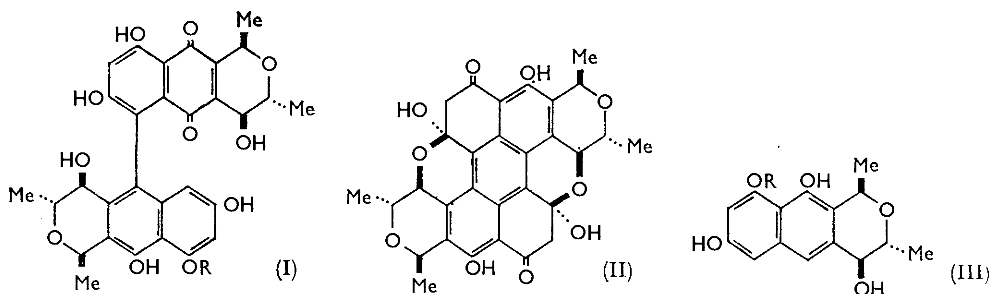
804. *Colouring Matters of the Aphididae. Part XXIV.*[1] *The Enzymic Conversion of Protoaphin into Xanthoaphin*

By D. W. Cameron, H. W.-S. Chan, and D. G. I. Kingston

Permethylation followed by acid hydrolysis established that the protoaphins are D-glucopyranosides. Nevertheless they are not hydrolysed in the presence of commonly available β- or of α-glucosidases. The only enzyme systems to effect this hydrolysis have been extracts of protoaphin-containing insects, *e.g.*, the bean aphid *Aphis fabae*, which convert protoaphin-*fb via* its aglucone into xanthoaphin-*fb*; towards simple aryl glucosides they act as α-glucosidases and have no significant β-glucosidase activity. However enzymic methods could not be used to determine the configuration of the glucosidic linkage in protoaphins because of the influence of *peri*-hydroxyl groups on the specificity of hydrolysis. The protoaphin-*fb* aglucone has been isolated both by chemical and enzymic methods. It is very susceptible to atmospheric oxidation but is otherwise stable, except to prolonged treatment with acid or in the presence of insect extracts, when it undergoes conversion into xanthoaphin-*fb*. At least two enzymic functions therefore must be involved in the overall protoaphin–xanthoaphin conversion.

In an earlier Paper[2] of this Series we discussed briefly the conversion of protoaphin-*fb* (I; R = D-glucose) into xanthoaphin-*fb* (II). This characteristic enzyme-catalysed process can be brought about either *in vivo* by crushing protoaphin-containing species of aphids or *in vitro* by treatment of protoaphin with extracts of crushed insects.[3] It was assumed to involve initially enzymic hydrolysis of the glucosidic linkage, followed by condensation of a naphthaquinone carbonyl group with the newly formed "resorcinol" ring, and formation of the hemiketal links. Whether these last two stages were also enzyme-catalysed was not established. The overall process has no obvious chemical parallel and the present Paper is concerned with defining its course in greater detail.

Non-enzymic conversion of protoaphin into xanthoaphin or derived products has previously been brought about only in poor yield, *e.g.*, treatment with acid[3] yielded a mixture of D-glucose and an intractable amorphous "aglucone" together with only traces of erythroaphin. In attempting to effect this conversion under milder conditions we first studied the action of glucosidases of known specificity, assuming as before that glucoside hydrolysis was an early stage of the process. Both protoaphin-*fb* (I; R = D-glucose)



and the naphthalenic glucoside (III; R = D-glucose) derived from it[4] are resistant to commonly available β- (almond emulsin) and α-glucosidases (from yeast[5] and horse

[1] Part XXIII, D. W. Cameron, R. I. T. Cromartie, D. G. I. Kingston, and G. B. V. Subramanian, *J.*, 1964, 4565.
[2] A. Calderbank, D. W. Cameron, R. I. T. Cromartie, Y. K. Hamied, E. Haslam, D. G. I. Kingston, Lord Todd, and J. C. Watkins, *J.*, 1964, 80.
[3] B. R. Brown, T. Ekstrand, A. W. Johnson, S. F. MacDonald, and A. R. Todd, *J.*, 1952, 4925.
[4] D. W. Cameron, R. I. T. Cromartie, D. G. I. Kingston, and Lord Todd, *J.*, 1964, 51.
[5] H. Halvorson and L. Ellias, *Biochim. Biophys. Acta*, 1958, **30**, 28.

serum [6]), despite the fact that these enzymes effect the hydrolysis of the respective anomeric phenyl glucosides (see later). Permethylation [7] of glucoside (III; R = D-glucose) followed by acid hydrolysis yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, characterised as the corresponding *N*-*p*-tolylglucosylamine, as the only isolable product. Protoaphin-*fb* is therefore a D-glucopyranoside and the failure of both β- and α-glucosidases to effect its hydrolysis must stem in part from some structural feature in the aglucone. (Periodate titration could not be employed in determining the size of the glucose ring because of the ease with which the oxygenated naphthalene nucleus was itself attacked by this reagent.)

This was further investigated by comparing the action of the glucosidases above and of extracts of the bean aphid *Aphis fabae* [3] towards synthetic glucosides of known configuration. Results are shown in the Table. The glucosides were chosen so as to bear an increasing structural resemblance to the environment of the glucosidic residue in protoaphin itself. The phenyl and 1-naphthyl glucosides were synthesised by Helferich's method.[8] With the latter, separation of the α-isomer, which was difficult to achieve by the usual method of fractional crystallisation of the mixed tetra-acetates, could readily be effected by prolonged alkaline treatment. This converts aryl β-glucosides into a mixture of the phenol and 1,6-anhydro-β-D-glucopyranose, the α-anomers being stable and readily separable from the mixture.[9] Attempts to apply Helferich's method to the synthesis of 8-hydroxy-1-naphthyl glucosides from naphthalene-1,8-diol led only to traces of unidentifiable material. In the absence of forcing conditions, monoalkylation of this diol is generally observed; [10] the present failure to obtain a product is presumably due to complex formation between the two peri-oxygen atoms and the Lewis acid catalyst employed, or simply to decomposition of the diol during the reaction. Application of the Koenigs–Knorr procedure,[11] however, readily yielded an 8-hydroxy-1-naphthyl glucoside. This was formulated tentatively as the β-anomer, since α-glucosides are not usually obtained by this method and molecular models suggest no obvious reason why the usual order of anomeric stability should be reversed in this case. The product's negative rotation ($[\alpha]_D$ −96°) was consistent with this assignment. The product also underwent hydrolysis in the presence of emulsin though, as will be dicussed, results of enzymic experiments in this system must be interpreted with caution. Several attempts, by a variety of other procedures,[12] were made to synthesise the corresponding α-anomer but all were unsuccessful.

Enzymic hydrolyses of aryl glucosides
(% yield of aglucone)

	Phenyl		1-Naphthyl		8-Hydroxy-1-naphthyl
	α	β	α	β	β
A. fabae extracts	78	—	96	—	90
Yeast α-glucosidase	80	—	94	—	—
Horse serum α-glucosidase	70	—	96	—	—
Almond emulsin	—	85	—	94	90

— indicates a yield less than 5%.

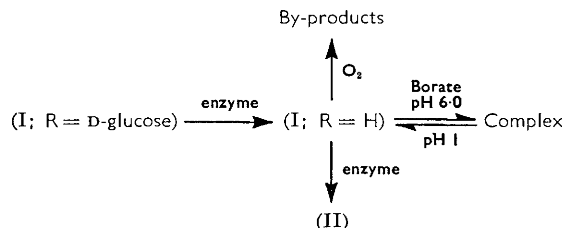
Towards both phenyl and 1-naphthyl glucosides, insect extracts behave as α-glucosidases and have no significant β-glucosidase activity. Unexpectedly, however, they also catalyse the hydrolysis of 8-hydroxy-1-naphthyl-β-D-glucoside, as does emulsin, but not yeast or horse serum α-glucosidases. This provides an interesting example of two glucosidases of different specificities, apparently catalysing the hydrolysis of the same substrate,

[6] I. Liebermann and W. H. Eto, *J. Biol. Chem.*, 1957, **225**, 899.
[7] R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, 1955, **67**, 32.
[8] B. Helferich and E. Schmitz-Hillebrecht, *Ber.*, 1933, **66**, 378.
[9] C. E. Ballou, *Adv. Carbohydrate Chem.*, 1954, **9**, 59.
[10] Elsevier's " Encyclopedia of Organic Chemistry," Elsevier, New York, 1950, Series 3, vol. XII, p. 1993.
[11] A. Robertson and R. B. Waters, *J.*, 1930, 2730.
[12] T. G. Bonner, E. J. Bourne, and S. McNally, *J.*, 1962, 761; W. J. Hickinbottom, *J.*, 1930, 1338; E. Pascu, *Ber.*, 1928, **61**, 1508; E. Fischer and L. von Mechel, *ibid.*, 1916, **49**, 2813.

a situation to which we know of no obvious analogy. Since the peri-hydroxyl group significantly influences enzymic hydrolyses in this case, it is likely to affect the hydrolysis of protoaphin and also to be a factor in its resistance to both β- and α-glucosidases, as described earlier. For this reason the configuration of the glucosidic linkage in protoaphin cannot at present unequivocally be determined by enzymic methods alone. To do so would require knowledge of the role of peri-hydroxyl groups in these reactions; this is under investigation.

We can now consider the later stages in the protoaphin–xanthoaphin conversion. These do not involve any spectroscopically identifiable intermediate in appreciable concentration [2] and can best be studied by isolation of the protoaphin-*fb* aglucone (I; R = H). This substance cannot be obtained directly by treatment of protoaphin-*fb* with insect extracts; evidently it does not attain a sufficiently high concentration in the mixture before conversion into xanthoaphin-*fb*. However it is readily obtained in solution by acid hydrolysis of protoaphin-*fb* under nitrogen. The two compounds have virtually identical light absorption but are readily distinguishable by their partition behaviour. The aglucone could not be obtained crystalline on account of its expected instability to air but when treated under nitrogen with extracts of *A. fabae* was converted into xanthoaphin-*fb* in good yield. If it was left in air for some hours before such treatment, in contrast to protoaphin itself, no xanthoaphin was obtained.

The aglucone (I; R = H) has also been obtained by a modified enzymic procedure. If protoaphin-*fb* is treated with extracts of *A. fabae* under nitrogen in the presence of a large excess of buffered boric acid (pH 6·0), the aglucone once formed is trapped as its borate complex involving the two peri-oxygens.[13] Model experiments with naphthalene-1,8-diol show that at this pH, equilibrium strongly favours formation of the complex as indicated in the scheme below. The rate of xanthoaphin production is substantially reduced, though a good overall yield can eventually be obtained if the reaction is kept for several hours under nitrogen. The effect of adding boric acid to the mixture is to bring about the accumulation of a large concentration of aglucone as its complex. The mixture may then be brought to pH 1, hydrolysing the complex, inactivating the enzyme, and precipitating the aglucone, which can then readily be extracted.



The isolation of aglucone by this enzymic procedure supports the assumption that it is the first formed intermediate between protoaphin-*fb* and xanthoaphin-*fb*. Attempts were made to convert it into xanthoaphin or the derived erythroaphin by chemical methods. Treatment with Lewis acid, alkali, or heat were unsuccessful, producing only traces of erythroaphin; however the prolonged action of mineral acid gave an improved yield [3] of 20%. This condensation stage of the protoaphin–xanthoaphin conversion is, clearly, also enzyme-controlled and capable of being effected non-enzymically only under forcing conditions. Extracts of *A. fabae* therefore must possess at least two enzymic functions associated with the overall process. Possibly two distinct, separable enzymes are involved. In view of the geometry of the system it seems unlikely that isolable intermediates will be found between the protoaphin aglucone and xanthoaphin and we regard this part of the process as being probably concerted.

[13] J. Böeseken, *Rec. Trav. chim.*, 1939, **58**, 3.

Experimental

Unless otherwise stated, infrared spectra were measured in Nujol mulls and ultraviolet and visible spectra in ethanol.

2,3,4,6-Tetra-O-methyl-D-glucose.—A solution of glucoside (III; R = D-glucose; 570 mg.) in purified dimethyl formamide (10 ml.) was shaken with freshly prepared silver oxide (3·2 g.) and redistilled methyl iodide (3·3 g.) for 18 hr.[14] The mixture was then filtered and the filtrate and washings diluted with chloroform (100 ml.), washed thoroughly with water, dried, and evaporated under reduced pressure to yield a brown solid. This was treated at 100° with sulphuric acid (3N; 20 ml.) in dioxan (30 ml.) for 3 hr. Dioxan was evaporated, a brown precipitate filtered off, and the aqueous filtrate further evaporated to about 10 ml. and then extracted with chloroform (3 × 50 ml.). The extract was chromatographed on a column of silica gel (B.D.H.) containing water (3 : 1), 1 cm. in diameter and 40 cm. in height. Elution with chloroform gave an initial brown fraction (20 ml.), followed by a pale yellow fraction (200 ml.), which gave a positive Molisch test. Evaporation of the latter yielded a brown oil (120 mg.), which slowly crystallised over calcium chloride *in vacuo*. On recrystallisation from light petroleum containing a trace of ether, it yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, as needles, m. p. 90—92°, undepressed in admixture with authentic material [15] and having identical infrared absorption, $[\alpha]_D^{20}$ +84·5° (*c* 0·6 in water). For authentic material $[\alpha]_D^{20}$ +93 ⟶ +84° (*c* 0·6 in water).

The *N-p*-tolylglucosylamine derivative, prepared by heating the product (20 mg.) with *p*-toluidine (60 mg.) at 100° for 2 hr., removal of excess of reagent *in vacuo*, and recrystallisation from petroleum, had m. p. 142—144·5°, undepressed in admixture with authentic material [16] and having identical infrared absorption, $[\alpha]_D^{20}$ +208 ⟶ +63° (*c* 0·6 in methanol).

Phenyl Glucosides.—Phenyl tetra-*O*-acetyl-α-D-glucopyranoside, prepared by the Helferich procedure,[8] had m. p. 113—114°, $[\alpha]_D^{20}$ +162° (*c* 0·2 in benzene). Deacylation by the Zemplen procedure [17] gave the free α-glucoside, m. p. 157°, $[\alpha]_D^{20}$ +187° (*c* 0·2 in water).

In the β-series [8] the tetra-acetate had m. p. 124—125°, $[\alpha]_D^{20}$ −30° (*c* 0·2 in benzene), and the free glucoside m. p. 170—172°.

1-Naphthyl Glucosides.—Redistilled 1-naphthol (37 g.) and β-D-glucose penta-acetate (25 g.) were allowed to react according to the Helferich procedure,[18] to yield a syrupy mixture of anomeric tetra-acetates (9 g.). Trituration with ether followed by two recrystallisations from ethanol yielded 1-naphthyl tetra-*O*-acetyl-β-D-glucoside (1 g.), m. p. 178°, $[\alpha]_D^{20}$ +69° (chloroform), which on deacylation gave the free β-glucoside, m. p. 177° (from water), $[\alpha]_D^{20}$ −90·4° (*c* 0·6 in water).

Unlike Hurd and Bonner [18] we were unable to isolate the tetra-acetyl α-derivative by direct crystallisation of the mixture (9 g.), which instead was refluxed in aqueous potassium hydroxide (1·3 N; 600 ml.) for 12 hr. The reaction mixture was brought to pH 6, washed with small volumes of ether, and then extracted with butanol. Evaporation of the extract yielded a syrup (2 g.) which solidified on trituration with ethanol. Crystallisation (ethanol) yielded *1-naphthyl α-D-glucoside*, m. p. 169—170°, which was analysed immediately after being dried (Found: C, 62·8; H, 5·4. $C_{16}H_{18}O_6$ requires C, 62·7; H, 5·9%); $\lambda_{max.}$ 219, 287, and 319 mμ (log ε 4·65, 3·76, and 3·11); $\lambda_{infl.}$ 283 and 304 mμ (log ε 3·76 and 3·43); $\nu_{max.}$ 3350br, 1595, and 1580 cm.$^{-1}$; $[\alpha]_D^{20}$ +110° (*c* 0·33 in water). This compound was also obtained by deacylation of the pure tetra-acetate below.

Treatment of this glucoside (300 mg.) with acetic anhydride (1 g.) and anhydrous pyridine (1·2 g.) at room temperature for 18 hr. yielded the tetra-acetate (240 mg.), m. p. 114° (from ethanol), $[\alpha]_D^{20}$ +190° (*c* 0·2 in chloroform). This compound has twice previously been reported.[18,19] Our physical constants agree only with those quoted by the former authors; the melting point (178—179·5°) given by the latter is close to that of the β-anomer.

8-Hydroxy-1-naphthyl β-D-Glucoside.—Naphthalene-1,8-diol was prepared by alkali fusion

[14] H. G. Walker, M. Gee, and R. M. McCready, *J. Org. Chem.*, 1962, **27**, 2100.
[15] E. W. West and R. F. Holden in "Organic Syntheses," Wiley, New York, 1940, vol. XX, p. 97.
[16] G. P. Ellis and J. Honeyman, *J.*, 1952, 2053.
[17] G. Zemplen and E. Pacsu, *Ber.*, 1929, **62**, 1613.
[18] C. D. Hurd and W. A. Bonner, *J. Org. Chem.*, 1945, **10**, 603.
[19] T. Kariyone, M. Takahashi, and K. Takaishi, *J. Pharm. Soc. Japan*, 1952, **72**, 13 (cf. *Chem. Abs.*, 1952, **46**, 11,114h).

of commercially available sodium 8-hydroxynaphthalene-1-sulphonate (8·8 g.) at 290° for 35 min.[13,20,21] The product (3 g.), m. p. 140°, was obtained in an improved yield of 60%.

A mixture of naphthalene-1,8-diol (1 g.) and tetra-*O*-acetyl-α-D-glucopyranosyl bromide (5·3 g.) in redistilled quinoline (6 ml.) was made into a paste and stirred while freshly prepared silver oxide (3 g.) was added. After 15 minutes' agitation the mixture was set aside for 1 hr., extracted with acetic acid (25 ml.), filtered, and the filtrate poured into ice-water (120 ml.). The resulting brown oil was dissolved in ether, and the solution filtered and evaporated to yield 8-*hydroxy*-1-*naphthyl tetra*-O-*acetyl*-β-D-*glucoside* (2·2 g.), which after crystallisation from ethanol had m. p. 167—168° (Found: C, 58·8; H, 5·3. $C_{24}H_{26}O_{11}$ requires C, 59·1; H, 5·1%); $[\alpha]_D^{20}$ $-55°$ (c 0·5 in chloroform); $\lambda_{max.}$ 226, 300, 316, and 331 mμ (log ε 4·66, 3·78, 3·73, and 3·74); $\lambda_{infl.}$ 290, 308, and 323 mμ (log ε 3·68, 3·68, and 3·52); $\nu_{max.}$ 1748 cm.$^{-1}$.

The tetra-acetate (350 mg.) on deacylation yielded the free β-*glucoside* (130 mg.), m. p. 148—150° (from water) (Found, on a sample analysed immediately after being dried: C, 59·7; H, 6·0. $C_{16}H_{18}O_7$ requires C, 59·6; H, 5·6%); $[\alpha]_D^{20}$ $-96°$ (c 0·4 in water); $\lambda_{max.}$ 225, 302, 316, and 331 mμ (log ε 4·69, 3·81, 3·75, and 3·75); $\lambda_{infl.}$ 293, 308, and 324 mμ (log ε 3·75, 3·69, and 3·55); $\nu_{max.}$ 3420, 3280br, 1633, 1612, and 1589 cm.$^{-1}$.

Enzymic Hydrolyses.—Extracts of *A. fabae* were prepared as previously described,[3] samples of crystalline emulsin and of horse serum were obtained commercially, while a crude extract (200 ml.) of brewers' yeast (100 g.) was prepared (without fractionation) by the method of Halvorson and Ellias.[5]

In general, solutions of glucosides (5·0 mg. in 10 ml.) in phosphate buffer, pH 6·6, were treated with insect extracts (1 ml.), horse serum (2 ml.), yeast extract (2 ml.), or emulsin (2 mg.). The solutions were set aside at room temperature for 8 hr., the phenols produced being then estimated spectroscopically using Gibb's reagent.[22] In the case of 8-hydroxy-1-naphthyl β-D-glucoside, the reaction mixture was extracted with ether, the extracts washed with small volumes of water, and the aglucone estimated directly by optical density measurements. For this compound it was necessary to carry out the hydrolysis under nitrogen; otherwise decomposition of the product occurred, forming a precipitate in the reaction mixture.

Protoaphin-fb *Aglucone* (I; R = H).—(*a*) Protoaphin-*fb* (20 mg.) was dissolved in a mixture of aqueous methanol (80%; 3·5 ml.) and sulphuric acid (N; 5 ml.), refluxed under nitrogen for 20 min., then cooled and, still under nitrogen, extracted with ether. The extract, which contained the *aglucone* (I; R = H) and whose visible spectrum was almost identical with that of protoaphin, was washed with dilute acid and with water. Phosphate buffer, pH 6·0 (20 ml.), was then added and the ether allowed to evaporate in a stream of nitrogen. Not all the material dissolved immediately but the mixture was treated with insect extracts (3 ml.) and set aside for 3 hr. Extraction with chloroform yielded a solution whose visible spectrum was identical with that of xanthoaphin-*fb*.[1] This was heated with formic acid and the resulting erythroaphin-*fb* estimated spectroscopically;[23] the yield was 6·3 mg. (60%). Alternatively the ethereal solution of aglucone above was evaporated to dryness. It was treated under nitrogen with aqueous 2N-sodium hydroxide, or with aluminium chloride in chloroform, with or without heating in either case. No more than a trace of erythroaphin could be detected. Short contract with mineral acid gave a similar result but prolonged treatment was more successful (see below).

(*b*) A suspension of protoaphin-*fb* (20 mg.) in buffer (20 ml.) containing 0·5M-boric acid and 0·2M disodium hydrogen phosphate solutions (24 : 1) was treated under nitrogen with extracts of *A. fabae* (3 ml.) for 1·5 hr. The protoaphin dissolved to give a red solution which was washed with chloroform, thereby extracting xanthoaphin-*fb* (2·5 mg., estimated spectroscopically [1]). The aqueous solution was then immediately acidified and extracted with ether under nitrogen to yield a solution of aglucone. This was spectroscopically identical with the product from (*a*) and was washed and suspended in buffer pH 6·0 as above and treated further with insect extract (2 ml.). After 3 hr. extraction with chloroform yielded xanthoaphin-*fb* (6 mg., estimated spectroscopically). It was identical in paper chromatographic behaviour with authentic material [2] and with formic acid it yielded erythroaphin-*fb* (6 mg.), whose identity was confirmed by visible and infrared spectra and R_F value.

Non-enzymic Formation of Erythroaphin-fb.—To a solution of protoaphin-*fb* (20 mg.) in

[20] A. P. Lurie, G. H. Brown, J. R. Thirtle, and A. Weissberger, *J. Amer. Chem. Soc.*, 1961, **83**, 5018.
[21] H. E. Fierz, *Helv. Chim. Acta*, 1920, **3**, 318.
[22] F. Wild, " Estimation of Organic Compounds," Cambridge, 1950, p. 90.
[23] J. P. E. Human, A. W. Johnson, S. F. MacDonald, and A. R. Todd, *J.*, 1950, 477.

aqueous ethanol (60%; 3 ml.), was added dilute sulphuric acid (3N; 6 ml.) and ethanol (1·5 ml.) and the mixture was refluxed gently under nitrogen for 17 hr. It was cooled, diluted with water (2 ml.), and extracted with chloroform (2 × 10 ml.). The extract was washed with aqueous sodium hydrogen carbonate (2 × 10 ml.) and dried. The resulting solution was spectroscopically identical with erythroaphin-*fb* and was estimated spectrophotometrically to contain 2·7 mg. (20%).

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