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Aromatic-Alcohol-Oxidase Activity in the Growth Medium of *Polystictus versicolor*

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To gain a better understanding of the changes which plant lignins undergo in the soil the fungal breakdown of aromatic compounds related to lignin has been studied. Previous work has shown that the wood-rotting basidiomycete *Polystictus versicolor* is able to reduce many aromatic acids to the corresponding alcohols, or to mixtures of aldehydes and alcohols (Farmer, Henderson & Russell, 1959). The persistence of aldehydes after complete reduction of the acids appeared to be due to an extracellular oxidase acting on the alcohols, and this was confirmed for *p*-methoxybenzyl alcohol. The properties and substrate specificity of this system are more fully examined here.

MATERIALS AND METHODS

Organism. *Polystictus versicolor* as used previously (Farmer *et al.* 1959) was isolated from fructifications collected from a tree stump.

Buffers. Sørensen's phosphate (Clark, 1928) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (Gomori, 1955) were used.

Production of enzyme. The fungus was grown in 250 ml. conical flasks containing 100 ml. of mineral salts solution ($NaNO_3$, 0·3%; $MgSO_4$, 0·05%; KH_2PO_4, 0·1%; KCl, 0·05%) to which were added 1·0 g. of glucose and 0·5 g. of yeast extract. Each flask was inoculated with four disks, cut from the periphery of a culture of the fungus on potato-dextrose–agar, and incubated at 22°, usually for 10–12 days. Hyphae from these disks spread over the surface of the medium to form a firm mat. During growth of the fungus the enzyme accumulated in the growth medium, and this solution was used directly in many experiments. A further supply of enzyme could be obtained by pouring off the medium at the end of the growth period, washing the mats three times with sterile water and then pouring 100 ml. of sterile water under them. After further incubation enzyme diffused from the mycelium into the water.

An enzyme concentrate was obtained by saturating growth solutions with $(NH_4)_2SO_4$. The saturated solution was allowed to stand overnight and the resulting precipitate was centrifuged down at 38 000 *g* for 20 min. It was then suspended in 3–5 ml. of water and dialysed for 21 hr. against Sørensen's phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 6·2) at 5°. Under these conditions the volume did not change appreciably.

Enzyme activity. The activities of enzyme solutions were compared spectrophotometrically by measuring the rates at which they oxidized *p*-methoxybenzyl alcohol to the aldehyde, and are reported as μmoles of aldehyde produced by 1 ml. of enzyme solution in 1 hr. (μmoles/ml./hr.). The assay solution contained 1 ml. of 0·067 M-phosphate buffer (pH 6·2), 4 μmoles of *p*-methoxybenzyl alcohol, *x* ml. of enzyme solution and water to 3 ml. The change in absorption per minute (ΔE/min.) was measured at 290 mμ in

1 cm. cells with a Beckman DU spectrophotometer. The temperature of the cell housing was not controlled. The reference cells contained buffer, water and either enzyme or alcohol. The activity, in terms of μmoles of aldehyde/ml./hr., is given by $1{\cdot}8 \times 10^5$ (ΔE/min.)/ϵx (ϵ, the molar extinction coefficient of *p*-methoxybenzaldehyde at 290 mμ, being taken as 15 000). Similar methods were used to estimate the activity of enzyme solutions towards other aromatic alcohols, differing wavelengths and appropriate ϵ values being used (Table 1). As the aldehydes have carbonyl groups conjugated with the aromatic ring, they all absorb at longer wavelengths than the alcohols, and therefore small amounts could be detected in the presence of excess of the alcohols.

Enzyme activity was also indicated by oxygen uptake in the Warburg apparatus at 25°. Enzyme solution (1 ml.) and 1 ml. of phosphate buffer, pH 6·2, were added to the main compartment of the flasks, and 0·5 ml. of 0·01 M solutions of alcohols were tipped in from the side arms. β-Naphthylcarbinol, because of its low solubility, was added in aqueous suspension. This method was less sensitive than the photometric method. Oxidation of 1 μmole of *p*-methoxybenzyl alcohol/hr. in a 3 ml. assay solution causes a change in absorption at 290 mμ of 0·0833/min., and an oxygen uptake of 11·2 μl./hr., assuming that oxygen is used only in oxidizing alcohol.

Enzymes and coenzymes. The following preparations were also used: alcohol dehydrogenase and horseradish peroxidase (C. F. Boehringer und Söhne, Mannheim, Germany); glucose oxidase and triphosphopyridine nucleotide (TPN; Sigma Chemical Co., St Louis, Mo., U.S.A.); diphosphopyridine nucleotide (DPN; L. Light and Co. Ltd., Colnbrook).

Chemicals. Most of the compounds examined were commercial samples, recrystallized where necessary, or were prepared from commercial samples. Melting points reported are uncorrected. Reduction of the aldehydes by $LiAlH_4$, by the procedure of Larsson (1950), gave β-naphthylcarbinol, m.p. 79°, *p*-hydroxybenzyl alcohol, m.p. 116°, *m*-methoxybenzyl alcohol (b.p. 90–100°/0·01 mm. Hg) and 3:4-dimethoxybenzyl alcohol, b.p. 140°/0·5 mm. Hg. This last was an oil which slowly changed on standing into a crystalline solid without hydroxyl groups, thought to be the di-ether (Lindgren, 1950). By a procedure used to reduce acetylated ethyl ferulate (Allen & Byers, 1954), ferulic acid was reduced directly by $LiAlH_4$ to give 4-hydroxy-3-methoxycinnamyl alcohol, m.p. 71–73°, in poor yield. Reduction of the appropriate aldehydes and ketones (1 g.) in aqueous 2% (w/v) NaOH (plus ethanol where necessary to achieve complete solution) by $NaBH_4$ (0·45 g.) at room temperature (Adler & Hernestam, 1955) gave 4-hydroxy-3-methoxybenzyl alcohol, m.p. 113–114°, 1-(4-hydroxy-3-methoxyphenyl)ethanol, m.p. 100–102°, 1-(*p*-methoxyphenyl)ethanol and 1-(3:4-dimethoxyphenyl)-ethanol. The two last-named were oils which were not further purified because of their instability (Stedman & Stedman, 1929). Reductions by $NaBH_4$ were followed spectrophotometrically, and were all complete within 18 hr. Phenolic aldehydes and ketones were more slowly reduced than the fully methylated compounds (Smith, 1955). Melting points and boiling points of the various preparations were close to published values, except for *p*-hydroxybenzyl alcohol (Heilbron & Bunbury, 1946, quote 125°).

RESULTS

Activity of preparations. Activity in growth solutions reached a maximum after 10 days (Fig. 1, curves *A*). Most of the work was done with solutions obtained after 12 days' growth, whose activity, i.e. rate of oxidation of *p*-methoxybenzyl alcohol, ranged from 0·6 to 1·4 μmoles/ml./hr. On replacement of the growth solution with water, a further amount of enzyme diffused from the mycelial mat as shown in Fig. 1, curves *B*. Such solutions were generally used after 7 days' incubation under the mat, when they showed activities ranging from 0·1 to 1·6 μmoles/ml./hr.: most were near 0·3 μmole/ml./hr. Concentration of the enzyme from 300 ml. of growth solution by precipitating it with $(NH_4)_2SO_4$, followed by resolution in 5 ml. of water, gave activities ranging from 8 to 38 μmoles/ml./hr. However, only about 38% of the total initial activity was recovered by this technique. On one occasion, 28% of the initial activity was found to be in the supernatant after $(NH_4)_2SO_4$ saturation. Enzyme activity was also found in an extract of a mycelial mat of which the cells had been disrupted. A 14-day-old mat was well washed, ground with silver sand and extracted with 7 ml. of phosphate buffer, pH 6·2. After centrifuging and filtering, the supernatant showed an activity of 0·53 μmole/ml./hr. initially. This enzyme preparation differed from the others in that its rate of oxidation was not linear, but fell off to only one-seventh of its initial value after 28 min.

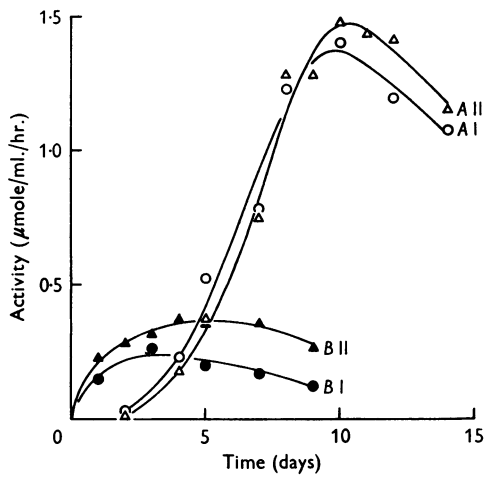

Fig. 1. Results of two experiments, I and II, showing the development of oxidase activity from *Polystictus versicolor*: *A*, in growth solution; *B*, in water to which the mycelium was transferred after 14 days' growth. Activity was estimated spectrophotometrically as described in the Materials and Methods section.

Table 1. *Relative rates of oxidation of various aromatic alcohols by the extracellular oxidase from* Polystictus versicolor

The aldehydes or ketones formed were estimated at wavelengths [λ (mμ)] where they had molar-extinction coefficients ϵ.

Alcohol	Concn. (mM)	λ (mμ)	ϵ	Relative oxidation rates (μmoles/hr.)
p-Methoxybenzyl alcohol	1·33	290	15 000	1·00
m-Methoxybenzyl alcohol	1·77	310	2 550	0·32
3:4-Dimethoxybenzyl alcohol	1·11	310	10 300	0·056
4-Hydroxy-3-methoxybenzyl alcohol	1·33	310	8 800	0·12
o-Hydroxybenzyl alcohol	1·33	245	7 200	0·003
p-Hydroxybenzyl alcohol	1·33	300	8 880	0·0007
Benzyl alcohol	1·33	240	9 600	0·036
4-Hydroxy-3-methoxycinnamyl alcohol	1·33	342·5	56 800	0·008
β-Naphthylcarbinol	0·67	245	34 000	1·14
1-(3:4-Dimethoxyphenyl)ethanol	1·33	300	8 340	<0·0004
1-(4-Hydroxy-3-methoxyphenyl)ethanol	1·33	305	8 050	<0·00001
1-(4-Methoxyphenyl)ethanol	1·33	290	11 250	<0·00001

Range of substrates. The enzyme was found to dehydrogenate all the primary aromatic alcohols examined, and also the unsaturated alcohol, 4-hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol), although at very different rates. No activity was detected with secondary aromatic alcohols, even when enzyme concentrates were used (Table 1). The formation of *o*- and *p*-hydroxybenzaldehyde, vanillin and 4-hydroxy-3-methoxycinnamaldehyde from the alcohols was confirmed by the appearance of their characteristic ultraviolet-absorption maxima in the range 330–400 mμ on making the test solutions alkaline (Lemon, 1947; Aulin-Erdtman, 1953). In the Warburg apparatus oxygen uptakes of 83, 52 and 6 μl. in 30 min. were obtained with *p*-methoxybenzyl alcohol, β-naphthylcarbinol and water. The uptake for *p*-methoxybenzyl alcohol exceeds the theoretical maximum (56 μl.), probably owing to the formation of hydrogen peroxide (see below). The enzyme concentrates gave no oxygen uptake with glucose, the primary alcohols ethanol and butanol or the L-forms of the amino acids leucine, methionine and proline. Conversely, glucose oxidase, which gave an oxygen uptake of 324 μl. in 25 min. in the presence of 0·1 M-glucose, did not oxidize *p*-methoxybenzyl alcohol, nor did the DPN-coupled alcohol dehydrogenase of yeast. The relative rates of oxidation of 0·2 M-ethanol and 0·66 mM-*p*-methoxybenzyl alcohol by the yeast dehydrogenase were found to be not less than 4000:1.

General properties. The rate of oxidation of *p*-methoxybenzyl alcohol followed the Michaelis equation up to substrate concentrations of 1·3 mM, but inhibition by substrate occurred at higher concentrations. As a result, the rate of oxidation of alcohol was almost independent of the concentration between 1·3 and 5·0 mM (Fig. 2). An alcohol concentration of 1·33 mM was selected for enzyme assay, as at higher concentrations absorption by the alcohol itself became troublesome at 290 mμ, where the aldehyde formed was estimated. The rate of oxidation of *p*-methoxybenzyl alcohol was directly proportional to enzyme concentration in the usual working conditions. Under these conditions, the oxidation of alcohol was linear with time, but at very high enzyme concentrations the rate of oxidation decreased with time, perhaps due to exhaustion of dissolved oxygen in the test solution. At such high enzyme concentrations, the formation of aldehyde was followed at 310 mμ, where the aldehyde absorbs less strongly. Measurements at this wavelength were also used to show inhibition

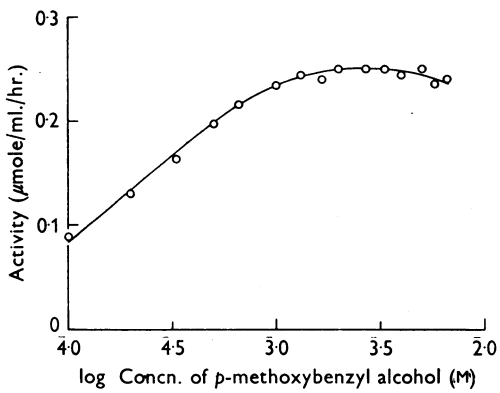

Fig. 2. Effect of the concentration of *p*-methoxybenzyl alcohol on its rate of oxidation by the oxidase from *Polystictus versicolor*. The source of enzyme was water to which the mycelium had been transferred from growth solution 5 days previously. Rates of oxidation were estimated spectrophotometrically, as described in the Materials and Methods section. The alcohol concentration was varied while the enzyme concentration remained constant.

of the oxidation of *p*-methoxybenzyl alcohol by the presence of *p*-methoxybenzaldehyde (Fig. 3). The enzyme showed its maximum activity between pH 6·0 and 6·5 in 0·022 M-phosphate buffer (Fig. 4). Activity fell off rapidly above pH 7·0, but was higher in tris buffer than in phosphate buffer.

Enzyme solutions were stable, and could be kept at room temperature for many days without marked loss in activity. Heating for 5 min. at temperatures up to 45° had no effect, but above this temperature activity rapidly fell off, the enzyme being totally inactivated after heating to 55° for 5 min. Activity was unaffected by 0·67 mM-*p*-chloromercuribenzoate in the test solution, indicating that SH groups do not contribute to the activity. Also no heavy-metal ions appear to be involved, as activity fell by only 12% in the presence of 2 and 4 mM-KCN, and by only 9% in the presence of 2 and 4 mM-NaN_3. No diffusible cofactor is involved, as the activity of an enzyme solution was unaffected by dialysis against 0·067 M-phosphate buffer for 24 hr. The activity of the enzyme was found to be unaffected by the addition of 0·15 μmole of DPN or TPN to the usual test solution.

Formation of hydrogen peroxide. The formation of hydrogen peroxide during oxidation of *p*-methoxybenzyl alcohol by the enzyme was indicated by the appearance of a red–brown when *o*-dianisidine and peroxidase were present (Huggett & Nixon, 1957). To 1·5 ml. of reagent solution [1–2 mg. of peroxidase and 0·5 ml. of 1% (w/v) *o*-dianisidine in 95% (v/v) ethanol, in 29·5 ml. of phosphate buffer, pH 7·0] were added 0·5 ml. of 5 mM-*p*-methoxybenzyl alcohol and 1 ml. of growth solution of activity 0·74 μmole/ml./hr. After 1·5 hr. the test solution had an extinction of 1·015 at 420 mμ. No colour developed in a control from which *p*-methoxybenzyl alcohol was omitted. Oxidation of *p*-methoxybenzyl alcohol by the enzyme ceased after bubbling nitrogen through the usual test solution for 15 min. On adding 0·15 μmole of DPN or TPN to the anaerobic solution, no formation of reduced DPN or TPN could be detected by absorption measurements at 340 mμ. On bubbling oxygen through an anaerobic solution, oxidation began again. In solutions saturated with oxygen at atmospheric pressure, the rate of oxidation was only 10% higher than when saturated with air.

These results indicate that the enzyme transfers hydrogen from aromatic alcohols directly to molecular oxygen, but cannot transfer hydrogen to

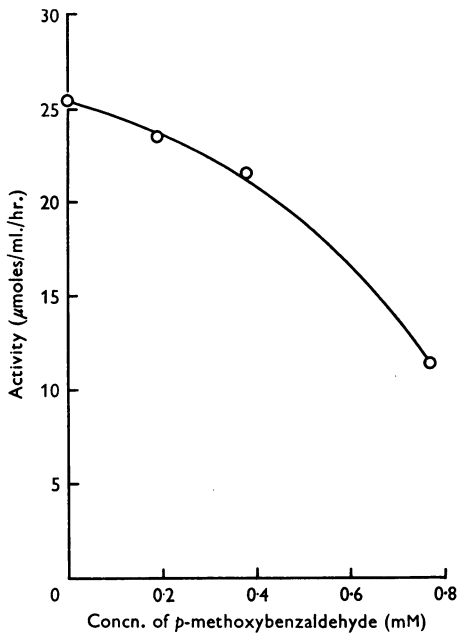

Fig. 3. Inhibitory effect of *p*-methoxybenzaldehyde on the rate of oxidation of *p*-methoxybenzyl alcohol by oxidase from *Polystictus versicolor*. The source of enzyme was a 50-fold dilution of a concentrate prepared from a 10-day-old growth solution. The rate of oxidation was determined spectrophotometrically, aldehyde being added to the usual assay solution before making up to 3 ml. (see Materials and Methods section).

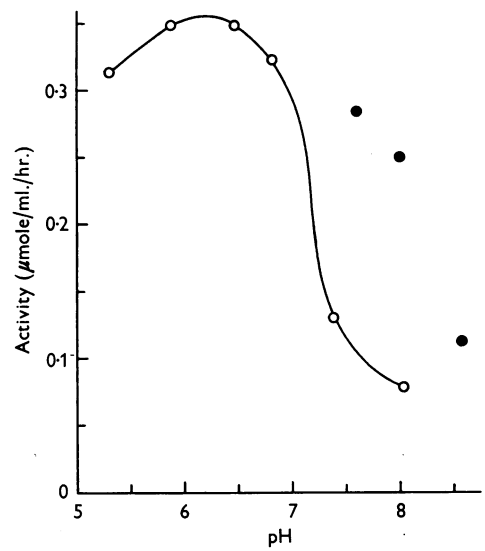

Fig. 4. Effect of pH on the rate of oxidation of *p*-methoxybenzyl alcohol by the oxidase from *Polystictus versicolor*, the source of enzyme being water to which the mycelium had been transferred from growth solution 7 days previously. The buffers used, Sørensen's KH_2PO_4–Na_2HPO_4 (○) and tris (●), were substituted for the usual phosphate buffer in the assay solution used for spectrophotometric determination of oxidation rates.

DPN or TPN. Transfer of hydrogen to methylene blue was observed under anaerobic conditions. A portion (1 ml.) of enzyme concentrate was added to 1 ml. of phosphate buffer, pH 6·2, 0·5 ml. of 0·534 mM-methylene blue and 0·5 ml. of 0·04 M-*p*-methoxybenzyl alcohol or 0·5 ml. of water in Thunberg tubes, after evacuating and filling the tubes with nitrogen three times. After incubation for 4 hr. at 22° in daylight or in the dark the methylene blue was reduced 60 % in the presence of the alcohol and was unchanged in the control. The rate of transfer of hydrogen to methylene blue by the enzyme was only about one-fiftieth of the rate of transfer to oxygen.

Polyphenoloxidase activity was absent from a growth solution and an enzyme concentrate. The former was tested by the method of Dion (1952), with 1 ml. of 10 days' growth solution and 5 ml. of 0·1 % (w/v) solutions of catechol, guaiacol, phenol and *p*-cresol. After incubation for 24 hr. at 25° no coloured products were formed. The enzyme concentrate was tested for oxygen uptake in the Warburg apparatus. Portions (1 ml.) of enzyme solution and 1 ml. of phosphate buffer, pH 6·2, were added to the main compartments of the flasks and 0·5 ml. of 0·02 M solutions of catechol and *p*-cresol were tipped in from the side arms. No oxygen uptakes were recorded nor were any coloured products formed.

DISCUSSION

The aromatic-alcohol oxidase of *Polystictus versicolor* differs from alcohol dehydrogenase in not oxidizing ethanol and butanol and in being independent of DPN. Ose & Hironaka (1957) reported an alcohol dehydrogenase which reduced benzaldehyde and which was not identical with, but was very similar to, ethanol dehydrogenase. Gillette (1959) presented evidence of an aromatic-alcohol dehydrogenase, from rabbit liver, which oxidized *p*-nitrobenzyl alcohol to *p*-nitrobenzaldehyde, but this enzyme was also dependent on DPN. Various aromatic aldehydes, including some which have been examined in the present work, have been found to be reduced to give the corresponding alcohols when added to cultures of brewer's yeast which were vigorously fermenting sugars (Neuberg, 1949; Higuchi, Kawamara & Ito, 1955). The reduction is linked with the fermentation.

The enzyme of *Polystictus versicolor* has not yet been obtained in sufficient concentration and purity to identify its prosthetic group with certainty. However, its general properties suggest that it is a flavoprotein, as they closely resemble those of known enzymes of this type, such as the amino acid oxidases, and glucose oxidase. Like these it is scarcely inhibited by cyanide or azide, and transfers hydrogen directly to molecular oxygen to give hydrogen peroxide. It transfers hydrogen to methylene blue rather slowly, as does, for instance, the D-amino acid oxidase of kidney (Krebs, 1935). The L-amino acid oxidase of kidney is known to oxidize α-hydroxy acids, including the aromatic phenylglycollic acid (Ratner, 1955), but it is certainly distinct from the enzyme examined here, which does not oxidize L-amino acids.

The release of this aromatic-alcohol oxidase into solutions in contact with mats of the fungus provides an explanation of previous observations made with the Kluyver technique (Farmer *et al.* 1959). It was then found that solutions of *m*- and *p*-methoxybenzoic acid and of 3:4-dimethoxybenzoic acid were completely reduced in the presence of fungal mats to give mixtures of the corresponding alcohols and aldehydes, but benzoic acid gave only the alcohol. These observations can be interpreted as being the result of a dynamic equilibrium in which the aldehydes were reduced to alcohols within the cells of the fungus, while the alcohols were re-oxidized to the aldehydes by the extracellular enzyme. The relative rates of these two processes must determine the ratio of aldehyde to alcohol at equilibrium. Our present observations give information only on the rates of oxidation of the alcohols, so these do not exactly parallel the amounts of aldehyde formed on reducing the corresponding acids by fungal mats.

Polystictus versicolor is a white rot, attacking the lignin of wood. Henderson (1955) found that vanillic acid and syringic acid could be extracted from a hardwood sawdust after this fungus had been growing on it for some weeks, but that only vanillic acid could be extracted from a softwood sawdust, in agreement with the known aromatic structure of the lignin of these woods (Brauns, 1952). The initial attack on the lignins must be by extracellular enzymes, causing a degradation of the lignin to water-soluble substances which can be absorbed by the fungal cells. The possibility that the extracellular enzyme examined here plays some part in the breakdown of the lignin is being further investigated.

SUMMARY

1. Growth media of *Polystictus versicolor*, or water left in contact with mycelial mats of this fungus, contained an enzyme system which transferred hydrogen from aromatic alcohols to molecular oxygen, with the formation of aromatic aldehydes and hydrogen peroxide. No phenol-oxidase activity was detected.

2. Activity was estimated either by spectrophotometric estimation of the rate of aldehyde formation, or by oxygen uptake.

3. The enzyme system could be concentrated from growth media, though with some loss, by ammonium sulphate precipitation.

4. Of the substances tested, all the primary aromatic alcohols, which included β-naphthylcarbinol, benzyl alcohol and seven other ring-substituted benzyl alcohols, were oxidized, but three secondary 1-phenylethanols were not. Glucose, ethanol, butanol and the L-amino acids tested were not oxidized.

5. Enzyme activity was little affected by *p*-chloromercuribenzoate, cyanide or azide ions.

6. There was no evidence for pyridine nucleotide participation in the reaction.

We are indebted to Dr D. M. Webley for valuable discussions during the course of this work.

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Plant Polyphenols

1. ANTHOCYANIN PRODUCTION IN THE CULTIVATED POTATO

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Earlier studies of the biochemical effects of single gene substitutions upon anthocyanins in plants (Scott-Moncrieff, 1936; Lawrence, 1950) have neglected a detailed consideration of both the glycosidic nature of the pigments and the structure of the related polyphenols, e.g. flavones and cinnamic acids, that were also present. Even in more recent studies, e.g. of the flavonoids of known genotypes of *Antirrhinum majus* (Geissman, Jorgensen & Johnson, 1954), no information was obtained about the genetical control of glycosidation. A study of the glycosidic pattern of anthocyanins, flavones and related polyphenols in genetically analysed plants would therefore add considerably to our understanding of the role of glycosidation and other gene-controlled processes in flavonoid biosynthesis.

For this purpose colour mutants of the cultivated diploid potato were particularly suitable. Dodds & Long (1955) had shown that anthocyanin production was controlled by the presence of two genes. The gene *P* controlled production of an acylated petunidin glycoside and a gene *R* controlled the production of an acylated pelargonidin glycoside in the tuber and a cyanidin glycoside in the flower. Later, a third factor, *Ac*, controlling the acylation of the anthocyanins, was found (K. S. Dodds & D. H. Long, unpublished results). The detailed structures of the anthocyanins present were not investigated. The only previous detailed examination of the anthocyanins of the cultivated potato was that of Chmielewska (1936), who studied pigments present in the skin and flesh of a purple–black variety called Negresse. A malvidin 3-rhamnosylglucoside, acylated with *p*-coumaric acid (negretein), and a 3-monoglucoside (tuberin) of a supposedly unknown anthocyanidin, tuberinidin, were reported.

In the present work, ten anthocyanins have been found in tubers or flowers of cultivated potatoes.