C to reduce oxidation of the lipids. The column was washed with 3 column volumes (each of 80 ml.) of chloroform, before addition of the extract in 5 ml. of chloroform. The eluents used are in Table 2.

TABLE 2

Solvent sequence for silica-gel column.

Fraction	Eluent	Eluate vol.ml.
1	Chloroform	240
2	Chloroform/methanol 98:2	350
3	Chloroform/methanol 95:5(i)	132
4	Chloroform/methanol 95:5(ii)	162
5	Chloroform/methanol 9:1	210
6	Methanol	100

The presence of galactosyl diglycerides in certain fractions was detected after concentration and chromatography of fractions on Whatman silica-gel loaded paper, SG 81, developed with "Analar" di-isobutyl ketone/"Analar" acetic acid/distilled water (40:25:5) containing 0.05% 2:6 di-tert-butyl p-cresol (butylated hydroxytoluene) as antioxidant. Chromatograms were dried for 2 hr.

in an air stream, sprayed with 0.1% Rhodamine 6G in aqueous solution and inspected under U.V. light at 260 and 360 mμ. Lipids containing unsaturated fatty acids fluoresced yellow or orange.

HYDROLYSIS OF LIPIDS

Samples to be hydrolysed, details of which are given later (pages 79 & 89) were dried in vacuo by rotary evaporation at 35°C, taken up in 100 ml. 4N methanolic hydrochloric acid (conc. HCl./absolute methanol/glass distilled water; 40:35:35; v/v) and heated for 3 hr. in a steam bath under a reflux condenser. After cooling, an equal volume of distilled water was added. The fatty acids were removed by triple extraction with equal volumes of diethyl ether/petroleum spirit (1/1; v./v.). The ether/petroleum spirit extract was washed X3 with equal volumes of distilled water, dried by rotary evaporator in vacuo at 35°C, taken up in 10 ml. chloroform, and stored at -20°C under N₂.

CHROMATOGRAPHY OF WATER SOLUBLE HYDROLYSIS PRODUCTS

The aqueous portion from the hydrolysis was freed of water and acid by drying by rotary evaporator in vacuo at 45°C, taken up in 3 ml. distilled water and 0.05 ml. chromatographed (descending) on Whatman No. 1 paper with pyridine/ethyl acetate/distilled water (2/5/5, v./v. upper phase). Standard markers for comparison were glycerol, galactose and glucose applied as 5 µl. of solution containing 7 µg. per µl.

Galactose and glycerol were detected by spraying the chromatograms with a freshly prepared mixture of 2% sodium metaperiodate/1% potassium permanganate (2/1, v./v.). The reactive compounds appeared first as yellow spots on a purple background. After 5/10 minutes the chromatograms were washed in water whereupon the spots appeared brown on a white background. (Sastry and Kates 1964a).

ISOLATION OF FREE FATTY ACIDS

In order to estimate free fatty acid levels in leaves (page 92) and in aqueous leaf homogenates (page 95) it was first necessary to isolate the fatty acids because phospholipids interfere with the colorimetric method used for the estimation of free fatty acids.

Extracts of intact leaves or aqueous homogenates were made in 80% ethanol, washed with petroleum spirit, evaporated almost to dryness in vacuo, taken up in chloroform and washed with water. The chloroform solution was then evaporated to dryness in vacuo and the residue dissolved in 5 ml. diethyl ether per g. fresh weight of leaves and extracted X6 with 2 vol. 10% Na_2CO_3 . The carbonate solution was bulked and acidified to pH 3.0 with conc. phosphoric acid, and then extracted X6 with half volumes of diethyl ether/petroleum spirit

(1/1; v./v.). The combined ether/petroleum spirit extract was washed with distilled water X3 and dried by rotary evaporator in vacuo at 35°C.

ESTIMATION OF FATTY ACIDS

The method and reagents for estimating the amount of fatty acids were based on those of Itaya and Ui (1965). The fatty acids were taken up in a volume of chloroform sufficient to bring readings within the range of a standard curve (Fig. 3). Six ml. of this solution was pipetted into a 50 ml. separatory funnel together with 3 ml. of copper-triethanolamine reagent. (1 M triethanolamine/1 N acetic acid/6.45% $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$; 9:1:10; v./v.). The mixture was shaken X30 and left to settle for at least 15 min. The chloroform layer was filtered through silicone treated filter paper, Whatman No. 4 ST, into a 25 ml. beaker in a chloroform atmosphere. Two drops of 0.2% "Analar" sodium diethyl dithiocarbamate in n-butanol were added to the chloroform solution, the sample mixed and the transmittance measured at 440m μ on a Beckman spectrophotometer. The standard curve was obtained in this manner using 15 - 37.5 μ equivalents palmitic acid per litre of chloroform.

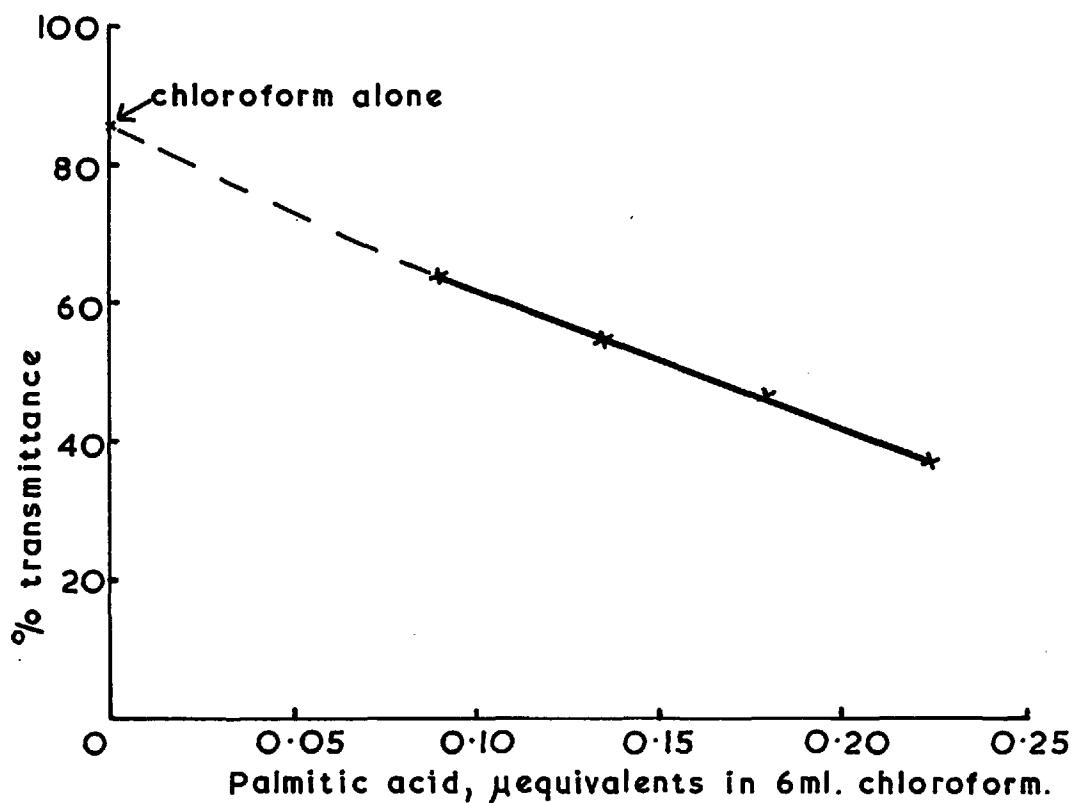


Figure 3. Standard curve for colorimetric estimation of free fatty acid by method of Itaya & Ui (1965).

GAS-LIQUID CHROMATOGRAPHY

Some of the gas-liquid chromatography was performed by the author and some by Dr. V. A. Knivett, Miss J. Cullen and Mr. M. Jackson of Twyford Laboratories Ltd., London N.W.10.

The author's procedure was as follows. A chloroform solution containing between 10 and 20 mg. of the extract to be methylated was dried in a stream of N_2 in a McCartney bottle. The extract was dissolved in 1 ml. boron trifluoride-methanol reagent (BF_3 about 51%, B.D.H. Ltd.) and the bottle was capped tightly, the rubber cap liner having been covered by a layer of "Saran-wrap" (Dow Chemical Company). The bottle was heated in a water bath at $60^{\circ}C$ for 30 min. This hydrolysed the lipids and methylated the fatty acids. After cooling for 10 min. 1 ml. distilled water was added and the methyl esters were extracted with three 1 ml. portions of Analar petroleum spirit. This solution was dried in a weighed bottle under a stream of N_2 and taken up in chloroform which was evaporated to about 0.3 ml. and weighed. Fifty μ l. of this solution were applied to a column of polyethylene glycol adipate (PEGA) at $184.5^{\circ}C$. on a Pye Series 104 chromatograph (W. G. Pye Ltd., Cambridge). The PEGA

was on 100-120 mesh celite (10% wt./wt.) and the carrier gas was N_2 at a flow rate of 50 ml. per min. and input pressure of 40 lb. per sq. in.

The technique used by Knivett et al. was essentially the same except that they used BF_3 -methanol containing 65 g. BF_3 per litre. Their analyses were carried out on a Pye Argon chromatograph, on most occasions using the same type of column as the author but at $180^\circ C.$, but on a few occasions using Apiezon L. on 80-120 mesh celite at $197^\circ C.$ Initially Knivett et al. used Apiezon L. but changed to PEGA when it was found that extracts contained linoleic and linolenic acids which are not resolved separately on Apiezon L., but are separated on PEGA. Whichever stationary phase was used the flow rate of Argon was 52.4 ml./min.

The peaks were identified by comparison with published retention values, (Burchfield and Storrs, 1962) or in the case of the cyclopropane acids encountered by Knivett et al. in analysis of the bacterial cell wall by comparison with standards.

Knivett et al. calculated percentage composition by the method of Carroll (1961) but the author used the following method. Peak heights were measured and the base widths measured after extrapolation of the peak sides to zero. 96% of the peak area is given by the

multiplication of peak height by $\frac{1}{2}$ base width. This area was adjusted to 100% and the total peak area of the chromatogram calculated. The percentage of each fatty acid present was then found by calculation of the percentage of total peak area covered by the peak produced by each fatty acid.

In tables recording the results of gas chromatograms fatty acids are referred to by their "carbon number". Absolute retention times of fatty acid methyl esters on gas-chromatograms are often variable on different occasions due to a steady decrease in retention times as a column is repeatedly used and because small differences in pressure, temperature or other operating conditions of the column. For these reasons it has become the practice to quote relative retention times referred to one component of the mixtures. This does not always permit comparison of retention values from different laboratories or when different reference compounds are used, without re-calculation of results. Woodford and van Gent (1960) have devised the following procedure enabling such comparisons to be made more easily. The retention times of the saturated straight-chain esters of which a number are normally present in natural mixtures, are

plotted against chain-length on semi-logarithmic paper and the best straight line joining them is constructed. A value corresponding to the retention time of any other peak can then be read off from the graph, and gives the chain length of the hypothetical saturated straight-chain ester which would be eluted at that point. This figure is called the "carbon-number" of the ester on the stationary phase in question. The carbon-number is characteristic of a particular ester on a particular stationary phase. It is dependent on the temperature at which the column is run but is sufficiently constant over small temperature ranges (at least 20°) to allow comparison with other runs on the same stationary phase made in the same temperature region.

GALACTOLIPID HYDROLASE ASSAY

The method for demonstrating enzymatic hydrolysis of galactosyl diglycerides was that of Sastry and Kates (1964b), which was based on the acyl ester estimation method of Snyder and Stephens (1959).

A known amount of mono- or di-galactosyl diglyceride (MGD, DGD) (extract of 4g. or 5g. fresh wt. leaves - see table 32) was dried out of chloroform solution in a stream of N₂ and suspended in 0.05 ml. methanol followed by 0.1 ml. of 0.5 M phosphate buffer. If MGD hydrolase

was to be assayed the buffer was pH.7.0, if DGD hydrolase, pH.5.6. The enzyme source was a homogenate prepared by grinding equal weight of 10 day old monofoliolate leaves with distilled water at 3°C. After filtering through 4 layers of muslin, 0.7 ml. of homogenate were added to the galactolipid sample and the mixture incubated, with shaking at 30°C for 60 min. In some instances no galactolipid was added in which case the homogenate was assayed alone.

The reaction was stopped by adding 2 ml. of methanol and placing the samples in a water bath at 30°C for 30 seconds. Lipids in the reaction mixture were then extracted by the procedure of Bligh and Dyer (1959). One ml. of "Analar" chloroform was added, the mixture shaken and after 30 minutes a further 1 ml. of chloroform plus 1 ml. of distilled water were added. The mixture was shaken and centrifuged for 10 min. at 500 xg. 0.3 ml. of the chloroform phase were withdrawn and dried under N₂. One ml. of freshly prepared hydroxylamine solution was added (2g. hydroxylamine hydrochloride dissolved in 2.5 ml. H₂O made up to 50 ml. with ethanol + 4 g. sodium hydroxide dissolved in 4 ml. H₂O made up to 50 ml. with ethanol. Mixed in equal volumes and centrifuged to remove NaCl).

After shaking, tubes were placed in a water bath at 65°C for 2 min. then allowed to cool for 5 min. 2.5 ml. freshly made ferric perchlorate reagent were added. (Reagent ferric perchlorate - 4 ml. stock ferric perchlorate + 3 ml. 70% perchloric acid made up to 100 ml. with cold ethanol.

Stock ferric perchlorate - 5 g. ferric perchlorate (non-yellow) dissolved in 10 ml. 70% perchloric acid diluted to 100 ml. with cold ethanol).

Tubes were shaken and left for 30 min. after which the absorbance was measured at 530m μ . in a Beckman DB spectrophotometer. Control samples were substrate with buffer and homogenate with buffer.

PREPARATION AND EXTRACTION OF BACTERIAL CELL WALLS

Cells were grown in liquid shake-culture for two days and separated from the nutrient broth by passing through a continuous action rotor at 22,000 xg. (M.S.E. High-Speed 18 centrifuge).

Cells were washed twice in distilled water by centrifugation at 10,000 xg., suspended in distilled water and subjected to ultrasonic vibrations from a 60 watt. M.S.E. Ultrasonic Disintegrator, using a 6 mm. probe for 15 min. The suspension was cooled to 0-2°C by keeping the tube in an iced water bath,

and the probe was cooled by a water jacket. Whole cells were removed by centrifugation at 1,500 xg. for 30 min. Cell walls and cytoplasm were suspended in 1 M NaCl (by the addition of solid NaCl) to aid the separation of cytoplasm from cell walls. Cell walls were separated from remaining cytoplasm by centrifugation at 20,000 xg. for 45 min. This procedure for disruption of cells and separation of cell walls is adapted from that of Hunt et al. (1959) who show electron micrographs to demonstrate purity of cell-wall fractions.

The walls were taken up in distilled water and spread thinly in petri dishes. Water was removed by placing over calcium chloride in vacuo for 12 hr. The dried material was powdered and placed in fat-free extraction thimbles which were extracted by refluxing in a Soxhlet apparatus with chloroform/methanol (2/1, v./v.) for 24 hr. under reduced pressure at 45°C to avoid possible breakdown of the extracted lipids by heat or oxidation. For the same reasons the extracts were dried in vacuo at 35°C by rotary evaporator.

ANALYSIS OF CELL WALL EXTRACT

Phospholipids were precipitated by acetone. After

drying in vacuo the phospholipid-free extract was placed on a column of silica-gel (Sastry and Kates, 1964a) as described previously, using the solvent sequence shown in Table 2. Fractions were analysed by paper chromatography using Whatman S.G.81 with di-isobutyl ketone/acetic-acid/distilled water (40/25/5) as described under fractionation of leaf lipids (page 26).

Reagents Petroleum spirit used throughout was a fraction with boiling range 40° - 60°C, from Esso Petroleum.

Fatty acids were "Laboratory Biochemical" or "Specially pure" grade from B.D.H. Poole, Dorset. No "Technical" grade fatty acids were used.

Unless otherwise stated chloroform was "General Purpose" grade throughout. Methanol and ethanol were absolute "Analar" grade.

"Tween 80" is a trade name for sorbitan mono-oleate produced by Honeywill-Atlas Ltd.

Glass distilled water was used except for the making of culture media, in which water was from a metal still.

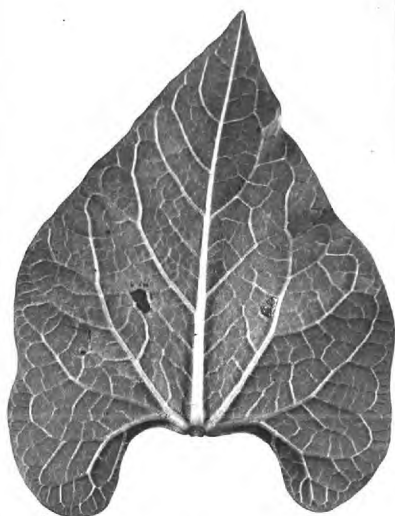
RESULTS

PART 1

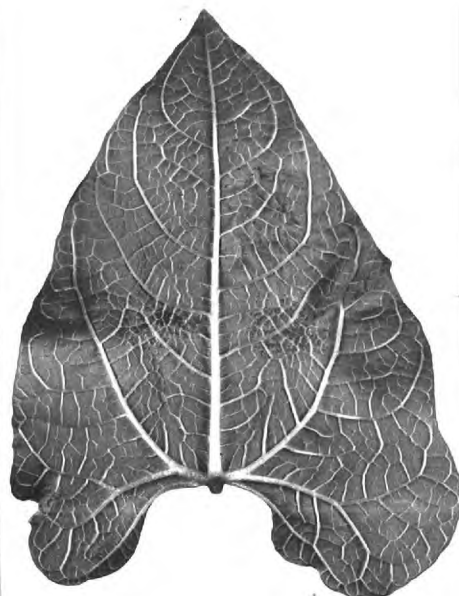
Chapter 1. Physiologic races of *Pseudomonas phaseoli-* *cola*

Suspensions of isolates from the National Collection of Plant Pathogenic Bacteria were used to inoculate Red Mexican, resistant to race 1, and two varieties, Kinghorn Wax and Highgrade, susceptible to race 1. After three days at 20°C, bronzing and desiccation had occurred at the inoculation sites on Red Mexican leaves, but on the susceptible leaves there were green water soaked lesions. Chlorosis occurred in newly emerging leaves of susceptible plants, but this symptom was absent from similar leaves of resistant plants (see figure 4). The disease reactions of isolates tested are shown in Table 3 (i.e. whether resistant or susceptible), along with allocation to race 1 or 2 depending upon virulence on Red Mexican. (Table 3 overleaf).

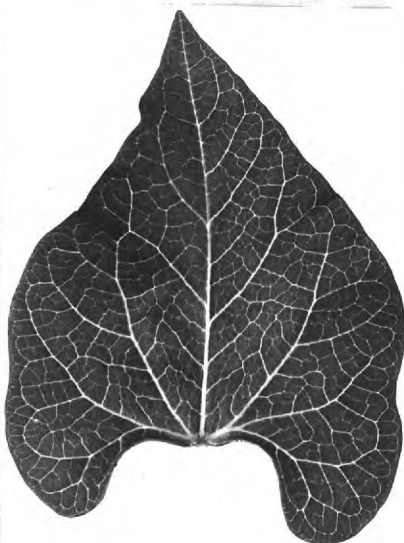
In an attempt to find other resistant varieties the following varieties were tested but none was resistant to either race:- Tendergreen, Guernsey Runner, Prince, Comtesse de Chambord, Canadian Wonder, Bounteous, Top Crop, Mont d'Or, Masterpiece, Premier, Black Prince, Brown Dutch, Blue Lake.



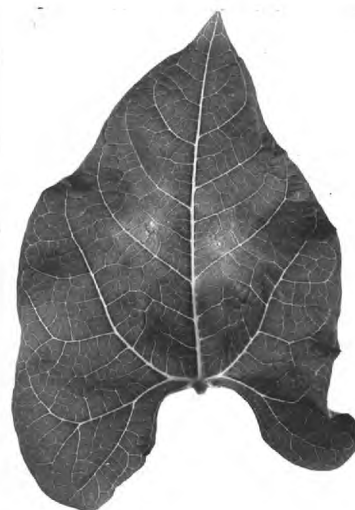
RESISTANT



SUSCEPTIBLE



RESISTANT



SUSCEPTIBLE

Above, lower surface; Below, upper surface

Figure 4. Disease reactions on resistant and susceptible leaves.

TABLE 3 Race designation of N.C.P.P.B. isolates of P. phaseolicola.

N.C.P.P.B. No.	Disease reaction			Race	Country of origin
	Red Mexican	Kinghorn Wax	Highgrade		
380	S	-	S	2	Sweden
604	R	-	S	1	U.K.
605	R	S	-	1	U.K.
1057	R	-	S	1	Ethiopia
1098	R	-	S	1	New Zealand
1103	R	-	S	1	Hungary
1321	S	S	-	2	U.K.
1341	S	S	-	2	Tangan- yika

R = resistant S = susceptible - = not tested

It can be concluded that races 1 and 2 of P. phaseoli-
cola are widespread, that both occur in this country
(see Table 3) and that many British varieties of
Phaseolus vulgaris are susceptible to both races of the
pathogen.

PART 2

NATURE OF OXIDATION REACTION

Chapter 2. Oxygen uptake by homogenates of healthy resistant and susceptible leaves.

Comparison of oxygen uptake by homogenates of resistant and susceptible lines derived from the cross between Red Mexican and Kinghorn Wax (see page 9). were made using on each occasion a pair of lines grown at the same time under identical conditions. (See Materials and Methods, Plant Culture). Leaves used were monofoliates, 2-3 days after emergence. Deverall and Daly (1964) point out the necessity of using leaves at the same stage of development and show that differences in oxygen uptake between resistant and susceptible leaves are most easily seen when leaves are very young.

Oxygen uptake of homogenates was measured as described on page 20 of Materials and Methods. Comparison of oxygen uptake of resistant and susceptible leaf homogenates after the first hour is shown in Table 4. The uptake of each of the three replicate treatments is given. Each flask contained 0.4 g. fresh wt. leaf tissue in 3 ml. buffer at pH. 5.2. Deverall and Walker (1963) performed experiments to elucidate the effect of pH on oxygen uptake and found the optimum to be about pH 5.

TABLE 4

Oxygen uptake of resistant and susceptible homogenates in first hour. Readings in $\mu\text{l. O}_2$. One resistant and one susceptible line tested on each occasion.

RESISTANT					SUSCEPTIBLE				
Line No.	Replicates				Line No.	Replicates			
	1	2	3	Mean		1	2	3	Mean
61	102	99	97	99.3	229	80	73	75	76
61	113	122	118	117.7	129	67	74	71	71.6
141	85	83	89	85.6	12	71	68	70	69.6
120	99	94	96	96.3	122	71	75	68	71.3
Red Mexican	123	120	127	123.3	Canadian Wonder	93	95	90	92.6

Figure 5 shows the course of oxygen uptake for one resistant and one susceptible line over the first hour. The range of scatter of the replicate readings is indicated by the vertical bars. It can be seen that the difference between the mean readings of resistant and susceptible leaf homogenates at 15 min. is 23 $\mu\text{l.}$, and at 60 minutes is 23.3 $\mu\text{l.}$ Thus, nearly all the difference in oxygen uptake developed in the first 15 minutes, later oxygen absorption by the homogenates merely maintaining this initial difference.

Table 5 shows the difference between oxygen uptake readings of resistant and susceptible varieties during the course of oxygen uptake over 60 minutes and the figures indicate that although some variation occurs most of the difference in readings occurs in the first 15 to 30 min.

TABLE 5 Differences between oxygen uptake of homogenate of resistant and susceptible leaves over 60 minutes.

Readings in $\mu\text{l. O}_2$.

Time, min.	Resistant and susceptible lines compared	
	RM - CW	141 - 12
15	12.6	12.3
20	17.6	13.0
30	25.3	14.7
45	29.6	no reading
60	30.7	16.0

This point is reinforced by figure 6 which shows a comparison of varieties over 300 min. The early difference in oxygen uptake begins to diminish after about 180-200 min.

Thus, the oxygen uptake reaction is initially a rapid reaction which quickly slows down but oxidation is not completed for many hours. There is a

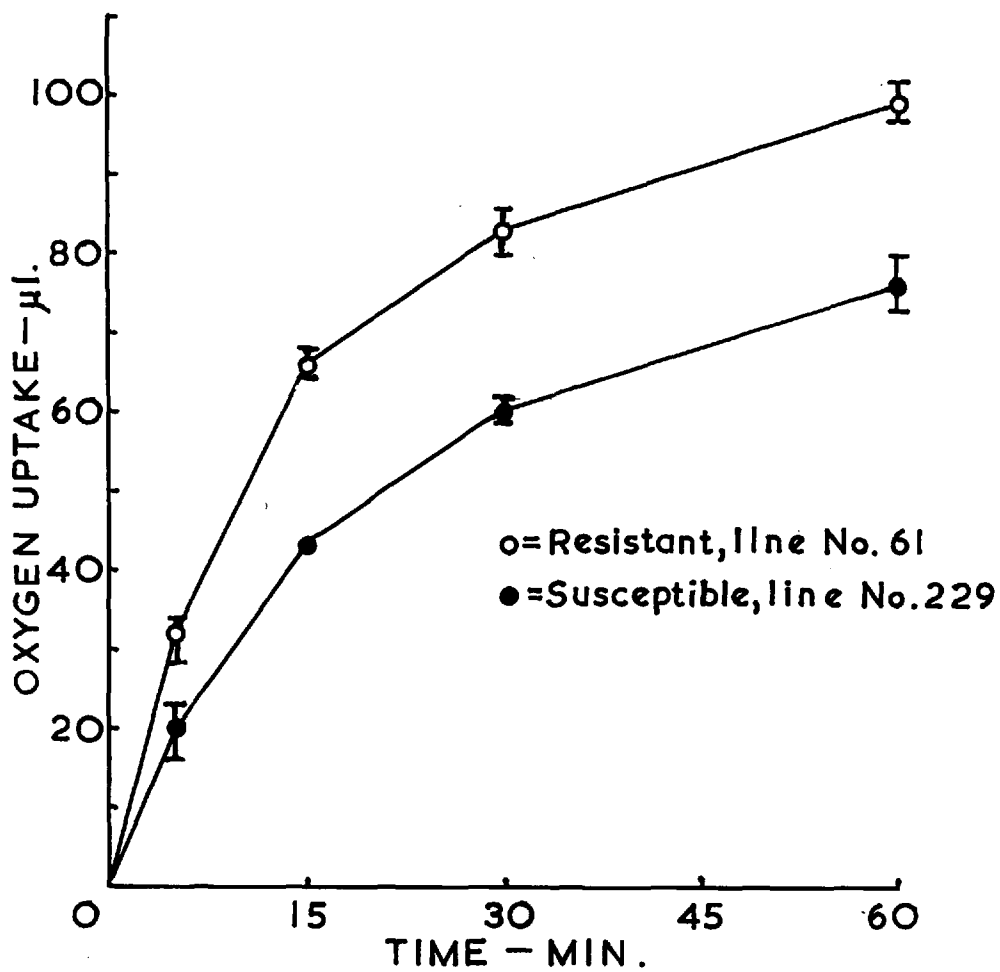


Figure 5. Course of oxygen uptake by 0.4 g. fresh wt. leaf homogenates of resistant and susceptible lines of Phaseolus vulgaris over first hour.

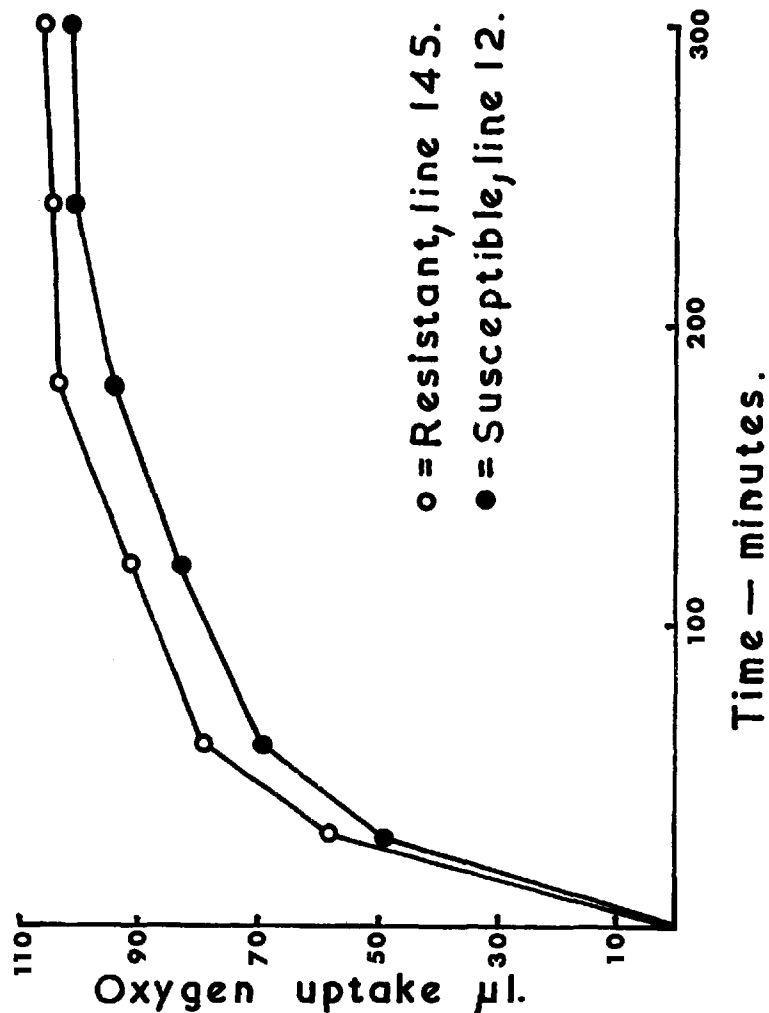


Figure 6. Course of oxygen uptake by 0.4g. fresh wt. leaf homogenates over 5hr. for resistant and susceptible lines of Phaseolus vulgaris.

considerable difference between the oxygen uptake of resistant and susceptible varieties during the first 15 min. of measurement by Warburg manometry. The volume of this difference remains unchanged for about 3 hr. during which oxidation of resistant and susceptible homogenates continues at the same rate in both lines, the rate gradually decreasing.

Further remarks on these results are made in the discussion.

Chapter 3. Development of method of extracting
substrate of oxidation reaction.

An indication that most of the oxygen uptake by homogenates was a result of enzymic activity was given by inhibition of the reaction by various inhibitors. This removed the possibility that the reaction was only due to autooxidation of a substance. Details of these inhibition results are given later (Table 22). Therefore it was expected that destruction of either enzyme or possibly substrate also would be brought about by heating a homogenate. If only the enzyme were destroyed by heat this might provide a method of separating enzyme and substrate. Other possible means of separating enzyme^{and}/substrate were dialysis or solvent extraction of homogenates after inactivation of the proteins. Ethanol appeared to be suitable for this as it would inactivate and precipitate many proteins as well as possibly extracting the substrate from the rest of the homogenate. Initially only aqueous homogenates were extracted, whole leaf extraction following on from this later. The method finally adopted is recorded on page 23.

a) Dialysis.

An attempt was made to extract the substrate of the homogenate oxidation reaction by dialysis.

a homogenate was made by grinding equal weights of leaves and distilled water in the cold. The whole homogenate was poured into dialysis tubing and placed in a beaker of distilled water for about 24 hr. in a refrigerator at 7°C. The dialysate was concentrated until it was $\frac{1}{10}$ of the volume of the homogenate. This was tested for ability to stimulate oxygen uptake of a fresh homogenate using 1 ml. of concentrated dialysate per reaction flask containing 2 ml. homogenate. No stimulation of oxygen uptake occurred. At the time of this experiment the result was taken to mean that the substrate was not dialysable. When the substrate of oxidation was later identified as linolenic acid it was realised that although it should have been dialysable it would probably have undergone lipoxidation or autoxidation over a few hours while in the dialysis tube at 7°C.

b) Boiling

A homogenate, filtered through muslin after grinding with pestle and mortar, was boiled and concentrated to about $\frac{1}{2}$ its original volume and added to a fresh homogenate in a Warburg flask to test its ability to stimulate oxygen uptake. As no stimulation occurred it was assumed that the substrate was heat labile. Now that the substrate has been identified it seems more likely that failure to stimulate oxygen uptake was due to the oxidation of linolenic acid after homogenisation but before the temperature of the homogenate was high enough

to inactivate the lipoxidase.

c) Solvent extraction of aqueous homogenates.

Having failed to extract the substrate by dialysis and to separate it from inactivated enzyme an attempt was made to do so with organic solvents using a variety of techniques. First an attempt was made by homogenising 10 g. fresh wt. leaves in 10 ml. phosphate buffer, pH. 3.6 in a cold room at 4°C. The homogenate was immediately poured into 40 ml. absolute ethanol, so that a final concentration of 80% ethanol was obtained. The extract was filtered, the filtrate washed twice with 50 ml. 80% ethanol, the supernatant and washings combined and evaporated to dryness by rotary evaporation at 35°C in vacuo. The extract was suspended by shaking with 5 ml. 0.1 M phosphate buffer, pH. 4.7. Volumes of this suspension representing an extract of 1g. and 2 g. fresh wt. leaves were added to fresh homogenates in duplicate Warburg flasks. Oxygen uptake was measured by the general procedure given under "Oxygen uptake measurement", page 20. The oxygen uptake of a portion of fresh homogenate from the sample extracted was also measured. Results are in Table 6.

Bearing in mind that the uptake of 78 μ l. by a fresh portion of the extracted homogenate does not represent the possible uptake of lg. tissue because homogenates are filtered through muslin before pipetting into Warburg flasks, the results suggest that extraction is 100% efficient as the boost in O₂ uptake caused by the addition of extract from lg. fresh wt. leaves was about 100 μ l.

The boost of about 175 μ l. caused by the addition of extract from 2g. tissue suggests that the limiting factor in O₂ uptake is probably the amount of substrate present and not the amount of enzyme.

TABLE 6 Oxygen uptake stimulation by 80% ethanol extract of aqueous homogenate.

Vol. fresh homogenate	Added extract from fresh wt. leaves	Oxygen uptake μ l. of two replicates		
		1	2	Mean
2 ml.	none	76	75	75.5
2 ml.	1 g.	177	172	174.5
2 ml.	2 g.	254	245	249.5

O₂ uptake homogenate of lg. fresh wt. leaves from sample, rest of which was extracted = 78 μ l. in 60 min. (one flask only)

Footnote - volumes in flasks made up to 3 ml. where necessary with 0.1 M KH₂PO₄, pH 4.7.

It appeared from the results in Table 6 that extraction of substrate was achieved efficiently by 80% ethanol. Further comment on this result is made in the discussion.

d) Solvent extraction of whole leaves.

Having found that ethanol extraction of homogenates appeared to be efficient in extraction of substrate an attempt was made at direct extraction of whole leaves by 80% ethanol. In preparing the dilution of ethanol consideration was given to the water content of the leaves.

Various techniques for breaking up leaf tissue were compared by preparation of solvent free buffer suspended extracts and the addition of these extracts to fresh homogenates, oxygen uptake stimulation being taken as a measure of efficiency.

Comparison was made of the efficiency of extraction of leaves in 80% ethanol by a Kenwood Blender and by hand grinding using a pestle and mortar containing a small amount of fine acid washed sand. Extracts were filtered and dried in vacuo at 35°C before suspension in buffer.

The liquidiser was found to be slightly more efficient in extraction and easier in operation. Detailed results are given in Table 7.

TABLE 7 Comparison of extraction of tissue in 80%
ethanol by sandgrinding and mechanical blending

TREATMENT	μl. O ₂ uptake at 60 min.		
	Replicates		Mean
	1	2	
Fresh homogenate alone	36.8	36.9	36.85
Homogenate + sand extract of lg. fresh wt. leaves	56.8	58.0	57.4
Homogenate + blender extract of lg. fresh wt. leaves	63.0	67.3	65.1

Using the blender 80% ethanol and chloroform extraction were compared for efficiency. Chloroform appeared unsatisfactory as it was seen to be immisible with the damaged leaf tissue because of the water content of the latter. Oxygen uptake was measured by solvent removal from the supernatant and suspension of the extracts in buffer, which were then used in oxygen uptake stimulation tests, the results of which are shown in Table 8.

TABLE 8 Comparison of extraction by chloroform and
80% ethanol.

ADDITION TO HOMOGENATE	μl. O ₂ uptake at 60 min.			
	Replicates			Mean
	1	2	3	
Homogenate alone	33.0	30.0	30.0	31.0
Chloroform extract of 1.6 g. fresh wt. leaves	47.3	47.3	47.3	47.3
80% ethanol extract of 1.6 g. fresh wt. leaves	60.8	58.6	58.0	59.1

Comparison of extraction under air and under nitrogen using 80% ethanolic extraction in the blender showed that there was no advantage in using an inert atmosphere. Results are shown in Table 9.

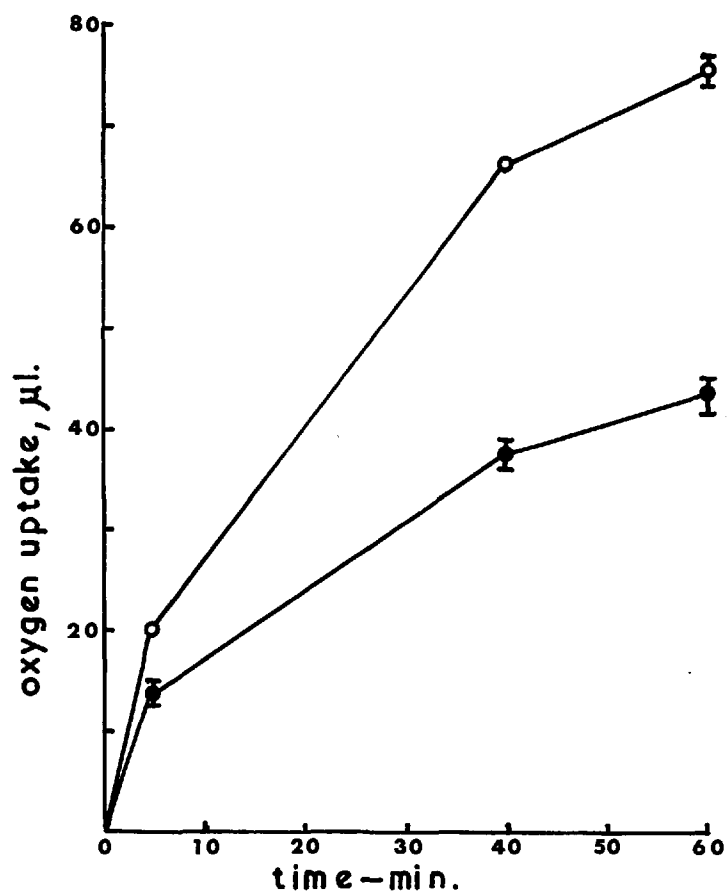
TABLE 9 Comparison of extraction in 80% ethanol under
air and under nitrogen.

TREATMENT	μl. uptake at 60 min.			
	Replicates			Mean
	1	2	3	
Homogenate alone	36.8	39.8	38.0	38.2
Homogenate + air extract of 1.8 g. fresh wt. leaves	55.2	57.1	58.0	56.8
Homog. + N ₂ extract of 1.8g. fresh wt. leaves	47.0	47.0	47.1	47.0

Therefore the method of extraction decided upon, on the evidence of the preceding experiments was that of extraction in air, by 80% ethanol, using a Kenwood blender. The method is recorded in full on page 23.

Figure 7 shows the course of oxygen uptake by a homogenate of mixed resistant and susceptible leaves with and without the addition of an 80% ethanol extract suspended in buffer. Flasks containing extract without homogenate did not show a measurable amount of oxygen uptake indicating that autoxidation of the substrate is insignificant over a few hours at 25°C.

Investigation of the properties of the substrate were performed by drying portions of 80% ethanol whole leaf extract in test tubes. Each portion was an extract of 2g. fresh wt. leaves to which, after evaporation of 80% ethanol, 2 ml. of each solvent was added. After gentle shaking for 2 min. the solvent was poured off and evaporated under a stream of N₂. The residue left after evaporation of the various solvents was then suspended in 1 ml. of 0.1 M phosphate buffer, pH 4.7 and added to Warburg flasks containing 2 ml. of fresh homogenate. Oxygen uptake was measured and results are shown in Table 10.



Bars or spots include replicate scatter.

● Homogenate of mixed Red Mexican and Canadian wonder, 0.4g. fresh wt./flask.

○ above homogenate + 80% ethanol extract of 0.4g. Canadian wonder leaves.

Figure 7. O_2 uptake of homogenate with and without crude 80% ethanol extract.

TABLE 10. Extraction of dried 80% ethanol extract into various solvents.

No replication of treatments.

Additions to homogenate	μl. O ₂ uptake at 80 ² min.	μl. O ₂ stimulation of homogenate by extracts.
Homogenate alone	114	-
Crude 80% ethanol extract	256	142
H ₂ O soluble	184	70
ethanol "	159	45
acetone "	102	0
butanol "	149	35
ether "	235	121
petroleum spirit "	237	123
benzene "	214	100

The different levels of O₂ uptake stimulation might imply that either there were several substrates with differing solubilities or that the substrate was being taken up to different degrees by different solvents.

After suspension of a dried 80% ethanol extract in water, portions of this suspension were extracted with ether and chloroform separately, evaporated to dryness in vacuo at 35°C and resuspended in pH 4.7, 0.1 M

phosphate buffer solution. A portion of the suspension before extraction was also dried in vacuo at 35°C. and resuspended in buffer solution. The three suspensions were then tested for ability to stimulate oxygen uptake of a fresh homogenate. The level of stimulation was the same in all samples indicating that the substrate was recovered completely when aqueous suspensions were extracted with ether or chloroform. This was repeated using acid, neutral and alkaline aqueous suspensions and it was found that recovery of substrate was 100% under all conditions of pH. Results are shown in Table 11.

TABLE 11 Extraction of substrate from aqueous suspensions under various pH conditions.

Treatment	μl. O ₂ uptake at 60 min.
Homogenate alone	71
+ H ₂ O suspension	160
<u>neutral extraction</u>	
chloroform	158
ether	153
<u>acid extraction</u>	
about pH 2.0, ether	156
<u>basic extraction</u>	
about pH 12.0, ether	144

The results indicate that, although there may be several substrates, they are behaving as one class of ether soluble compounds. It was later realized that the slightly lower O_2 uptake following ^{basic} extraction was due to the presence of some free fatty acids removed by alkaline conditions.

In order to obtain an extract free from chlorophyll an 80% ethanolic extract of leaves was washed x3 in equal volumes of petroleum spirit (boiling range $40^{\circ} - 60^{\circ} C.$) Chlorophyll passed into the petroleum spirit portions and these were bulked and evaporated in vacuo at $35^{\circ}C.$ The ethanol portion was also evaporated to dryness and the dry extract and the chlorophyll fraction both taken up in pH 4.7, 0.1 M phosphate buffer. These extracts were added to fresh homogenates in Warburg flasks and oxygen uptake was measured. All substrate remained in the ethanol fraction and results are shown in Table 12. (over)

TABLE 12 Extraction of chlorophyll from 80% ethanol
extract of leaves.

Addition* to homogenate	ul. uptake at 60 min		
	Replicates		Mean
	1	2	
Homogenate alone	36.8	36.9	36.85
Whole 80% ethanol extract	63.0	67.3	65.1
petroleum spirit washing	36.0	36.8	36.4
washed 80% ethanol extract	64.0	71.6	67.8

* each addition was extract of 1g. fresh wt. leaves.

An experiment was performed to find an adsorbent which would aid in the purification of the substrate. Samples of an 80% ethanol leaf extract were shaken for 5 min. with 1 g. charcoal and with 1 g. silica gel, M.F.C. grade. The ethanol was filtered off, the adsorbents washed with more 80% ethanol and the washings combined with the filtrates. After evaporation of the ethanol and water in vacuo at 35°C the residues were taken up in buffer and added to homogenates to test ability to boost oxygen uptake. Results are shown in Table 13.

TABLE 13 Loss of substrate from extracts on to
adsorbents.

There was no replication.

Treatment	$\mu\text{l. O}_2$ uptake at 60 ² min.	$\mu\text{l. O}_2$ uptake stimulation of homogenate
Homogenate alone	51	-
+ untreated extract	131	80
+ charcoal treated extract	45	0
+ silica gel treated extract	74	23

The results showed that both charcoal and silica gel were capable of adsorbing the substrate.

Kates and Eberhardt (1957) give details of fractionation procedure for bean leaf lipids using a chromatography column of silica gel and celite. Their method was used, as recorded on page 24, and the presence of substrate in each solvent sequence tested by ability to stimulate oxygen uptake of a fresh homogenate, after removal of solvent and suspension of the residue in buffer. Results are given in Table 14, being of two experiments.

These results and those in the preceding pages of this chapter presented good evidence that the substrate behaved as a lipid or group of similar lipids which were

separable from other material in the 80% ethanol extraction by silica gel chromatography with chloroform methanol mixtures as eluents.

TABLE 14 Stimulation of O₂ uptake by silica gel/celite column fractions

Extract from this fresh wt. g.	Fraction added to homogenate	μl. O ₂ uptake in 60 min.		
		Replicates		Mean
		1	2	
-	homogenate alone	22.3	24.0	23.1
15	chloroform	57.0	57.6	57.3
15	chloro/meth, 1/1	47.0	50.0	48.5
15	methanol	34.6	42.2	38.4
-	homogenate alone	87.9	88.1	88.0
4.5	chloro/meth 5/1	327.2	345.0	336.2

Chapter Footnote - When extraction by 80% ethanol and boiling iso-propanol were compared it was found that 80% ethanol extracted a greater dry-weight of material but that the iso-propanol extract contained more "active" material than the 80% ethanol extract.

Extraction with 80% ethanol was made in the Kenwood liquidiser at maximum speed for 3 minutes. The ethanol

had been chilled to about 4°C and the leaves frozen at -20°C. Leaves were powdered with a pestle and mortar at -20°C before extraction by pouring the frozen leaf powder into boiling iso-propanol. Both samples were centrifuged to remove insoluble material and the isopropanol extract was evaporated to dryness in vacuo at 35°C. The residue was taken up in 80% ethanol. Chlorophyll was removed from both extracts by washing x4 with equal volumes of petroleum spirit. Samples were removed for dry weight estimation and the remaining extracts were evaporated to dryness in vacuo at 35°C. The dried extracts were then each taken up in buffer and oxygen uptake stimulation of a fresh homogenate was measured as described previously under "Oxygen uptake measurement."

The result was that the 80% ethanol extract of stimulated homogenate O₂ uptake/36.8 µl. to 57.4 µl. over 60 min. while the iso-propanol extract stimulated homogenate O₂ uptake to 67.0 µl. over the same period of time.

The dry weight of an extract from lg. fresh wt. tissue was 0.0190 g. with 80% ethanol extraction and 0.0134 with iso-propanol extraction.

Unfortunately, this comparison which shows iso-propanol extraction to be slightly more efficient than

80% ethanol extraction of the substances sought, was carried out after the extraction procedure recorded below had been devised and used in a number of experiments. There are only very few results which are affected by using the less efficient extraction procedure and these results will be discussed later in this thesis.

Chapter 4. Fractionation of bean leaf lipids.

From the results recorded in the previous chapter evidence accumulated that suggested that fractionation of the 80% ethanol leaf extract could be achieved by following the procedure of Kates and Eberhardt (1957), details of which are given on page 24. As shown in the preliminary test of ability to pass through a silica gel/celite column, results of which are in table 14, oxygen uptake stimulating activity was almost entirely restricted to the chloroform/methanol, 5/1 eluate. Therefore further fractionation of this was undertaken by dividing up the eluate into fractions of 30 ml. In these initial experiments the column volume was 30 ml. and the column was eluted with 10 column volumes of each solvent. Each fraction was dried in vacuo at 35°C., taken up in phosphate buffer, pH 4.7 and added to a fresh homogenate to test ability to stimulate oxygen uptake. Results are shown in Table 15 and it can be seen that the substrate was present only in fractions 2 and 5. These fractions were still contaminated with a brown pigment and in order to achieve further purification a column was devised which it was hoped would give better purification of substrate by further fractionation of fractions 2-5 of the silica gel/celite column.

TABLE 15 Oxygen uptake stimulation by silica gel/
celite column fractions eluted by chloroform/
methanol, 5/1.

Original extract in 80% ethanol from 40g. fresh wt. leaves. Each addition to homogenate is extract of 4.5 g. fresh wt. leaves. No replication.

Fraction added to homogenate	μ l. O ₂ uptake at 60 min.	Dry wt. of fraction mg.
Homogenate alone	87.9	-
1	84.0	19.5
2	246.2	109.8
3	170.5	33.9
4	123.4	38.4
5	104.4	18.3
6	90.0	24.0
7	90.0	25.5
8	90.2	25.8
9	91.3	28.5
10	86.9	lost

The author constructed a column of silica gel G of 10 cm. length and 1 cm. diameter. Some success in purification was achieved by elution with 3 column volumes of chloroform/methanol, 20/1, followed by 3 column volumes of chloroform/methanol, 2/1 substrate

being eluted by the latter, but separation from contaminating material was inadequate. Improvement of separation was attempted by elution with 3 column volumes successively of mixtures of chloroform/methanol, 20/1, 10/1, 5/1, and 1/2. Although contaminants were separated from the substrate, there was considerable loss of substrate probably caused by the oxidation of this on the surface of the silica gel particles. At this time Sastry and Kates (1964a) published details of fractionation, on a silica gel column, of lipids which had previously been eluted from the silica gel/celite column of Kates and Eberhardt (1957) by chloroform/methanol, 5/1. Therefore the chromatographic method of Sastry and Kates (1964a) was adopted for further fractionation of the extract. Their method for acetone precipitation of phospholipids was also adopted, this being performed before the extract was put on the silica gel column. Complete practical details are given on pages 25 and 26.

The precipitated phospholipids and the supernatant after removal of all solvent were taken up in phosphate buffer and tested for substrate presence. Oxygen uptake readings are given in Table 16. The phospholipid portion was almost entirely free of substrate and this was confined to the non-phospholipid portion.

TABLE 16 Testing of phospholipid and non-phospholipid fractions for ability to cause oxygen uptake stimulation of homogenates.

Each addition to homogenate is an extract of 5g. fresh wt. leaves.

Fraction added to homogenate	μl. O ₂ uptake at 60 min.		
	Replicates		Mean
	1	2	
Homogenate alone	105.8	107.5	106.7
phospholipid	108.0	112.3	110.1
non-phospholipid	245.0	235.7	240.4

After removal of phospholipids, fractionation of the active fractions from the silica gel/celite column was performed on the silica gel column of Sastry and Kates (1964a) using the solvent sequence in Table 2. All fractions from this column were tested for substrate activity (results in Table 17) and chromatogrammed on Whatman SG 81, silica gel loaded paper, in di-isobutyl ketone/acetic acid/water, (40/25/5: v./v.).

Each spot was the extract of 2g. fresh wt. tissue the exact weights varying between fractions. The weight of each fraction applied can be derived from Table 5. After development chromatograms were inspected and photographed under U.V. light (Figure 9).

TABLE 17. Oxygen uptake stimulation of homogenates by fractions from silica gel column. Addition to homogenate represent extract from 5g. fresh wt. leaves. Homogenate is mixed Red Mexican/Canadian Wonder pH 5.2.

Fraction added to homogenate	μl. O ₂ uptake at 60 min.		
	Replicates		Mean
	1	2	
Homogenate alone	88.3	93.3	90.8
chloroform	88.0	92.0	90.0
C/M, 98/2	98.6	99.5	99.0
C/M, 95/5 (i)	101.2	99.0	100.1
C/M, 95/5 (ii)	108.0	106.7	107.4
C/M, 9/1	135.4	132.9	134.2
Methanol	138.0	141.5	139.7

Footnote - It was realised later, as will be explained further on in this chapter, that although MGD is present in chloroform/methanol 98/2 and 95/5 fractions it failed to stimulate homogenates very much as optimum pH for its hydrolysis is pH 7.0.

The substances shown on the chromatograms were identified tentatively as indicated in the key, by reference to the results of Sastry and Kates (1964a) as known standards of these substances were not available.

TABLE 18 Dry weights of extracts at stages of fractionation of bean leaf lipids.

Letters in brackets refer to stages shown in figure 8.

Weights are in mg.

Stage	Resistant (Red Mexican)		Susceptible (Highgrade)	
	from 40g. fresh wt.	per g. frsh wt.	from 40g. fresh wt.	per g. frsh wt.
Dry wt. leaves	3,292.0	82.30	3,300.0	82.50
a) 80% ethanol	1,393.2	34.83	1,396.4	34.91
b) washed ethanol	1,238.2	30.95	1,252.0	31.30
c) petroleum spirit	155.0	3.87	144.4	3.61
d) washed chloroform	496.6	12.41	526.2	13.15
e) water	738.4	18.46	725.2	18.13
f) column 1, chloroform	80.6	2.01	92.0	2.30
g) " C/M, 5/1	362.0	9.05	392.8	9.32
h) " C/M, 1/1	47.0	1.17	50.4	1.26
i) acetone soluble	221.2	5.53	243.6	6.09
j) phospholipid ppt.	lost	lost	128.0	3.2
k) column 2, chloroform	41.2	1.03	47.4	1.18
l) " C/M, 98/2	42.8	1.07	48.0	1.2
m) " C/M 95/5 (i)	27.0	0.67	32.4	0.81
n) " C/M 95/5 (ii)	15.6	0.39	14.2	0.35
o) " C/M 9/1	20.0	0.50	21.4	0.53
p) " methanol only	26.6	0.66	31.6	0.79

FRACTIONATION OF BEAN LEAF LIPIDS

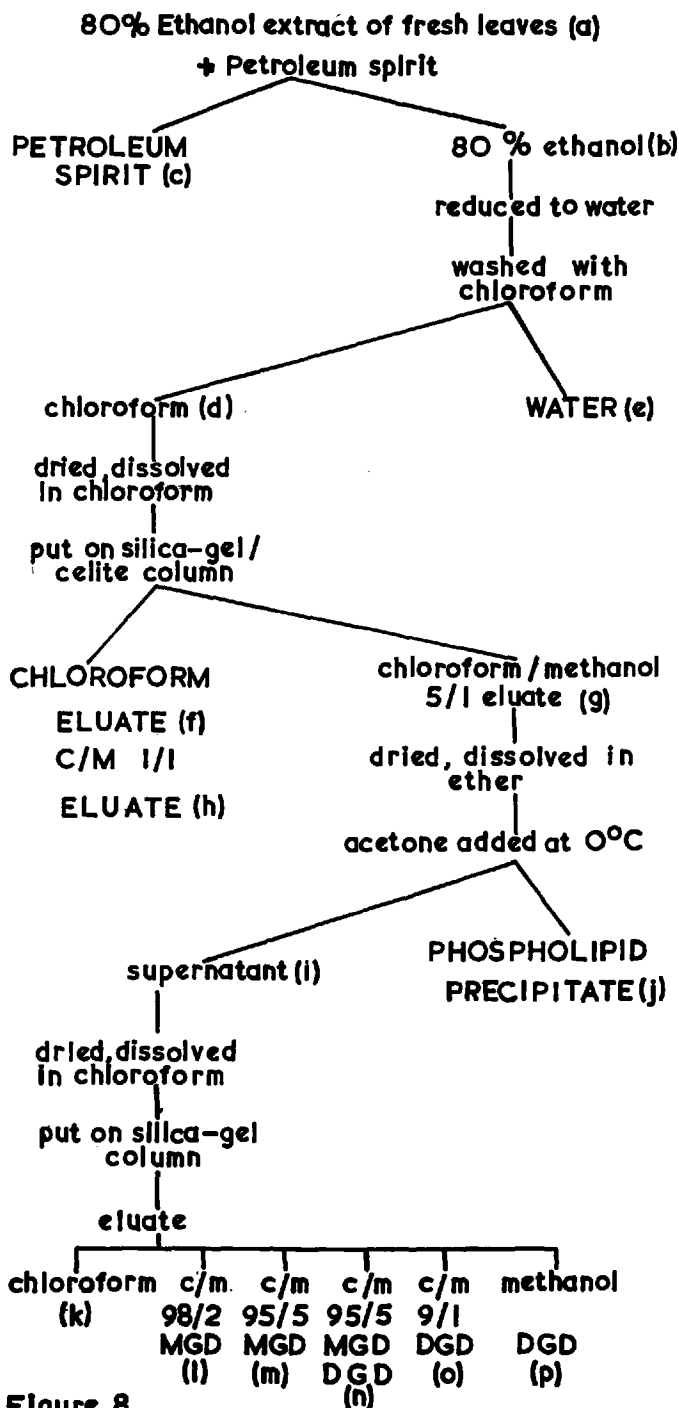


Figure 8

Letters in brackets refer to dry weights in table 18

Key to figure 9.

Spot No.	Compound	Rf.	Spot colour under U.V. light with Rhodamine 6G	Insolubility*
1	Sulpholipid and phosphatidyl inositol	0.1	Blue	a
2	Digalactosyl diglyceride	0.13	Orange fluorescence	
3	Phosphatidyl glycerol and phosphatidyl serine	0.18	Blue	a b
4	Phosphatidyl ethanolamine	0.23	Orange fluorescence	a
5	Diphosphatidyl glycerol	0.32	Blue	a
6	Monogalactosyl diglyceride	0.37	Orange fluorescence	
7	Phosphatidic acid	0.8	Blue	b

* a = insoluble in acetone
b = insoluble in methanol

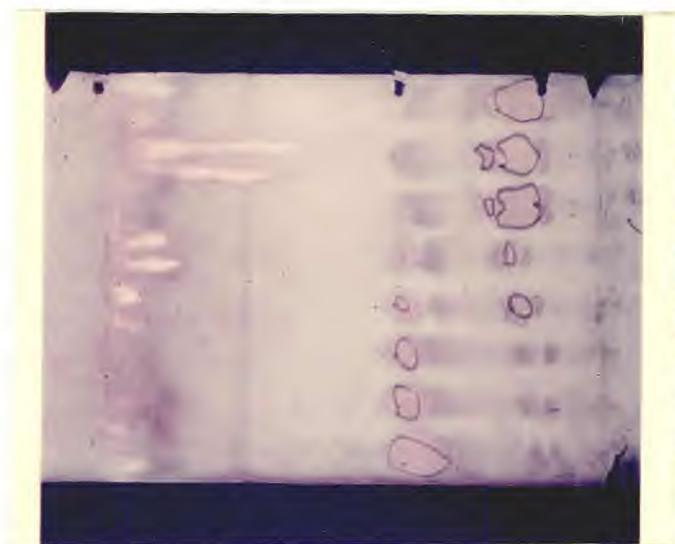
Footnote -

a) Colour photographs

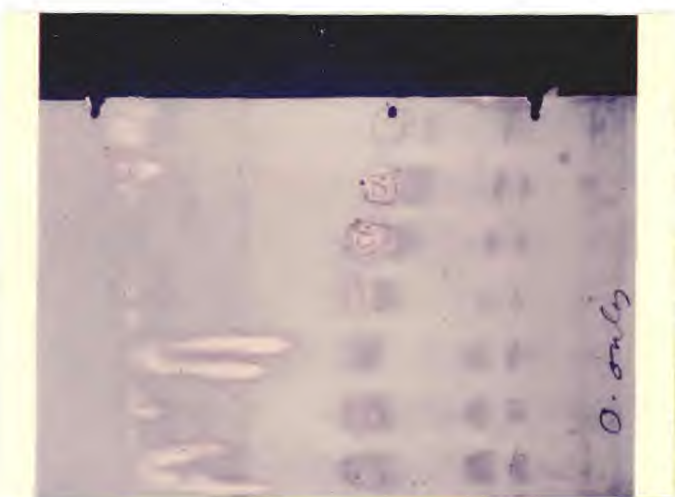
Spot colours given refer to colour immediately after spraying. When chromatograms were photographed colours had changed with age.

b) Black and white photographs

White spots are fluorescence, dull patches absorption. Pencil lines encircle MGD and DGD.



Eluent	Vol, ml.	Var.
M	100	RM
C/M, 9/1	210	CW
C/M, 9/1	210	RM
C/M, 95/5	162	CW
C/M, 95/5	162	RM
C/M, 95/5	132	CW
C/M, 95/5	132	RM
C/M, 98/2	150	CW



C/M, 98/2	150	RM
C/M, 98/2	100	CW
C/M, 98/2	100	RM
C/M, 98/2	100	CW
C/M, 98/2	100	RM
C	240	CW
C	240	RM

Front

Origin

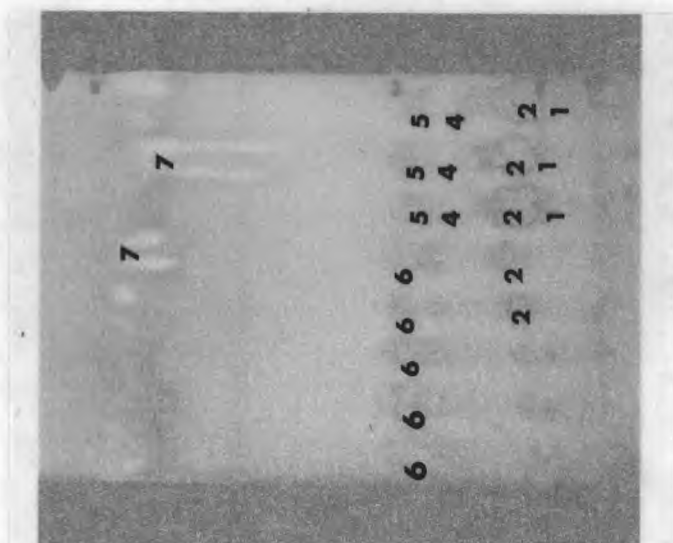
C = chloroform

M = methanol

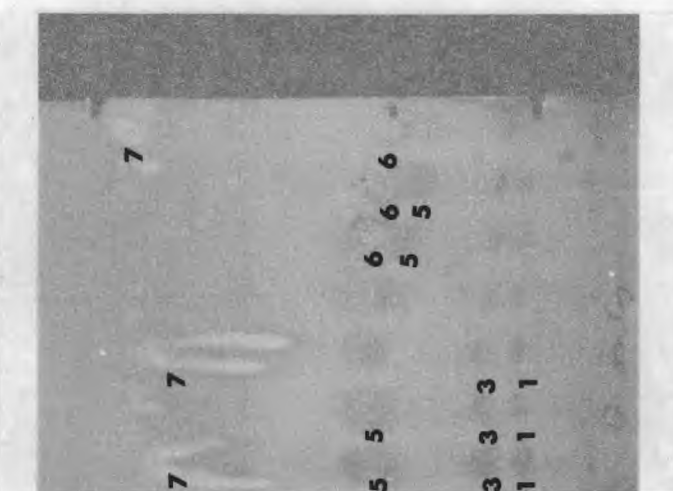
RM = Red Mexican

CW = Canadian Wonder

Figure 9. Photographs of chromatograms of silica gel column fractions under U.V. light.



Eluent	Vol, ml.	Var.
M	100	RM
C/M, 9/1	210	CW
C/M, 9/1	210	RM
C/M, 95/5	162	CW
C/M, 95/5	162	RM
C/M, 95/5	132	CW
C/M, 95/5	132	RM
C/M, 98/2	150	CW



C/M, 98/2	150	RM
C/M, 98/2	100	CW
C/M, 98/2	100	RM
C/M, 98/2	100	CW
C/M, 98/2	100	RM
C	240	CW
C	240	RM

Front

Origin

C = chloroform

M = methanol

RM = Red Mexican

CW = Canadian Wonder

Figure 9. Photographs of chromatograms of silica gel column fractions under U.V. light.

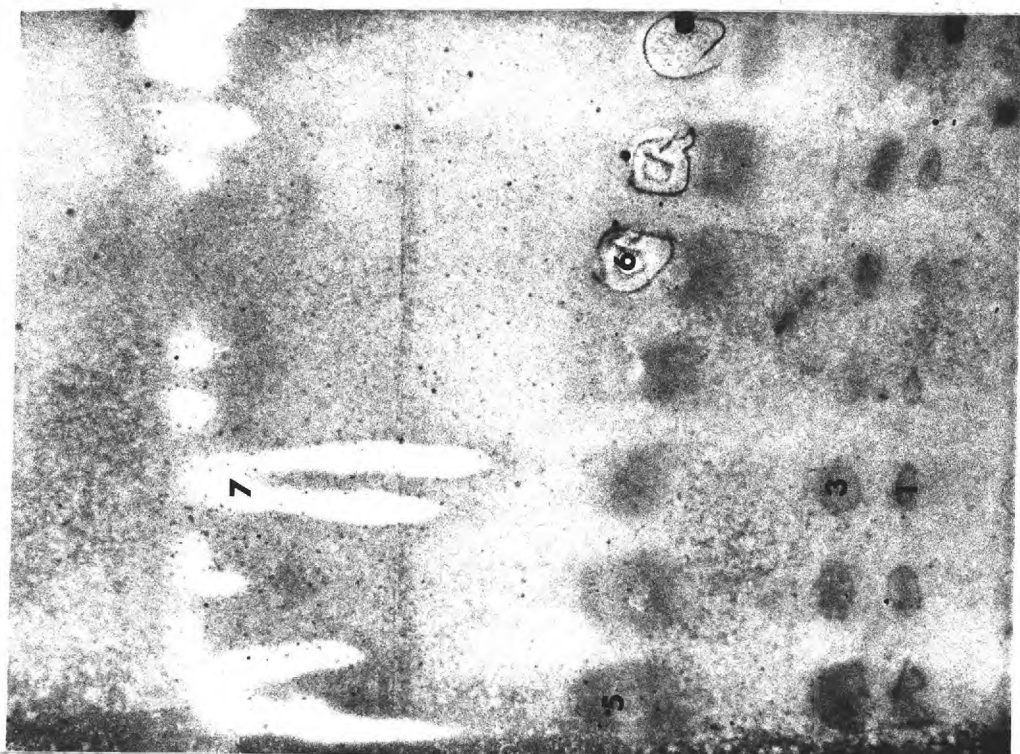
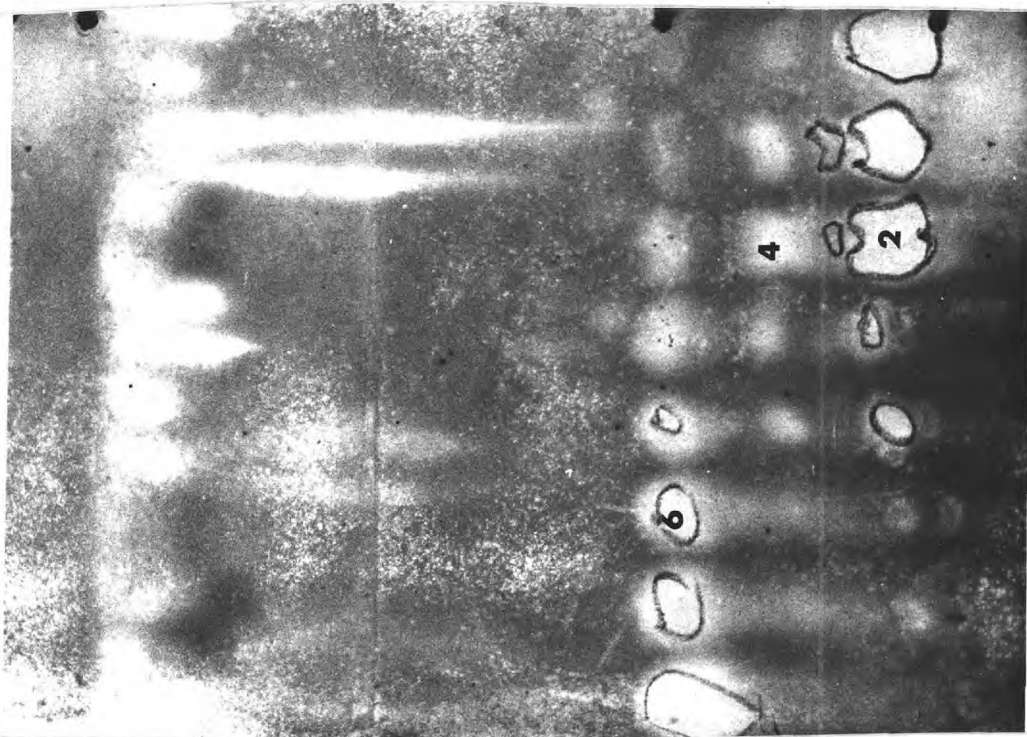


Figure 9. For order of fractions see previous page.

The fractions with substrate activity were those containing compounds 6 and 2 (figure 9), which corresponded in colour - reaction and order of migration with the galactolipids, mono and di-galactosyl diglycerides (MGD and DGD). The complete fractionation procedure recorded on pages 23 - 27 is summarised in figure 8. The dry weights of the extracts from a resistant and a susceptible variety at all stages of fractionation are given in Table 18, and cross reference to these stages is given on figure 8.

On the chromatograms Rf. values were lower than those published by Sastry and Kates (1964a) who prepared their own silica-gel loaded paper (Marinetti, 1962). Commercially available paper has greater adsorptive properties which probably caused the lower Rf. values (Marinetti 1965).

Lipids other than MGD and DGD were removed from substrate active fractions by virtue of their insolubility in methanol or acetone as recorded in the key to figure 9. Fractions were dried in a stream of N_2 and taken up in methanol if soluble. Precipitate was removed by filtration, drying repeated and fractions taken up in acetone and filtered. This did not result in the loss of substrate activity by the portion of the fraction soluble in methanol and acetone. MGD and DGD

extracted and purified in this way stimulated oxygen uptake of fresh homogenates of resistant leaves as follows: MGD from 1g. fresh wt. leaves stimulated oxygen uptake of variety Red Mexican at pH 7.0 by 51% and DGD from 2g. fresh wt. leaves stimulated oxygen uptake of line 141 at pH 5.6 by 150%. The pH stated in each case is the optimum for hydrolysis of each galactolipid. (For volume of O_2 uptake see Table 30).

Chapter 5. Confirmation of composition of active fractions of lipids.

Confirmation that the substrates of oxidation were MGD and DGD was obtained by acid hydrolysis (method page 28) of the purified active fractions which had been tentatively identified by paper chromatography (Figure 9). The water soluble hydrolysis products were chromatogrammed each spot being equivalent to an extract of 2g. fresh wt. leaves. (Method, page 28).

The only substances showing on the chromatograms were glycerol and galactose, identified by comparison of Rf. values with those of marker spots.

Ether soluble hydrolysis products were analysed by gas chromatography after methylation. Results of the analysis shown in Table 19 show that linolenic acid is the predominant fatty acid. The content of linolenic acid is a reasonable means of confirming the nature of the fats as Sastry and Kates (1964a), who purified MGD and DGD to a greater extent, found that linolenic acid represented 96% and 97% respectively of the fatty acids present after hydrolysis of MGD and DGD from runner bean leaves.

Identification of major components of the hydrolysed active fractions as galactose, glycerol and linolenic acid supported the view that the substrates were the galactosyl diglycerides.

TABLE 19 Fatty acid analysis of MGD and DGD.

Fatty Acid	Carbon numbers, Apiezon L.	% Total fatty acid methyl esters	
		MGD	DGD
	15.7	0.9	1.4
Palmitic	16.0	7.4	13.8
Linolenic (and linoleic*)	17.55	90.0	83.5
Stearic	18.0	1.7	2.0

Numerous small peaks before carbon number 15.7 are not included.

*Performed by Knivett et al. (see page 32) who stated that less than 5% linoleic acid is included in the linolenic figures.

Furthermore it was found that the fatty acids obtained by hydrolysis of substrate active lipid extracts retained substrate activity. An unhydrolysed lipid extract of 5g. fresh wt. tissue stimulated oxygen uptake by a fresh homogenate by 93% over the first 60 min. while an identical extract, after acid hydrolysis of the contained lipids, stimulated a portion of the same fresh homogenate by 114% over the same period of time. Readings are shown in Table 20.

TABLE 20. Retention of substrate activity by hydrolysed active extract.

Addition to homogenate	μl. O ₂ uptake at 60 min.		
	Replicates		Mean
	1	2	
Homogenate alone	79.1	77.9	78.5
Unhydrolysed extract of 5g. fresh wt. leaves	151.5	152.1	151.8
Hydrolysed extract of 5g. fresh wt. leaves.	167.2	168.8	168.0

Sastry and Kates (1964b) demonstrated that specific galactolipases liberated linolenic acid from MGD and DGD instantly upon breaking cells in bean leaves during homogenisation. Their method was used successfully to demonstrate galactolipase activity in leaves of Red Mexican. Full details will be given in Chapter 10 but it is worthy of mention that the content of linolenic acid increased at least 8-fold in resistant leaf homogenates and about 4-fold in susceptible leaf homogenates in the sixteen minutes following leaf homogenisation (detailed results in tables 28 and 29).

Therefore it seemed very likely that homogenisation of leaves permitted hydrolysis of the galactolipids liberating linolenic acid as a substrate for oxidation by lipoxidase.

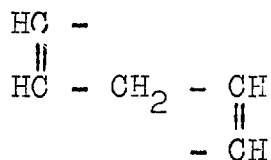
Chapter 6. Fatty acid oxidation by leaf homogenates.

Following the demonstration of the substrate action of galactolipids containing linolenic acid in stimulating O_2 uptake by homogenates (page 77), the effect of a number of different fatty acids on O_2 consumption by homogenates was investigated. Fatty acids were added to homogenates as 10 mg. amounts in 1 ml. of buffer containing Tween 80.

The results, in Table 21, show that a marked increase in oxygen uptake occurred only after the addition of linoleic and linolenic acids, and not of oleic acid or saturated fatty acids.

The enzyme responsible for the oxidation of unsaturated fatty acids in corn (Fritz and Beevers, 1955) and sunflower seedlings (Gamborg and Zalik, 1958) is lipoxidase. It has been shown (Holman and Bergström 1951) that lipoxidase will only oxidize unsaturated fatty acids with the configuration of double bonds shown in Figure 10, as found in linoleic, linolenic and arachidonic acids. (See Figure 1, page 13).

FIGURE 10



Unsaturated fatty acids undergoing oxidation have characteristic U.V. absorption spectra (Holman and Burr, 1945, Farmer et al. 1943). These absorption spectra were detected by ^{oxidation of} linoleic and linolenic acids suspended in buffer and shaken in air at 20°C for 10 min. Absorption maxima were found at 272 and 310 mμ. in an ethanol extract of the oxidized acids (Figure 11a).

TABLE 21 Homogenate oxygen uptake stimulation by various fatty acids. No replication.

Fatty acid	No. Carbon atoms	No. Double bonds	O ₂ uptake μl. in 30 min
Caproic	6	0	78
Myristic	14	0	73
Oleic	18	1	95
Linoleic	18	2	172
Linolenic	18	3	175
Arachidic	20	0	76
Linoleic without homogenate			2
Linolenic without homogenate			1
Homogenate alone			81
Homogenate plus Tween 80			86

Peaks were detected at the same wavelengths when ethanol extracts were prepared from homogenates (6g. fresh wt. tissue in 30 ml. pH 4.7 phosphate buffer), shaken at 20°C for 1 hr. (Figure 11b). Samples of

0.6 g. fresh wt. tissue were withdrawn, extracted by addition of absolute ethanol to a final concentration of 80% ethanol, filtered, diluted x30 and scanned at intervals during this period, and gradually increasing absorption maxima were found in samples at 20, 40 and 60 minutes. Figure 11b shows the spectrum of the 60 min. sample. Further evidence for the presence of lipoxidase activity was found by the addition of linolenic acid to homogenates, which were shaken at 20°C and extracted with ethanol. The absorption maxima of the ^{extracts} ethanol/are shown in Figure 12. This clearly suggests rapid enzymic action in the homogenate, causing oxidation of the linolenic acid.

Results in Table 22 show that partial inhibition of oxygen uptake in homogenates was obtained by the addition of catechol and α -naphthol to a mixed 1:1 homogenate of resistant and susceptible leaves. Catechol and α -naphthol are known to inhibit lipoxidase activity (Holman and Bergström, 1951). Sodium azide and sodium cyanide have relatively little effect at a concentration at which they might be expected to completely inhibit metal containing oxidases. (James, 1953).

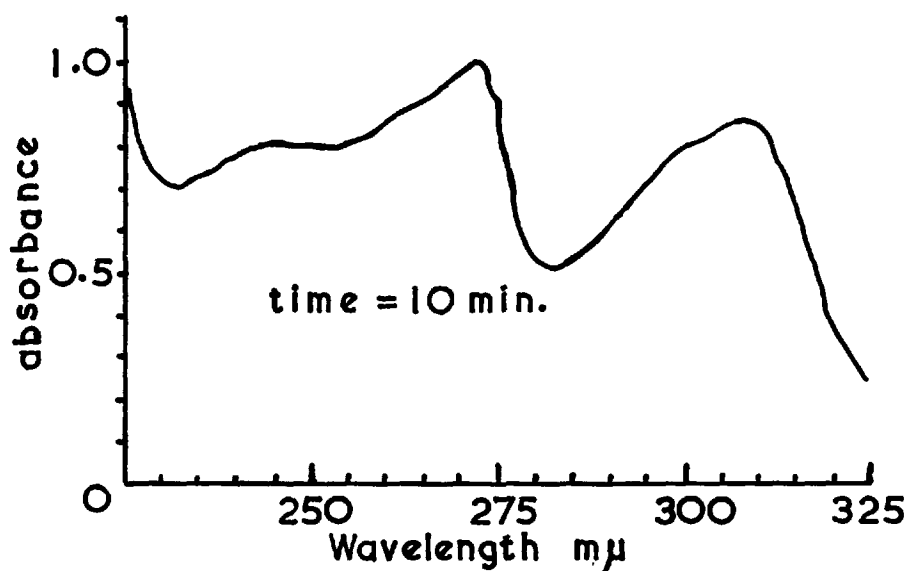
TABLE 22 Inhibition of oxygen uptake of homogenate.

Concentration of inhibitors was 10^{-2} .M.pH. 4.4.

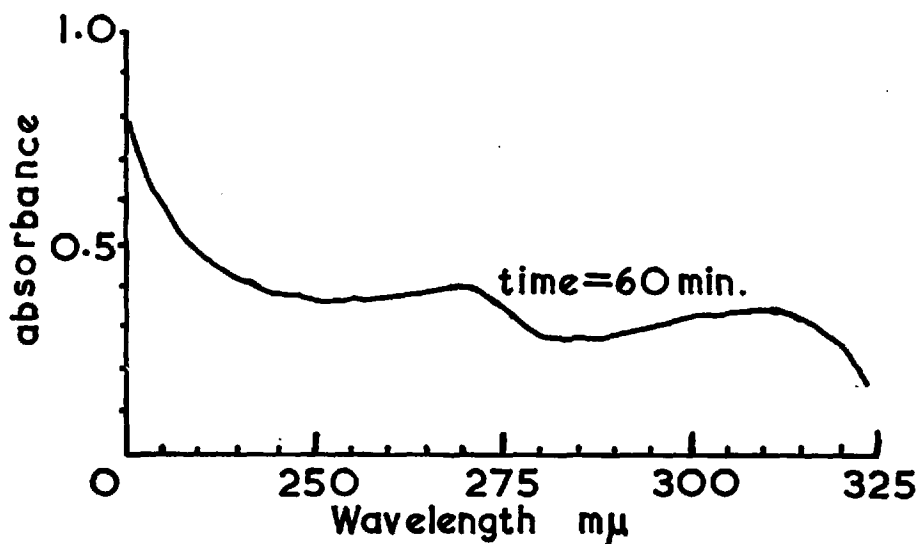
Inhibitor	% inhibition*	μ l. O_2 uptake. Mean of 3 ² replicates.
Sodium azide	22.8	39.0
Sodium cyanide	31.3	34.7
Homogenate without KOH in centre well	--	50.5
α -naphthol	55.5	34.0
Catechol	70.2	22.8
Homogenate	--	76.5

*%inhibition of total O_2 uptake after 2 hr.

Therefore the substrate specificity, appearance of distinctive absorbance peaks and response to inhibitors are all consistent with the theory that the endogenous oxidation in homogenates is caused in the most part by the action of lipoxidase on linolenic and linoleic acids.

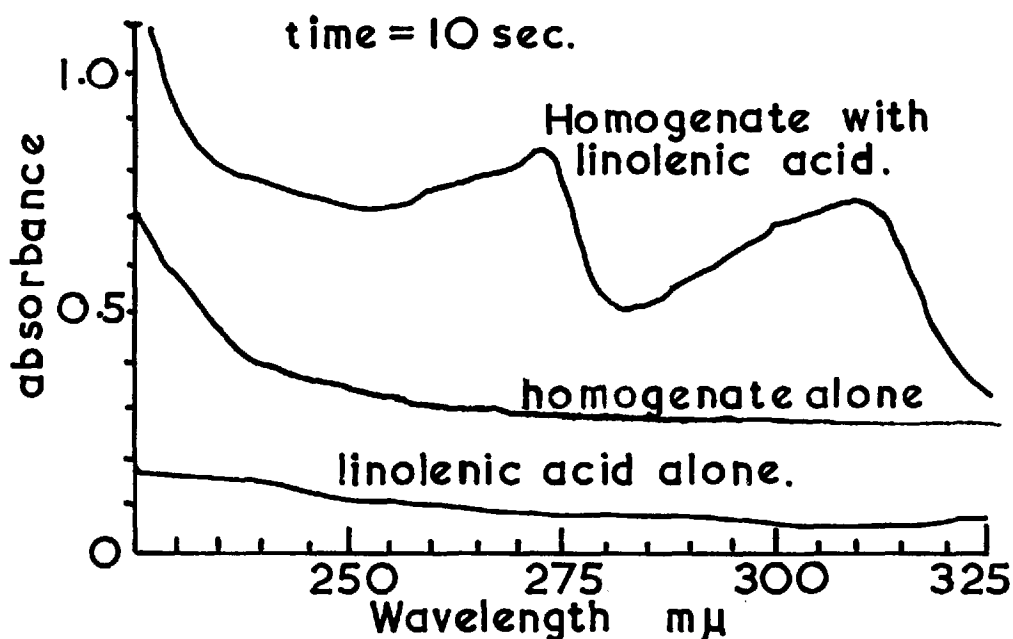


(a) SPECTRUM OF OXIDIZED LINOLENIC ACID



(b) SPECTRUM OF ETHANOL EXTRACT OF
HOMOGENATE UNDERGOING OXIDATION

Figure 11.



SPECTRUM OF ETHANOL EXTRACT OF
HOMOGENATE UNDERGOING OXIDATION
WITH ADDED LINOLENIC ACID.

Figure 12.

PART 3

NATURE OF DIFFERENCE BETWEEN RESISTANT AND SUSCEPTIBLE LEAVES

Having elucidated the nature of the endogenous oxidation in homogenates it was necessary to find the point in this hydrolytic and oxidative pathway at which resistant and susceptible leaves differ.

These were possible points of difference:-

- 1) activity of lipoxidase.
- 2) presence or absence of an antioxidant.
- 3) different levels of substances which undergo coupled oxidation with linolenic acid.
- 4) initial levels of linoleic and linolenic acid in intact leaves.
- 5) levels of MGD and DGD.
- 6) activity of galactolipid hydrolases.
- 7) presence or absence of hydrolase inhibitor.

Chapter 7. Comparison of Lipoxidase activity

A suspension of linolenic acid in phosphate buffer plus Tween 80, pH.4.7 was added to homogenates of resistant and susceptible leaves, Red Mexican and Canadian Wonder. The stimulation of oxygen uptake above the control levels was identical in resistant and susceptible leaves as recorded in Table 23.

TABLE 23

Stimulation of oxygen uptake of resistant and susceptible lines by linolenic acid

	μl. O ₂ uptake in 15 min.	
	Red Mexican (res.)	Canadian Wonder(susc.)
Homogenate alone	31	18
Homogenate + 9 mg. linolenic acid	173	164
Increase in O ₂ uptake caused by linolenic acid	142	146

This suggested that the activity of lipoxidase was the same in resistant and susceptible varieties, and also that there were no differences between the varieties in levels of antioxidant or substances undergoing coupled oxidation (e.g. Carotenes, Sumner and Sumner, 1940).

Chapter 8 Levels of linoleic and linolenic acids in
extracts after acid hydrolysis in vitro.

80% ethanolic extracts were made from 10g. fresh wt. leaves of each of the lines 61 and 129, which were respectively resistant and susceptible. These extracts which contained both free fatty acids and lipids were hydrolysed in methanolic HCl (page 28) and the resulting fatty acids were subjected to gas chromatography (page 33) and estimation of total fatty acid content (page 30). The results of these experiments and the weight of each fatty acid calculated from these results are given in Tables 24 and 25.

It can be seen from the results that in this pair of lines linoleic and linolenic acid are at a slightly higher level in the susceptible than in the resistant line. Therefore unsaturated fatty acid contents cannot explain the greater rate of O_2 uptake in the resistant leaf homogenates.

In addition, it seems unlikely that the amount of galactolipids varies between resistant and susceptible lines as most linolenic acid in Phaseolus vulgaris is contained in these lipids (Sastry and Kates, 1964a), and the above results show that there is not more linolenic acid in resistant than in susceptible leaves.

TABLE 24 Gas chromatography of acid hydrolysed leaf extracts.
Performed by Knivett et al. on PEGA column at 180°C.

LINE NUMBER:-			61 (Res.)		129 (Sus.)	
FATTY ACID			% of total fatty acid	µg./g. frsh.wt. leaves	% of total fatty acid	µg./g. frsh.wt. leaves
Name	Equiv. Wt.	C No. PEGA 180°C				
Myristic	228.36	14.0	trace	-	trace	-
	231	14.2	0.3	1.6	1.0	6.1
	235	14.5	0.3	1.6	0.9	5.5
	242	15.0	0.2	1.1	0.5	3.0
	249.7	15.55	0.2	1.1	0.4	2.4
Palmitic	256	16.0	20.2	108.3	17.2	104.9
	254	16.55*	3.8	20.4	4.3	26.2
	268.2	16.86	1.2	6.4	2.7	16.5
	274	17.3	trace	-	0.4	2.4
Stearic	284.47	18.0	2.7	14.5	3.0	18.3
Oleic	282.45	18.27*	2.2	11.8	3.3	20.1
Linoleic	280	18.88*	11.1	59.5	10.2	62.2
Lindenic	278	19.63*	57.5	308.2	56.0	341.6

* Unsaturated acid esters, disappeared on bromination.

TABLE 25 Total fatty acid content, lines 61 and 129.

Line No.	µ. equiv./g. fresh wt. leaves	Average equiv. wt. from Table 10	Total fatty acid µg./g. fresh wt.
61	1.98	271.5	536
129	2.25	271.3	610

Furthermore, comparison of spot size of MGD and DGD in chromatograms of lipids of resistant and susceptible leaves agree with this conclusion (figure 9).

Chapter 9 Levels of free linoleic and linolenic acids
in vivo.

A known weight, between 2.0 g. and 3.0 g. fresh wt. of leaves of resistant and susceptible lines or varieties were frozen and extracted with 50 ml. 80% ethanol in a Kenwood liquidizer at maximum speed for 3 min. Insoluble material was removed by filtration and the extract evaporated to dryness by rotary evaporator in vacuo at 35°C. The dry extract was dissolved in 5ml. diethyl ether/g. fresh wt. leaves and fatty acids were extracted as described on page 29. On one occasion the fatty acids extracted were divided into two portions which were used for analysis by gas chromatography (page 32) and estimation of total fatty acid content (page 30). On another occasion only total fatty acid content was measured. Results are shown in Tables 26 and 27.

TABLE 26 Free fatty acid content in vivo.

Resistant		Susceptible	
Var. or line	μ equiv. fatty acid/g. fresh wt. leaves	Var. or line	μ equiv. fatty acid/g. fresh wt. leaves
141	0.525	229	0.300
Red Mexican	0.550	Highgrade	1.020

TABLE 27 Gas chromatography of in vivo free fatty acids performed by author on PEGA at 184.5°C.

FATTY ACID			LINE: NO: 141 (Resistant) % of total free fatty acids	229 (Susceptible) % of total free fatty acids
Name	C No. PEGA 184.5°C	Equiv. wt.		
Myristic	14.0	228.36	4.9	3.7
Palmitic	16.0	256	20.7	18.2
	16.45	254	4.8	3.5
	16.7	268.2	2.5	1.8
Stearic	18.0	284.47	5.6	4.3
Oleic	18.3	282.45	7.1	6.0
Linoleic	18.85	280	9.5	13.2
Lindenic	19.6	278	35.1	42.3
	ca. 21	326	8.9	6.1

From these figures it has been calculated that the linolenic and linoleic acid content of 141 and 229 in vivo are as follows:

TABLE 28

Line No.	Linoleic acid	Linolenic acid
141	13.6 µg.	50.2 µg.
229	10.8 µg.	34.6 µg.

Allowing 2 molecules O_2 per linoleic and 3 per linolenic acid molecule the amount of oxygen that could be taken up by these fatty acids is 15.16 μ l. for 141 and 10.27 μ l. for 229. However, although in this instance the resistant line would initially take up more oxygen than the susceptible line in the comparison of Red Mexican and Highgrade, assuming the same fatty acid content, oxygen uptake by these unsaturated fatty acids would be approximately twice as high in the susceptible than in the resistant variety. It does not therefore appear that the consistently higher oxygen uptake of homogenates of resistant lines can be explained by the in vivo free fatty acid content of intact leaves. Also the amount of free unsaturated fatty acids present in vivo is not of sufficient magnitude to take up the volume of oxygen measured by Warburg experiments with homogenates. (Page 44).

Chapter 10. Levels of free fatty acids after homogenisation.

Bearing in mind the findings of the previous chapter it was thought that changes may occur in fatty acid levels as a result of aqueous homogenisation.

Four g. of 2 day old leaves of resistant and susceptible varieties were separately homogenised at 4°C in 20 ml. pH 4.7, 0.1 M phosphate buffer and maintained at 4°C to avoid lipoxidation. At various time intervals samples representing 1g. fresh wt. leaves were withdrawn and added to a volume of absolute alcohol sufficient to give a final concentration of 80% ethanol. After filtration the extract was dried in vacuo by rotary evaporation at 35°C and dissolved in 15 ml. diethyl ether. Fatty acids were extracted as described previously (page 29) and total free fatty acid content was estimated as before (page 30). The result is shown in figure 13. The zero-time reading on the graph is the free fatty acid content of intact leaves, (i.e. before homogenisation, Chapter 8) which was measured on the same occasion. It is obvious that fatty acids are being released from other compounds at a much greater rate in the resistant than in the susceptible homogenate.

The composition of this released fatty acid was found by repeating the above procedure using lines 141

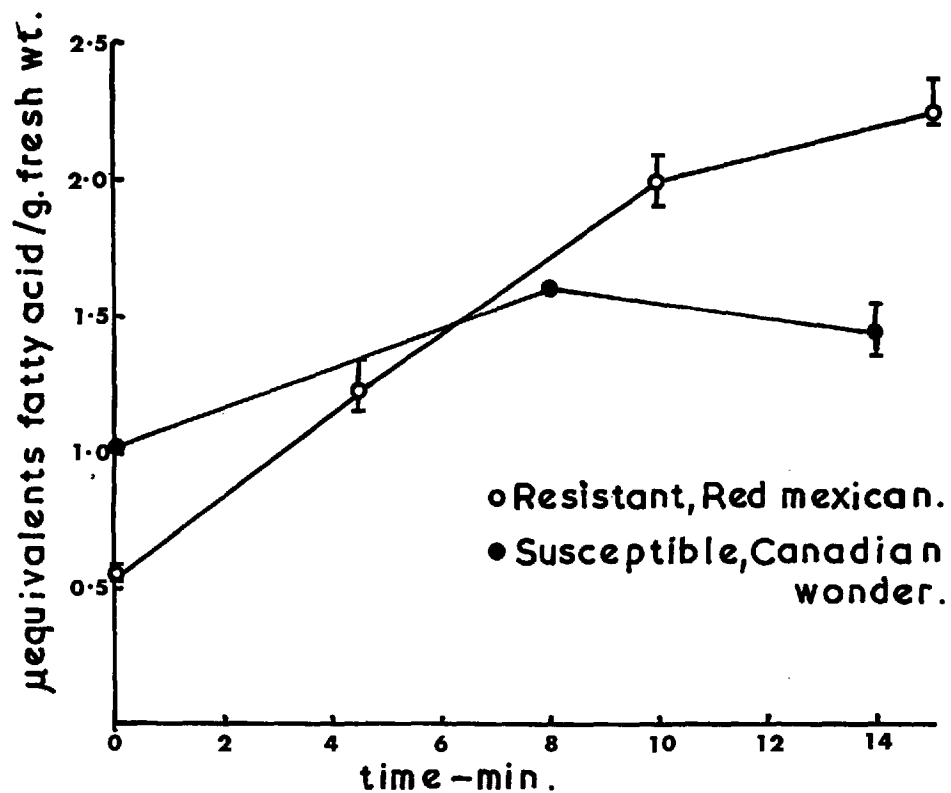
and 229, respectively resistant and susceptible. Samples were not removed at various time intervals but the whole sample of each line was extracted with 80% ethanol after 16 minutes at 4°C. The extracted fatty acids were used for total fatty acid estimation and analysed by gas chromatography by the author using a PEGA column at 184.5°C. (Details, page 32).

The change in total fatty acid content with time is shown in figure 14. The zero-time reading is of fatty acid content of intact leaves.

One of the gas chromatograms obtained is shown in Figure 15 and a comparison of the percentage composition and weights of fatty acids of resistant and susceptible leaf homogenates present as free fatty acids after 16 minutes at 4°C is given in Table 29. (see next page).

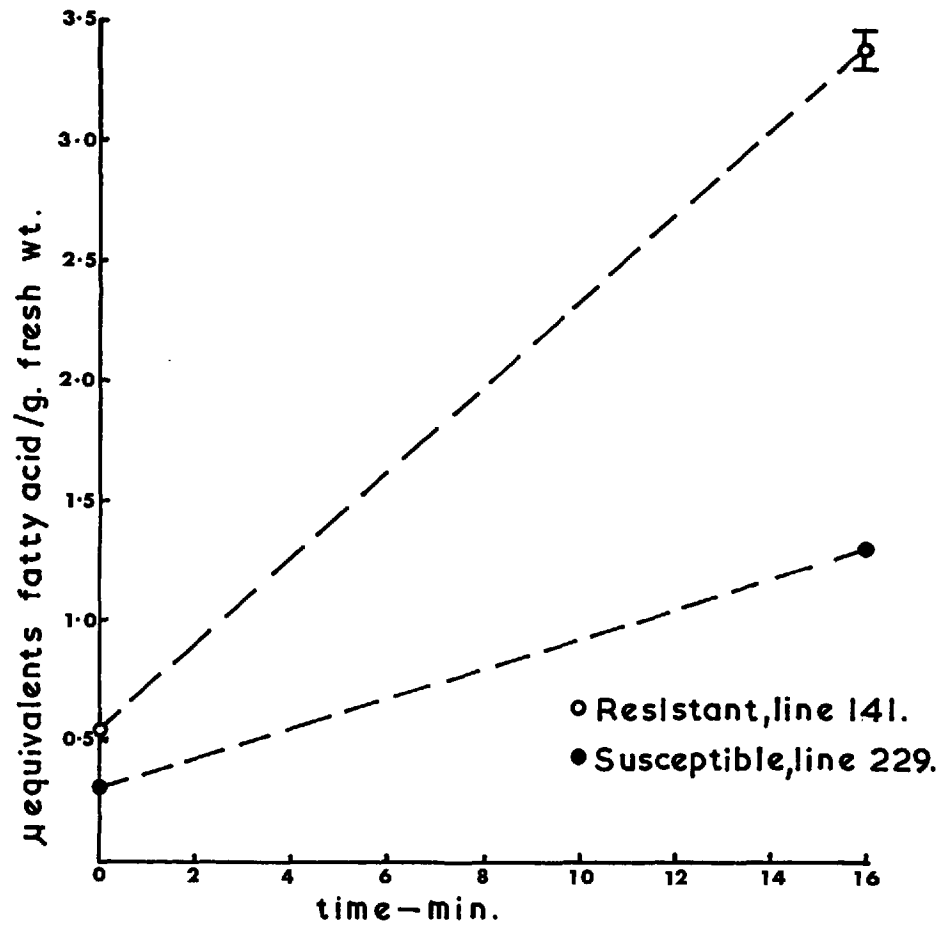
TABLE 29 Analysis of free fatty acids in resistant and
susceptible leaf homogenates 16 min. after
homogenisation.

LINE NO:-			141(Resistant)		229(Susceptible)	
FATTY ACID			% of total fatty acids	µg/g.frsh wt.leaves	% of total fatty acids	µg/g.frsh wt.leaves
Peak No. Fig. 15	Name	C No. PEGA 184.5°C				
1	Myristic	14.0	1.9	17.2	2.9	10.1
2		14.3	2.2	19.9	2.9	10.1
3		15.0	2.0	18.1	2.4	8.3
4		15.5	2.3	20.8	2.8	9.7
5	Palmitic	16.0	19.6	177.6	19.7	68.4
6	Palmito- leic	16.45	3.1	28.0	4.5	15.6
7		16.7	3.0	27.2	2.6	9.0
8	Stearic	18.0	3.8	34.4	5.4	18.7
9	Oleic	18.3	5.1	46.2	7.5	26.0
10	Linoleic	18.85	11.2	101.5	11.1	38.5
11	Lindenic	19.6	45.1	408.6	37.6	130.6



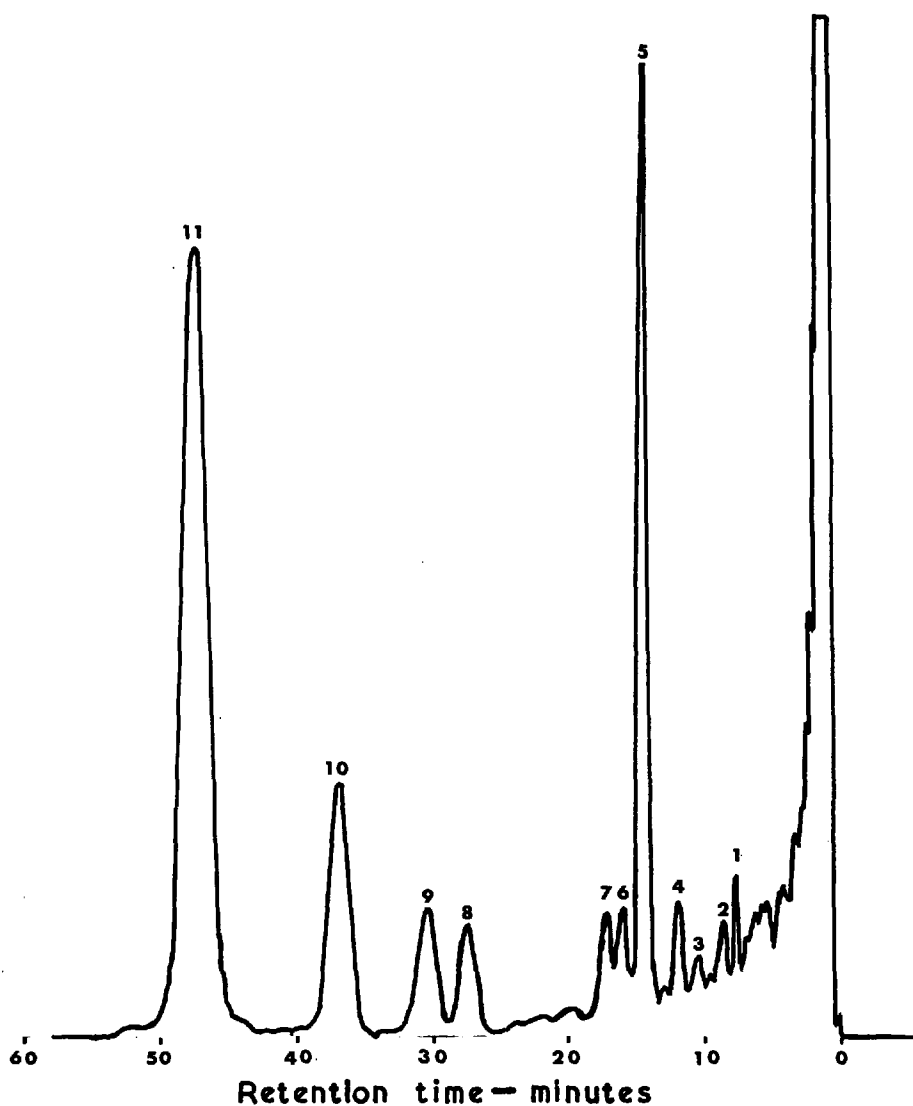
bars or spot include replicate scatter.

Figure 13. Change in free fatty acid content after leaf homogenisation.



bars or spots include replicate scatter.

Figure 14. Change in free fatty acid content during 16 min. after leaf homogenisation.



Retention time - minutes
Figure 15. Gas chromatogram of esters of free fatty acids of leaf homogenate of line 141 (res.) after 16 min. at 4°C. Key, table 29

Chapter 11. Cause of difference in oxygen uptake of resistant and susceptible leaf homogenates.

In earlier chapters it has been shown that the main substrate of endogenous oxidation of homogenates of bean leaves is linolenic acid (Chapter 6) and it was suggested that most of the linolenic acid is liberated from galactolipids (last paragraph, Chapter 5.). The results set out in Chapter 10 show that during the first 16 minutes after homogenisation a greater amount of unsaturated fatty acids is released from the lipids of resistant than from those of susceptible leaf homogenates giving rise to the suggestion that this difference is a result of a difference in the activity of galactolipid hydrolases.

To test the activity of these hydrolases in homogenates, extracted galactolipids were added to homogenates of resistant and susceptible leaves as described on page 22. The results are shown in Table 30.

It can be seen that in all cases galactolipids stimulate O_2 uptake in resistant more than in susceptible homogenates.

To confirm that the galactolipid hydrolase system is more active in resistant lines an assay for enzyme activity by measuring lipid destruction was performed (Method, page 35).

TABLE 30 Stimulation of oxygen uptake of homogenates after 1 hr.
a) by addition of DGD. H. optimum 5.6 (Sastry and Kates, 1964b).

pH	DGD from this frsh. wt. (g) leaves	Resistant			Susceptible			Stim of susc. Stim of Res. x 100
		Line or var.	Replicates	Mean	Line or var.	Replicates	Mean	
5.2	None	RM*	76.6, 77.0 74.3	76	CW ^W	105.5, 104 103.1	104.2	24.3%
"	5	"	148, 146.2	147.1	"	120, 123, 122.5	121.8	
			Stim.	71.1		Stim.	17.6	
5.6	None	141	21.6, 23.4 20.6	21.5	229	13.8, 13.4 13.9	13.7	11.8%
"	2	"	40, 35.8, 42	39.3	"	14, 17, 16	15.6	
			Stim.	17.8		Stim.	2.1	
3.6	None	RM	46.8, 47 45.3	46.4	124	31.5, 33.8 32.2	32.5	40.5%
"	2	"	70, 72.3 71	71.1	"	44, 42 41.6	42.5	
			Stim.	24.7		Stim.	10.0	
5.6	None	120	38.3, 34	36.1	122	23.9, 22.8	23.3	60.9%
"	5	"	96.5, 94	95.2	"	63, 55.5	59.3	
			Stim.	59.1		Stim.	36.0	

* Red Mexican ■ Canadian Wonder

continued overleaf

TABLE 30 (continued from previous page)

pH	MGD from this frsh. wt. (g) leaves	Resistant			Susceptible			Stim of susc. Stim of Res. x 100
		Line or var.	Replicates	Mean	Line or var.	Replicates	Mean	
7.0	None	RM*	57.5, 52.9 61	57.1	CW ^W	57.1, 57.7 57.9	57.5	
"	1	"	84, 86.1, 89.5	86.5	"	64, 71.7, 70	68.2	
			Stim.	29.4		Stim.	10.7	36.4%
7.0	None	120	41.2, 42	41.6	122	41.8, 40.6	41.2	
"	5	"	78.8, 82.6	80.7	"	50, 52.9	51.5	
			Stim.	39.1		Stim.	10.3	26.4%
7.0	None	RM	51.1, 52.3	51.7	CW	47.3, 49	48.2	
"	5	"	98.5, 100.4	99.5	"	71.0, 64.7	67.8	
			Stim.	47.8		Stim.	19.6	41.0%

* Red Mexican W Canadian Wonder Stim. = stimulation

Results in Table 31 show that there is a reduction in lipid content over 60 min. in both resistant and susceptible homogenates. The reduction of lipid content is greater in resistant than in susceptible leaf homogenates as can be seen from the table. Table 31 also shows the reduction in level of lipids of the susceptible variety as a percentage of that in the resistant variety.

TABLE 31 Reduction in level of lipids in bean leaf
homogenates over 60 minutes.

Var. or line *	Res. or Sus.	pH	Absorbance 530 mμ		Reduction after 60 min.	Redn. of Sus. Redn. of Res x 100
			0-time	60 min.		
RM	R	7.0	0.470	0.430	0.040	} - 62.5%
CW	S		0.490	0.465	0.025	
141	R	5.6	0.365	0.304	0.061	} - 70.4%
129	S		0.365	0.322	0.043	
RM	R	5.6	0.477	0.376	0.101	} - 43.6%
124	S		0.410	0.366	0.044	

* One resistant and one susceptible variety tested on each occasion.

These results indicated that a hydrolase enzyme was more active in resistant than in susceptible lines. The assay was not specific for galactolipids, however, so to confirm that the difference in hydrolase level was mainly a difference in galactolipid hydrolase, galactolipids were added to homogenates and the same assay was carried out. Results are shown in Table 32.

TABLE 32 Reduction in level of MGD and DGD added to bean
leaf homogenates over 60 minutes.

Var. or line*	Res. or Sus.	Galacto- lipid from this fresh wt.	pH	Absorbance 530 mμ		Redn. after 60 min	redn. of sus. reln. of Res. x 100
				0-time	60 min.		
RM	R	MGD	7.0	0.787	0.672	0.115)	57.3%
CW	S	5g.		0.812	0.746	0.066)	
141	R	DGD	5.6	0.500	0.373	0.127)	43.3%
129	S	5		0.499	0.444	0.055)	
RM	R	DGD	5.6	0.594	0.459	0.135)	62.8%
124	S	4		0.535	0.450	0.085)	

* One resistant and one susceptible variety tested on each occasion.

It can be seen from these results, especially by comparison of the reduction in absorbance in Tables 31 and 32 that hydrolysis of the added galactolipids occurred, and did so at a greater rate in resistant than in susceptible leaf homogenates.

It is this difference which results in the faster release of unsaturated fatty acids and therefore in the greater endogenous oxygen uptake of resistant varieties and lines. Thus it may be inferred that some phase of this hydrolysis and resulting release of unsaturated fatty acids may be instrumental in conferring resistance

to race 1 of Pseudomonas phaseolicola on some varieties and lines of Phaseolus vulgaris when leaf cells are disrupted by bacterial activity.

CHAPTER 12

PART 4

Demonstration of Inhibition of *P. phaseolicola* with linolenic acid in vitro.

(i) Race 1

a) Cup-Plate method

The first attempt to bring about inhibition of race 1 with linolenic acid in vitro was by means of the cup-plate method, similar to that used in assaying antibiotics. Nutrient agar (page 16) was sterilized in test tubes (15 ml./tube) and allowed to cool to 45°C. One ml. of a suspension of race 1 of *P. phaseolicola* containing 3×10^{10} cells/ml. was added to each tube of agar which was shaken and immediately poured into a sterile petri dish. When the agar had set four equidistant holes of 1 cm. diam. were cut in the agar using a sterile cork borer. Into each hole was pipetted 0.3 ml. of one of the following solutions:-

- a) sterile distilled water
- b) sterile Tween 80 soln. (0.05 ml./100 ml. H₂O)
- c) sterile Tween 80 soln. as in (b) containing 0.1 g./100 ml. linolenic acid.
- d) sterile Tween 80 soln. as in (b) containing 0.5g./100 ml. linolenic acid.

Linolenic acid was suspended by ultrasonic vibrations. (page 22).

Plates were stored at 7°C for 24 hr. to permit diffusion of the linolenic acid into the agar and were then transferred to an incubator at 23°C. Some plates which had been seeded with bacteria had no additions of Tween or linolenic acid but were given the same incubation treatment.

Plates were inspected at 2 day intervals up to 10 days but no plates showed any bacterial growth. This is thought to be due to the hot agar causing the death of the bacteria when the agar was seeded before pouring the plates.

b) By measurement of oxygen uptake

Two ml. of a suspension of P. phaseolicola, line 1098, race 1, containing 2×10^{10} cells/ml. suspended in 0.75% glucose in 0.15 M phosphate buffer pH 5.5 were pipetted into each of nine Warburg flasks containing 0.2 ml. 10% KOH solution in their centre wells. To each three flasks in turn were added one of the following:-

- a) 1 ml. distilled H₂O
- b) 1 ml. soln. of Tween 80 (0.05 ml./100 ml. H₂O)
- c) 1 ml. 7.5 µM linolenic acid in Tween 80 solution

A tenth flask, the thermobarometer contained only 3 ml. phosphate buffer and 0.2% KOH in the centre well. Oxygen uptake was measured by standard Warburg technique. No significant variation was found between any treatments

all flasks taking up between 240 μ l. and 260 μ l. O_2 over the first 30 minutes.

The manometer taps were opened and the flasks left on the Warburg bath for 5 hr. after which the taps were closed and uptake was again measured. No variation was found between treatments, all flasks taking up between 101 μ l. and 120 μ l. O_2 over 60 minutes.

- c) By measurement of O_2 uptake following prolonged incubation with linolenic acid.

Bacteria of race 1, isolate 1098, were grown in stationary liquid culture on nutrient medium in mechanical flats. Each bottle contained 19 ml. of the medium the composition of which is given on page 17. After 4 days incubation at $25^\circ C$ the bottles were divided into three groups and one of the following additions made to the bottles in each group:-

- a) 1 ml. sterile distilled H_2O
- b) 1 ml. sterile soln. of Tween 80 in distilled water (0.05 ml. in 100 ml. H_2O)
- c) 1 ml. sterile soln. of Tween 80 as in (b) + 50 μM linolenic acid (≈ 1.39 g./100 ml.).

Thus the addition of 1 ml. of soln. (c) to the liquid cultures gave a final concentration of 2.5 μM /g. medium. The solution incorporated Tween 80 to aid in suspension of the linolenic acid which was suspended by

ultrasonic vibrations (page 22). This necessitated the introduction of a control containing Tween 80 (solution (b)).

The concentration of linolenic acid is similar to that found in leaf tissue about 15 to 16 min after homogenisation.

This concentration was used as it was similar to that which bacteria may meet after enzymic disruption of the leaf cell contents.

After the above additions had been made, cultures were incubated for 4 days at 25°C under fluorescent lights. The bacteria were harvested by centrifugation for 20 min at 5,000 x g. resuspended in distilled water, centrifuged and suspended in 0.1 M phosphate buffer, pH 5.5 containing 0.5% glucose. A haemocytometer count of the bacteria was taken and the bacterial concentration adjusted so that all three treatments contained 3.2×10^{10} cells in 3 ml. the capacity of the Warburg flasks in which their oxygen uptake was then measured. Results are shown in Table 33.

When this experiment was repeated using isolate 604 of race 1, procedure was identical except that the bacteria were incubated for 48 hr. before addition of Tween 80 and linolenic acid, and then incubated for a further 48 hr. before harvesting for oxygen uptake measurement.

In this experiment each Warburg flask contained 4×10^{10} cells. Results are shown in Table 33.

TABLE 33 Effect of linolenic acid on oxygen uptake of
race 1 of *P. phaseolicola*

Isolate	Conc. cells/ flask	Time min.	O ₂ uptake - μ l.		
			+ dist. H ₂ O	+ Tween 80	+ linolenic acid and Tween 80
1098	3.2×10^{10}	30	62.4	41.9	0
"	" "	180	*	224.0	0
604	4×10^{10}	30	151.6	116.3	15.4

* Volume of manometer exceeded, reading lost.

It can be seen from the results that after 4 days incubation with linolenic acid, at a concentration probably very similar to that in infected leaves, respiration of the bacteria has ceased. When exposed to linolenic acid for only two days respiration has been greatly reduced. Tween 80 also appears to have some effect on respiration rate. Further comment on these results will be made in the discussion.

ii) Race 2. Effect of Tween 80 of race 2 of *P. phaseo-*
licola.

While observing the effect of linolenic acid on race 1 of *P. phaseolicola*, a race 2 isolate, 1341 was

treated in the same way as isolate 1098, as reported in this chapter, section C. The oxygen uptake of the replicates incubated with distilled water was 64.5 μ l. over 30 min. almost identical to that of race 1, but replicates incubated with Tween 80 solution, or with Tween 80 + linolenic acid, all had oxygen uptakes between 8.0 μ l. and 8.7 μ l. over 180 min. Over 30 min. O_2 uptake was negligible.

When an attempt was made to repeat this experiment, due to an error in manipulation all bacteria were killed before their oxygen uptake was measured.

CHAPTER 13. Comparison of lipid constituents of the cell wall of *Pseudomonas phaseolicola* races.

Before the experiments on inhibition of bacterial respiration were performed (Chapter 12) it was thought possible that galactolipid hydrolase may have an effect on the cell wall of race 1, resulting in loss of virulence.

The cell walls of about 25g. fresh wt. of each race, grown in vitro (page 17) were removed from the cells by ultra-sonic vibrations and extracted for their constituent lipids (details, page 37). Lipids were separated on a silica-gel column identical to that used for the separation of galactolipids (page 25), after phospholipids had been precipitated from the cell wall extract. The fresh and dry wt. of the extracted bacteria and the wt. of each fraction from the column is given in Table 34.

TABLE 34 Extraction and chromatography of cell wall lipids of *P. phaseolicola*.

Race	1	2
Isolate	1098	1341
Fresh wt.	21 g.	25 g.
Dry wt. of cell walls after lipid extraction	2.97 g.	3.67 g.
(Chloroform	96.5 mg.	272.5 mg.
Column frac- (C/M 98/2	157.5 mg.	lost
tions. Volumes- (C/M 95/5	189.2 mg.	105.6 mg.
see Table 2, (C/M 9/1	11.5 mg.	26.8 mg.
page 26. Methanol	19.0 mg.	68.7 mg.

Each column fraction was dried in vacuo, taken up in 10 ml. chloroform and known volumes between 50 μ l. and 400 μ l. applied to each spot on silica-gel loaded chromatography paper, Whatman SG 81, together with marker spots of MGD and DGD. The volume of each fraction applied was determined by trial chromatography of varying volumes; the volumes finally used are stated on the photographs of the chromatograms shown in figure 16a. The chromatograms were developed in di-isobutyl ketone/acetic acid/water (40:25:5) containing 0.05% 2:6 di-tert-butyl p-cresol as antioxidant, stained with rhodamine 6G, and inspected and photographed under U.V. light.

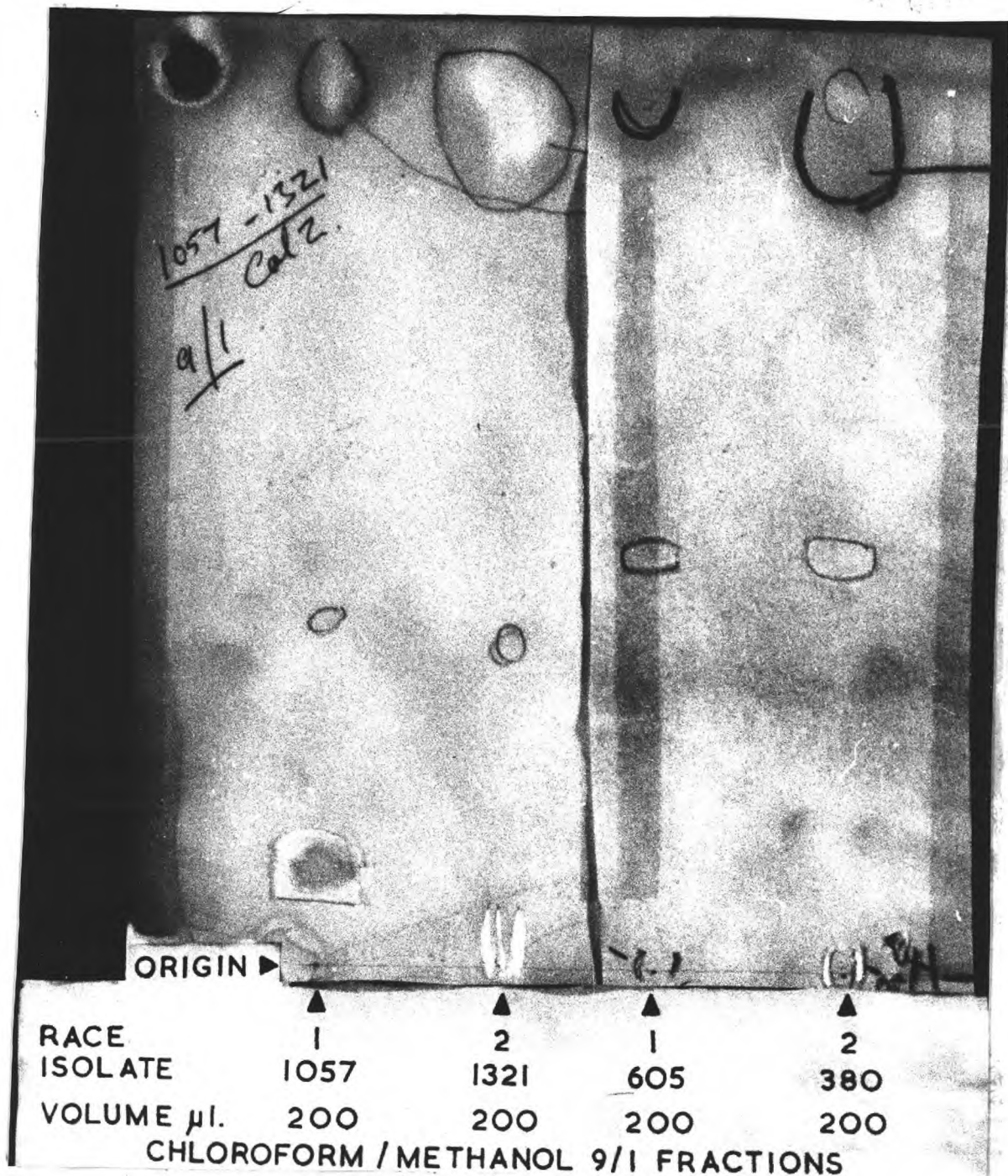
MGD and DGD were absent from the cell wall extracts. Fractions from races 1 and 2 were chromatographically identical except for the chloroform/methanol, 9/1, fractions which differed in the size of the only large spots in the fraction. These spots were near the solvent front and fluoresced orange under U.V. The race 2 spot was large and on some chromatograms appeared to be divided into two smaller spots.

The 9/1 spot size difference on the chromatograms of isolates 1098 and 1341 seemed to be reflected in the dry weights of the 9/1 fractions recorded in Table 34. Although other fractions did not differ in spot size

between the two isolates (Fig. 16a) they showed great differences in dry weights of column fractions (Table 34). This could not be explained and further evidence was sought by extraction and fractionation of the cell wall lipids of two more isolates, 605 and 380, respectively race 1 and race 2. On this occasion only the dry 9/1 fraction was weighed and this weight is given in Table 35 together with the corresponding figures for isolates 1098 and 1341. These results show that cell wall lipids of race 1 contain about half the weight of material eluted by 9/1 chloroform/methanol when compared with the weight of this material in the cell wall lipids of race 2.

TABLE 35. Dry weight of 9/1 chloroform/methanol column fractions of cell wall lipids of race 1 and race 2 of *P. phaseolicola*.

Race	Isolate	Wt. 9/1 fraction per g. dry wt. bacterial cell wall.
1	1098	3.87 mg.
2	1341	7.30 mg.
1	605	3.77 mg.
2	380	7.07 mg.



1057 and 1321, white spots = fluorescence,
 dull areas = absorbance.
 605 and 380, photographed when dry so spots did not
 fluoresce.

Figure 16b. Chromatograms of lipids of cell walls
 of *Pseudomonas phaseolicola* stained with Rhodamine
 6G and viewed under ultraviolet light. Isolates
 1057, 1321, 605 and 380.

The central streak is caused by Sellotape backing, and
 the dark spot, bottom left, by Indian Ink.

The 9/1 fractions of isolates 605 and 380 were chromatogrammed and this is shown in figure 16b. The same difference in spot size can be seen in this chromatogram as occurred in the chromatogram of isolates 1098 and 1341. Although weights were not measured identical chromatographic differences were found in comparison of isolates 1057, race 1 and 1321, race 2. (Fig. 16b)

The fractions 9/1 and 98/2 from the column chromatograms of lipids from cell walls of isolates 1098 and 1341 were hydrolysed and analysed by gas chromatography by Knivett et al. (method, page 32). The results of this analysis are shown in Table 36.

TABLE 36. Analysis of fatty acids in cell walls of races 1 and 2 of *P. phaseolicola*.

Column Fractions; chloroform/methanol			9/1		98/2	
Race			1	2	1	2
Isolate			1098	1341	1098	1341
Fatty acid	Shorthand Desig'n *	Carbon No. Apiezon. L.	%	%	%	%
Myristic	14:0	13.7	2.9	2.3	-	tr.
	14:0	14.0	1.9	0.8	tr.	tr.
	15:1	14.7	tr.	tr.	-	tr.
Palmitoleic	15:0	15.0	0.6	0.8	-	tr.
	16:1	15.7	10.8	9.7	9.9	8.6
Palmitic	16:0	16.0	62.2	62.1	40.7	41.4
	17:▽	16.75	11.5	15.2	34.6	38.0
Vaccenic	18:1	17.65	10.1	9.1	11.9	9.3
Stearic	18:0	18.0	-	-	tr.	-
Lacto- bacillic	19:▽	18.7	tr.	-	2.5	2.7

* First figure = No. carbon atoms
 Second figure = :▽ cyclopropane rings
 :0 no double bonds
 :1 one double bond

tr. = trace
 - = absent

The only major differences between the races in the fatty acid composition of the fractions analysed is that race 2 / contained a higher percentage of 17:∇ fatty acid.

The possible relevance of this and of the difference in the 9/1 fractions is considered in the discussion.

The analysis showed that linoleic and linolenic acids were absent from the cell wall fractions analysed.

It seems unlikely that galactolipid hydrolases could act on other cell wall lipids as Sastry and Kates (1964b) demonstrated the restricted activity of galactolipid hydrolases, showing that they had slight activity towards leaf lecithin and a diglyceride derived from lecithin, but that there was no activity toward galactolipids in which all fatty acids were saturated. However it must be conceded that it may be possible for the hydrolases to act on lipids containing Palmitoleic or Vaccenic acids as they are unsaturated fatty acids.

DISCUSSION

This discussion will be developed under chapter heading referring to the chapters of the results section and will be concluded by a summary of the findings of this work and the problems remaining.

Chapter 2 Differences between the oxygen uptake of leaf homogenates of resistant and susceptible lines of beans reported by Deverall and Walker (1963) and Deverall and Daly (1964) are confirmed. The oxygen uptake was measured by Warburg manometry, usually over 60 minutes and it was observed that most of the oxygen uptake resulting in the higher uptake of resistant leaf homogenates occurred in the first 15 to 30 min. After this time the difference was simply maintained by parallel rates of oxygen uptake in both resistant and susceptible lines.

Deverall and Walker (1963) using an oxygen electrode found much more rapid oxygen uptake and greater differences between that of resistant and susceptible leaf homogenates. They found that monofoliate leaf homogenates of resistant leaves frequently took up 47 μ l. O₂ in 12 - 18 seconds while identical susceptible leaf homogenates took 72 to 186 seconds to take up the same volume of oxygen. This would suggest that

Warburg manometry must miss the observation of the early and fastest part of the oxidation reaction as no equilibration time was allowed when the oxygen electrode readings were taken by Deverall and Walker (1963) but Warburg manometry demands 5 min. equilibration of flask temperatures, before measurement of oxygen uptake is commenced.

It would appear from the results in this chapter that perhaps two oxidation mechanisms are operating in homogenates; (i) one bringing about a very rapid oxygen uptake for the first few minutes after homogenisation, this uptake being at a much greater rate in resistant than in susceptible homogenates and ceasing when substrate is exhausted. (ii) the other proceeding at the same rate in resistant and susceptible lines and taking many hours for completion at a comparatively slow rate. Alternatively the oxidation reaction may be due to one enzyme reaction which is initially rapid but slows down, perhaps due to enzyme saturation or inactivation.

Chapter 3 In the development of the extraction procedure it can now be seen that errors occurred which would have been avoided had the nature of the substrate been better understood.

As a result of experiments where aqueous homogenates were extracted with 80% ethanol it was thought that 80%

ethanol was very efficient in substrate extraction (table 6). It was only realized much later that this type of extraction permits hydrolysis of lipids before hydrolases are inactivated by 80% ethanol (Chapter 10). This hydrolysis results in the release of free fatty acids and it is these that are extracted by 80% ethanol when used to extract aqueous homogenates. Therefore this test of efficiency of extraction of substrate, by 80% ethanol did not test the efficiency of extraction of galactolipids but of free fatty acids. It seemed reasonable to extract whole leaves in the same way and it was not then understood that this type of extraction removed the substrate from the leaves while still in the form of an intact galactolipid. Eventually it was demonstrated that galactolipid extraction is achieved more effectively with boiling isopropanol (end of Chapter 3).

Figure 7 shows that the addition of galactolipid substrate (i.e. obtained by whole leaf 80% ethanol extraction) greatly increased the total O_2 uptake after 1 hr. suggesting that it is not enzyme inactivation but substrate limitation which causes the rapid decline in oxygen uptake rate after 15 to 30 min. referred to in Chapter 2.

Table 10 indicates that petroleum spirit was very efficient in dissolving dried substrate from a dry 80% ethanol whole leaf extract. This is not contradictory

to the result in Table 12 because in the latter experiment chlorophyll was separated from the substrate which was in ethanol solution, not in the dry condition.

In Table 11 it is seen that extraction of substrate from an alkaline aqueous suspension resulted in a slightly lower oxygen uptake. This probably results from the fact that a small amount of linolenic acid is free in vivo (Chapter 9) and this would probably form a compound with the alkaline molecule, in this case sodium hydroxide yielding sodium linolenate which would not be extracted by the ether.

The method used for lipid extraction by Sastry and Kates (1964a) consisted of fragmentation of leaves under liquid nitrogen, the resulting powder being extracted with boiling isopropanol in a Waring blender. After filtration the residue was washed a number of times with hot isopropanol and isopropanol/chloroform mixtures. The extract was water washed a number of times, dried in vacuo and chromatogrammed on the silica-gel/celite column described by Kates and Eberhardt (1957) and used in the present study. The method used by the author employed 80% ethanol for initial extraction of frozen leaves in a blender and was derived from the demonstration that substrate was extracted from aqueous homogenates by pouring into cold ethanol. Both of these methods

have the advantage of avoiding activation of phosphatidase C which catalyses the hydrolysis of glycerolphosphatides to free nitrogenous bases and phosphatidic acid. Phosphatidase C is activated by the use of diethyl ether in extraction procedures (Kates, 1957).

Chapter 4 As has been stated in the description of development of the extraction procedure (Chapter 3) after much of the work in this thesis had been completed it was found that boiling isopropanol was slightly more efficient in extraction of substrate of oxidation than 80% ethanol. Calculation of theoretical oxygen uptake by homogenates based on the total fatty acid content of leaves shown in Chapter 8, are therefore impossible. Some estimation of the theoretical oxygen uptake resulting from oxidation of unsaturated fatty acids is given in the discussion of Chapter 10.

Another difference from the extraction method of Sastry and Kates (1964a) was that in the present study the solution in 80% ethanol was washed with petroleum spirit to remove chlorophyll and some other pigments before column chromatography. This had the beneficial effect of reducing the load on the column, without loss of substrate, tests having been made for substrate activity of the discarded pigment portion during development of the fractionation technique.

With the silica-gel column it was found satisfactory to take fewer, larger fractions to separate the two galactolipids than those of Sastry and Kates (1964a) who were concerned with separating nine substances from each other.

The photographs of chromatograms of column fractions (Fig. 9) show a major difference in the amount of phosphatidic acid present in the two varieties. This reflects a difference in the efficiency of the phospholipid precipitation (page 25) probably due to slight differences in temperature of the acetone used in the precipitation from the extracts of the two varieties. It can be seen from the photos that otherwise the loading of the chromatogram was the same for both varieties.

Chapter 5 Stimulation of oxygen uptake of homogenates by an acid hydrolysed lipid extract was greater than that by the same amount of unhydrolysed extract. This was probably because all the unsaturated fatty acid in the acid hydrolysed extract was immediately available for lipoxidation when added to the fresh homogenate whereas release of unsaturated fatty acids from the lipids in the unhydrolysed extract would only have occurred at the rate permitted by the galactolipid hydrolase level in the fresh homogenate to which extract was added. The acid hydrolysed extract contained all the unsaturated fatty

acid from the lipids extracted including that from lecithin. It is possible that low activity of lipases in homogenates prevented liberation of all bound unsaturated fatty acids in the unhydrolysed extract.

Chapter 6 As mentioned briefly in the introduction, rapid oxygen uptake by freshly prepared plant homogenates has been reported by Fritz and Beevers (1955), working with corn seedlings and by Gamborg and Zalik (1958) with sunflower seeds and seedlings. Both groups suggested that lipoxidation of certain unsaturated fatty acids accounts for most of the oxygen uptake in homogenates. Their conclusions were based on the lipoidal nature of the substrate, specificity of added fatty acids and absorption spectra of oxidation products. Further evidence was obtained by addition to homogenates of oxidase inhibitors. Fritz and Beevers (1955) obtained respectively 95 and 98% inhibition by α -naphthol and catechol, working with corn seedlings at pH. 5.0. Gamborg and Zalik (1958) inhibited oxidation in homogenates of sunflower seedlings by 100% with α -naphthol but only by 28% with catechol.

Similar evidence concerning substrate specificity, light absorption by oxidation products and response to inhibitors has been presented in this thesis to show that endogenous oxidation in bean leaf homogenates is caused by the oxidation primarily of linolenic acid.

Inhibition by α -naphthol and catechol was less complete than that described above in storage or etiolated tissues. This may be because of loss of inhibitors by their reactions with other components of homogenate of leaves. Alternatively lipoxidation may represent only part of the oxidation in the leaf homogenates.

Additional evidence presented in this thesis, but not by other workers, is the presence of linolenic acid in the homogenates taking up oxygen (Chapter 10).

Chapter 8 The conclusion has been drawn in this chapter that similar amounts of galactolipids, and so fatty acids bound in these lipids, are found in resistant and susceptible varieties. This does not disturb the hypothesis that resistance is conferred by high fatty acid levels following lipid hydrolysis. The fatty acids are bound until cell disruption occurs (except for small amounts as recorded in Chapter 9), and it has been reported by Spoehr et al. (1949) that glycerides lacked antibacterial activity, even though they contained fatty acids were antibacterial.

Chapter 9 It must be stressed that the extraction of fatty acids from leaf tissue was made directly into ethanol which stopped the action of galactolipid hydrolase instantaneously and so the fatty acid extracted must have been that which was free in the tissue.

The variation between the two susceptible varieties in fatty acid content, recorded in Table 26 may be explained by intervarietal variation but further determinations are necessary to elucidate this point.

Chapter 10 Figures 13 and 14 clearly show a more rapid release of fatty acids from fats in resistant than in susceptible lines.

The results in Figure 14 were obtained without taking any intermediate readings between zero-time and 16 min. so the graph does not show any possible fluctuations in free fatty acids which can be seen in Figure 13. In Figure 13 it can be seen that ^{in the susc. var.} after approximately 8 min. a reduction in the level of fatty acids occurs which may be due to lipoxidation as this most probably occurs at a slow rate even at 4°C. Alternatively this decrease may be due to the enzymatic degradation of other free fatty acids.

Gas chromatography showed which fatty acids were increasing in concentration, and whether any were increasing preferentially. Comparison of zero time and 16 minute samples in Tables 27 and 29 respectively, shows that the percentage of linoleic and linolenic acids increase preferentially in the resistant variety homogenate but decreases in the susceptible variety homogenate.

Comparison of the weight of fatty acids present, shown in Tables 28 and 29 indicate much faster release of linolenic acid in resistant homogenates.

In the resistant homogenate linolenic acid increased by about 815% over 16 min. while in the susceptible homogenate it increased by 377%.

Table 29 shows that the concentration of other fatty acids is higher in the resistant than in susceptible homogenate. Some of this increase results from the fact that fatty acids other than linoleic and linolenic acids will have been released from the galactolipids. Sastry and Kates (1964a) showed that in runner-bean leaves MGD contained 2.3% palmitic acid, and traces of stearic, palmitoleic and oleic acids, and DGD contained 4.5% palmitic acid, 1% stearic acid and traces of palmitoleic and oleic acids. Thus, all these will increase at a similar rate to that of linoleic and linolenic acids during hydrolysis of MGD and DGD. Perhaps other fatty acids are present in higher concentration in resistant than in susceptible varieties due to enzymic processes such as β -oxidation of longer chain fatty acids, which it has been shown are present in high concentration as a result of galactolipid hydrolase activity.

The figures given in Table 29 for weight of linoleic and linolenic acids enable calculation of the oxygen

which may be taken up by complete oxidation of this amount of fatty acid. These figures for content of linoleic and linolenic acids do not represent the total amount of these acids that would eventually be yielded by hydrolysis of galactolipids but do give some idea of the amount liberated over the first 16 min. after a homogenate is prepared.

Figures are for lg. fresh wt. leaves.

	<u>Resistant</u>	<u>Susceptible</u>
Wt. Linoleic acid	101.5 μ g.	38.5 μ g.
Equiv.wt.	280	280
μ equivalents present	0.36	0.14
Probable number of μ equivalents O_2 taken up per μ equivalent fatty acid	2	2
Wt. Linolenic acid	408.6 μ g.	130.6 μ g.
Equiv. wt.	278	278
μ equivalents present	1.47	0.47
Probable number of μ equivalents O_2 taken up per μ equivalent fatty acid	3	3

1 μ g. equivalent O_2 at 25°C occupies 24.45 μ l. at 760 mm. Hg pressure.

$\therefore$ Linoleic takes up	17.6 μ l.	6.85 μ l.
Linolenic takes up	108.0 μ l.	34.50 μ l.
Total O_2 uptake	125.6 μ l.	41.35 μ l.

∴ 0.4 g. fresh wt. leaves (wt. used in Warburg flask)
would take up:-

Resistant = 50.2 μ l.

Susceptible = 16.5 μ l.

However, it must be remembered that in this experiment which provided these figures on fatty acid content, hydrolysis occurred at 4°C. to prevent lipoxidation from oxidizing unsaturated fatty acids. Oxygen uptake is measured at 25°C. at which temperature hydrolysis may occur at a much greater rate.

Other factors preventing a direct comparison of theoretical and actual oxygen uptake are (i) that the homogenate is given 5 min. to reach 25°C. while equilibration is occurring on the Warburg bath and during this time oxygen must be taken up by the homogenate and cannot be measured. (ii) Inhibition studies (Table 22, Chapter 6) show that oxidation of unsaturated fatty acids accounts for between 55% and 70% of endogenous oxygen uptake over 2 hr. using a mixed resistant and susceptible leaf homogenate.

Although a direct comparison of actual and theoretical oxygen uptakes cannot be made the calculations shown above indicate that estimates of oxygen uptake based on free fatty acid content 16 minutes after homogenisation are

of a similar order to the values obtained by Warburg measurements. It can be seen from figure 5, Chapter 2, that a resistant line has taken up 67 $\mu\text{l. O}_2$ and a susceptible line 44 $\mu\text{l. O}_2$ over the first 16 min. of measurement.

It is not known how a difference in concentration of unsaturated fatty acid can result in resistance or susceptibility. It would appear that a moderate amount of fatty acid is freed on galactolipid hydrolysis in susceptible leaves. Perhaps a biochemical mechanism gradually converts the fatty acid or its oxidation product to a form non-toxic to bacteria, but in the resistant leaves, release of fatty acids is at such a high rate that removal may not be sufficiently rapid to prevent accumulation of the toxic material. An indication of the possibility of this occurring is in Figure 13 in which a reduction in free fatty acids is shown about 8 min. after homogenisation, indicating a change in the nature of some of the free fatty acids.

Chapter 11 Sastry and Kates (1964a) showed that a rapid consequence of cell damage was the contact of galactolipids and their corresponding hydrolases and the liberation of linolenic acid.

Present results suggest that there is greater activity of galactolipid hydrolase in resistant leaves, resulting

in a higher free linoleic and linolenic acid content in these than in susceptible leaves. This appears to be the basis of the difference in rate of liberation of fatty acids in homogenates (Chapter 10) causing the difference between oxygen uptake of resistant and susceptible leaf homogenates. However, whole homogenates have been used as an enzyme source and acyl ester breakdown was observed after addition of MGD and DGD. It is possible that enzymes other than the respective hydrolases may be instrumental in the acyl ester breakdown observed and proof should be sought by the isolation and assay of the individual galactolipid hydrolases. Rudolph and Stahmann (1966) carried out polyacrylamide disc electrophoresis of proteins extracted from bean leaves before and after infection by Pseudomonas phaseolicola in an investigation of changes in hydrolase contents. They found that a bacterial acid phosphatase appeared after infection and that several esterase bands decreased. Their failure to notice any difference in the level of the galactolipid hydrolase between resistant and susceptible varieties was probably caused by the lack of galactolipid substrate and a suitable test staining technique.

The possibility also remains that the absence of activity in susceptible varieties may be the result of

the presence of a hydrolase inhibitor and that no difference exists in enzyme content.

Leaf homogenates of at least twelve resistant lines resulting from one cross, tested by the author and by others (Deverall and Walker, 1963; Deverall and Daly 1964) have shown higher oxygen uptake than a similar number of susceptible lines grown and tested together. In the present study two resistant lines tested for enzyme activity have been shown to have a higher galactolipid hydrolase activity than the three susceptible lines tested on the same occasions. (One resistant line was used twice). Other evidence of greater galactolipid hydrolase activity in resistant lines comes from (a) the greater stimulation of oxygen uptake in resistant than in susceptible leaf homogenates, by the addition of identical amounts of galactolipids to leaf homogenates of three resistant and three susceptible lines, and (b) from the more rapid release of fatty acids in resistant leaf homogenates, a major constituent of these fatty acids being linolenic acid which is probably derived from galactolipids.

The concluding sentence of Chapter 11 suggests how resistance may be conferred by some phase of the hydrolysis of galactolipids and the resulting release of unsaturated fatty acids following cell disruption by bacterial activity.

This disruption is assumed to occur and to bring the various substrates and enzymes into contact, as occurs when leaves are homogenised. Omer (1966) presented evidence that in the early stages of the disease cell walls remained intact and bacteria did not enter the cells. Williams and Keen (1957) and Keen, Williams and Walker (1967a, b.) have isolated a protease produced by Pseudomonas lachrymans in cucumber leaves, while causing angular leaf spot disease. They suggest that the pathogen is bringing about proteolysis by means of the protease and that the initial water soaking symptoms of the disease are the result of the action of the protease on the permeability of host plasma membranes. It is possible that P. phaseolicola produces a similar protease as similar water-soaked lesions are characteristic of the early disease symptoms, and that this protease disrupts other cell membranes bringing galactolipids and their hydrolases into contact.

Chapter 12 There are many reports of antibiotic effects of fatty acids. Riley and Miller (1948) isolated an antibiotic from Penicillium crustosum after observing the antibacterial activity of the mycelium. The substance was identified as linoleic acid which had bactericidal activity against the gram-positive Mycobacterium.

A mixture of fatty acids, mainly linoleic and linolenic acids composed an antimycotic substance isolated from Rye seedlings by Honkanen and Virtanen (1960). Kodicek (1955) has shown that linoleic acid has a bacteriostatic effect against gram-positive bacteria, but failed to show this against the two gram-negative species tested. (P. phaseolicola is gram-negative). Testing a number of fatty acids he found that bacteriostatic activity increased with the number of double bonds.

In the light of these reports inhibition of Pseudomonas phaseolicola was attempted using linolenic acid.

(i) Race 1. The cup-plate method (Chapter 12 a) failed, probably due to the death of the bacteria caused by hot agar. Neither could inhibition of the bacteria be demonstrated by oxygen uptake observations made immediately on mixing fatty acid with the bacteria. (Chapter 12b).

Prolonged incubation of bacteria with linolenic acid inhibited their respiration (Chapter 12 c). The success of this technique could be explained by two hypotheses: (i) the incubation period was the time taken for the linolenic acid to penetrate the cell wall: (ii) during the incubation period a slow change occurred in the linolenic acid molecule to produce a toxic product.

The first hypothesis is strengthened by reports of cellular damage caused by the direct action of linolenic acid. McCarty and Jagendorf (1965) observed damage to bean and spinach chloroplasts in the form of breakdown of limiting membranes and of uncoupling of electron transport from phosphorylation, caused by the destruction of the component lipids, MGD and DGD, and also by the action of their decomposition product, linolenic acid. Pressman and Lardy (1956) found that linoleic and linolenic acid reduced the efficiency of mitochondrial oxidative phosphorylation by stimulating the activity of mitochondrial ATP-ase. Björntorp, Ells and Bradford (1964) demonstrated the linoleic acid inhibited respiration of isolated mitochondria, but that this damage could be prevented by the presence of albumin, a significant point in view of the findings of Dubos (1947) that albumin protects acid-fast organisms from the toxic action of unsaturated fatty acids.

Mitochondria are not present in bacteria but Stanier and van Niel (1962) express the opinion that the cytoplasmic membrane of the gram-negative cell is functionally analogous to the internal membrane system of the mitochondrion, and state that electron transport is intimately associated with the membrane which also

carried certain enzymes of the tricarboxylic acid cycle. Possibly linolenic acid can be deleterious to the gram-negative cell as it is to sub-cellular particles in higher plants.

The second hypothesis suggesting antibacterial activity only after some change in the molecule of fatty acid is supported by the work of Spoehr et al. (1949) who found antibacterial activity displayed by unsaturated fatty acids extracted from Chlorella but state that this only occurred after photo-oxidation of the fatty acid. Spoehr et al. (1949) tested a number of unsaturated fatty acids, including linoleic acid, for antibacterial activity and in all cases showed that there was no effect on the bacterium immediately on exposure to the fatty acid, but only after 2 to 4 days photo-oxidation.

The mechanism of oxidation of linoleic and linolenic acids is uncertain, whether it occurs by autoxidation (Farmer et al. 1943) or by lipoxidation (Holman and Bergström, 1951) but it is generally agreed that during the course of oxidation a peroxide is formed and the end product is a hydroperoxide. Spoehr et al. (1949) destroyed all the peroxides in the photo-oxidized fatty acids and showed that bacterial inhibition still occurred in the absence of peroxides. This

indicates that the toxic substance may be the hydroperoxide or an intermediate oxidation product.

It must be conceded that the inhibition of respiration of race 1 of P. phaseolicola apparently brought about by linolenic acid may, in fact, be due to the synergistic action of linolenic acid and Tween 80, the chemical name of which is sorbiton mono-oleate. It is possible that during the incubation period some sorbiton mono-linolenate is produced which is more toxic to the bacterium than linolenic acid alone or Tween 80 alone. Further work is needed using other means of dispersion of linolenic acid to eliminate this possibility.

(ii) Race 2. Effect of Tween 80 on race 2 of P. phaseolicola. If repetition of the experiment reported confirms the results obtained, it may be possible to use these results to further elucidate the difference in cell wall composition between races 1 and 2 with the eventual objective of determining how race 2 overcomes the resistance found in Red Mexican, to which race 1 succumbs.

Chapter 13 Possible causal relations between galactolipase activity and resistance were considered. The first possibility was that the hydrolase acted upon galactolipids or similar lipids in the cell wall of race

1 but not of race 2, of the bacterium.

In lipids from the cell walls of race 1 and 2, galactolipids were absent, and no linoleic or linolenic acids were found in the samples analysed. There have been no reports of linolenic or linoleic acids being found in bacteria (Hilditch 1956), and it has been stated that bacteria do not contain any polyunsaturated fatty acids (Scheuerbrandt and Bloch, 1962).

Therefore it is not possible to suggest any direct interaction between host galactolipid hydrolase and bacterial cell wall lipid.

Chromatography of the lipids of cell walls of races 1 and 2 showed them to be very similar in the two races, with the exception of a lipid in the chloroform/methanol 9/1, column fractions. (fig. 16). The dry weight of these fractions was compared and it was found that the weight of race 2 fractions was almost twice that of race 1 fractions (Table 35). Gas chromatography of the constituent fatty acids suggested that there was also a qualitative difference in this lipid between the races, the race 2 fraction containing 15.2% of a 17-carbon cyclopropane fatty acid, whereas the race 1 fraction contained 11.5% of this acid. Law et al. (1963), investigating the metabolism of a 17-carbon

cyclopropane acid state that the biosynthesis of a cyclopropane ring in bacterial fatty acids involves the insertion of a methylene bridge across the double bond of a fatty acid and suggest that the formulation of cyclopropane fatty acids may serve to "remove some undesirable metabolic product such as ... unsaturated acids". Perhaps the larger quantity of cyclopropane acids in race 2 cell walls reflects an enhanced ability of race 2 to synthesise these acids from unsaturated fatty acids, an ability which may serve to remove unsaturated fatty acids from the environment of the bacterium before damage is caused to the cell.

It has been suggested above that bacterial respiration is inhibited by oxidized unsaturated fatty acids. Law et al. (1963) suggest as an alternative to their hypothesis mentioned in the previous paragraph that conversion of unsaturated fatty acids to cyclopropane acids could prevent peroxidation. This may occur, with the result that the unsaturated fatty acids are converted to cyclopropane acids before oxidation to the inhibitory form.

CONCLUSION

A basic problem in this study is that of linked characters. The approach adopted in the present study is a comparison of a number of homozygous resistant and susceptible lines derived from one cross between parental varieties, in one of which resistance is monogenic. A degree of independent assortment of characters had occurred so that resistance could be compared with susceptibility against a background of morphological and pigmental characters. Viewed thus Deverall and Walker (1963) and Deverall and Daly (1964) found that high endogenous oxidation in homogenates was always associated with resistance and not with height, growth habit, etc. It is still possible that there is gene linkage on one chromosome between genes conferring resistance and controlling galactolipase action. This can be tested only by examining more lines from the same cross or by testing other varieties of bean, which are susceptible to race 1. Apart from Red Mexican and the progeny of the cross between Red Mexican and Kinghorn Wax, only Canadian Wonder has been tested for galactolipase activity. Canadian Wonder is susceptible to race 1 and has low galactolipase activity. Sastry and Kates (1964b) found that

an unspecified variety of Phaseolus multiflorus Willd. possessed marked galactolipase activity, and Patel and Walker found that P. coccineus L. (= P. multiflorus Willd.) was susceptible to race 1. This may contradict the other findings relating resistance to race 1 and galactolipase activity, but, besides doubts concerning varieties of bean used, there is also no method of relating level of galactolipase activity as expressed by Sastry and Kates (1964b) and that detected in present comparisons of resistant and susceptible lines. This problem remains for exploration.

It seems probable that resistance to race 1 of Pseudomonas phaseolicola is brought about by the action of linoleic and linolenic acids or their oxidation products on bacteria infecting the leaves of variety Red Mexican U.I. No. 3 or its resistant progeny. It seems likely that fatty acids become available in the leaf cells due to the hydrolysis of galactolipids by specific hydrolases which are present in greater abundance in resistant than in susceptible leaves. Galactolipids and their hydrolases may be brought into contact as a result of the action of bacterial extracellular enzymes. No evidence has been sought as to whether these events occur on infection.

Little insight has yet been gained as to the means by which race 2 overcomes the resistance mechanism which is active against race 1 but it may be important that a lipoidal difference exists between the cell wall composition of races 1 and 2. A greater level of 17-carbon cyclopropane acid in race 2 cell walls may be associated with a mechanism of protection of the cytoplasmic membranes in cells of race 2 against the deleterious action of unsaturated fatty acids. Future work may be assisted by the discovery of the fact by Patel and Walker (1965) that some other species of Phaseolus show the same type of hypersensitive resistance to race 2 as Red Mexican has to race 1.

The importance of this research is that it has defined the biochemical basis for the previously reported physiological difference between resistant and susceptible lines of bean. This will permit direct tests to be made of the activity of galactolipid hydrolases in different varieties of bean, and thus to eliminate the possibility of gene linkage between factors for resistance and galactolipid hydrolase action. The biochemical understanding also allows the construction of the hypothesis concerning the action of linolenic acid on its oxidation products on the active surface membranes of race 1 of Pseudomonas phaseolicola.

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ACKNOWLEDGEMENTS

I am indebted to Dr. B. J. Deverall for his guidance and advice.

I wish to thank Mr. S. Draper of Manchester University Botany Department for instructing me in the technique of gas chromatography and Dr. V. A. Knivett, Miss J. Cullen and Mr. M. Jackson of Twyford Laboratories Ltd. for performing some of the gas chromatographic analyses.

I am grateful to Mr. G. Grange of Manchester University for photography, in particular for his efforts in overcoming the difficulties of photographing under ultra-violet light.

My thanks are especially due to my wife who cut the stencils and duplicated the thesis.

Most of the work was carried out during the tenure of a Studentship awarded by the Science Research Council, whom I wish to thank.