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CARBOHYDRATE METABOLISM IN HEALTHY AND RUSTED LEAVES OF COLTSFOOT

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Summary

Infection of leaves of *Tussilago* by *Puccinia poarum* results in decreased export of photosynthetic products and, when infection is severe, in increased import. Photosynthetic products move to infection sites where host metabolites are converted to specific fungal products, including mannitol, arabitol, trehalose, glycogen, glucomannan and lipids. More previously fixed ^{14}C is translocated to the fungus in the dark than in the light since, in the dark, degradation of starch contributes to net movement of ^{14}C.

Introduction

In order to understand the nutrition of biotrophic fungi *in vivo*, it is necessary to investigate the metabolism and translocation of potential fungal nutrients in host tissues, the transfer of specific nutrients to the fungi and the metabolism of these nutrients after their absorption. With respect to carbohydrates, progress has been made with the first and third of these processes with a variety of host–symbiont combinations (see reviews by Thrower, 1965a; Daly, 1967; Durbin, 1967; Yarwood, 1967; Smith, Muscatine and Lewis, 1969; Scott, 1972). The transfer of specific compounds has not been studied in detail.

We have used the host–parasite combination of the rust, *Puccinia poarum* Niels., and leaves of coltsfoot, *Tussilago farfara* L., as a model system in our investigations of the nutrition of biotrophic fungi. Following analyses of the gross carbohydrate composition of healthy and infected leaves (Holligan, Chen and Lewis, 1973), we have investigated the synthesis and translocation of carbohydrates in the host and their fate in the fungus. These studies, reported here, are a necessary prelude to the investigation of the transfer of specific nutrients between the symbionts.

Materials and methods

Healthy and infected plants

Healthy leaves of *Tussilago* and leaves infected by *Puccinia poarum* were either collected from the field or grown under controlled conditions as previously described by McGee *et al.* (1973). Intact plants for experiments on long-distance transport were grown and infected under growth room conditions. Unless stated otherwise, pustules with approximately 50% of the aecia dehisced were used (stage 3 as defined by Holligan *et al.* (1973)).

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Methods of supplying $^{14}CO_2$

All experiments were conducted in a growth room at 20° C with a light intensity of 1100 lux from fluorescent tubes supplemented by tungsten lamps.

Supply of $^{14}CO_2$ to attached leaves. $^{14}CO_2$ was supplied to single leaves of healthy and rusted plants from a diethanolamine–HCl–$NaH^{14}CO_3$ buffer, permitting a maximum CO_2 concentration of 0.2% in the vapour phase (Macfadyen, 1970). A glass Petri dish containing 5.0 ml buffer and 50 μCi $NaH^{14}CO_3$ was held horizontally in a clear polythene bag, which was then placed over the leaf and sealed around the petiole.

Supply of $^{14}CO_2$ to detached leaves and leaf discs. Rusted leaves were placed inside a clear plastic box with the petiole projecting from one end into a beaker of water. Leaf discs, 12 mm diameter unless stated otherwise, were excised from healthy and diseased leaves and placed abaxial surface upwards on moistened filter paper in 14-cm Petri dishes. Discs from diseased leaves either were taken from host tissue away from pustules or included a single central pustule.

Both types of container were sealed with petroleum jelly. $^{14}CO_2$ was released by injecting 10% v/v lactic acid through small holes in their lids into small glass dishes containing 10–50 μCi $NaH^{14}CO_3$. At the end of the feeding period, residual $^{14}CO_2$ was absorbed into 2 N KOH injected into a second glass dish.

In contrast to the method used for attached leaves, $^{14}CO_2$ was thus fed for short periods at a high specific activity.

Treatment of leaf tissue after feeding with $^{14}CO_2$

Attached and detached leaves and leaf discs were dried for autoradiography or extracted for chemical analysis either immediately after incubation with $^{14}CO_2$ or after a period in the light or dark to allow further metabolism and translocation. During this period, all tissues were exposed to air to permit normal light and/or dark fixation of CO_2.

Extraction of leaf tissue. All samples were normally extracted in methanol–chloroform–water (12:5:3 v/v) or aqueous ethanol followed by water and 1.5 N H_2SO_4 as described by Holligan *et al.* (1973). However, in one experiment, instead of the aqueous extraction, the tissue was homogenized and treated with amyloglucosidase (Sigma type III, 0.5 mg/ml in water) at 45° C for 2 h. This procedure extracts α-glucans, which are hydrolysed to glucose, and water-soluble polysaccharides (see Holligan, McGee and Lewis (1974) for details). In another experiment, intact discs, after extraction in aqueous ethanol were further extracted with 0.1 N oxalic acid for 1 h before treatment with 1.5 N H_2SO_4.

Analysis of extracts. Free sugars in the alcoholic, aqueous and acid hydrolysate fractions were resolved by paper chromatography and located either as radioactive spots (see below) or with silver nitrate–sodium ethoxide (Trevelyan, Proctor and Harrison, 1950).

Detection of radioactivity

Tissue extracts. Aliquots, dried on planchets or mixed with a toluene/Triton X-100 scintillant (Turner, 1968), were counted in a Nuclear-Chicago gas flow or scintillation counter respectively. Samples on planchets were assumed to be of infinite thinness and no corrections for self absorption were made. For scintillation counting, appropriate quench corrections were made.

Paper chromatograms. Radioactive substances were located by autoradiography as

described below or with a Nuclear-Chicago Actigraph III scanner. Each was then isolated by cutting out the appropriate segment and elution with water. Radioactivity in these aqueous extracts was assayed by scintillation counting.

Dried leaf tissue. Whole leaves and discs, before or after extraction of soluble substances, were dried at 55° C between sheets of filter paper, covered with 'Melinex' and exposed to Kodak Kodirex KD54T X-ray film. After suitable periods (usually 2–14 days), the autoradiographs were developed using standard techniques. Since soluble substances were largely removed by direct drying, both procedures essentially show the distribution of ^{14}C-labelled insoluble compounds. There was little or no difference between autoradiographs of upper and lower surfaces of samples (Yuen, 1969).

Results

Long-distance transport in whole plants

As in other plants, the products of photosynthesis in mature, healthy leaves of *Tussilago* are translocated away in the dark to the root/rhizome system, to the shoot apex and developing leaves (Table 1, treatment 1). In plants infected by *Puccinia poarum*, the export of photosynthate from rusted leaves is almost completely inhibited (Table 1, treatment 2). When infection is slight (pustules $< 10\%$ surface area of leaf) as in treatment 3 of Table 1, no significant import to the infected leaf was observed. However, other experiments have shown that, when infection is severe (pustules $>25\%$ surface area), an infected mature leaf imports up to 20% of the photosynthate from a healthy younger leaf.

Short distance transport in detached leaves and leaf discs

Experiments with detached whole leaves and leaf discs gave very similar results (Plates 1 and 2). Immediately after exposure to $^{14}CO_2$, the pustules appear as clear areas on the autoradiographs where little or no radioactivity has been fixed into insoluble compounds (Plate 1, No. 1, Plate 2, No. 5). Following a subsequent period of translocation in the dark, the infected areas became heavily labelled (Plate 1, No. 2; Plate 2, No. 6). This is especially marked in mature aecia as shown but occurs at all stages of pycnial and aecial development (Yuen, 1969). Extraction of discs with oxalic and sulphuric

Table 1. *Translocation of photosynthetically fixed ^{14}C in intact plants of healthy and infected* Tussilago

Treatment	Total ^{14}C recovered by extraction (ct/min × 10^{-3})	Sample	Dry wt (mg)	Distribution of extracted ^{14}C (% total ^{14}C)
(1) Healthy plant: ^{14}C supplied to youngest, fully expanded leaf	380	Shoot apex and immature leaves	286	23
		Leaf fed with $^{14}CO_2$	308	47
		Mature leaves	865	3
		Roots and Rhizomes	717	27
(2) Plant with single infected leaf: ^{14}C supplied to infected leaf	319	Shoot apex and immature leaves	271	<1
		Infected leaf fed with $^{14}CO_2$	283	97
		Mature leaves	1432	<1
		Roots and Rhizomes	1189	3
(3) Plant with single infected leaf: ^{14}C supplied to healthy leaf immediately above (i.e. younger than) infected leaf	453	Shoot apex and immature leaves	235	11
		Leaf fed with $^{14}CO_2$	281	32
		Infected leaf	206	1
		Mature leaves	615	2
		Roots and Rhizomes	1084	54

acids prior to autoradiography shows that some, but not all, of the insoluble radioactive compounds are removed by these hydrolytic treatments (Plate 2, No. 8a–c). In this experiment, the distribution of the total ^{14}C recovered immediately after photosynthesis was 68% in the aqueous alcoholic fraction, 29% in the oxalic acid fraction and 3% in the sulphuric acid extract. Twenty-four hours later, the total extractable radioactivity had fallen to 54% of the original and the distribution in the 3 fractions was 63%, 27% and 10% respectively.

When infected leaves are kept in the light instead of the dark after a pulse of $^{14}CO_2$, the pustules become comparatively less heavily labelled and, around each pustule, there is a marked halo (Plate 1, No. 3). Light appears to have at least two effects: firstly, a lower proportion of the labelled photosynthate is transferred to pustules and, secondly, in the host tissue around each pustule, there is either a more rapid turnover of the insoluble labelled compounds or a greater retention of radioactivity in soluble compounds which are lost during the drying process (see 'Materials and methods'). In contrast to the leaf kept in the dark, less radioactivity is present in the major veins.

Although exposure to $^{14}CO_2$ in the dark clearly shows that the fungus is capable of dark fixation (Plate 1, No. 4, Plate 2, No. 7), this aspect of fungal metabolism has not been investigated further here.

Products of $^{14}CO_2$ fixation and translocation

In healthy discs, most of the radioactivity was incorporated into sucrose and an insoluble polymer that yielded glucose on hydrolysis (Plates 3a and 4a). The free monosaccharides, glucose and fructose, and the trisaccharide, sucrosyl fructose (isokestose), also became labelled though to a lesser extent. During the subsequent translocation period, radioactivity in the glucose polymer was retained in the light (Plate 4b) but disappeared almost completely in the dark.

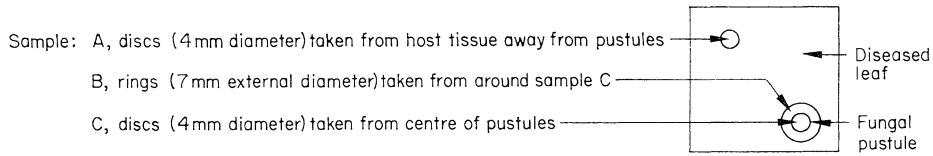
In infected discs, sucrose and a glucan were also the major products of photosynthesis (Plates 3b and 4c). However, in the soluble fraction, some ^{14}C was incorporated into the fungal metabolites, mannitol, arabitol and trehalose, within the 2-h feeding period and, after a further period of 18 h in the light or dark, these compounds were the main labelled products (Plate 3b–d). As in the insoluble hydrolysate from healthy discs, radioactivity is retained in glucose during the translocation period in the light (Plate 4e). However, in the dark in contrast to healthy discs, considerable ^{14}C remains in glucose (Plate 4d). Also in contrast to healthy discs, mannose becomes labelled within 2 h and continues to incorporate radioactivity especially in the dark (Plate 4c–e).

The synthesis and translocation of carbohydrates was studied in more detail by feeding detached infected leaves with $^{14}CO_2$ for 15 min and analysing three regions of the leaf: (A) healthy tissue away from pustules; (B) rings around pustules that included some fungal tissue; and (C) pustule centres (see Table 2). Samples were taken immediately after the 15-min pulse of $^{14}CO_2$ and after 24 h either in the dark or the light. The distribution of radioactivity in the tissue samples (Table 2) confirm in general terms the results of the whole leaf autoradiographs (Plate 1). The movement of labelled substances from the host to the fungus is most marked in the dark and radioactivity accumulates in the chloroform-soluble fraction (lipid and pigments) and acid hydrolysate (mainly mannose) as well as in soluble sugars ($>90\%$ of ^{14}C in aqueous methanol fraction) and α-glucans.

The soluble sugars formed during $^{14}CO_2$ assimilation are sucrose and, especially in host tissues around the pustules, glucose and fructose (Table 3). A considerable amount

Table 2. *Distribution of radioactivity in infected detached leaves of* Tussilago *following exposure to* $^{14}CO_2$

Treatment	Sample	Total ^{14}C in extracts (ct/min/cm^2)	Percentage total ^{14}C in individual extracts: Chloroform*	Aqueous* methanol	Amyloglucosidase†	H_2SO_4 hydrolysate
15 min exposure to $^{14}CO_2$ in light	A	48	2	71	18	9
	B	46	69		24	7
	C	24	3	74	17	6
15 min exposure to $^{14}CO_2$ in light followed by 24 h in dark	A	18	6	62	15	17
	B	18	74		17	9
	C	194	16	31	17	35
15 min exposure to $^{14}CO_2$ followed by 24 h in light	A	35	36		57	7
	B	36	58		38	4
	C	75	67		17	16



* For four treatments only, the methanol/chloroform/water extract was split into chloroform and aqueous phases which were counted separately. For the remainder, aliquots from the original extract were assayed.

† Tissue was ground and extracted in an aqueous solution of amyloglucosidase (see 'Materials and Methods') so that this fraction contained water-soluble polysaccharides and hydrolysed α-glucans.

Table 3. *Distribution of radioactivity in the soluble sugars of infected detached leaves of* Tussilago *following exposure to* $^{14}CO_2$ (*for notation of samples, see Table* 2)

Treatment	Sample	^{14}C in sugar as percentage total in neutral soluble fraction*		
		Sucrose	Glucose + fructose	Arabitol + mannitol
15 min exposure to $^{14}CO_2$ in light	A	89	8	1
	B	42	55	0
	C	57	13	17
15 min exposure to $^{14}CO_2$ in light followed by 24 h in dark	A	51	12	15
	B	45	13	26
	C	4	6	72
15 min exposure to $^{14}CO_2$ in light followed by 24 h in light	A	46	17	6
	B	54	10	8
	C	11	7	36

* Sucrosylfructose, trehalose and unidentified compounds, adhering to base-line of chromatograms comprise remainder to 100%.

of radioactivity in sucrose was also recovered from pustules themselves. The synthesis of mannitol and arabitol by the pathogen from labelled substrates occurred within 15 min and continued during the translocation period, especially in the dark. Radioactive polyols were also recovered from the samples of host tissue.

DISCUSSION

The data reported here confirm that leaves of *Tussilago* infected with *Puccinia poarum* represent a very suitable model system for the investigation of biotrophic nutrition. Holligan *et al.* (1973) showed that the general pattern of carbohydrate composition of this association was comparable with other biotrophic host-parasite combinations involving 'higher' fungi. Here, we show that infection of *Tussilago* by *Puccinia poarum* exerts both long- and short-distance effects on patterns of translocation such that, on infection, export from mature leaves is decreased (Table 1), and, especially when heavily infected, their capacity to import is enhanced (cf. Livne and Daly (1966) and reviews cited in the 'Introduction'). The large size of individual pustules produced by *P. poarum* facilitates the investigation of the transfer process and, following photosynthesis, marked accumulation occurs in individual sites of infection in both detached leaves and leaf discs as noted earlier by others for several biotrophic pathogens (see e.g. Shaw and Samborski, 1956; Thrower, 1965b; von Sydow, 1966a; Gaunt and Manners, 1971; Thrower & Lewis, 1973).

A significant qualitative difference between infection of *Tussilago* by *Puccinia poarum* and those previously studied is the accumulation, in tissue of *Tussilago* at infection sites, of polyfructan (inulin) instead of the stimulated synthesis of polyglucose (starch) as in tissues of hosts to other biotrophs (Holligan *et al.*, 1973). Despite this accumulation, fructose polymers, particularly if of high molecular weight, incorporate very little ^{14}C during short term photosynthesis in $^{14}CO_2$. Instead, sucrose and starch, which is labile in the dark but stable in the light, become most heavily labelled. This starch, present at low levels compared with the fructans and extractable from leaves by oxalic acid and amyloglucosidase, is characterized and discussed further by Holligan *et al.* (1974). The retention of radioactivity in starch in the light is shown by both autoradiography and chemical analysis (Plate 1 and Table 2) and appears to account for the smaller

amount of radioactivity which moves from host to parasite in the light. Under these conditions, unlabelled sugars from continuing photosynthesis are available to the fungus. The halo around pustules may be due to greater lability of starch in this region, possibly through the production by the fungus of an activator of β-amylase, as shown by Schipper and Mirocha (1969) for urediospores of the rust, *Uromyces phaseoli*, or of a diffusible amylase, as shown for some strains of the wheat stem rust in axenic culture by Maclean (1971) (see Scott, 1972).

Recently synthesized photosynthate, particularly in the dark when starch is utilized, passes rapidly to the fungus where it is transformed to polyols (Table 3), lipids (Table 2), a glucomannan and glycogen (see Holligan *et al.*, 1974), together with the products of intermediary metabolism and insoluble compounds not analysed by the methods used (see Plate 2, No. 8c). (Details of the accumulation of specifically fungal lipids will be given in a subsequent paper.) The presence of ^{14}C-polyols away from pustules may be due either to leakage from spores and hyphae or to the presence of very young pustules not visible to the naked eye. The significance of polyols in biotrophic symbionts has been discussed by Smith *et al.* (1969).

A feature of the transfer of sucrose from host to parasite appears to be its hydrolysis before absorption (Yuen, 1969; Lewis, 1970). This is supported by the absolute increase in amount of hexoses in infected regions of *Tussilago* (Holligan *et al.*, 1973) and the increased incorporation of radioactivity into them (Table 3). Similar phenomena have also been reported for other host parasite combinations (Jain and Pelletier, 1958; Gervitz and Durbin, 1960; von Sydow, 1966b). In a subsequent paper, studies on the role of invertase and on changes in its activity during infection will be described.

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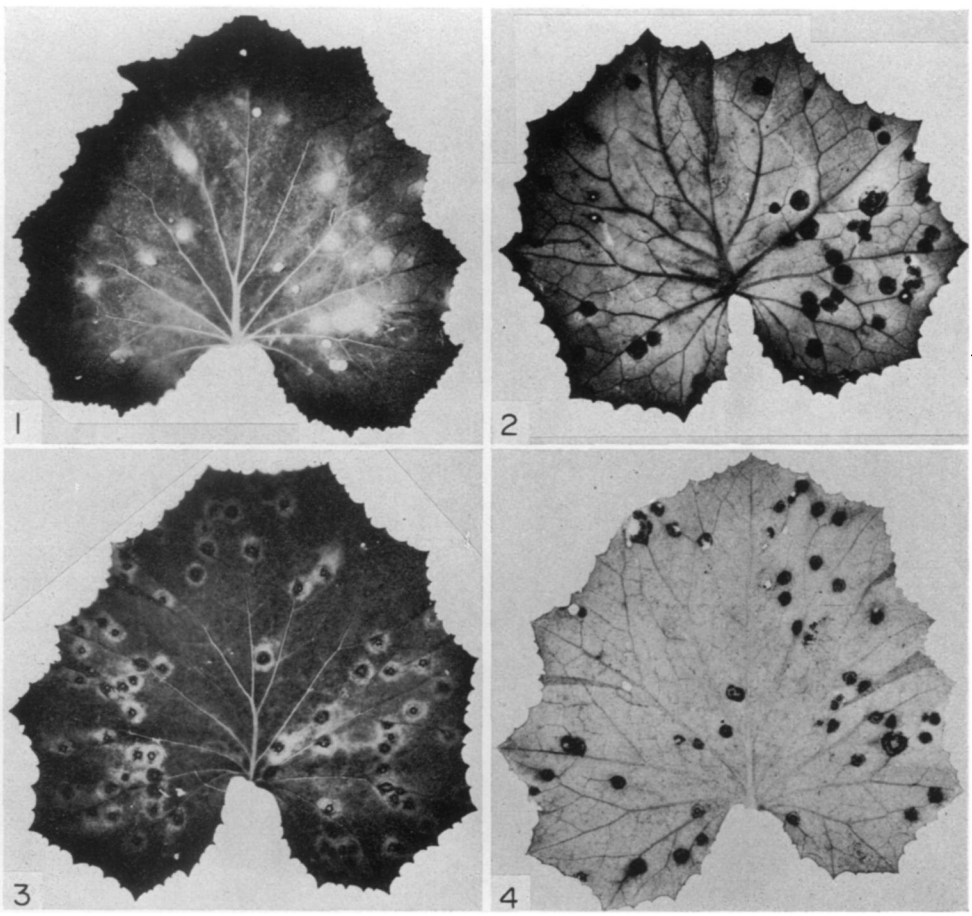

Autoradiographs of detached leaves of *Tussilago* infected by *Puccinia poarum*.
No. 1. Immediately after 15-min exposure to $^{14}CO_2$ in the light.
No. 2. 15-min exposure to $^{14}CO_2$ in the light followed by 20 h in the dark.
No. 3. 15-min exposure to $^{14}CO_2$ in the light followed by 20 h in the light.
No. 4. After exposure to $^{14}CO_2$ in the dark for 20 h.

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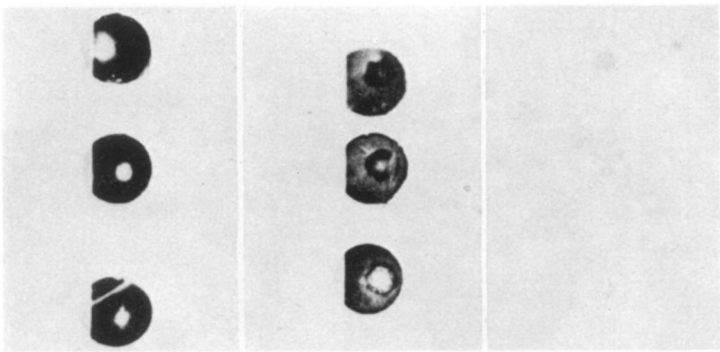

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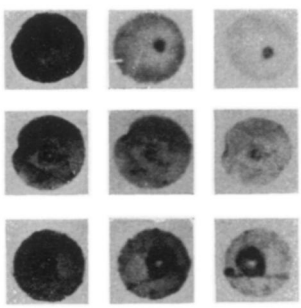


(a) (b) (c)

8.

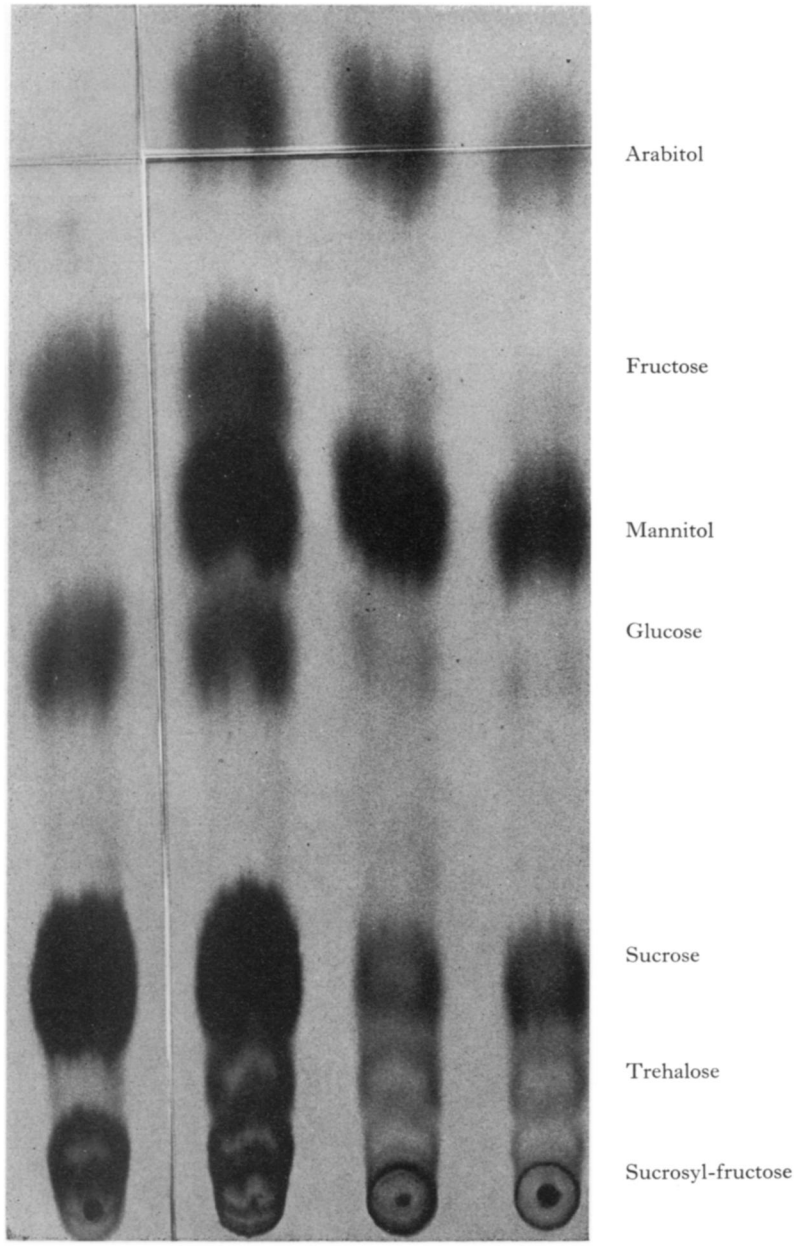
Autoradiographs of leaf discs of *Tussilago* infected by *Puccinia poarum*.

No. 5. Immediately after 30-min exposure to $^{14}CO_2$ in the light.

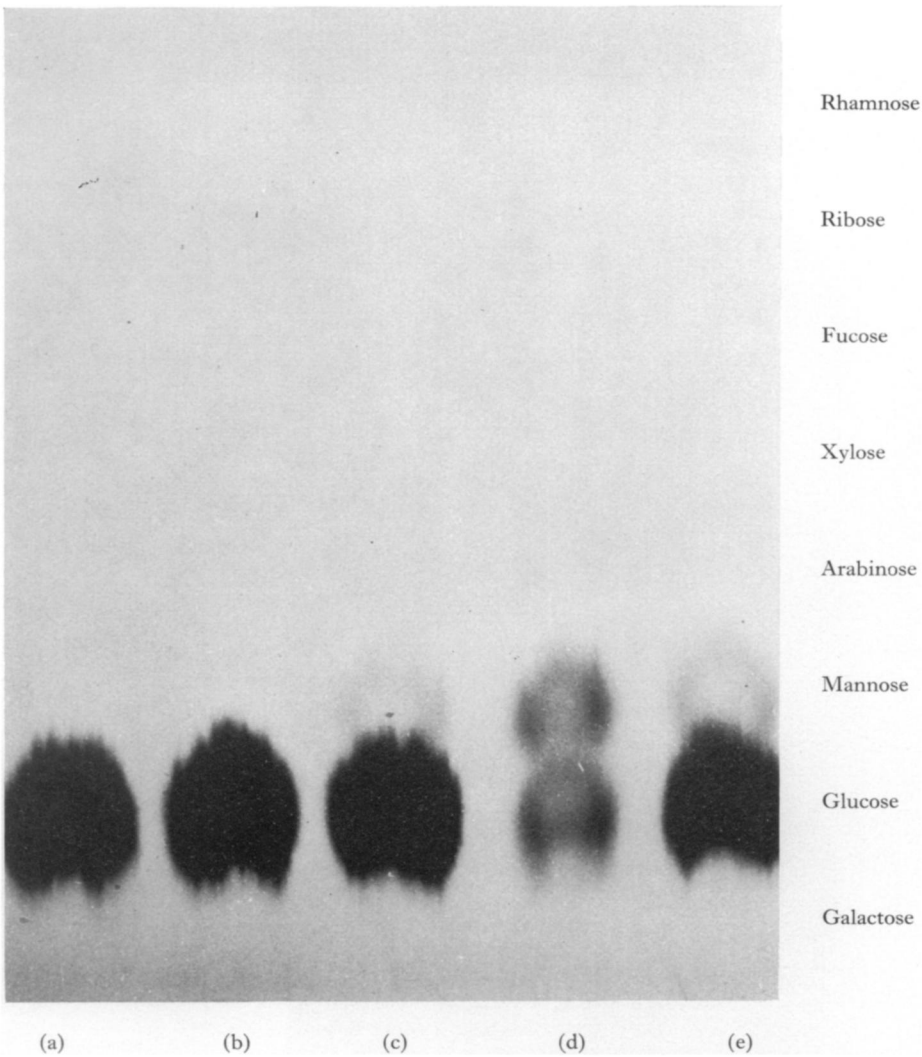
No. 6. Thirty-minute exposure to $^{14}CO_2$ in the light followed by 4 h in the dark.

No. 7. After 4-h exposure to $^{14}CO_2$ in the dark.

No. 8. Autoradiographs of same discs after sequential extraction in (a) aqueous ethanol, (b) oxalic acid and (c) sulphuric acid (see 'Materials and methods'). Discs exposed to $^{14}CO_2$ for 3 h, followed by 24 h in the dark.



Autoradiograph of chromatograms of neutral soluble extracts (solvent: ethyl–acetate–acetic acid–water 14:3:3) (a) from healthy leaf discs exposed to $^{14}CO_2$ in the light for 2 h; (b) from infected leaf discs exposed to $^{14}CO_2$ in the light for 2 h; (c) from infected leaf discs exposed to $^{14}CO_2$ in the light for 2 h, followed by 18 h in the dark; (d) from infected leaf discs exposed to $^{14}CO_2$ in the light for 2 h, followed by 18 h in the light.



Autoradiograph of chromatograms of neutral fraction of insoluble hydrolysate (solvent: ethyl acetate–acetic acid–water, 14:3:3) (a) from healthy leaf discs exposed to $^{14}CO_2$ in the light for 2 h; (b) from healthy leaf discs exposed to $^{14}CO_2$ in the light for 2 h, followed by 18 h in the light; (c) from infected leaf discs exposed to $^{14}CO_2$ in the light for 2 h; (d) from infected leaf discs exposed to $^{14}CO_2$ in the light for 2 h, followed by 18 h in the dark; (e) from infected leaf discs exposed to $^{14}CO_2$ in the light for 2 h, followed by 18 h in the light.