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THE USE OF A NEW, RAPID MICRO-METHOD FOR ANALYSING CHANGES IN THE CARBOHYDRATE FRACTIONS OF POTATO TUBER TISSUE AFTER INVASION BY *PHYTOPHTHORA INFESTANS*

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Abstract—The development of a rapid micromethod for separation and estimation of carbohydrate fractions of potato tuber tissue allowed a re-examination of certain changes following the invasion of thin tissue discs by *Phytophthora infestans*. Invasion is followed by a considerable depletion in 80% ethanol-soluble carbohydrate, and a lesser depletion of starch levels. A "pectic extract" isolated by treatment with alkali followed by a *P. infestans* glycosidase preparation, showed decreases by comparison with uninoculated tissue, but the decreases were more marked when the proportion of this fraction hydrolysed by the fungal glycosidases was examined. As expected the final residue of the extraction sequence showed greater increases in infected discs. The relation of these changes in the pathogenic interaction is discussed.

INTRODUCTION

IN ORDER to assess the role of cell-wall degrading enzymes in the invasion of a plant tissue by a fungal parasite it is necessary to know to what extent host wall components are affected by invasion. Friend and Knee[1] attempted to provide this information for the interaction of *Phytophthora infestans* and potato tuber tissue. Analysis of the carbohydrate composition of tuber cell walls showed that approximately 50% of the dry wt. of wall could be extracted by water at 98°; this "pectic fraction" contained 57% galactose, 31% galacturonic acid, 9% arabinose and 3% rhamnose residues. After removal of this material the residue was largely α-cellulose with a small amount of alkali-soluble polysaccharides containing galactose, uronic acid, arabinose, xylose, mannose and glucose units. The only monosaccharide detected after hydrolysis of ethanol-extracted mycelium of *P. infestans* was glucose and it was concluded that the major polysaccharide component of the hyphal wall of this species is an alkali-insoluble glucan as it is in *P. cinnamomi* and *P. parasitica*.[2] The evidence obtained that galactan was degraded during invasion of potato tuber tissue by *P. infestans* was inconclusive, despite the fact that galactanase activity could be demonstrated in infected tissue. Alkali-insoluble glucan, presumably fungal wall, accumulated rapidly in infected tuber tissue, as did a lignin-like polymer which may have limited the spread of fungal hyphae.

It was thought that galactan degradation might be more extensive if the balance of the interaction were shifted in favour of the fungus. This was achieved by using a zoospore inoculum and tissue discs 0·1 cm thick, as opposed to a sporangial inoculum and 0·5-cm discs in the earlier work. Because of the suggestion in the earlier work that host glucan synthesis was arrested by the presence of the fungus, changes in the common carbohydrate supply of soluble sugars and changes in starch levels were examined.

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[1] J. FRIEND and M. KNEE, *J. Exptl. Botany* **20**, 763 (1969).
[2] S. BARTNICKI-GARCIA, *J. Gen. Microbiol.* **42**, 57 (1966).

This work necessitated the development of analytical methods applicable to small amounts of tissue. Specific enzymic methods seemed attractive for this purpose; but as Lamport[3] has noted, commercially available preparations of plant cell-wall degrading enzymes, referred to as "pectinase", "hemicellulase", or "cellulase" invariably contain a wide range of activities, which limits their utility unless a specific method of estimation of a degradation product is employed, as in the established procedure for estimation of wall polyuronide.[4] Culture filtrates of *P. infestans* contain galactanase activity[5] which will liberate a high proportion of the galactose residues present in potato tuber cell walls prepared by a suitably mild procedure.[6,7] Uronic acid residues were not released by the action of culture filtrates on tuber cell walls or on a soluble potato pectin preparation,[5,6] and this was thought to be in keeping with the failure to detect polygalacturonase activity of any kind in culture filtrates of *P. infestans*.[8] However, A. L. J. Cole (personal communication) has confirmed an earlier finding[9] that *P. infestans* produces polygalacturonase (though not polygalacturonate lyase) in liquid culture. In the work described here an enzyme concentrate from *P. infestans* culture filtrates was used to release pectic material from the alcohol-insoluble residue of tuber tissue, prior to estimation in terms of total carbohydrate extracted and proportion degraded by the fungal enzymes. The results have been interpreted in relation to the later findings.

RESULTS

Optimum conditions for infection. In view of the limited extent of experimental infection in earlier work the progress of invasion of tuber tissue by *Phytophthora infestans* was examined in free-hand sections of discs prepared in various ways. Discs of different thickness inoculated with zoospores or sporangia and incubated at 22° were tried. Sporangia at 22° germinate directly, while at 10° they liberate motile zoospores which encyst and germinate (indirect germination). Consequently discs incubated at 10° for 15 hr after inoculation with sporangia were also examined histologically.

After 24 hr incubation hyphae could be seen entering the tissue between cells. Where sporangia had germinated directly the infection sites were sparsely scattered, but where zoospores had been produced from a similar number of sporangia before inoculation, or where there had been indirect germination of a sporangial inoculum, the infection sites were comparatively dense.

The further progress of infection was dependent on the thickness of the tuber discs. In discs as thin as 2 mm (ten cells thick), invasion of the upper three cell layers occurred within 48 hr and further progress through the disc was slow. Discs 1 mm thick were infected throughout by 48 hr and this infection was especially intense after an inoculation with zoospores or with sporangia that had germinated indirectly.

In subsequent experiments a combination of 1 mm thick tuber discs inoculated with zoospores was analysed after 24 and 48 hr and compared with corresponding uninoculated discs. Twenty discs was a convenient sample for analysis and since this amount of tissue contains only 10 mg of cell wall the analytical procedures employed in earlier work were inappropriate.

Development of analytical procedure. An analytical procedure was sought which would

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[6] M. KNEE and J. FRIEND, *J. Gen. Microbiol.* **60**, 23 (1970).
[7] M. KNEE, Ph.D. Thesis, University of Hull (1968).
[8] D. D. CLARKE, *Nature* **211**, 649 (1966).
[9] F. GROSSMAN, *Naturwiss.* **50**, 721 (1963).

include the following steps: (1) ethanol extraction and estimation of soluble sugars; (2a) saponification of pectic methoxyl groups and estimation of the methanol formed; (2b) treatment with a glycosidase preparation from *P. infestans* and estimation of the carbohydrate liberated; (3) solubilization and estimation of starch; (4) estimation of the carbohydrate remaining after this extraction sequence.

Total carbohydrate was estimated throughout using a modification of Devor's method[10] with sulphonated α-naphthol reagent, because previous experience had demonstrated the general applicability of this method.

Satisfactory conditions for the step involving glycosidase treatment were established using a larger sample of tissue (50 g) than intended in the final scheme. The progress of release of carbohydrate by a glycosidase preparation from the ethanol extracted and saponified material is shown in Fig. 1 in terms of total carbohydrate, reducing sugar and uronide material.

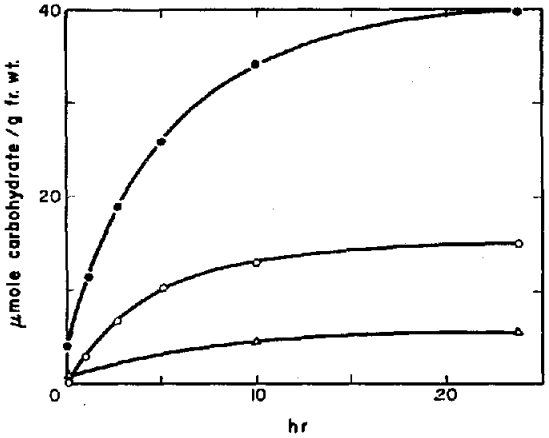

FIG. 1. RELEASE OF CARBOHYDRATE FROM ETHANOL EXTRACTED AND SAPONIFIED POTATO TUBER TISSUE BY A *Phytophthora* GLYCOSIDASE PREPARATION.

Tuber tissue (50 g) extracted with 80% ethanol gave 81 μmoles soluble carbohydrate/g fr. wt. Saponification with 0·05 N NaOH released methanol estimated[11] in a distillate as 5·3 μmoles/g fr. wt. (Total uronide = 7·22 μmoles, degree of esterification = 73%.) Material then suspended in 200 ml dilute buffer (pH 4·5) and 2·0 ml of a glycosidase preparation added. Samples (5·0 ml) filtered at time intervals, filtrates boiled and carbohydrate estimated as follows: Total carbohydrate[10] (galactose equivalents) ●——●; Reducing sugar[12,13] (as galactose) ○——○; Uronide[4] (as galacturonic acid) △——△.

The release of these components seemed to reach completion at about 24 hr. The release of uronide was unexpected, and at the time was thought to be due to the inevitable breakage of glycosidic bonds that accompanies alkaline de-esterification of pectinic acids.[14] Since, as noted above, *P. infestans* is now known to produce polygalacturonase this enzyme may have been responsible for the solubilization of uronide. However, this material forms a small proportion of the total carbohydrate liberated and is not the whole of the uronide present in the tissue (more can be liberated by treatment with a commercial "pectinase" preparation). The predominant monosaccharide residue in the extracts should, on the basis of earlier

[10] A. W. DEVOR, *J. Am. Chem. Soc.* **72**, 2008 (1950).
[11] R. N. BOOS, *Analyt. Chem.* **20**, 964 (1948).
[12] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).
[13] M. SOMOGYI, *J. Biol. Chem.* **195**, 19 (1952).
[14] B. VOLLMERT, *Makromol. Chem.* **5**, 110 (1950).

work, be galactose. The sequence of alkali and glycosidase treatment solubilizes a part of the pectic fraction of the whole tissue which is in turn partly degraded so that substantial amounts of reducing sugar are found in the extracts. This "enzymatically hydrolysable fraction", estimated as reducing sugar, is regarded also as the portion of the pectic fraction which can be degraded by *P. infestans* when it invades potato tuber tissue. Its chemical nature is discussed below.

Further experiments showed that the saponification procedure extracted small amounts of carbohydrate which then escaped detection. Thus, in the final procedure, saponification was carried out prior to the glycosidase treatment without intervening filtration. It proved impossible to estimate methanol reliably at the low concentration encountered in these more dilute extracts.

Trial of analytical procedure. The procedure as finally elaborated was tried out on three batches of twenty discs from a single potato tuber, prepared as for an infection experiment. As can be seen from Table 1, reproducible results were obtained. The amount of ethanol-

TABLE 1. ANALYSIS OF POTATO TUBER DISCS BY EXTRACTION PROCEDURE, FOLLOWED BY CARBOHYDRATE ESTIMATION

Carbohydrate fraction	μmoles/g fresh wt.
(1) 80% ethanol soluble	46·2 ± 0·5
(2) Pectic extract	36·6 ± 0·6
Enzymatically hydrolysable	14·2 ± 0·2
(3) Starch	963 ± 24
(4) Residue	38·7 ± 0·3

Estimations in duplicate on each extractive from three batches of twenty discs, as glucose except for pectic extract and enzymatically hydrolysable fraction which were as galactose. Standard deviations shown.

soluble carbohydrate was much less than in the intact tissue, probably due to leaching of this fraction into the water used to wash the discs. As expected, the starch fraction proved far larger than the others in carbohydrate content; this estimation was somewhat less reliable than the others.

As a further check on the procedure, tuber cells walls were prepared, hydrolysed with acid and component monosaccharides in hydrolysates were estimated. The same cell-wall preparation was analysed by the procedure for discs, omitting the ethanol extraction. The results are shown in Table 2.

It has been shown that the final products of the reaction of a *P. infestans* glycosidase preparation with a soluble potato pectin substrate include galactose and probably short galactose oligomers (though not galacturonic acid). For this reason estimates of reducing sugar in the products are about 25% less than the galactose found in acid hydrolysates of the substrate.[6,7] It can be seen from Table 2 that the enzymatically hydrolysable fraction of the pectic extract accounts for about 75% of the galactose found in tuber cell walls after acid hydrolysis. This would suggest that the enzyme reaction had followed a similar course to that with the soluble substrate, and that degradation of wall polyuronide was limited in extent. The galactose residues found in acid-hydrolysed cell walls would account for a large proportion of the pectic extract especially when allowance is made for losses through acid hydrolysis which would not be incurred in the extraction sequence. A portion of the galacturonic acid

TABLE 2. ANALYSIS OF A TUBER CELL-WALL PREPARATION BY CONVENTIONAL MEANS AND BY EXTRACTION PROCEDURE FOLLOWED BY CARBOHYDRATE ESTIMATION

(*a*) Monosaccharide composition of hydrolysates		(*b*) Carbohydrate content of extractives	
Sugar residue	μmoles/mg dry wt.	Fraction	μmoles/mg dry wt.
Uronic acid	0·76	(2) Pectic extract	2·79
Galactose	1·62	(2) Enzymatically hydrolysable	1·17
Arabinose	0·28		
Xylose	0·10	(3) Starch	0·37
Mannose	0·11	(4) Residue	1·42
Glucose	1·58		

(*a*) Samples (20 mg) of tuber cell wall were dissolved in 72% H_2SO_4, diluted to 2·5% H_2SO_4 and hydrolysed by autoclaving at 15 psi for 60 min.[15] The acid was removed by shaking the products with a solution of *N,N*-bis-*n*-octylmethylamine in $CHCl_3$.[16] Neutral monosaccharides in the hydrolysate were separated by descending paper chromatography in EtOAc–pyridine–H_2O (8:2:1). Chromatograms were dipped in aniline hydrogen phthalate reagent and heated at 105° to visualize spots which were cut out and eluted in acid ethanol for spectrophotometric estimation.[17] Average recovery of known amounts of monosaccharides subjected to the entire procedure was 85%. Galacturonic acid residues were estimated by the carbazole method using 20-mg samples of cell wall which were saponified with alkali and treated with pectinase to solubilize the polyuronide.[4]

(*b*) Samples (20 mg) were saponified with alkali and treated sequentially with *P. infestans* glycosidase, pH 7·0 buffer at 100°, amylase, and 72% H_2SO_4 as described in the Experimental.

residues would be found in the pectic extract and since arabinose, galactose and uronic acid almost certainly form a co-polymer in the intact wall, arabinose residues would probably occur also in this extract. Cell-wall preparations of this kind always contain between 0·1 and 0·2 μmoles/mg glucose equivalent of water-soluble glucan which is thought to be starch and would be found in the pectic extract.

Boiling at pH 7·0 would presumably liberate any uronide remaining in the wall after the first extraction. The small amount of carbohydrate extracted from the walls in this step reacted with the sulphonated α-naphthol reagent to give a brown colour and a high ratio of absorbance at 480 nm to that at 555 nm, known to be characteristic of uronide. The fraction soluble in 72% H_2SO_4 is easily accounted for as the bulk of the glucan with small amounts of xylan and mannan which are the more insoluble wall components.

Analysis of infected and uninfected tissue. Potato tubers sometimes contain latent microbial infections which become apparent after incubation of cut tissue.[18] Any discs cut from such tubers will contaminate a whole batch when discs are pooled; therefore discs cut from single tubers were kept separate throughout the course of the experiment. Because of the variation in carbohydrate composition between tubers, the results of an infection experiment (Fig. 2) are expressed in terms of the average percentage change from an initial sample for respective tubers.

Taking account of the standard deviation for each estimation in the analytical sequence (Table 1), significant changes are as follows. In the uninfected tissues ethanol-soluble carbohydrate showed a marked increase by 24 hr but by 48 hr was less than in the initial samples.

[15] J. F. SAEMAN, W. E. MOORE, R. L. MITCHELL and M. A. MILLETT, *Tappi* **37**, 336 (1954).
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[17] C. M. WILSON, *Analyt. Chem.* **31**, 1199 (1959).
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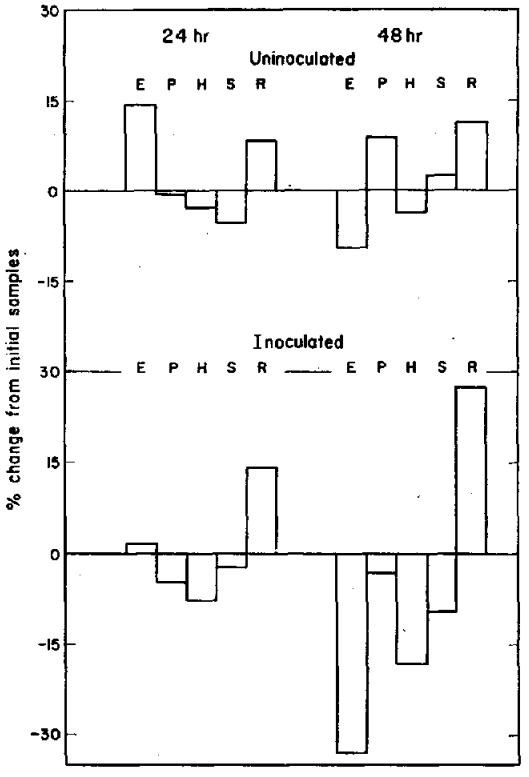

FIG. 2. CHANGES IN CARBOHYDRATE COMPOSITION OF THIN POTATO TUBER DISCS, UNINOCULATED AND AFTER INOCULATION WITH ZOOSPORES OF *Phytophthora infestans*.
E, 80% ethanol soluble; P, pectic extract; H, enzymatically hydrolysable fraction; S, starch; R, residue.

Conversely starch decreased at first and then increased slightly. The pectic extract showed no change by 24 hr but had increased substantially at 48 hr. There were slight decreases in the enzymatically hydrolysable fraction indicating that the increases in the pectic extract involved some material not hydrolysed by the fungal glycosidases. The residue increased steadily throughout the experiment.

In the infected discs there was no initial increase in alcohol-soluble carbohydrate and by 48 hr this fraction was much lower than in the first samples. Starch decreased more consistently than in uninfected discs. The pectic extract showed slight decreases in all infected samples, but the losses from the enzymatically hydrolysable fraction were much greater so that there was probably a counter-balancing synthesis of non-hydrolysable pectic material as in the uninfected discs. The residue increased more strongly in infected discs.

DISCUSSION

The analytical procedure described above was developed for a particular purpose. If the pectic extraction were improved to liberate uronide quantitatively the method would be generally applicable for the separation and estimation of carbohydrate fractions in potato tissue.

The interpretation of the analytical results depends upon the evidence presented for the composition of the carbohydrate fractions of potato tuber tissue, separated and estimated in the analytical scheme. The ethanol-soluble fraction would consist mainly of the free sugars of the tissue, glucose, fructose and sucrose. The pectic extract must comprise the bulk of galactan with a proportion of the polyuronide and araban present in the cell wall; these components may form a copolymer rather than distinct homoglycans.[19] The enzymatically hydrolysable fraction is probably an indication of the galactan content of the pectic extract. It is thought that under the conditions of extraction the action of the polygalacturonase component of the *Phytophthora infestans* glycosidase preparation was limited and that polyuronide and araban largely constitute the non-hydrolysable portion of the extracts. The starch extraction step devised for the whole tissue released from a cell-wall preparation a small amount of material which seemed to be mainly polyuronide left in the wall by the preceding extraction. However, the starch fraction in the whole tissue is so large that this polyuronide would form a negligible proportion of it. The final extraction residue must be largely α-cellulose with a small amount of hemicellulosic polysaccharides. In infected tissue this fraction would include the fungal wall polysaccharide which is a glucan, though not entirely in the crystalline form of α-cellulose.[2]

The results of the infection experiment amplify those of Friend and Knee,[1] who used more traditional analytical methods. Their suggestion that hypha and host cell are in competition for carbohydrate supplies is borne out by the greater losses of alcohol-soluble carbohydrate in infected tissue. While starch decreases after infection there remains a huge amount by comparison with other carbohydrate fractions, and it is suggested that the degradation of starch to alcohol-soluble sugars is a limiting step in the interaction of host and pathogen.

The conspicuous decreases in the enzymatically hydrolysable fraction of the pectic extracts from infected tissues suggests that the fungal glycosidases and particularly galactanase are active during penetration and establishment of infection and, indeed, may aid these processes. The increase of non-hydrolysable pectic components, presumed to occur in infected tissue, agrees with the earlier finding that polyuronide and araban were synthesised in similar circumstances.[1] The greater increase of the residue fraction in infected discs also confirms the earlier work where it was attributed to a synthesis of fungal wall.

In these and the preceding experiments[1] discs of tuber tissue were used largely because it was felt necessary to provide aseptic conditions for the development of infection by *P. infestans*. However, it has to be remembered that excision of pieces of tissue from storage organs has a stimulative effect on their metabolism which, in turn, may alter their interaction with a pathogen. It is thought that the enhanced metabolic activity of thick discs enables them to respond rapidly to invasion, by lignification and thus limit the spread of fungal hyphae; hence the evidence of cell-wall degradation in these circumstances is slight. On the other hand, invasion of thin discs is accomplished before lignification can occur and there is more dramatic evidence of cell-wall degradation and accumulation of fungal wall; the latter occurs at the expense of the carbohydrate resources of the host, both free sugars and, indirectly, starch. These two types of infected disc are model systems; neither represents infection of the susceptible whole tuber exactly, but they show in terms of carbohydrates that this must be a dynamic interaction, particularly at the host-parasite interface in the cell wall. It is at this interface that cell-wall degradation and lignification occur in susceptible and in resistant tubers;[1] hypersensitive resistance introduces another element to the interaction, the produc-

[19] A. J. Barrett and D. H. Northcote, *Biochem. J.* **94**, 617 (1965).

tion of fungitoxic metabolites by the host tissue.[20,21] It has been shown that this type of resistance is not expressed if slices of tuber tissue less than ten cells in thickness are inoculated with an incompatible race of *P. infestans*.[22] The timing of lignification in relation to the progress of the fungal hyphae through the tissue may be crucial in both compatible and incompatible interactions. Asada and Matsumoto[23] have suggested that resistance depends firstly upon fungitoxic substances which are effective against the growing tips of hyphae, and secondly upon lignification which isolates the infection site preventing a renewal of hyphal growth.

EXPERIMENTAL

Sources of Biological Material

Potatoes (var. Majestic) were grown at the University Botanic Garden and stored in clamps until required for use.

An isolate of *Phytophthora infestans* from infected leaves of Duke of York potatoes was maintained as stock cultures on frozen bean agar at 10°. Sporangia were obtained by washing slope cultures, in 2·4 cm dia. tubes grown for 7 days at 22°, with 10 ml sterile water. Sporangial suspensions were incubated at 10° for 1 hr and held at room temperature for 5 min to promote liberation of zoospores.

Preparation of Tissue

Whole sound potatoes were dipped in ethanol and flamed. Under aseptic conditions, outer tissue was cut away to leave a block which was surface-sterilized in saturated aq. calcium hypocholrite for 5 min; a cork borer (1·0 cm dia.,) was pushed at right angles to one face, three-quarters through the block, in four positions. Slices (0·1 cm thick) were cut from this face using an instrument similar in design to a culinary vegetable slicer. The first slice was rejected and the discs were separated from the remainder, washed four times with water and divided into batches of twenty-five in 9·0-cm petri plates.

A batch of discs from each tuber used in the experiment was kept at −20° for subsequent analysis. Of the remainder, half were inoculated with 2·0 ml of a suspension of zoospores (2×10^5/ml) and the other half with 2·0 ml water. After incubation at 22° for 24 and 48 hr three batches of discs from each series were selected at random and kept at −20° for analysis.

Preparation of P. infestans *Glycosidase*

The isolate of *P. infestans* mentioned above was grown in bean medium containing 0·2% casein hydrolysate, in flat glass bottles as described previously.[6] Glycosidase activity in culture filtrates was concentrated by precipitation with 80% saturated ammonium sulphate and dialysis.[5]

Fractionation Procedure

(1) *Ethanol-soluble carbohydrate.* Twenty discs (initial fresh wt. = 1·54 g) were disintegrated in 25 ml 80% ethanol using an Ultra Turrax TP 18/2 homogenizer (Janke & Kunkel, Staufen i. Br., Germany). Twenty-five ml 80% ethanol was used to wash the homogenizer blades and vessel. The combined ethanolic suspensions were then refluxed for 30 min, filtered on sintered glass (porosity 3) and washed with a further 25 ml 80% ethanol.

(2) *Pectic extract.* The residue, after washing with a small volume of water to remove remaining ethanol, was resuspended in 4·0 ml water to which was added 0·2 ml N NaOH. After 30 min at room temp. the suspension was neutralized with N HCl and 5·0 ml citrate–phosphate buffer (pH 4·5) including 0·01 M EDTA, 0·2 ml of a glycosidase preparation from culture filtrates of *P. infestans* and 4 drops of toluene were added. After incubation with continuous agitation for 20 hr at 25°, the suspension was filtered and the residue washed three times with 2·0 ml water.

(3) *Starch.* The residue was suspended in 10 ml 0·1 M sodium phosphate (pH 7·0) including 0·05 -M KCl and boiled for 10 min. The suspension was made up to approx. 100 ml and 1·0 ml of a salivary amylase preparation[24] and 0·5 ml toluene were added. After incubation for 20 hr, as above, the suspension was filtered through a clean sintered-glass crucible (porosity 3) and the residue washed three times with 10 ml water.

[20] N. F. ROBERTSON, J. FRIEND, M. A. AVEYARD, J. BROWN, M. HUFFEE and A. L. HOMANS, *J. Gen. Microbiol.* **54**, 216 (1968).

[21] K. TOMIYAMA, T. SAKUMA, N. ISHIZAKA, N. SATO, N. KATSUI, M. TAKASUGI and T. MATSUMUNE, *Phytopathology* **58**, 115 (1968).

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[23] Y. ASADA and I. MATSUMOTO, *Phytopathology* **57**, 1339 (1967).

[24] S. A. OLAITIN and D. H. NORTHCOTE, *Biochem. J.* **82**, 509 (1962).

(4) *Final residue.* The residue was washed with ethanol and ether and left for 48 hr at room temp. It was allowed to dissolve in 1·5 ml 72% (w/v) H_2SO_4 for 24 hr.

Carbohydrate Estimation

The solutions obtained at each step were made up to an appropriate volume with water. Total carbohydrate in each was estimated using a slight modification of Devor's procedure:[10] 4·0 ml reagent was added to a 2·0 ml sample cooled in ice, mixed well and heated for 10 min at 98°; after cooling in ice, absorbance at 555 nm was measured and related to a standard sample of glucose or galactose, depending upon which was the predominant sugar in the unknown sample.

In the extract from step (2) reducing sugar was estimated in terms of galactose by a standard procedure.[12,13] To allow for the slower reaction of galactose, samples were heated 15 min with copper reagent. This gave an estimate of the enzymatically hydrolysable fraction.

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