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THE OXIDATION OF ARBUTIN BY ISOLATED CHLOROPLASTS OF ARBUTIN-CONTAINING PLANTS

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Abstract—A new explanation is proposed to explain the blackening of *Pyrus* leaves. The phenolic glucoside arbutin present in the leaves is oxidized directly by enzymes in the chloroplasts, but not present in chromoplasts, leucoplasts or cytoplasm. This enzyme is also present in the chloroplasts of other arbutin-containing plants, for instance, *Pyrola japonica* and *Saxifraga stolonifera*. The oxidation produces successively six chromatographically-detected glucosides by two independent pathways. One of the first products is shown to be 3,4-dihydroxyphenyl-β-D-glucoside. The two independent pathways are controlled by two different parts of chloroplasts; the one being a protein fraction dissociable with cholate, while the other is firmly bound to the structure of the chloroplasts.

INTRODUCTION

THE LEAVES of certain varieties of *Pyrus* turn black when damaged or after abscission in autumn. This has long been attributed to the indirect oxidation of arbutin (*p*-hydroxyphenyl-β-glucoside) which is present in *Pyrus* leaves in comparatively large amount. It has been supposed that arbutin is first hydrolysed by β-glucosidase (β-D-glucoside glucohydrolase) and the quinol liberated is then oxidized to black pigments by an enzyme of laccase type in the tissue, or by atmospheric oxygen. The fact that the leaves of other varieties of *Pyrus* first develop a yellow and then a black colour has been similarly ascribed to the indirect oxidation of another glucoside, methylarbutin, which is found in larger amounts than arbutin in these varieties.[1,2]

According to the results of the present experiments, it appears that the above explanation is not entirely adequate. It seems that instead arbutin is directly oxidized without hydrolysis to give a coloured substance or substances by an oxidase present in the chloroplasts. The distribution of such oxidases in arbutin-containing plants, some properties of the chloroplast enzyme, and the nature of the oxidation products have been investigated.

RESULTS

Several plants are known to contain arbutin in the tissues, for example, *Pyrus* varieties, *Pyrola japonica*, *P. rotundifolia*, *P. nephrophylla*,[3] *Saxifraga stolonifera*[4] and *Gaultheria miqueliana*.[3] The isolated chloroplasts from all these were investigated but only those from a *Pyrus* variety, *Pyrola japonica* and *Saxifraga stolonifera* were shown to be capable of oxidizing arbutin (Fig. 1, Table 1).

The chloroplasts from these three species were, however, all incapable of oxidizing quinol, the aglucone of arbutin, methylarbutin, *ortho*-substituted monophenols and *meta*-dihydric

[1] E. BOURQUELOT and A. FICHTENHOLZ, *J. Pharm. Chim.* 7, 3 (1911).
[2] M. BRACKE, *Ann. soc. roy. sci. méd. nat. Bruxelles* 164 (1925).
[3] J. L. VAN RIJN, *Die Glykoside*, Berlin (1931).
[4] H. DANNER, *Botan. Arch.* **41**, 168 (1940).

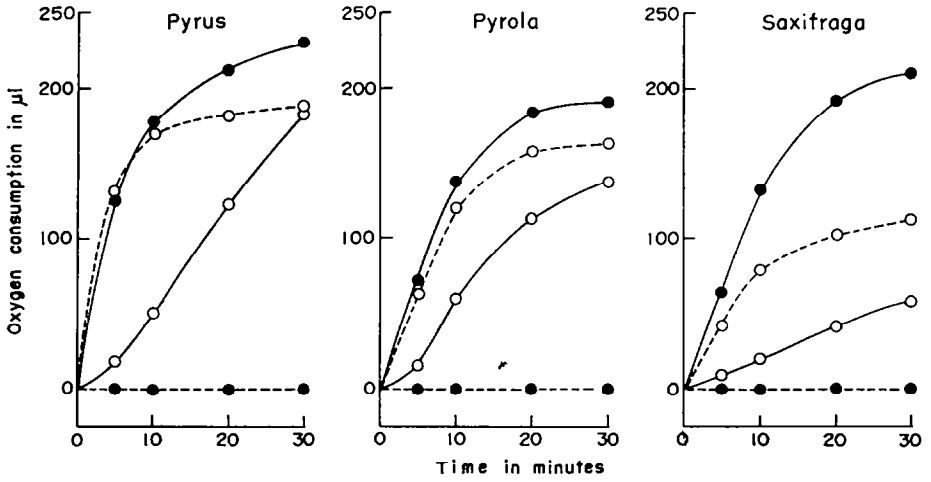

FIG. 1. OXYGEN UPTAKE BY CHLOROPLASTS FROM *Pyrus* VARIETY, *Pyrola japonica* AND *Saxifraga stolonifera* WITH ARBUTIN (—○—), *p*-CRESOL (—●—), CATECHOL (--○--) AND QUINOL (--●--). See Experimental.

phenols. On the other hand, all the *para*-substituted mono-phenols and *ortho*-dihydric phenols tested were oxidized. Thus the enzymic activity of the chloroplasts of these three plants is limited to *para*-substituted mono-phenols and *ortho*-dihydric phenols which are typical substrates of so-called tyrosinase (*o*-diphenol:O_2 oxidoreductase).*

TABLE 1. OXYGEN UPTAKE* BY VARIOUS PLASTID PREPARATIONS, WITH SEVERAL PHENOLIC SUBSTRATES

	Source of plastid							
	The *Pyrus* variety				*Pyrola japonica*			*Saxifraga stolonifera*
Substrate	Leaf	Bark	Unripe fruit	Ripe fruit	Leaf	Unripe fruit	Rhizome	Leaf
Arbutin	82	36	7	0	73	30	0	23
L-Tyrosine	34	16	4	0	15	4	0	9
p-Cresol	188	157	158	93	154	158	69	123
Quinol monomethylether	282				129			81
Catechol	231	247	200	167	154	58	77	104
Chlorogenic acid	267	185	159	146	128	25	42	33
Quinol	0	0	0	0	0	0	0	0
Guaiacol	0				0			0
Resorcinol	0				0			0
Methylarbutin	0				0			0

* O_2 µl. in 10 min., with 50 mg chloroplasts (in dry weight).

* Although this is the recommended nomenclature of the Commission on Enzymes of the I.U.B. it would be better if the hydroxylating activity of the enzyme was also mentioned (e.g. tyrosine-3-hydroxylase, cf. 1.99.1.1) ED.

The crude enzyme obtained from the cytoplasm of the leaves of the three plants was not capable of oxidizing arbutin at all. The *Pyrus* enzyme showed activity with catechol, chlorogenic acid, L-tyrosine and *p*-cresol, but not with quinol (*p*-diphenol:O_2 oxidoreductase, laccase). Hence laccase has nothing to do with blackening of *Pyrus* leaf. The preparations from *Pyrola* and *Saxifraga*, although showing low activity toward catechol and *p*-cresol, did not oxidize quinol, tyrosine or chlorogenic acid.

In order to examine the distribution of arbutin oxidizing activity in organs other than the leaf, the plastids from *Pyrus* bark, green rind of unripe fruit of *Pyrus*, unripe fruit of *Pyrola*, ripe fruit of *Pyrus* and rhizome of *Pyrola* were tested (Table 1). Of these five preparations, the first three, which have chloroplasts and chromoplasts, were able to oxidize arbutin. The last two, on the other hand, which do not contain chloroplasts, did not attack arbutin, although they oxidize other substrates (Table 1).

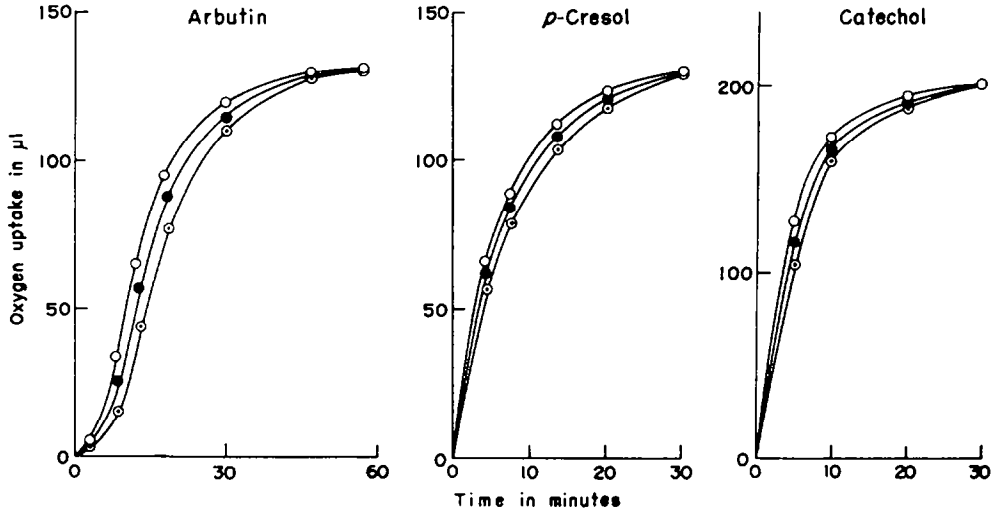

FIG. 2. OXYGEN UPTAKE BY THE *Pyrus* WHOLE CHLOROPLASTS, AND TWO DEPLETED CHLOROPLAST PREPARATIONS WITH ARBUTIN, *p*-CRESOL AND CATECHOL; WHOLE CHLOROPLASTS, —○—; *DC-I*, —●—; *DC-II*, —⊛—. See Experimental.

As mentioned above, the chloroplasts of *Pyrola rotundifolia*, *P. nephrophylla* and *Gaultheria miqueliana* did not oxidize arbutin at all, although they showed activity with catechol, chlorogenic acid and *p*-cresol. A partially purified "tyrosinase" from dahlia tuber (*Dahlia variabilis*) also lacked arbutin oxidizing ability.

Two chloroplast fractions freed from stroma were prepared from *Pyrus* by blending with sodium cholate and centrifugation (*DC-II* and *DC-I*). The oxygen uptake curves are shown in Fig. 2. The initial rates do not differ significantly in the three preparations, and it appears that the enzyme is not present in stroma, but firmly bound to the structure of the chloroplasts and not dissociated with cholate.

The chromatogram of phenolic substances which appeared after one-day incubation of a solution of arbutin with chloroplasts from a *Pyrus* variety, *Pyrola japonica* and *Saxifraga stolonifera* are shown in Fig. 3. A mixed chromatogram of the three products showed no separation and these three chloroplasts are regarded as giving the same products. Since none of these products appeared under anaerobic conditions, oxygen is indispensable for at least some steps of the reaction. On exposure to air, unsprayed chromatogram gradually develops

a violet colour at the place corresponding to *A*, and a brown to *D* and *F* (Table 2), showing these three substances are autoxidizable.

With chloroplasts from *Pyrus* and *Pyrola*, the substances produced were independent of the substrate concentration, but with a substrate concentration less than 0·02 M the chloroplasts of *Saxifraga* did not give rise to *D*, *E*, or *F*, although *B* and *C* were found together with a small amount *A*.

Chloroplasts from *Saxifraga* were found to contain a small amount of chlorogenic acid (0·7 mg/g dry) and this accounted for the variation in the kinds of the products from arbutin

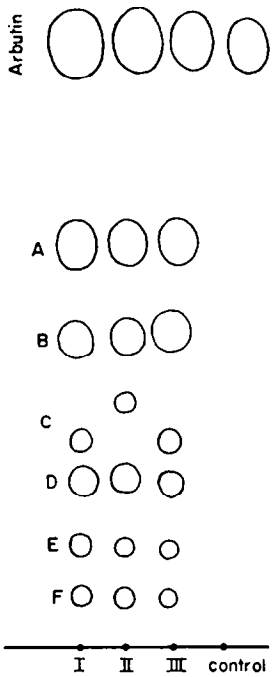

FIG. 3. CHROMATOGRAM OF OXIDIZED ARBUTIN USING CHLOROPLASTS OF THE *Pyrus* VARIETY (I), *Pyrola japonica* (II) AND *Saxifraga stolonifera* (III). Substrate is 0·02 M, with chloroplasts (50 mg dry wt) in 1 ml solution for overnight. Solvent is n-butanol–acetic acid–water (4:1:2), chromatogram developed with reagent (I).

upon the substrate concentration. Fragments of *Saxifraga* chloroplasts freed from chlorogenic acid by dialysis, produced *A* and *D* independently of arbutin concentration, while the addition of a small amount of chlorogenic acid inhibited the formation of *A* and *D*, especially the latter, and enhanced the production of *B* and *C* with substrate concentration more than 0·02 M. From these facts it is obvious that chlorogenic acid has an inhibitory effect on the production of *A* and *D* from arbutin. Chloroplasts from the *Pyrus* variety and *Pyrola japonica* did not contain any chlorogenic acid.

It was doubtful whether all the products (*A* to *F*) were enzymically produced, since the reaction time used was very long, and it appeared probable that some of them might have been non-enzymically produced from arbutin or products of arbutin oxidation. In order to clarify this point, the compounds produced in the early reaction stage were examined, and it can be seen (Fig. 4) that *A* and *D* can be regarded as the main products of enzymic oxidation. When ascorbic acid was added to the solution just as it was developing an orange colour, *A*

TABLE 2. REACTION OF THE OXIDATION PRODUCTS OF ARBUTIN WITH VARIOUS REAGENTS

Substance	$R_{arbutin}$*	Reagents†				Autoxidation
		I[a]	II[b]	III[c]	IV[b]	
Catechol		+	+	–	+	—
Resorcinol		+	–	Green	–	—
Quinol		+	–	–	+	—
Arbutin	1·00	+	–	–	+	—
A	0·68	+	+	–	+	Violet (then brown)
B	0·52	+	–	Pink	–	—
C	0·33	+	–		–	—
D	0·26	+	+	–	+	Brown
E	0·13	+				—
F	0·07	+				Brown

* In n-BuOH:HOAc:H_2O (4:1:2).

† I, 1% ferric chloride–1% potassium ferricyanide; II, alkaline 0·5% potassium ferricyanide solution (pH = 8·0); III, alkaline catechol solution; IV, 1% phosphomolybdic solution; [a] on chromatogram; [b] on chromatogram and in test tube; [c] in test tube.

accumulated and further formation of *D* was suppressed. *A*, together with an intermediate coloured quinoid compound which is easily reduced back to *A* by ascorbic acid, must therefore be regarded as being formed before *D*. After several hr incubation, substances *B* and *C*

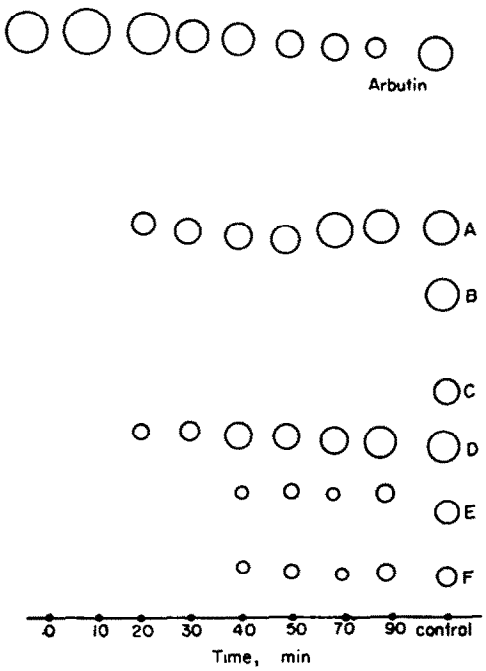

FIG. 4. A CHROMATOGRAM SHOWING THE TIME COURSE OF ARBUTIN OXIDATION. (Using *Pyrus* and *Pyrola*, chloroplasts gave chromatograms of similar type.) Substrate is 3·3 mM chloroplasts, (50 mg dry wt) in 1 ml solution.

appeared in that order. This sequential appearance was clearly demonstrated by using *Saxifraga* chloroplasts with 0·01 M arbutin. Because *B* and *C* are stable and do not undergo any oxidation, they may be other enzymic products.

When a two-dimensional chromatogram of the products was dried in the air for several hr after running in the first direction, and then run in the other direction with the same solvent, it was easily seen that *A* was converted into *D*, *E* and *F* (especially *E*), while *D* gave rise to *F* (Fig. 5). From this experiment it was clearly established that *A* was converted into *F* via *D*,

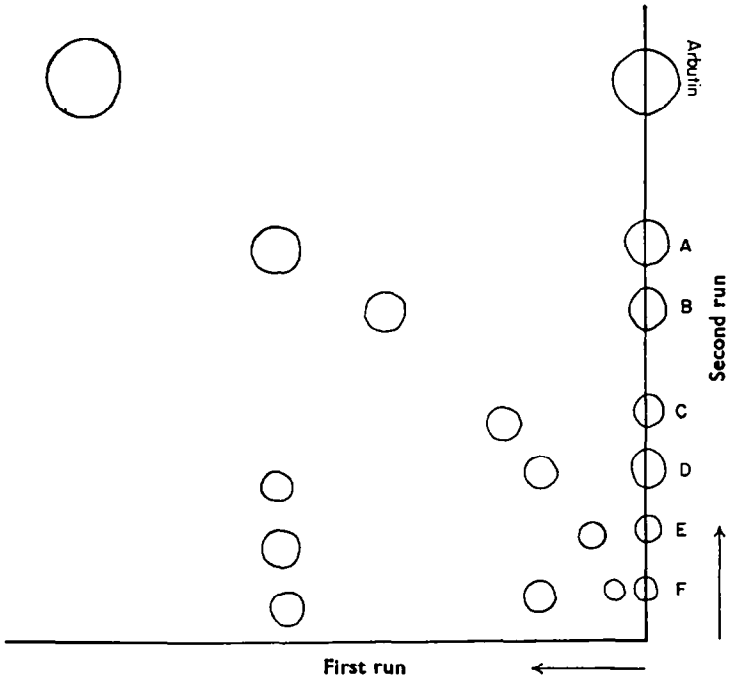

FIG. 5. A TWO-DIMENSIONAL CHROMATOGRAM OF THE REACTION MIXTURE OF ARBUTIN OXIDATION WITH *Pyrus* OR *Pyrola* CHLOROPLASTS, INDICATING THE NON-ENZYMIC TRANSFORMATION BETWEEN THE PRODUCTS. Chromatogram dried for several hr in the air between the first and second runs (solvent, n-butanol–acetic acid–water (4:1:2) both ways).

or into *E*, non-enzymically. The route from *A* to *F* was confirmed when it was found that the addition of ascorbic acid to a reaction mixture which was deep brown induced the accumulation of *D* and *A*, and the suppressed production of *F*.

To gather more information about the chemical nature of the products, attempts were made to isolate them with large-scale chromatography, and *A*, *B*, and *D* were obtained. The absorption curves of *A*, *B* and *D* (Fig. 6), λ_{max} 283, 283 and 292 mμ, respectively show no extra conjugation over that of arbutin (282 mμ). Hydrolysis of these products with either 1 N HCl at 100° for 10 min, or with β-glucosidase (in 0·05 M phosphate buffer, pH = 6·0) at room temperature for 1 hr showed that they were glucosides, glucose being detected with Horrock's reagent after chromatography of the hydrolysate. Although this test was carried out only with *A*, *B* and *D*, it is probable that the other products also contain glucose. In the hydrolysate of *A* treated with β-glucosidase, a spot corresponding to 1,2,4-trihydroxybenzene was found. The hydrolysate of *B* and *D* also gave phenolic aglucones, but their chemical natures were not determined.

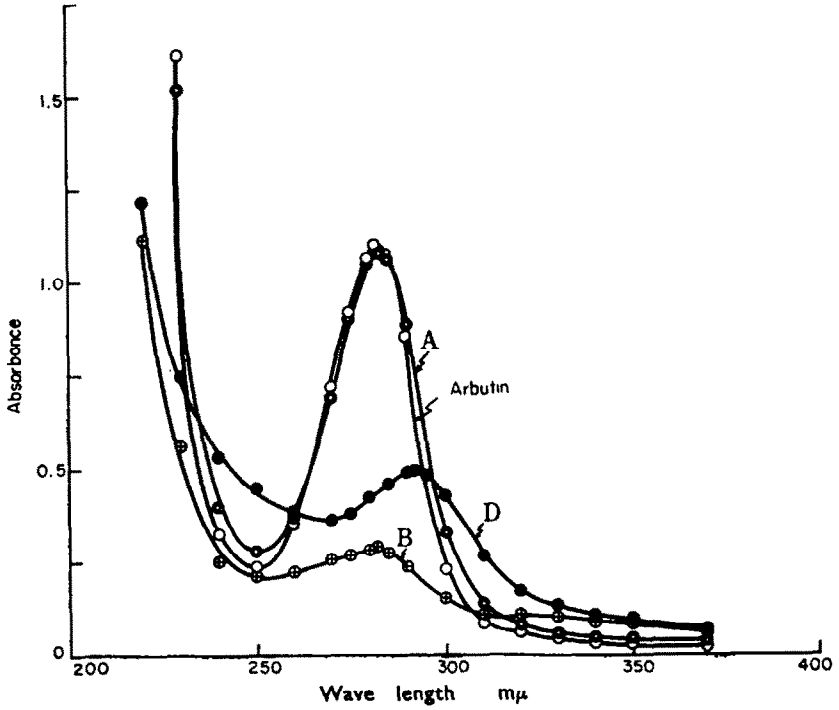

FIG. 6. U–V ABSORPTION CURVES OF *A*, *B*, *D* AND ARBUTIN.

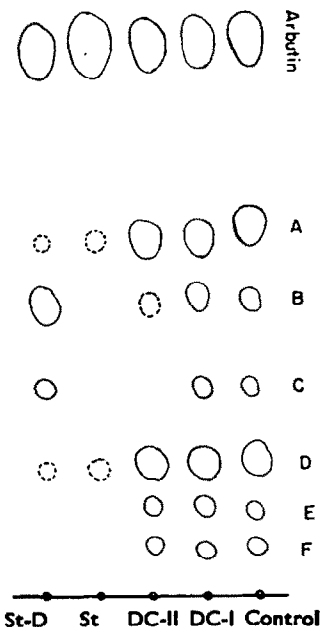

FIG. 7. CHROMATOGRAM OF THE PRODUCTS OF ARBUTIN OXIDATION BY VARIOUS FRACTIONS OBTAINED FROM THE *Pyrus* WHOLE CHLOROPLASTS. Abbreviations, see text.

The oxidation products by various fractions obtained from *Pyrus* whole chloroplasts, when incubated in arbutin solution for 2 hr at 30°, were compared and the results are shown in Fig. 7. The products by *DC-I* were the same with those obtained by the whole chloroplasts, whereas stroma (*St*) gave no significant reaction. It was found, however, that the formation of *B*, and possibly *C*, was catalysed by enzyme dissociated by cholate from the chloroplasts (*St-D*), while *A* and *D* were formed by the enzyme that remained in the chloroplasts (*DC-II*) after treatment with cholate (Fig. 7).

DISCUSSION

From the results shown above, the postulated hypothesis on blackening of *Pyrus* leaves mentioned previously[1,2] is obviously not valid. The crude enzyme from *Pyrus* leaf cytoplasm showed no oxidizing activity against quinol, and the idea of an "indirect" oxidation as suggested by previous authors is not supported.

The present data also show that neither the production nor the oxidation of quinol could take place in *Pyrus* leaf chloroplasts. The lack of activity in the chloroplasts of oxidation of methylarbutin also shows the complete absence of any β-glucosidase since its aglucone (quinol monomethylether) is very rapidly oxidized.

The chloroplasts were, however, found to oxidize arbutin itself, and the products of this oxidation were shown to contain glucose. It should be pointed out that there have been a few reports regarding discoloration of plant tissue caused by the hydrolysis of the glucoside *in situ*, for example, aucubin in *Aucuba japonica*[2,5] and probably furcatin in *Vibrunum furcatum*,[6] but none regarding discoloration due to the direct oxidation of the glucoside itself without prior hydrolysis.

It may thus be concluded that the *Pyrus* leaf chloroplasts oxidize arbutin and this leads to blackening in the leaf. This direct oxidation of arbutin is also carried out by chloroplasts of *Pyrola japonica* and *Saxifraga stolonifera*, both of which contain this glucoside (Fig. 1 and Table 1), but others such as *Pyrola nephrophylla*, *P. rotundifolia* and *Gaultheria miqueliana* lack this ability. Experiments with chloroplasts of some arbutin-deficient plants showed that they too were unable to attack arbutin.

Of the plastid preparations from the various organs of the *Pyrus* variety, *Pyrola japonica* and *Saxifraga stolonifera*, only those also containing chloroplasts are able to oxidize arbutin, no activity being found in the chromoplasts of *Pyrus* fruit and leucoplasts of *Pyrola* rhizome. The crude enzymes from the leaf cytoplasm of the three plants were also unable to attack this glucoside. It can be concluded therefore that arbutin is oxidized only by chloroplasts, and only by those from some of the plants that contain this glucoside in their tissue.

Of the six products of arbutin oxidation (Fig. 3), *A* and *D* are regarded as the main enzymic products (Fig. 4) and *A* is considered to be converted into *D* via an intermediary quinoid substance. Under non-enzymic conditions it was found that *A* was largely transformed into *E*, and to a smaller extent into *F* via *D* (Fig. 5). However, *E* and *F* might be formed also by enzymic action to some extent. *B* on the other hand, is directly formed from arbutin, and transformed into *C* by the action of an enzyme, as is shown by experiments using substrate concentrations less than 0·02 M with *Saxifraga* chloroplasts. Both *B* and *C* are stable and do not seem to be produced non-enzymically.

A was shown to be 3,4-dihydroxyphenyl-β-D-glucoside: the reaction of *A* to various reagents (Table 2) showed it was a phenolic substance with ene-diol group(s), and hydrolysis

[5] S. HATTORI and M. KURIHARA, *Misc. Repts. Research Inst. Nat. Resources (Tokyo)* **17–18**, 163 (1950).
[6] H. Imaseki, *Botan. Mag. (Tokyo)* **72**, 323 (1959).

gave 1,2,4-trihydroxybenzene. It was reported that stems of young beans immersed in a solution of quinol and glucose, or young shoots of beans fed with a solution of arbutin, produce a phenolic glucoside which was presumed to be 3,4-dihydroxyphenyl-β-D-glucoside.[7] It also seems to correspond to the compound produced by feeding *Vicia faba* with 1,2,4-trihydroxybenzene.[8]

D also has ene-diol group(s) but determination of its chemical nature has not been carried out. By analogy with the fact that laccase from mushroom produced a substance 2,3-dihydroxydiphenyl dioxide from *ortho*-benzoquinone which was an intermediary product from catechol,[9,10] it is probable that oxidative condensation of two molecules of the intermediary quinone from *A* might produce such a dioxide. The structures of *B*, *C*, *E* and *F* must await further investigation. The path of arbutin transformation can be summarized as shown in Fig. 8.

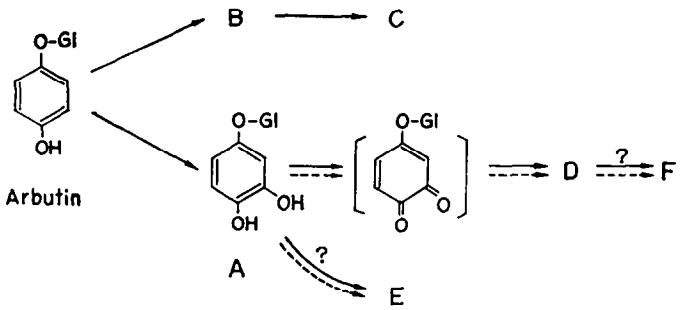

FIG. 8. SCHEME OF ARBUTIN OXIDATION (ENZYMIC, ⟶ NON-ENZYMIC, ⟿).

From the result shown in Fig. 7, it is obvious that the enzyme responsible for arbutin oxidation are not in stroma, but in the structural parts of the chloroplasts. By treating the chloroplasts with cholate it was established that an enzyme concerned with the production of *B* and *C* is extractable, while the enzyme responsible for the formation of *A* and *D* is in the chloroplast fragments.

The enzyme is firmly built-in to some uncharacterized structure of the chloroplasts, and treatment with cholate could not dissociate it. Previous workers have also reported the binding of phenolase to particulate fractions in the cell.[11–13]

Since the chloroplasts from *Pyrus* and *Pyrola* gave only *A* and *D* after half an hour incubation, the oxygen uptake determined manometrically must come largely from the oxidation of arbutin into *A* and thence into *D* via a corresponding quinone. It has been supposed that the oxidation of *para*-substituted monohydric phenols and that of *ortho*-dihydric phenols are catalysed by one enzyme (tyrosinase).[14–17] So the oxidation of arbutin and *A* may be catalysed by the same enzyme.

[7] J. B. PRIDHAM, *Nature* **182**, 795 (1958).
[8] J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **87**, 218 (1963).
[9] W. G. C. FORSYTH and V. C. QUESNEL, *Biochim. Biophys. Acta* **25**, 155 (1957).
[10] W. G. C. FORSYTH and V. C. QUESNEL, *ibid.* **37**, 322 (1960).
[11] P. H. LI and J. BONNER, *Biochem. J.* **41**, 105 (1947).
[12] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).
[13] W. D. BONNER, *ibid.* **30**, 30 (1955).
[14] H. S. MASON, *Nature* **177**, 79 (1956).
[15] H. DRESSLER and C. R. DAWSON, *Biochim. Biophys. Acta* **45**, 508 (1960).
[16] H. DRESSLER and C. R. DAWSON, *ibid.* **45**, 515 (1960).
[17] G. FÅHRAEUS and H. LJUNGGREN, *ibid.* **46**, 22 (1961).

From the fact that the chloroplasts were able to oxidize a series of typical tyrosinase substrates (tyrosine, *p*-cresol, catechol and chlorogenic acid) as well as arbutin the chloroplasts seem to possess a tyrosinase-like enzyme which convert arbutin into *A* and *D*. Many other chloroplast preparations examined however did not oxidize arbutin, though they showed activity to the other typical tyrosinase substrates. Furthermore, a partially purified tyrosinase preparation from dahlia tuber was unable to oxidize arbutin, and it has been reported that the tyrosinase prepared from *Bombyx mori* also could not oxidize this compound.[18] It may be supposed therefore that another type of enzyme, different from tyrosinase, might exist in the chloroplasts. In view of the results shown in Fig. 2, however, it does not seem that the oxidation of arbutin, *p*-cresol, and catechol is catalysed by different enzymes, and it is possible that the chloroplast enzyme may be regarded as merely having a different substrate specificity from the other tyrosinase preparations.

MATERIALS AND METHODS

The Preparation of Whole Chloroplasts

Chloroplasts were prepared by a modified method of Granick[19] and Arnon.[20] Fresh leaves of the plants (20 g) were blended in 200 ml of an equal volume of 0·5 M sucrose and 0·2 M Tris buffer (pH = 7·0–7·1) in the cold for 5 min. The homogenate was squeezed through silk cloth to remove cell debris and the filtrate centrifuged at $3000 \times g$ for 10 min, the residue being suspended in cold 0·3 M sucrose solution. The procedure was repeated three or four times to purify the chloroplast fraction. The preparation was finally subjected to two further centrifugations at $1000 \times g$ for 10 min and the supernatant was discarded to remove the mitochondria, chloroplast fragment or grana.

In *Pyrus* leaf homogenate, however, pectin-like substances prevented the chloroplasts from being separated using low speeds, so a high speed centrifugation ($12{,}000 \times g$) for 30 min was required. The subsequent procedures were the same as above.

The procedures for obtaining the whole plastid preparations from other organs were essentially the same as above.

The Preparation of Crude Enzyme from Leaf Cytoplasm

The leaves were blended with cold water, and the homogenate was centrifuged, at $1500 \times g$ for 10 min in *Pyrola* and *Saxifraga*, and at $12{,}000 \times g$ for 30 min in *Pyrus*, for removal of cell debris. The supernatant was cooled to 5°, and 2 vols. acetone at −15° added. (In the case of *Pyrus*, 1 vol. acetone was added first, the floating material discarded, and the second vol. of acetone then added.) The precipitate produced was collected by centrifugation, suspended in water, and dialysed against cold water for overnight.

The Preparation of Dahlia Tyrosinase

From dahlia tuber (*Dahlia variabilis*) a partially purified tyrosinase was prepared essentially according to the method of Dawson.[21]

Fractionation of the Pyrus Whole Chloroplasts

(*a*) The whole chloroplasts of *Pyrus* were blended in cold 0·05 M phosphate buffer (pH = 6·5), and the resulting homogenate was centrifuged for 20 min at $10{,}000 \times g$. Depleted

[18] S. SHO and T. NAKAMURA, *35th Annual Meeting of Japan Biochemical Society*, Tokyo (1962).
[19] S. GRANICK, *Am. J. Botan.* **25**, 558 (1938).
[20] D. I. ARNON, *Ann. Rev. Plant Physiol.* **7**, 325 (1956).
[21] C. R. DAWSON and W. B. TARPLEY, *The Enzymes*, New York, Vol. II, p. 462 (1951).

chloroplasts devoid of "stroma" was thus obtained from the whole chloroplasts and named *DC-I*. To the supernatant were added 2 vols. acetone at −15°, and the precipitate consisting of "stroma" protein was gathered by centrifugation and designated as *St*.

(*b*) Whole chloroplasts were blended in 1% (w/v) sodium cholate (the pH being adjusted to 6·5 with 0·05 M phosphate buffer) and left overnight. The suspension was divided into two fractions by centrifugation for 30 min at 10,000 × *g*. The pellet, which represented depleted chloroplasts devoid of stroma and that part of the bound protein which had been dissociated with cholate, was named *DC-II*, and the precipitate obtained from the supernatant by adding 2 vols. acetone at −15°, and consisting of the stroma protein and that part of chloroplast protein which had dissociated with cholate was given the name *St-D*.

DC-I and *DC-II* represent 65 and 58 per cent, respectively, of the dry weight of the whole chloroplasts.

Manometric Procedures

In the Warburg flask was placed a suspension of chloroplasts (50 mg dry weight) in 1 ml 0·04 M phosphate buffer (pH = 6·5) and 0·5 ml 0·01 M substrate in the side arm. The temperature was 30°, and the flask was oscillated 100 times/min.

Condition for Isolating Reaction Products

Preliminary experiments showed the pH optima of the reaction was in the range from 5·5 to 7·0. A mixture of 1 ml 6·6 mM arbutin solution and 1 ml suspension of chloroplasts (100 mg in dry weight) was therefore allowed to react at 30° without buffer. When the reaction time was overnight, toluene was added to prevent contamination. After the reaction was over, the chloroplasts were filtered off, and the filtrate used for chromatographic examinations.

The presence or absence of isotonic mannitol solution for preventing destruction of the chloroplasts was shown to have no effect either on oxygen uptake or the products obtained.

Paper Chromatography

(*a*) Whatman No. 1 filter paper was used throughout with n-butanol–acetic acid–water (4:1:2) as solvent, chromatogram developed with reagent (I).

(*b*) Reagents: (I) Equal volumes of 1% aqueous solution of $K_3Fe(CN)_6$ and 1% alcoholic solution of $FeCl_3$ used as a dip.[9] (II) 0·5% potassium ferricyanide solution (pH = 8·0) gives a pink or red colour only with *ortho*- or *meta*-dihydric phenols. (III) To one drop of test solution, a small quantity of catechol was added and the mixture made alkaline with 0·33 M NaOH. A green colour shows the existence of *meta*-dihydric phenols. Other phenols do not react.[22] (IV) 1% phosphomolybdic acid gives a blue colour (via green) when *ortho*- or *para*-dihydric phenols are present.[23]

Substrates

1,2,4-trihydroxybenzene[24] and methylarbutin[25] were prepared according to the literature, other compounds were available in the laboratory and purified where necessary by recrystallization, sublimation or distillation.

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