959. *Colouring Matters of the Aphididæ. Part VI. The Glucosidic Nature of Protoaphin.*

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Acid hydrolysis of protoaphin-*fb* has yielded D-glucose, together with a brown amorphous material which can be converted either into xanthoaphin-*fb* by the action of an enzyme solution prepared from fresh insects, or into erythroaphin-*fb* by acid treatment. A similar series of reactions is described with protoaphin-*sl*. Protoaphin-*fb* is converted directly into erythroaphin-*fb*, although in poor yield, by the action of acids.

In earlier papers (Parts II, III, IV, V; *J.*, 1950, 477, 485, 3304; 1951, 2633), we described a remarkable group of colouring matters known as aphins, which can be obtained from a large number of dark species of Aphididæ. Apart from the primitive *Hamamelistes* species which are abnormal in type, all the aphin-containing species examined appear to be similar in that the hæmolymph of the living insect contains a water-soluble protoaphin, which is converted by enzymic action after death into a yellow fat-soluble fluorescent xanthoaphin, the latter compound undergoing further changes by chemical action to give an orange chrysoaphin and a red erythroaphin. Although in each insect species a similar series of conversions is observed, the individual pigments obtained from different species are not always the same; indeed, in the species we have so far closely examined it would appear on infra-red spectroscopic evidence that at least two distinct, although very closely related, series are found, and it has been customary, pending a complete understanding of their nature, to describe individual pigments by a type name followed by a suffix indicating the species of origin, *e.g.*, protoaphin-*fb* from *A. fabæ*, etc.

Protoaphins isolated from several species are golden-yellow crystalline compounds, whose aqueous solutions become reddish-violet at pH values above 5·5. In chemical behaviour they are very similar, and most of our work on protoaphin has been done on protoaphin-*fb* which is readily obtained from the common black aphid *A. fabæ* found on cultivated broad beans. Protoaphin differs from the other aphins (xanthoaphin, chrysoaphin, and erythroaphin) not only in its water-solubility and colour in alkaline solution, but also in its lack of fluorescence and in its ultra-violet absorption spectrum (Part II, *loc. cit.*); it is, moreover, considerably more stable to acids and alkalis than is either xanthoaphin or chrysoaphin. Protoaphin-*fb* gives analytical values which are in reasonable agreement with a molecular formula $C_{36}H_{44-46}O_{19}$ although in the absence of other supporting evidence this cannot be regarded as definitely established. In earlier studies (Part II, *loc. cit.*) it was shown that protoaphin could be converted smoothly into xanthoaphin by adding freshly crushed insects to its solutions at pH 6—7. It has now been possible to prepare pigment-free enzyme solutions from fresh insects which can be used to effect this conversion. Whether this conversion is brought about by one enzyme, or if more than one is involved, is at present unknown, since purification has not been carried beyond this point. The method used for preparing pigment-free enzyme concentrates, which is recorded in the Experimental section, utilises butanol-extraction (for its use with other enzymes, cf. Morton, *Nature*, 1950, **166**, 1092). That protoaphin is the precursor of the fluorescent aphins was also confirmed chemically, for when heated with mineral acids in aqueous dioxan for some time protoaphin-*fb* yielded, among other products, erythroaphin-*fb*. The yield of erythroaphin obtained in this way was variable and usually low, hot aqueous formic acid giving the best results. Since the conversion of xanthoaphin into erythroaphin in acid solution is practically quantitative, the low yield must be due to the initial protoaphin–xanthoaphin conversion being far from quantitative when carried out by chemical, as distinct from enzymic, action.

The properties of protoaphin-*fb* and its conversion into erythroaphin-*fb* (presumably *via* xanthoaphin-*fb*) by hot acids, suggested that it might be glycosidic in character. An examination of the action of dilute sulphuric acid on protoaphin-*fb* has confirmed this; in

addition to a small quantity of erythroaphin-*fb* there were produced a sugar and a brown amorphous material. The sugar has been identified as D-glucose by its behaviour on paper chromatograms and its optical rotation, and by preparation of its osazone and osotriazole. Estimation of glucose in the hydrolysis solution gave a value of 0·73 mol. per mol. of protoaphin; from this it is concluded that protoaphin is a monoglucoside, and presumably an *O*-glucoside from its ease of hydrolysis.

The brown amorphous material which forms the other main product of hydrolysis was at first thought to be the aglucone of protoaphin, but it is now known to be a mixture of probably related compounds, some of which are unstable, and it has not been possible to isolate any one of them in a crystalline condition. This brown material gives a brown solution in ether, a reddish-violet solution in sodium hydrogen carbonate, and is non-fluorescent. It is indeed very similar to protoaphin except for its greater solubility in ether and its higher acidity; its ultra-violet absorption spectrum, too, is like that of protoaphin, showing mainly end absorption. When heated with acids this product yielded some erythroaphin, and with the enzyme preparation used above for the protoaphin–xanthoaphin conversion it yielded a small amount of xanthoaphin, whose presence was demonstrated spectroscopically and confirmed by conversion into erythroaphin. The conversions into fluorescent aphins were far from quantitative, however.

The simplest explanation of these results is that the enzymic conversion of protoaphin into xanthoaphin is a two-stage process involving the production of the aglucone by hydrolysis, followed by some further reaction which yields the fluorescent pigment. When protoaphin is hydrolysed by acids, the true aglucone is doubtless first formed, but decomposes to substances which are not convertible into xanthoaphin; the brown amorphous product of hydrolysis probably contains some of the true aglucone which can undergo normal enzymic conversion into xanthoaphin and a number of these decomposition products. The analogy between the hydrolysis of protoaphin by acids and the action of the enzyme preparation must not be pressed too far at this stage, since they might involve quite different mechanisms. The explanation outlined above is, however, reasonable and it is consistent with the fact that on the provisional formula $C_{36}H_{44-46}O_{19}$ for protoaphin-*fb*, its aglucone should have a formula $C_{30}H_{34-36}O_{14}$, whereas xanthoaphin-*fb* gives values corresponding to a formula $C_{30}H_{28-30}O_{11}$.

It has been shown that the fluorescent aphins from *Tuberolachnus salignus* are not identical with those from *A. fabæ* (Part V, *loc. cit.*), and accordingly a similar series of experiments were carried out on a solution of protoaphin-*sl* prepared from a charcoal adsorbate (Part III, *loc. cit.*) by elution with 75% acetone. Similar results were obtained, D-glucose being identified in the hydrolysate; the difference between protoaphin-*fb* and protoaphin-*sl* thus resides solely in the non-carbohydrate portion of the molecule.

For the work recorded in this paper the preparation of substantial quantities of crystalline protoaphin-*fb* was necessary, and the preparative procedure described in Part II (*loc. cit.*) has been revised in that the amount and concentration of the acetone used have been restricted to minimise the difficulties due to the presence of fat and wax in the extracts.

Experimental

Protoaphin-fb (cf. Part II, *loc. cit.*).—The aphids *Aphis fabæ* (from bean plants) were killed on the plants in water at 70°, removed as before, and partly dried on a Buchner funnel under weak suction. A portion (135 g.) was ground with acetone (250 c.c.), and the solid material separated in a centrifuge. The extraction and separation was repeated first with 80% acetone (250 c.c.), and then with 50% acetone (250 c.c.). The combined extracts were kept at 0° overnight, then separated from any solids, and the filtrate was stirred for 10 minutes with charcoal (15 g.; Darco G60). The carbon was separated and the filtrate concentrated (to 125 c.c.) under a slight vacuum, protoaphin-*fb* separating as well-formed crystals (816 mg.). It was washed with water, dried by suction, and washed with acetone and ether. If necessary it could be further purified by dissolving it in N/100-sodium hydroxide, filtering, and acidifying the filtrate to pH4 with hydrochloric acid (N/100). Protoaphin is very sparingly soluble in water or acetone but easily soluble in mixtures of the two.

Hydrolysis of Protoaphin-fb.—(i) *Identification of glucose.* Protoaphin-*fb* (601 mg.) was heated at 75—80° during 1 hour with 60% dioxan (30 c.c.) and N-sulphuric acid (300 c.c.).

The mixture was extracted with ether until the ethereal layer remained colourless. The aqueous layer was neutralised with barium carbonate, filtered, and evaporated to small bulk; a positive Molisch test was then obtained. The solution was evaporated to dryness, and the residue dissolved in pyridine (10 c.c.), filtered, and evaporated to a syrup (100 mg.), $[\alpha]_D$ +44° in H_2O. On a paper chromatogram the syrup had R_F 0·20 (glucose, 0·21; sorbose, 0·21; fructose, 0·25; mannose, 0·25). The syrup gave no typical ketose reactions. An aqueous solution yielded an osazone (68 mg.) which separated from alcohol as yellow needles, m. p. 208° (decomp.) This was converted into the osotriazole (16·2 mg.) which separated from water as colourless needles, m. p. 193—194°, unchanged on admixture with the osotriazole from D-glucose (Found: C, 54·3; H, 5·5; N, 15·5. Calc. for $C_{12}H_{15}O_4N_3$: C, 54·3; H, 5·7; N, 15·8%). Hydrolysis of protoaphin and estimation of glucose by Somogyi's method (*J. Biol. Chem.*, 1945, **160**, 61) yielded the value 0·73 mole of glucose per mole of protoaphin-*fb* ($C_{36}H_{44}O_{19}$).

(ii) *The non-glucosidic products.* Protoaphin-*fb* (2·00 g.) was heated at 80° for 20 minutes with 60% methanol (100 c.c.) and N-sulphuric acid (250 c.c.). The cold mixture was extracted with ether, and the ethereal solution extracted twice with saturated aqueous sodium hydrogen carbonate. The ethereal layer showed the spectral lines of erythroaphin, and from it erythroaphin-*fb* (10 mg.) was isolated in crystalline form. Light absorption in chloroform: max. at 4210, 4470, 4840, 5220, 5610, and 5880 Å. The purplish-red bicarbonate solution was washed with ether until free from erythroaphin, and then acidified with dilute sulphuric acid. The solid product which separated was taken up in ether, and the solution dried and evaporated to small bulk. Addition of light petroleum precipitated the crude product (1·29 g.) as a brown powder. The compound was purified by extraction in a thimble with chloroform containing alcohol (1%) and precipitation by the addition of light petroleum, followed by dissolution in hot ethyl acetate, addition of light petroleum to turbidity, and cooling. The hydrolysis product was thus obtained as an amorphous brown powder, which did not melt below 300°. It was readily soluble in alcohol, acetone, ethyl acetate, ether, or carbon tetrachloride, but almost insoluble in light petroleum or alcohol-free chloroform. In aqueous alkalis it readily formed purplish-red solutions, but was sparingly soluble in aqueous acids.

Hydrolysis of Protoaphin-sl.—Protoaphin-*sl* from *T. salignus* (50 g.) was eluted from charcoal (Darco G60; 5 g.) with aqueous acetone (75%) (Duewell, Johnson, MacDonald, and Todd, *J.*, 1950, 485). The acetone was removed by evaporation and the resulting purplish-red aqueous solution of the protoaphin diluted (to 100 c.c.), made N with respect to sulphuric acid, and heated at 80° for 30 minutes. From the cooled liquid the precipitated hydrolysis product was extracted in ether.

The remaining aqueous solution was boiled with charcoal, filtered, neutralised with barium carbonate, again filtered, and then treated with successive amounts of charcoal until colourless. This solution gave a positive Molisch test. Evaporation yielded a colourless syrup, which had a positive optical rotation in water, and yielded an osazone (100 mg.) as yellow needles, m. p. 203—205° (decomp.). This gave the osotriazole which separated from water as colourless needles, m. p., alone and mixed with an authentic specimen of D-glucose osotriazole, 194—195°.

The brown ethereal solution was dried and evaporated to small bulk, and the crude hydrolysis product (320 mg.) isolated and partly purified as before. It separated as an amorphous orange powder which did not melt below 360°.

The solubility relations of the protoaphin hydrolysis product of the *sl* series are very similar to those of the *fb*-product.

Pigment-free Enzyme Solution from A. fabæ.—Washed insects (50 g.) were ground with phosphate buffer (50 c.c.; pH 6·5), the bulk of the solid material was removed by filtration through fine cloth, and the filtrate clarified by centrifuging and filtration through Supercel. The product (40 c.c.) was shaken with pure butanol (10 c.c.), the layers were separated in the centrifuge, and the aqueous layer was shaken and centrifuged again with butanol (15 c.c.). The aqueous solution was then filtered through Supercel and stored at 0°.

The extract (10 c.c.) was shaken with acetone (10 c.c.)–ether (10 c.c.), and the ethereal layer washed with water and then concentrated hydrochloric acid (to convert any aphins into erythroaphin). The ethereal layer was again washed with water and then concentrated (to 1 c.c.). Spectroscopic estimation of the erythroaphin by comparison with standard solutions gave a value of approximately 7×10^{-4} mg. of erythroaphin-*fb*.

Xanthoaphin-fb *from Protoaphin*-fb (cf. Part II, *loc. cit.*).—Protoaphin-*fb* (200 mg.) in phosphate buffer (50 c.c.; pH 6·5) and water (50 c.c.) was fermented with an enzyme extract prepared as above from *A. fabæ* (4 g.). After 40 minutes much xanthoaphin-*fb* had separated, and the mixture was extracted with ether. Concentration of the extract followed by addition of

a little chloroform caused crystallisation of the xanthoaphin-*fb* (Found: C, 63·7; H, 5·9. Calc. for $C_{30}H_{30}O_{11}$: C, 63·6; H, 5·35. Calc. for $C_{30}H_{32}O_{11}$: C, 63·4; H, 5·7%).

*Enzymic Conversion of the Non-glucosidic Hydrolysis Product of Protoaphin-*fb *into Fluorescent Aphins.*—(i) *Xanthoaphin-*fb. The hydrolysis product (2·3 mg.) from protoaphin-*fb* was dissolved in phosphate buffer (25 c.c.; pH 6·5), and an enzyme solution prepared from fresh aphids added. After 30 minutes the solution was extracted with chloroform (50 c.c.) to yield a bright yellow solution with a strong bluish-green fluorescence. The xanthoaphin-*fb* present (0·25 mg.) was estimated spectroscopically and showed the usual maxima at 3580, 3780, 4060, 4280, and 4600 Å.

(ii) *Erythroaphin-*fb. The hydrolysis product (74 mg.) in phosphate buffer (100 c.c; pH 6·5) was treated with the enzyme solution. After 1 hour, water (100 c.c.) was added, and the solution extracted with ether (3 × 100 c.c.). The ethereal layer was washed with saturated sodium hydrogen carbonate solution (3 × 50 c.c.), then with water, and dried. Evaporation yielded the crude mixed pigments which were taken up in chloroform, and converted into erythroaphin-*fb* with formic acid (Part II, *loc. cit.*). Purification by sulphuric acid partition and crystallisation from ethanol–chloroform gave erythroaphin-*fb* (13·6 mg.) as deep-red needles. Light absorption in chloroform: max. at 4210, 4470, 4870, 5220, 5620, and 5870 Å; log $\varepsilon_{max.}$ 4·46, 4·58, 3·99, 4·07, 4·26, and 3·88 respectively.

*Erythroaphin-*fb *from Protoaphin-*fb.—Protoaphin-*fb* (415 mg.) in water (160 c.c.) containing resorcinol (40 g.) and formic acid (9 c.c.) was heated under gentle reflux for 2 hours, and the solid product separated from the warm solution. The solid was washed with water and ethanol and dissolved in chloroform, and the solution shaken for 6 hours with saturated sodium hydrogen carbonate solution. The product was filtered and the chloroform layer concentrated, erythroaphin-*fb* being then obtained by the addition of hot ethanol. The product formed fine needles (16 mg.) from the same solvent (Found: C, 70·6; H, 4·7. Calc. for $C_{30}H_{22}O_8$: C, 70·6; H, 4·3%). The ultra-violet and infra-red spectra were identical with those of an authentic specimen of erythroaphin-*fb* (Parts II and V, *locc. cit.*).

Grateful acknowledgment is made to the Wellcome Trustees for a Fellowship (held by S. F. M.), and to the Royal Commissioners for the 1851 Exhibition for a Senior Studentship (held by B. R. B.). The absorption spectra were determined by Dr. R. H. Haszeldine, whom we thank.

University Chemical Laboratory, Cambridge. [*Received, September 2nd,* 1952.]