The Reaction of Carbohydrases with Phenolic Glucosides

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The biochemistry and physiology of phenolic glycosides in plants has been reviewed by Pridham (1960*a*). The biosynthesis of a phenolic diglucoside was first recorded by Miller (1941), who fed *o*-chlorophenol to *Gladiolus* corms and isolated the corresponding *o*-chlorophenyl β -gentiobioside. The enzymic formation of *p*-hydroxyphenyl β -gentiobioside from arbutin (*p*-hydroxyphenyl β -D-glucopyranoside) with a β -glucosidase preparation from aspen cambium (*Populus grandidentata*) has also been reported (Pridham, 1957, 1960*b*). With wheat-germ enzymes, however, the formation of this diglucoside from arbutin required uridine diphosphoglucose as a cofactor (Cardini & Yamaha, 1958, 1960). These wheat-germ extracts showed no β -glucosidase activity.

A study of the reaction of arbutin with enzyme preparations from almond (*Prunus amygdalus*) and broad bean (*Vicia faba* var. Johnson's Longpod) seeds suggests that β -glucosidase can catalyse the formation of a phenolic gentiobioside. When salicin (*o*-hydroxymethylphenyl β -D-glucopyranoside) was used as a substrate, *o*-hydroxybenzyl β -glucoside was produced, thus substantiating the early observations of Rabaté (1935), who investigated a similar reaction of salicin with an enzyme from willow (*Salix purpurea*) leaves.

MATERIALS AND METHODS

Ultraviolet-absorption spectra were measured in a Unicam SP. 500 spectrophotometer (1 cm. cell). Infrared measurements were made with a Unicam SP. 100 double-beam spectrophotometer. Control incubations were in all instances carried out with boiled enzyme preparations and all enzyme reactions were carried out in the presence of toluene.

Paper chromatography. Phenolic compounds and sugars were examined on Whatman no. 1 and no. 3 papers, respectively, by the descending technique. The solvent systems were: A, butan-1-ol-pyridine-water (6:4:3, by vol.); B, butan-1-ol-ethanol-water (40:11:19, by vol.) and C, ethyl acetate-acetic acid-water (9:2:2, by vol.). Diazotized *p*-nitroaniline- Na_2CO_3 (Swain, 1953) spray reagent was used to detect phenols and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), to detect the reducing sugars.

Paper electrophoresis. Whatman no. 3 paper and 0.1M-

sodium borate buffer (pH 10.0) was used as described by Pridham (1959). Mobilities are expressed as M_{SA} values, i.e. relative to the mobility of salicylic acid.

***p*-Hydroxyphenyl β -cellobioside.** This was synthesized by the method of Stánek & Kocourek (1953).

Enzyme preparations. Almond emulsin was prepared by the method described by Sumner & Somers (1947). Before use the enzyme solution was dialysed against running tap water (40 hr.) and then distilled water (5 hr.). Bean tissue was macerated with 0.07M-sodium phosphate (pH 7.0) buffer containing L-cysteine (0.5%, w/v) (about 1 g. of tissue/5 ml. of buffer) at 5°. The resulting extract was then centrifuged at 3000 rev./min. and the supernatant liquor dialysed against distilled water for 24 hr. at 5°. Enzyme preparations were made from (a) whole seeds (with testas removed) which had been soaked in water for 18 hr. at room temperature, (b) dormant embryos, (c) embryos from seeds treated as in (a), (d) embryos from germinating seeds which had been kept between layers of moist cotton wool for 6 days at room temperature, (e) cotyledons from seeds treated as in (a). Preparation (a) was fractionated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to give 50% saturation, the mixture was then put into a separating funnel and the precipitated protein allowed to rise to the surface. The clear solution was run off and the suspended protein dialysed against distilled water (24 hr., 5°) and freeze-dried. Preparations (c) and (e) were both dialysed successively against three changes of distilled water (4 days), 0.05M-sodium acetate, pH 5 (24 hr.) and 0.07M-sodium phosphate, pH 8.5 (24 hr.). The resulting solutions were then adjusted to pH 6.5 with acetic acid.

Each enzyme preparation was incubated with 2.0% (w/v) solutions of salicin and arbutin for 18 hr. at 27° and the reaction mixtures were examined on paper chromatograms for sugars and phenols. The incubations with arbutin were repeated in the presence of D-glucono-1 $\rightarrow$ 4-lactone (0.7%, w/v) for the emulsin and the enzyme preparations (a) and (b).

Compound (I). Arbutin (3 g.) was incubated with emulsin solution (50 ml.) for 5 days at 37°; the incubation mixture was deproteinized by the method of Sevag, Lackman & Smollens (1938) and then extracted with ether to remove quinol. The aqueous solution was then passed through a small charcoal-Celite column (Whistler & Durso, 1950) which was washed with water to remove glucose. Aqueous ethanol (50%, v/v) was used to elute the glycosides from the column and the eluate was then concentrated under reduced pressure to a thin syrup. A phenolic compound (I) (30 mg.) was obtained from the latter by chromatographic fractionation on Whatman no. 3MM paper (solvent B). Complete hydrolysis of (I) was effected

by heating with $N-H_2SO_4$ (100°; 2 hr.) and also by treating with emulsin (27°, 18 hr.). Heating with $N-HCl$ (100°, 10 min.) resulted in a partial hydrolysis of (I). Compound (I) (5 mg.) in water (1 ml.) was treated with bromine water (0.2M, 0.5 ml.) in the presence of Amberlite IR-4B (OH^- form) resin to neutralize any HBr formed. The solution was aerated to remove excess of Br_2 and then filtered and concentrated under reduced pressure to a syrup.

The optimum pH values for the formation of compound (I) with emulsin and with preparation (a) were estimated by incubating these enzymes (1 ml.) at 27° for 18 hr. with arbutin (5.0%, w/v) and either 0.1M-sodium citrate or 0.07M-sodium phosphate buffers (2 ml., pH range 3.0-8.0) and observing the rates at which compound (I) was formed, by paper chromatographic examination. Attempts to synthesize arbutin or compound (I), or both, were made by incubating 1 ml. of emulsin, preparation (a) or β -glucosidase (0.1%, w/v, aqueous solution; Sigma Chemical Co.) with quinol (20 mg.) and salicin, cellobiose, maltose or α -D-glucose 1-phosphate (100 mg. of each) in the presence of 0.05M-sodium acetate buffer (1 ml., pH 5.6) for 18 hr. at 27°.

Compound (II). Salicin (2 g.) was incubated with emulsin (66 ml.) for 7 days at 37°. The reaction mixture was then deproteinized and extracted with ether, as with compound (I), and the remaining solution concentrated and fractionated on Whatman no. 3MM paper (solvent B). Hydrolysis with $N-H_2SO_4$ and emulsin was carried out as with compound (I).

RESULTS

Paper-chromatographic examination of the products obtained by incubating arbutin with emulsin revealed the presence of glucose, quinol and a slower-moving component (I) (Table 1), which gave the same purple colour as arbutin with the diazonium spray reagent and also reacted with *p*-anisidine periodate (Bragg & Hough, 1958); small amounts of other compounds were also detected. In aqueous solution the spectrum of (I) resembled that of arbutin with an absorption maximum at 283 $m\mu$ (cf. Charlampowiczówna & Marchlewski, 1930). After addition of bromine water, this peak was replaced by two other maxima at 246 and 268 $m\mu$ which are characteristic of *p*-benzoquinone (cf. Braude, 1945). The solution after bromine treatment contained a compound which gave a brown colour with *p*-anisidine hydrochloride and co-chromatographed with authentic gentiobiose. (Gentiobiose was clearly distinguished

from all other glucopyranosyl disaccharides by solvent C.) This evidence suggested that compound (I) was *p*-hydroxyphenyl gentiobioside. Complete acid hydrolysis of the gentiobioside yielded glucose and quinol and a partial hydrolysis gave a disaccharide which was chromatographically identical with gentiobiose. Treatment of the gentiobioside with emulsin produced glucose, quinol, and arbutin, thus suggesting the presence of β -links in the molecule; this was confirmed by infrared measurements on compound (I), which showed a characteristic absorption band at 891 cm^{-1} (Barker, Bourne, Stacey & Whiffen, 1954). The behaviour of the β -gentiobioside (I) differed from that of authentic *p*-hydroxyphenyl β -cellobioside on paper chromatography and electrophoresis (Table 1).

Incubation of arbutin with enzyme preparations from broad-bean tissues also produced a compound identical with *p*-hydroxyphenyl β -gentiobioside and the pH optimum for the formation of this compound was between 5 and 6 for both the bean [preparation (a)] and emulsin. All enzyme preparations which were tested produced the β -gentiobioside when incubated with arbutin. They also hydrolysed the latter compound, and salicin, to glucose and the corresponding phenolic aglycone. This reversible β -glucosidase activity was inhibited by D-glucono-1 $\rightarrow$ 4-lactone with emulsin and enzyme preparations (b) and (a). The protein fraction (a) obtained by precipitation with 50% ammonium sulphate saturation retained its ability to synthesize *p*-hydroxyphenyl β -gentiobioside as did preparations (c) and (e). Unsuccessful attempts were made to prepare arbutin and *p*-hydroxyphenyl β -gentiobioside by incubating emulsin [commercial and Sumner & Somers (1947) preparations] and bean enzymes with quinol and various glucose derivatives. *p*-Hydroxyphenyl β -gentiobioside was not produced when bean seeds or shoots were allowed to take up solutions of quinol (0.25%) or arbutin (0.5%) under the conditions of Pridham (1958) and Pridham & Saltmarsh (1960).

When the almond or bean enzyme preparations were incubated with salicin, the following were formed: glucose, saligenin (*o*-hydroxybenzyl alcohol) and a phenolic compound (II). Saligenin and (II) gave the same red colour with the

Table 1. R_F and M_{SA} values of phenolic glucosides

Glucoside	R_F in solvents			M_{SA} in 0.1M-sodium borate buffer (pH 10)
	A	B	C	
Arbutin	0.75	0.40	0.51	—
<i>p</i> -Hydroxyphenyl β -gentiobioside (I)	0.55	0.07	0.17	0.28
<i>p</i> -Hydroxyphenyl β -cellobioside	0.61	0.10	0.24	0.22
Salicin	—	0.56	0.48	0
<i>o</i> -Hydroxybenzyl β -glucoside (II)	—	0.59	0.48	0.15

diazonium reagent and both had similar u.v.-absorption spectra with maxima at 275 and 274 $m\mu$, respectively (cf. Ramart-Lucas & Rabaté, 1933). In the presence of 0.002N-NaOH the absorption maximum of (II) shifted to 294 $m\mu$. Salicin had approximately the same R_f value as (II) (Table 1) but did not react with the diazonium reagent. However, these two compounds were clearly separated by paper electrophoresis (Table 1). The absorption maximum of salicin at 268 $m\mu$ (cf. Charlampowiczówna & Marchlewski, 1930) was not affected by the addition of alkali. Glucose and saligenin only were detected in the acid and emulsin hydrolysates of (II).

DISCUSSION

The results obtained show that compound (I) is *p*-hydroxyphenyl β -gentiobioside, which was formed directly from arbutin. That the transfer of β -D-glucosyl units by β -glucosidase is involved with

hydroxyl group *in vivo* and *in vitro* requires the presence of a 'high-energy' glucose donor and that simple transglucosylation reactions are of little importance for this process (Pridham & Saltmarsh, 1960).

Compound (II) is an isomer of salicin with the β -glucopyranosyl group attached to the primary hydroxyl group of the saligenin nucleus. The u.v.-absorption measurements and paper electrophoresis indicated the presence of a free phenolic hydroxyl group. In this instance β -glucosidase would appear to have catalysed the transfer of a glucosyl radical from the phenolic to the primary hydroxyl group in the side chain with the formation of *o*-hydroxybenzyl β -glucopyranoside. This glycoside is also formed when glucose is incubated with saligenin in the presence of emulsin (Bourquelot & Hérissay, 1913), by the action of a willow-leaf extract on salicin (Rabaté, 1935) and by allowing broad-bean shoots and seeds to take up solutions of saligenin (Pridham, 1958; Pridham & Saltmarsh, 1960).

both the almond and bean preparations is suggested by the fact that β -glucopyranosides were hydrolysed by all of the enzyme solutions tested and that this activity and the ability to synthesize the gentiobioside was inhibited by D-glucono-1 $\rightarrow$ 4-lactone, a competitive inhibitor of β -glucosidase (Conchie & Levvy, 1955). In addition, the pH optima for β -glucosidase activity and the formation of the gentiobioside are similar and the synthesis of a 1 $\rightarrow$ 6 β -link by D-glucopyranosyl transfer involving β -glucosidase might be expected from the observations of Peat, Whelan & Hinson (1952) and Barker, Bourne, Hewitt & Stacey (1955).

The fact that extensively dialysed enzyme solutions and a protein fraction obtained by ammonium sulphate precipitation all synthesized the phenolic gentiobioside makes it unlikely that uridine diphosphoglucose participates in the reaction although the possibility that this nucleotide is present in a bound form cannot be ignored (cf. Buell, 1952).

The biosynthesis of phenolic diglucosides *in vivo* is more likely to involve uridine diphosphoglucose than a simple glucose donor, e.g. a disaccharide, in conjunction with β -glucosidase. It also seems probable that monoglucosylation of a phenolic

The possibility of glycoside synthesis should be borne in mind when carrying out kinetic studies on glycosidases, with phenolic or alcoholic glycosides as substrates, otherwise anomalous results may be obtained.

SUMMARY

1. The incubation of almond emulsin, or enzyme preparations from broad bean, with arbutin produces *p*-hydroxyphenyl β -gentiobioside.
2. A transglucosylation reaction catalysed by β -glucosidase is involved with arbutin molecules serving as both donors and acceptors of D-glucopyranosyl residues.
3. Salicin is partially converted into *o*-hydroxybenzyl β -glucopyranoside by the almond and broad-bean preparations.

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The Acid-Soluble Nucleotides of Codling (*Gadus callarias*) Muscle

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The literature contains few references to the nucleotides of fish muscle. Tarr (1950) and Fujimaki & Kojô (1953) investigated a number of Pacific Ocean species by the fractionation of extracts with barium, and Partmann (1954) measured the adenosine 5'-triphosphate contents of tench and pike muscles. Steinbach (1949), Davison & Richards (1954) and Partmann (1954, 1957, 1958) have examined the apyrase activities occurring in fish muscle under different conditions. In preliminary communications (Jones & Murray, 1957; Murray & Jones, 1958; Jones, 1958) we described separations of the major constituents of the nucleotide fraction in codling muscle during exercise and after death. These studies were carried out by formate elution from anion-exchange resin columns (Hurlbert, Schmitz, Brumm & Potter, 1954). More recently, Saito & Arai (1958a, b) and Saito, Arai & Yajima (1959) have reported separations of the major nucleotides from carp and rainbow trout, using a chloride-elution technique.

This paper describes the identification of both the major and minor nucleotides of codling muscle in detail. Additionally, it extends our earlier quantitative data.

EXPERIMENTAL

Materials

Reference compounds for chromatography. Nucleotides were purchased from Sigma Chemical Co., St Louis, Mo. Guanosine and cytidine were purchased from Roche Products Ltd., Welwyn Garden City, Herts., adenosine, inosine, adenine, hypoxanthine, cytosine and guanine from L. Light and Co. Ltd., Colnbrook, Bucks., and uracil from British Drug Houses Ltd., Poole, Dorset.

Codling. These were caught off Aberdeen by trawl and kept in circulating sea water until landed. The fish were physically exhausted and some were killed in this condition. Others were kept ashore in aerated sea water for 8 weeks on a diet of squid. After this time, the fish were in a good nutritional condition and were well-rested when killed.

Methods

Killing. The fish, which were 18–24 in. long, were stunned by a blow on the head and dropped immediately into liquid oxygen. Preliminary experiments had ascertained that this procedure gave concentrations of labile phosphorus (measured as described below) comparable with those in the muscle of fish made comatose by immersion in sea water containing 1% (w/v) of urethane before killing. The concentrations of adenosine 5'-triphosphate (ATP) measured were significantly higher than those obtained by dropping the fish into liquid oxygen without stunning.