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Colouring Matters of the Aphidoidea XLII*. Purification and Properties of the Cyclising Enzyme [Protoaphin Dehydratase (Cyclising)] Concerned with Pigment Transformations in the Woolly Aphid *Eriosoma lanigerum* Hausmann (Hemiptera : Insecta)

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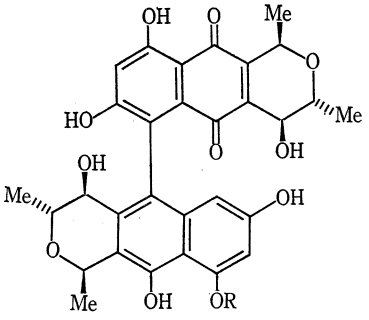
Abstract

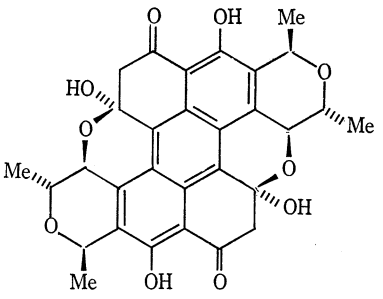
Dried extracts of woolly aphid were treated with n-butanol, then by chromatography on DEAE–Sephadex A50 and finally by filtration on Sephadex G150 to yield a substantially homogeneous protein catalysing the conversion of the aglucone of protoaphin into xanthoaphin. Traces of low-molecular-weight contaminants were removed by chromatography on Sephadex G100. The enzyme, which has a molecular weight of 120 000 ± 2 000 and a high content of β-structure, was inhibited by naphthoresorcinol. Its glycoprotein nature was indicated by amino acid analysis.

Introduction

A variety of species of the insect superfamily, Aphidoidea, including the woolly aphid which parasitizes apple trees, has been shown to produce the class of complex polycyclic pigments grouped together as the aphins (see Cameron and Todd 1967). The aphins occur in the haemolymph of the insects as the brownish-yellow glucoside, protoaphin (1), which is purple in mildly alkaline solution and is thus responsible for the dark colour of many of the relevant insect bodies. Also present in the insects is a system of two enzymes, a glucosidase and a condensing or cyclising enzyme, which, following insect damage, converts the protoaphin via its aglucone (2) irreversibly into



(1) R = $C_6H_{10}O_5$
(2) R = H



(3)

the highly fluorescent pigment, xanthoaphin (3). The latter has been shown to change slowly and non-enzymically at acid or alkaline pH into the more stable, highly aromatic erythroaphin by successive dehydration steps.

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Prior to the present investigation certain preliminary findings on the enzyme system had been reported (Cameron *et al.* 1965; Cameron and Craik 1968; Banks *et al.* 1969). Thus, although the protoaphins are not hydrolysed by the commonly available glucosidases they have been established by indirect methods as β-D-glucosides. Glucosidases in extracts of aphids containing protoaphins were found to be highly active towards the natural substrates but to show much reduced activity if the extracts were prepared in the presence of solvents such as n-butanol; incidentally, the cyclising activity appeared to remain unaffected by this treatment. By contrast the butanol-stable glucosidases in extracts of certain related aphid species (e.g. *Dactynotus jaceae* L.), while associated with a different group of pigments (Bowie and Cameron 1967), are yet able to hydrolyse protoaphins to the corresponding aglucones. In such cases no cyclisation occurs.

Advantage was taken of these qualitative observations in devising a method for the purification of the cyclising enzyme [4.2.1; protoaphin dehydratase (cyclising); see Discussion] and for its assay since initial experiments designed to isolate both enzymes from extracts of the woolly aphid, in satisfactory yields, had proved unsuccessful.

Materials and Methods

Insects

(i) *Eriosoma lanigerum*

Insects were collected, freed from small amounts of plant debris and processed as soon as possible. Owing to their waxy nature, extracts were preferably made by grinding the insects with sand and buffer rather than in a high speed blender. Two lots (each 143 g) were separately ground with acid-washed sand (14 g) and 0·02 M phosphate buffer, pH 6·6 (180 ml), allowed to stand at room temperature for 1·5 h and then centrifuged in a cold room at 12 500 rev/min for 0·5 h. The brown supernatant was filtered through glass wool and the insoluble material re-extracted with more buffer (210 ml), centrifuged and filtered. The combined extract from both lots (770 ml) was allowed to stand overnight at −10°C. It was then thawed and centrifuged for 0·5 h in a refrigerated centrifuge at 2 100 rev/min to separate dark residual material (*c.* 5 g). The clear brown supernatant was then dialysed against glass-distilled water and lyophilized, yielding a brown friable powder (15·6 g, 5·6%).

(ii) *Dactynotus jaceae*

A partially purified sample of glucosidase was prepared from a freeze-dried batch of insects collected in Cambridge (U.K.) in 1965 from knapweed and held in a sealed tube under nitrogen. The very dark insect bodies (10 g) were ground to a fine powder with sand and rubbed with 0·02 M phosphate buffer, pH 6·6 (80 ml). After standing overnight in the cold room the suspension was centrifuged for 15 min at 10 000 rev/min and insoluble material re-extracted with buffer (165 ml). The combined extracts were slowly stirred for 10 min with water-saturated n-butanol (25 ml) and centrifuged. After filtration through glass wool and separation of the butanol layer the aqueous part was stirred with ether (3 × 30 ml), then dialysed and lyophilized to give a light brown powder (502 mg). In the work that follows the unqualified term 'glucosidase' will refer to this material from *D. jaceae*, unless otherwise stated.

Although *D. jaceae* has not been described from Australia (Anon. 1973), another aphid (*A. nerii* Boyer de Fonscolombe) collected locally from oleander and processed as above also gave a glucosidase fraction which was, however, less active than that from *D. jaceae*.

Enzyme Assays

Since pure preparations of the aglucone (2) could not be isolated owing to atmospheric oxidation (Cameron *et al.* 1965), fractions of the cyclising enzyme could not be assayed directly. The aglucone was therefore produced *in situ* from protoaphin by the addition of the partially purified glucosidase. The protoaphin was from a stored sample of several grams which had been prepared by the method

of Brown *et al.* (1952) from *A. fabae*. When used as substrate the pigment was ground with 0·02 M phosphate buffer, pH 6·6 (1 mg/ml) at room temperature, centrifuged and decanted from a small residue.

(i) *Glucosidase*

Samples in phosphate buffer (1 ml) were incubated in glass-stoppered tubes (capacity 7·5–8 ml) together with a solution of protoaphin in the same buffer (1 ml), prepared as described above. The control (reference) tube contained buffer (1 ml) and protoaphin solution (1 ml). After a time interval, usually 1 h (at 20–22°C), ether (4·5 ml) was added and the tubes repeatedly inverted to effect the extraction of any aglucone formed. The ether extracts were checked for the absence of fluorescence and for the broad absorption band λ_{max} 430 nm (as in Fig. 4 curve *a*).

(ii) *Cyclising enzyme*

This was assayed as for the glucosidases except that the samples were either whole enzyme powders from the woolly aphid made up to 1 ml with phosphate buffer or fraction samples from the stages in the purification of the cyclising enzyme, with the addition of the appropriate amount (usually 1·5 mg) of glucosidase, diluted to 1 ml. Xanthoaphin formed was checked by its fluorescence and by noting particularly the main peak of absorbance at 375 nm which is also within an area of minimum absorption by the aglucone (see Fig. 4 curve *a*).

Column Chromatography

This was carried out by conventional procedures using the Sephadex ion exchanger, DEAE–A50 (3·5 ± 0·5 m-equiv/g), and the Sephadex gels, G100 and G150. Column fractions were assayed for protein by extinction at 280 nm.

Molecular Weight Determinations

Disc gel electrophoresis in sodium dodecyl sulphate (SDS) was performed by the procedure of Weber and Osborn (1969) as modified by Stocklosa and Latz (1974).

Sedimentation experiments were carried out in a Spinco model E analytical ultracentrifuge, the temperature being controlled and recorded by the rotor temperature indicator control unit. Sedimentation coefficients were corrected for solvent viscosity and temperature in the usual way (Svedberg and Pedersen 1940). Sedimentation equilibrium experiments were conducted by the meniscus depletion method of Yphantis (1964). Kel-F polymer oil was used as the inert base fluid in the standard six-channel cell. The time for the attainment of equilibrium was shortened by using the overspeed technique of Hexner *et al.* (1961). The final angular velocity was 21 740 rev/min and after 8 h the distributions of concentration were independent of time. Fringe displacements less than 100 μm were neglected in the analyses since they are subject to considerable error. The partial specific volume of the protein was assumed to be 0·73 ml/g.

Circular Dichroism

Circular dichroism measurements were made with a Jasco circular dichrometer modified from a J5 to the equivalent of a J20 configuration. A sensitivity setting of 10^{-4} ΔA unit/cm was chosen to maximize the signal to noise ratio. Cell path lengths of 0·5 mm in the near u.v. and 0·2 mm in the far u.v. enabled the photomultiplier voltage to remain within 'safe' limits such that spectral artefacts were not obtained. Spectra are presented in terms of mean residue ellipticities. The far u.v. spectrum was compared with theoretical spectra computed by the method of Saxena and Wetlaufer (1971).

Extinction Coefficient

Duplicate samples of the cyclising enzyme were dialysed exhaustively against 0·2 M phosphate buffer (pH 6·6). The refractive index difference between the solution and its solvent was measured in a Brice Phoenix differential refractometer. The protein concentration was determined using a value of $1{\cdot}8 \times 10^{-3}$ dl g^{-1} for the specific refractive increment. The optical density of the same solution was measured at 280 nm using a 1-mm path length cell with a Zeiss PMQII spectrophotometer.

Results

(*a*) *Purification of Cyclising Enzyme*

(i) *Treatment with n-butanol*

The dried extract of the woolly aphid (4 g) was dissolved with stirring and cooling in 0·02 M phosphate buffer, pH 6·6 (100 ml), in a stream of nitrogen. After 30 min, water-saturated n-butanol (50 ml) was added and stirring continued for a further 10 min. Following centrifuging at 10000 rev/min and separation of the solvent and aqueous layers, the insoluble residue was retreated with buffer (20 ml) and butanol (5 ml) and recentrifuged. The combined aqueous layers were washed with ether (2 × 40 ml) and solvent residues removed in a current of nitrogen. Following dialysis the extract was lyophilized to yield a light brown powder (2·05 g). This material was without effect on protoaphin unless glucosidase was added, in contrast to the original dried extract, indicating the destruction of *E. lanigerum* glucosidase by the butanol treatment.

(ii) *Ion exchange chromatography*

The butanol-treated sample (1 g) in 0·02 M tris–HCl buffer, pH 7·2 (10 ml), was applied to a DEAE–Sephadex A50 column (46 by 2·6 cm; volume 220 ml) and fraction 1 collected with the same buffer at the rate of 40 ml/h (240 ml). Fraction 2 was then eluted during 5 h with starting buffer containing 0·25 M sodium chloride (320 ml). Fractions 1 and 2 weighed, respectively, 622 and 70 mg after dialysis and lyophilization. When assayed in the presence of glucosidase, fraction 2 gave a strong spectrum of xanthoaphin whereas fraction 1 was inactive and appeared to consist essentially of glycogen. A sample (100 mg) was deproteinized by the method of Sevag according to Staub (1965) and yielded 80 mg [i.r. spectrum was identical with that recorded for yeast glycogen by Michell and Scurfield (1970) with the characteristic bands at 930, 850 and 760 cm^{-1}].

Although another sample of insects gave a comparable yield of enzyme fraction when extracted and chromatographed as above, the weight of glycogen fraction was much less (about 9%). This probably reflects the status of the original insects (cf. Wyatt 1967). Several samples with enzymic activity (i.e. fractions 2) were dissolved together in water (10 ml) and lyophilized for the next step.

(iii) *Gel Filtration with Sephadex G150*

The above fraction 2 (135 mg) was applied to a column (60 by 1·5 cm; volume 120 ml) in five separate filtrations, providing two fractions (2·1 and 2·2). These were collected without change of starting buffer (tris–HCl, 0·1 M; pH 7·2) at a filtration rate of 20–24 ml/h (see Fig. 1), then dialysed and lyophilized after each run. The total weight of active protein (34·5 mg; fraction 2·1) was about the same as that of fraction 2·2 (34·7 mg) which represented material of very low activity (Fig. 2). In a further comparative assay, fraction 2·2 (200 μg) showed even less activity than 1μg of fraction 2·1. Thus fraction 2·2 would appear to contain less than 0·5% of active enzyme. Both fractions consisted of colourless powders. The extinction coefficient ($E^{1\%}_{1cm}$) of fraction 2·1 was 15·0 ± 0·2 at 280 nm.

(*b*) *Assays of Cyclising Enzyme Fractions*

In Table 1 an evaluation is made of the degree of enhancement of the specific activity of the enzyme during the purification steps, within the limits of the assay

method. The values are based on the weight of enzyme fraction which gave an absorption peak at 375 nm comparable to that produced by 40 μg of the final product, fraction 2·1 (as in Fig. 2).

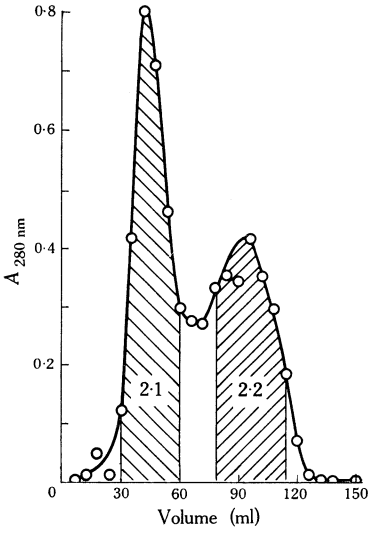

Fig. 1. Chromatography of partially purified cyclising enzyme (fraction 2) on Sephadex G150. Buffer: 0·1 M tris–HCl, pH 7·2. Fractions taken as hatched areas (2·1 and 2·2).

(*c*) *Molecular Weight*

SDS gel electrophoresis indicated that fraction 2·1 exhibited a strong main band of molecular weight 130 000–140 000 and two minor bands of lower molecular weight. Fraction 2·2 showed a trace of material corresponding to molecular weight 130 000–140 000 and two bands of molecular weight about 20 000 and 10 000–12 000.

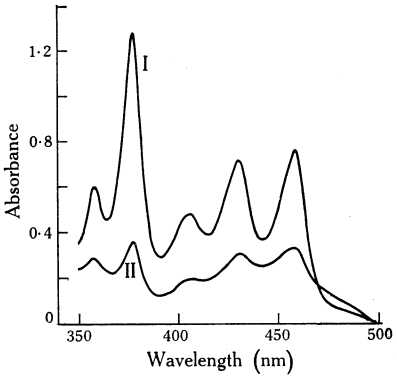

Fig. 2. Comparison of activities of fractions 2·1 and 2·2 following chromatography on Sephadex G150 (see Fig. 1). Substrates: protoaphin (1 mg) and glucosidase (1·5 mg). Time, 1 h; temperature 20°C. Buffer: 0·02 M phosphate, pH 6·6. Solvent: ether. Curve I: fraction 2·1 (40 μg) + glucosidase + protoaphin. Curve II: fraction 2·2 (200 μg) + glucosidase + protoaphin. Reference tube: protoaphin alone in buffer.

Sedimentation equilibrium analysis of fraction 2·1 indicated the presence of a small proportion of low-molecular-weight material. This was effectively removed by passage through Sephadex G100 (in 0·1 M phosphate buffer, pH 6·6). Analysis of this highly purified sample indicated a molecular weight of 120 000 ± 2 000. Plots of the logarithm of the fringe displacement *versus* the square of the radial distance were linear, indicating the presence of a single non-interacting species. Sedimentation velocity analysis of fraction 2·1 gave a single symmetrical Schlieren peak sedimenting

with a velocity of 6·1 S ($s_{20,w}$ at a protein concentration of 4·0 mg/ml). There was no indication of non-ideal behaviour in either equilibrium or velocity experiments, indicating that the shape of the molecule is not highly asymmetric.

Table 1. Recoveries of enzyme during purification steps

Treatment	Weight fraction (g)[A]	Weight in assay (mg)	Total 'units'	Purification factor
Dried extract	5·6	2·0	2800	—
Butanol-treated extract	2·87	1·0[B]	2800	2
DEAE–A50 (fraction 2)	0·185	0·125[B]	1480	16
Sephadex G150 (fraction 2·1)	0·047	0·040[B]	1175	50

[A] Actual weights adjusted to 100 g insect equivalence.
[B] Glucosidase added.

(*d*) *Circular Dichroism*

The circular dichroism spectra in the near and far u.v. are depicted in Fig. 3. The far u.v. spectrum shows the absence of the double minima (208 and 222 nm) that is characteristic of the α-helix. The single minimum at 220 nm suggests the presence of β-structure. A spectrum characteristic of 60% β-structure and 40% 'random' structure computed according to Saxena and Wetlaufer (1971) is superimposed on the experimental spectrum in Fig. 3. The near u.v. spectrum is dominated by a

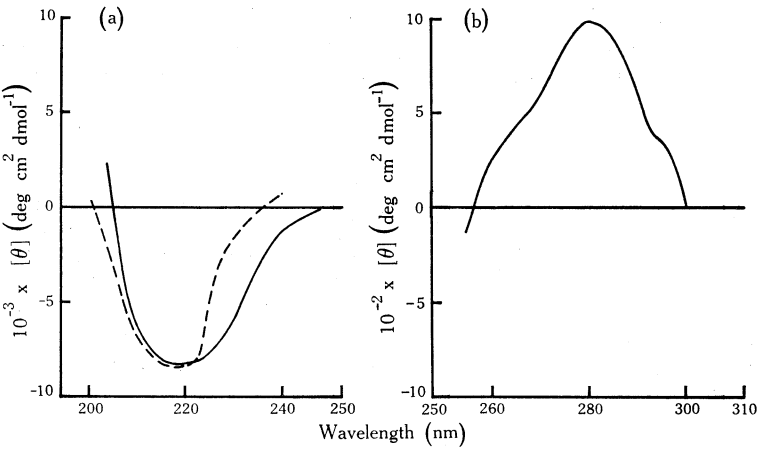

Fig. 3. Circular dichroic spectra of the purified cyclising enzyme in the far (*a*) and near (*b*) u.v. Ellipticities ([θ]) are expressed on a mean residue weight basis. The broken line is the spectrum computed for a molecule containing 60% β-structure and 40% 'random' structure according to the method of Saxena and Wetlaufer (1971).

contribution from tyrosyl and tryptophan residues at 280 nm, the shoulder at 292 nm being typical of the tryptophan ^1L_b transition. No spectral difference was found between fraction 2·1 and the purified sample used for sedimentation equilibrium analysis (see above).

(*e*) *Amino Acid Composition*

The results of the amino acid analyses are summarized in Table 2. Of particular interest is the presence of glucosamine, which thereby designates the enzyme as a glycoprotein. The possible occurrence of non-amino sugars was not investigated.

(*f*) *Inhibition Studies*

From a number of possible inhibitors naphthoresorcinol (1,3-dihydroxynaphthalene) was chosen for further study in view of its low absorbance in the region of the spectrum of xanthoaphin where measurements were taken.

Table 2. Amino acid composition of cyclising enzyme

Sample used was fraction 2·1 after passage through Sephadex G100. Hydrolyses were carried out in 6 M HCl–1% phenol *in vacuo* for 24, 48 and 72 h in duplicate and corrections applied for serine and threonine. Half-cystine was estimated after performic acid oxidation by the method of Moore (1963). Tryptophan was not determined. Actual mean values are given in parentheses

Amino acid	Residues/mole of protein[A]	Amino acid	Residues/mole of protein[A]
Lys	94 (93·6)	Ala	68 (67·6)
His	13 (13·0)	½Cys	7–8 (7·5)
Arg	31 (31·0)	Val	64 (63·9)
Asp	143 (143·3)	Met	21 (21·0)
Thr	64 (64·1)	Ile	69 (68·8)
Ser	72 (72·3)	Leu	72 (71·6)
Glu	101 (101·0)	Tyr	53 (53·2)
Pro	59 (58·8)	Phe	57–58 (57·5)
Gly	64 (63·9)	GlcN	14–15 (14·5)

[A] Molecular weight 120000.

Fig. 4 illustrates the results of a composite experiment from which naphthoresorcinol appears to be without effect on the activity of the glucosidase since the absorbance curve of the aglucone formed is unaltered (curves *a* and *b*—except in the region of low wavelength where the absorbance due to the inhibitor becomes superimposed). By contrast, with the addition of cyclising enzyme to aliquots of the incubated solutions the inhibitor caused a marked reduction in the formation of xanthoaphin (curves *c* and *d*).

Discussion

In the course of this investigation it has been shown that the glucosidase of *E. lanigerum*, a member of the family Pemphigidae, is inactivated by short contact with n-butanol, resembling, in this respect, the glucosidase of *Tuberolachnus salignus* Gmelin (family Lachnidae) but differing from those of *A. fabae*, and *D. jaceae* (family Aphididae) (Banks *et al.* 1969) and *A. nerii* (also family Aphididae). The glucosidases from *D. jaceae* and *A. nerii* have now been readily isolated as butanol-stable powders. Such differences in stability might lead to taxonomic or phylogenetic conclusions but a larger range of species would first have to be explored. The cyclising enzymes in *E. lanigerum*, *T. salignus* and *A. fabae* are all insensitive to butanol, minor differences in specificity between the last two having previously been noted (Banks *et al.* 1969).

The first stage in the purification sequence of the cyclising enzyme from *E. lanigerum* resulted from butanol treatment of the initial extract (Table 1). The final enzyme product (fraction 2·1) obtained by filtration through Sephadex G150 represented a purification factor of 50 with a recovery of 42% and appeared to consist essentially of one major component when examined by SDS gel electrophoresis

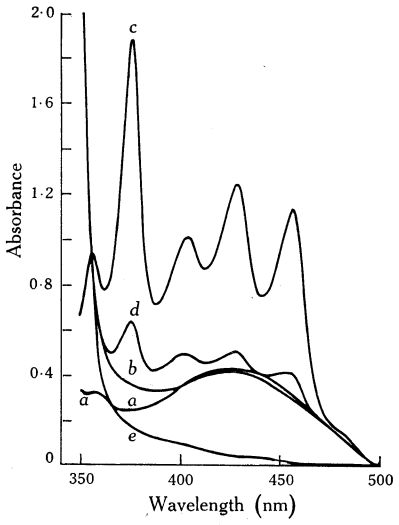

Fig. 4. Inhibition of cyclising enzyme (CE; fraction 2) by naphthoresorcinol (NR). Buffer: 0·02 M phosphate, pH 6·6.
Solutions used: *A* [glucosidase (G; 6 mg) in buffer (4 ml)]; *B* [NR in 0·01 M NaOH (30 mg/ml)]; *C* [protoaphin (Pa) in buffer (1 mg/ml)]; *D* [CE in buffer (2 mg/ml)].
Incubations for 1 h as for enzyme assays of: tube 1 [*A* (1·8 ml), *B* (0·2 ml), *C* (2 ml)], tube 2 [*A* (1·8 ml), 0·01 M NaOH (0·2 ml), *C* (2 ml)] and tube 3 [buffer (1·8 ml), 0·01 M NaOH (0·2 ml), *C* (2 ml)]. Aliquots (exactly 2 ml) were then taken from tubes 1, 2 and 3 and added to tubes 4, 5 and 6 respectively [tubes 4 and 5 also contained *D* (0·1 ml) and tube 6 buffer (0·1 ml)]. Ether (4·5 ml) was then added to the residual solutions in tubes 1, 2 and 3 and spectra examined while tubes 4, 5 and 6 were similarly treated *after* incubation for an additional 1 h.
Curve (*a*): Pa+G (tube 2 *v.* tube 3). Curve (*b*): Pa+G+NR (tube 1 *v.* tube 3). Curve (*c*): Pa+G+CE (tube 5 *v.* tube 6). Curve (*d*): Pa+G+CE+NR (tube 4 *v.* tube 6). Curve (*e*): NR alone (3 mg).

(molecular weight 130000–140000). After passage through a column of Sephadex G100 to remove minor low-molecular-weight contaminants, the molecular weight was 120000 ± 2000 as determined by analytical ultracentrifugation. The overestimate of the molecular weight found by SDS electrophoresis is a characteristic of glycoproteins (Bretscher 1971; Ward and Dopheide 1976). In the purified enzyme 14–15 glucosamine residues per mole of protein were found (Table 2). The purified protein has a high content of β-structure (approximately 60%) with little or no α-helix present, and the hydrodynamic conformation of the molecule is probably fairly symmetrical.

Although the enzyme is known to catalyse the conversion of the aglucone of protoaphin to xanthoaphin, no intermediates have yet been isolated from the complex reactions and rearrangements involved. A possible mechanism is discussed by Cameron and Todd (1967, pp. 232–4), the first stage involving reaction between the carbonyl group of the naphthoquinonoid moiety and the carbon directly opposite on the naphthalenic component of the aglucone (2). Stoichiometrically the key stage is the elimination of the carbonyl oxygen as water, a process that is likely to be favoured owing to the concomitant formation of the stable anthracenic system. In consequence, the enzyme could be considered as belonging to group 4.2.1 with a recommended name as protoaphin dehydratase (cyclising).

The mechanism of inhibition of the enzyme by naphthoresorcinol would seem to involve the two *m*-hydroxyl groups in the naphthalenic component of the aglucone,

rather than the corresponding hydroxyls in the naphthoquinonoid moiety, since the inhibitor is without effect in the presence of the glucosidase alone (Fig. 4).

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