94A. *Colouring Matters of the Aphididæ. Part II. Colouring Matters from* Aphis fabæ.

By J. P. E. Human, A. W. Johnson, S. F. MacDonald, and A. R. Todd.

An investigation leading to the isolation of four related colouring matters of a new type from the common bean aphid (*Aphis fabæ*) is described. The hæmolymph of the living insects contains the yellow *protoaphin*-fb, which is converted enzymically after the death of the insects into the fluorescent yellow *xanthoaphin*-fb. Protoaphin-*fb* can be isolated only from freshly collected insects which have been killed by a method (*e.g.*, quick heating) which simultaneously inactivates enzymes. Xanthoaphin-*fb* is unstable and undergoes ready conversion, particularly in presence of acids, first into the fluorescent, orange *chrysoaphin*-fb, and then into the fluorescent, carmine-red *erythroaphin*-fb. Preliminary chemical investigation of erythroaphin-*fb* indicates that it is a polycyclic quinone containing 2 phenolic hydroxyl groups; on oxidation with nitric acid it yields mellitic acid.

Very similar pigments are obtained from other species of Aphididæ, and the nomenclature adopted includes the use of suffix letters indicative of the species of origin.

In a preliminary communication (Duewell, Human, Johnson, MacDonald, and Todd, *Nature*, 1948, **162**, 759), which we regard as Part I of the present series of papers, we gave a general account of our initial investigations on a remarkable group of fluorescent colouring matters which occur in the hæmolymph of many species of Aphididæ, or which derive from precursors present in the hæmolymph. These pigments we propose to describe in general as *aphins*. In each species there are four aphins to be considered (*a*) the *protoaphin* present in the living insect, (*b*) the *xanthoaphin* into which protoaphin is converted by enzymic action on the death of the insect, (*c*) *chrysoaphin* produced when xanthoaphin is kept in solution, and (*d*) *erythroaphin* which is formed by a similar transformation of chrysoaphin and which represents the moderately stable end-product of the series. The conversions (*b*) ⟶ (*c*) ⟶ (*d*) occur spontaneously in crude extracts of the insects and appear to be independent of the presence of air, although they are enormously accelerated by heat or by acids or alkalis; the characteristic visible absorption spectra of the pigments make it easy to follow the conversions and to detect the individual pigments in solution by means of a hand-spectroscope. The existence of this series of pigments and their remarkable transformations were recorded in some detail by Sorby nearly eighty years ago (*Quart. J. Microscop. Sci.*, 1871, ii (N.S.), 352), but until now his work seems to have escaped the notice of other investigators. Thus Blount (*J.*, 1936, 1034) has reported the isolation of two aphid colouring matters lanigerin and strobinin (respectively a chrysoaphin and an erythroaphin on our nomenclature), and Schulz (*Biochem. Z.*, 1922, **127**, 122) has recorded some observations on the pigments of the woolly aphid (*Eriosoma lanigerum*) without apparently being aware of Sorby's pioneer work. Other colouring matters apparently unrelated to the aphins have also been detected by us in certain Aphididæ and will be described later; whether they are related to the pigments of *A. gossypii*, examined by Wall (*Ann. Entomol. Soc. Amer.*, 1933, **26**, 425), is at present unknown.

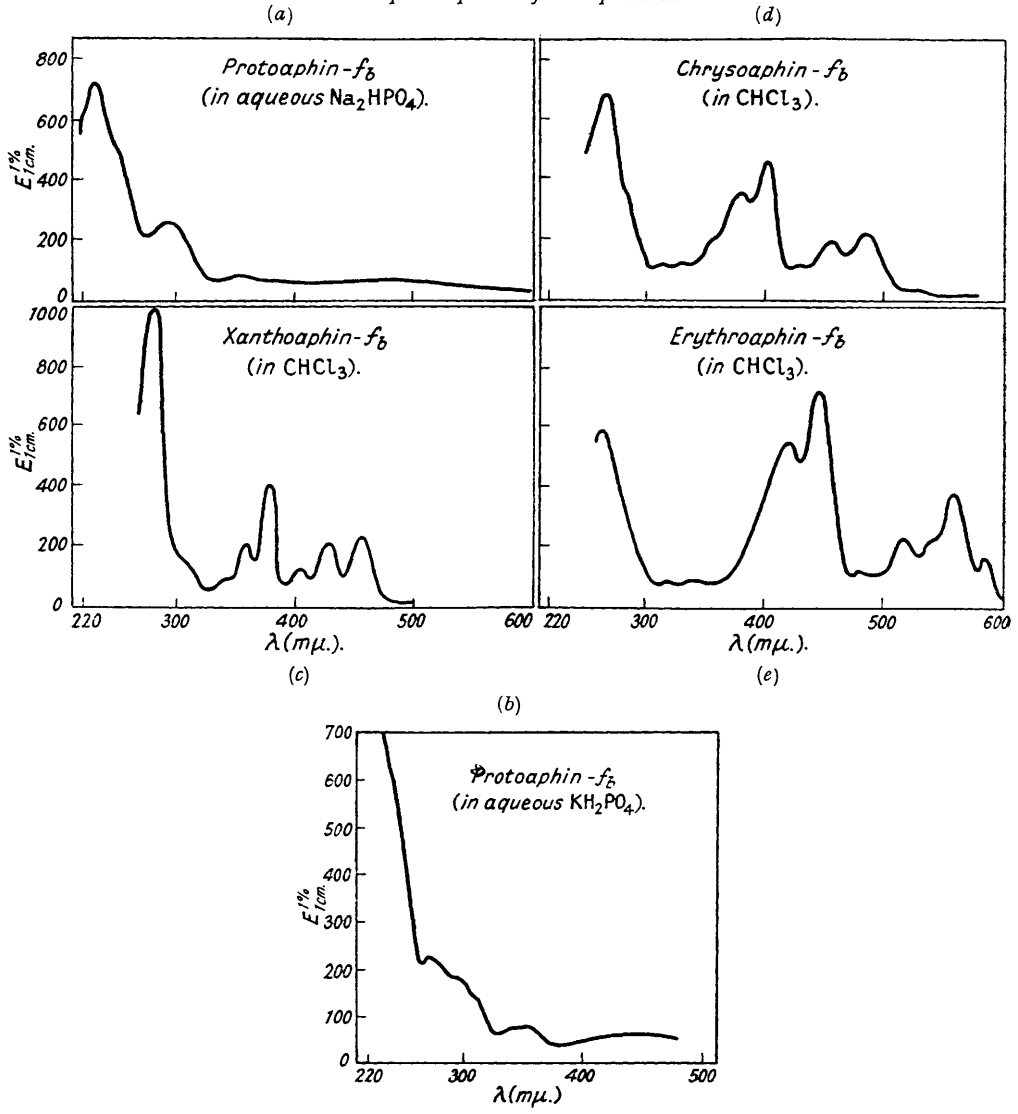
Although it is a simple matter to distinguish between the four aphins from any given aphid species by their visible and ultra-violet absorption spectra (cf. the spectra of the pigments from *Aphis fabæ* recorded in Fig. 1), it is not possible by this means to differentiate between the corresponding aphins from different species. Since, despite the close similarity in this and in many other respects, certain differences can nevertheless be detected in *X*-ray diffraction patterns and in infra-red absorption spectra, we have proposed that a two-letter suffix, indicative of the species of origin, be added to the name of each pigment until the question of identity or otherwise of corresponding pigments in the various species can be finally settled (Duewell, Human, Johnson, MacDonald, and Todd, *loc. cit.*). The present paper therefore deals with the isolation and preliminary examination of *protoaphin*-fb, *xanthoaphin*-fb, *chrysoaphin*-fb, and *erythroaphin*-fb, the pigments obtained from *Aphis fabæ*. This aphid is very prevalent in Britain and is the " black fly " which attacks cultivated broad beans (*Vicia faba*) during early

summer with depressing regularity; it also occurs on a variety of other hosts, *e.g.*, members of the beetroot family. In our experiments the insect was obtained mainly from heavily infested, cultivated broad bean plants in Cambridgeshire.

Although the actual pigment content of *A. fabæ* is *ca.* 0·8% of the weight of live insects, the development of suitable methods for isolating the various aphins in a pure condition has been a slow and tedious matter. This was caused in part by our failure to appreciate at the outset of

FIG. 1.

Absorption spectra of the aphins-fb.



our researches that we were dealing with a group of very labile substances capable of undergoing the series of interconversions mentioned above, and in part to the necessity of devising methods whereby insects could be collected as far as possible alive and undamaged. An added complication was the difficulty of obtaining the large amounts of insects necessary and the development of methods whereby, since they are seasonal in habit, the collected insects could be stored for later study without gross decomposition of the aphins. It is not proposed to discuss in detail the various methods we have from time to time employed during the progress of the work;

instead descriptions of methods will be confined essentially to those which we finally adopted for the isolation of the main bulk of *A. fabæ* pigments used in the present studies.

When undamaged specimens of *A. fabæ* are killed by quick heating to 70°, enzymes are simultaneously inactivated, and, if the insects are then crushed with aqueous acetone or aqueous methanol, extracts are obtained which show no characteristic bands in their visible-light absorption spectra and yield no fluorescent material on extraction with light petroleum. The colour of such crude extracts varies from red to greenish-brown in different batches of insects collected at different times. This is caused by the presence in the insects of varying amounts of a green material, apparently unrelated to the aphins. This green substance, which is probably a mixture of pigments, has not been closely examined, but its presence does not interfere with the isolation of the aphins; it is soluble in water, is unaffected by fermentation of the insect material, and is not extractable with light petroleum. When the filtered crude extracts are acidified and the acetone or methanol removed, protoaphin-*fb* separates as yellow needles. Protoaphin-*fb* contains only carbon, hydrogen, and oxygen, is yellow in acid aqueous solution and purplish-red at pH values >5·5. If, however, living specimens of *A. fabæ* are crushed with cold water and the suspension set aside at room temperature for an hour, the red colour of the protoaphin disappears and extraction of the resulting suspension with aqueous acetone gives bright yellow extracts with a strong bluish-green fluorescence. These extracts apparently contain no protoaphin-*fb*, since the yellow pigment they contain can be completely extracted from them with light petroleum; spectroscopic examination shows indeed that they contain mainly the water-insoluble xanthoaphin-*fb*. This conversion of protoaphin-*fb* into xanthoaphin-*fb* in crude extracts is best carried out by using, in place of water, buffer solution of pH 6·5. If the enzymic action occurs under unfavourable conditions, *e.g.*, on preserving dead but intact insects at temperatures >−10° for some weeks, the protoaphin-*fb* is degraded to various ill-defined products which can neither be extracted with light petroleum nor transformed into other aphins. Although the enzyme responsible for the conversion has not yet been isolated, there can be no doubt that protoaphin-*fb* is the precursor of xanthoaphin-*fb*. The crystalline protoaphin is converted into the xanthoaphin by treating solutions buffered to pH 6—7 with freshly crushed insects; under these conditions one crushed insect contains sufficient enzyme to convert some thirty times its own content of protoaphin. Again, although xanthoaphin-*fb*, chrysoaphin-*fb*, and erythroaphin-*fb* are all obtainable from dead fermented insects, they are not present to any appreciable extent in living insects. This follows from the facts that only protoaphin-*fb* is found in extracts of insects killed by being heated to 70° and that when living insects are crushed in concentrated sulphuric acid the solutions obtained do not show the strong absorption bands characteristic of the fluorescent aphins.

For the preparation of xanthoaphin, chrysoaphin, and erythroaphin, fermented insects are extracted with 80% acetone. Most of the fat in the extracts is separated by cooling and the solution is then shaken with light petroleum. Evaporation of the petroleum layer gives, as a rule, a granular precipitate of the mixed aphins in varying proportion, contaminated by some fat or wax which can be removed by re-extraction with light petroleum. Owing to the seasonal occurrence of *A. fabæ*, and indeed of all the Aphididæ, and the strictly limited period during which they can be collected in quantity, it was necessary to find some way of preserving the insects so that they could later be extracted. It has been found that this end can be achieved by storage at temperatures below −10° and that insects so stored for periods up to at least six months form suitable starting material for the preparation of chrysoaphin-*fb* and erythroaphin-*fb*, using a slight variation of the above method of extraction. For the xanthoaphin, fresh insects are preferable, and we have not been able to prepare protoaphin-*fb* in a pure state from preserved insects.

Separation and purification of the mixed aphins obtained by the above extraction procedures depend on the conversions xanthoaphin-*fb* ⟶ chrysoaphin-*fb* ⟶ erythroaphin-*fb* and on the fact that, although in most solvents the solubilities of the aphins decrease in the order named, this is reversed in carbon tetrachloride in which xanthoaphin-*fb* is the least soluble. The preparation of pure xanthoaphin-*fb* by direct crystallisation of the mixed aphins is satisfactory only when it is present in a relatively high proportion. It holds small amounts of solvents tenaciously and it is preferable to recrystallise it from ether immediately before analysis. Xanthoaphin-*fb* is unstable in solution or in the solid state in presence of light and changes into the orange chrysoaphin-*fb*; the change is greatly accelerated by heat or by acids, and it is advisable to carry out chemical manipulations with the substance rapidly and as far as possible in the absence of light.

Chrysoaphin-*fb* can be obtained by concentrating the carbon tetrachloride mother-liquor

obtained after xanthoaphin has been separated from the crude mixed aphins. It may also be prepared from the yellow crystalline xanthoaphin by mild acid treatment, *e.g.*, by warming it with commercial chloroform; mixtures of xanthoaphin and chrysoaphin may be worked up for the latter by similar treatment. Like xanthoaphin-*fb*, chrysoaphin-*fb* has no definite melting point and on heating appears to undergo partial conversion into erythroaphin-*fb* before decomposing.

Erythroaphin-*fb*, which forms beautiful carmine-red needles, can be isolated from the final crystallisation mother-liquors of the mixed aphins. It can also be conveniently prepared from xanthoaphin-*fb* or chrysoaphin-*fb*, or from mixtures of these pigments, by warming their solutions in ethyl acetate with a little formic acid or by treating them in ethanolic solution with mineral acid. Purification of crude erythroaphin-*fb* is normally effected by recrystallisation from ethanol–chloroform; chromatography has not been found very useful, as the pigment is too strongly adsorbed on most of the common adsorbents.

Erythroaphin-*fb*, being the end-product and the most stable member of the aphin-*fb* series, was the most readily available material in the earlier stages of our work and has therefore been the subject of most investigation so far. Like the other aphins, it has no melting point and on heating it gradually chars and decomposes above 250°; it cannot be sublimed even in a high vacuum. It is moderately soluble in chloroform, carbon tetrachloride, benzene, or acetone, and only sparingly so in most of the other common organic solvents. In dry pyridine it dissolves readily but solutions in this solvent undergo some chemical change on storage. Solutions in organic solvents or in concentrated sulphuric acid are red and show a red fluorescence, which is particularly intense in ultra-violet light. Solutions in aqueous pyridine, sodium hydroxide, and ammonia are bright green and deposit green precipitates on storage, and similar solutions are obtained under certain conditions with sodium carbonate or hydrogen carbonate. On treatment with acetic anhydride containing a drop of sulphuric acid, the solution, at first red, changes rapidly through green to pure blue with an intense red fluorescence. This striking colour reaction was first described by Blount (*loc. cit.*) for strobinin (erythroaphin-*st*) and is characteristic of all the erythroaphins we have examined. Since under the acid conditions used, xanthoaphins and chrysoaphins are converted into the corresponding erythroaphins, it may be regarded as a general reaction for all the fluorescent aphins.

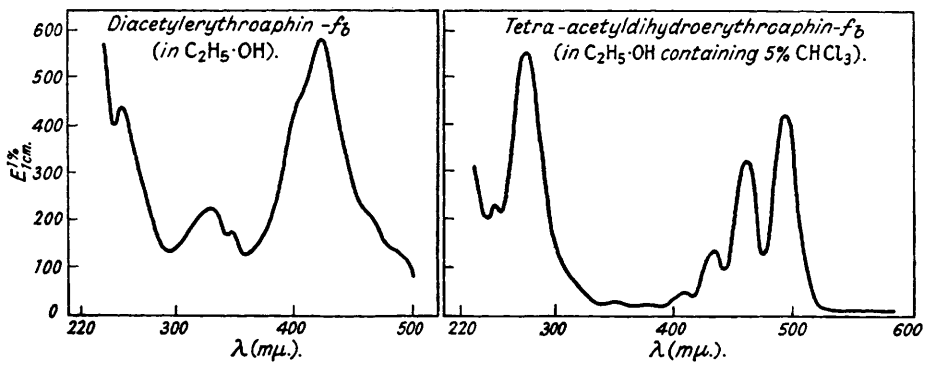
Erythroaphin-*fb* contains neither nitrogen, sulphur, nor halogens; it contains no methoxy-groups (Zeisel), and chromic acid oxidation (Kuhn–Roth) suggests the presence of several *C*-methyl residues. Difficulties have been encountered in obtaining consistent analytical values, but the results of a series of analyses indicate that it has a formula $C_{30}H_{22-24}O_8$. Attempts to determine the molecular weight of erythroaphin-*fb* by cryoscopic and ebullioscopic methods gave values ranging from 400 to 700, and similar inconsistent results were obtained using the Signer vapour-pressure method. Acyl derivatives of erythroaphin, although they seemed more suitable for these determinations on account of their greater solubility, also gave variable results. *X*-Ray crystallographic examination, however, indicates that erythroaphin-*fb* has a molecular weight of 260, 519, or 1038 $\pm$ 3% according to whether the unit cell contains 8, 4, or 2 molecules. Of these values only 519 $\pm$ 3% seems to be in accord with the behaviour of erythroaphin-*fb* on reduction and reductive acetylation and with the admittedly variable results of molecular-weight determinations by other methods. Acetylation yields *diacetyl-erythroaphin*-fb ($C_{34}H_{26-28}O_{10}$) which is insoluble in cold alkali and is readily hydrolysed to erythroaphin-*fb*. A *dibenzoyl* derivative has also been prepared, but attempted methylation failed to yield crystalline products. Hydrogenation of erythroaphin-*fb* in presence of a platinum catalyst or reduction with sodium dithionite gives a pale brown, fluorescent solution of a dihydroerythroaphin, which is rapidly re-oxidised in air to the original erythroaphin (identified by *X*-ray powder photography). These findings strongly suggest the presence of a quinonoid structure, and also the absence of non-reversibly reducing groups such as ethylenic linkages, aldehydo-groups etc. The presence of a quinonoid structure is confirmed by the behaviour of erythroaphin-*fb* or its diacetyl derivative on reductive acetylation, when the orange *tetra-acetyl-dihydroerythroaphin*-fb ($C_{38}H_{30-32}O_{12}$) is obtained. This derivative is insoluble in alkali and resists catalytic hydrogenation. Its formation eliminates a C_{15} formula for erythoaphin-*fb*, since reductive acetylation must introduce at least two acetyl groups. Evidence pointing to a C_{30} formula has also been obtained by quantitative microhydrogenation of erythroaphin-*fb* and its diacetyl derivative. A C_{60} formula (*i.e.*, *M*, 1038 $\pm$ 3%) would require the presence of two quinone groups and the absence of an intermediate absorption spectrum or of any break in the hydrogenation at the half-way stage is regarded as evidence against such a hypothesis. The absorption curves of diacetylerythroaphin-*fb* and tetra-acetyldihydroerythroaphin-*fb* are reproduced in Fig. 2.

In the erythroaphin-*fb* molecule, two oxygen atoms thus seem to be present in hydroxy-groups and two in a quinone system. The function of the remaining oxygens is still unknown, as is the nature of the nucleus. That the pigment is polynuclear would seem probable from the fact that oxidation with concentrated nitric acid yields mellitic acid, identified as its hexamethyl ester. In certain of its reactions erythroaphin-*fb* shows a resemblance to the photodynamic pigment of St. John's wort, hypericin, $C_{30}H_{16}O_8$ (Brockmann *et al.*, *Naturwiss.*, 1939, **27**, 550; *Annalen*, 1942, **553**, 1; Pace and McKinney, *J. Amer. Chem. Soc.*, 1941, **63**, 2570), and to the mould pigment oxypenicilliopsin (Oxford and Raistrick, *Biochem. J.*, 1940, **34**, 790). It is also of interest that Dhéré (*Compt. rend. Soc. Biol.*, 1939, **131**, 672; *Boissiera*, 1943, **7**, 423) has already remarked on the similarity between the ultra-violet fluorescence spectra of the lanigerin (chrysoaphin-*ln*) and strobinin (erythroaphin-*st*) of Blount (*loc. cit.*) and those of hypericin and oxypenicilliopsin. At present, however, there is insufficient chemical evidence to warrant any speculation on the nature of the relationship, if any, which may exist between erythroaphin-*fb* and these plant pigments.

So far little work has been done on the protoaphin, xanthoaphin and chrysoaphin from *A. fabæ*. The ready conversion of the last two into erythroaphin-*fb* complicates chemical study; thus, for example, when chrysoaphin-*fb* is acetylated the product is diacetyl-erythroaphin-*fb*. Elementary analysis gives values which correspond approximately to formulæ $C_{30}H_{30}O_{11}$ for xanthoaphin-*fb* and $C_{30}H_{26}O_9$ for chrysoaphin-*fb*. These formulæ,

FIG. 2.

Absorption spectra of derivatives of erythroaphin-fb.



although only provisional, are at any rate in agreement with our failure to detect any by-products in the conversion of them into erythroaphin-*fb* and suggest that the conversion involves dehydration with an increase in aromatic character.

Protoaphin-*fb* differs from the three fluorescent aphins derived from it both in its chemical properties and in its composition. Analytical results suggest for it a formula $C_{36}H_{44}O_{19}$. It is reversibly reduced by dithionite to a colourless, fluorescent material, and it is readily oxidised to colourless products by a variety of reagents. Whether the enzymic conversion of protoaphin-*fb* to xanthoaphin-*fb* is accompanied by loss of a carbohydrate residue (as a comparison of the provisional formulæ of the compounds might suggest) has not yet been determined, and a decision on this and other relevant points must await the results of further work now in progress. It must be emphasised that the formulæ given in this paper for protoaphin-*fb*, xanthoaphin-*fb* and chrysoaphin-*fb* are advanced merely to indicate that a simple relationship between them and erythroaphin-*fb* would not conflict with the analytical data, and they may require considerable revision when further evidence becomes available.

EXPERIMENTAL.

Collection of Material.—After trial of a variety of methods, that which involved collection of the infected portions of bean plants and removal of the insects by a washing procedure (see below) was adopted for general use. Although it is possible to store *A. fabæ* at temperatures $< -10°$ while still on the plants, it was found more satisfactory to remove them as soon as possible after the plants were collected and, after separating them from foreign insects, to keep the aphids in cold storage in glass bottles.

Protoaphin-fb.—Plant material bearing living insects was placed in a cylindrical basket of wire gauze ($\frac{1}{8}$" mesh) which fitted inside a beaker (10-l. capacity) containing sufficient water (usually 6 l.) at 70° to immerse the plants completely. After 3 minutes the beaker and contents were placed in a sink lined

with cotton gauze (cheese-cloth), and a strong jet of tap water was directed on one side of the basket. This had the effect of rotating the basket and washing the insects through the wire gauze, whereupon they passed out of the beaker with the stream of water and were collected on the cheese-cloth. After this operation, the beaker was removed and excess of water gently squeezed out of the insects before they were transferred to a beaker containing methanol (250 c.c. for each 100 g. of wet aphids).

The mixture of wet insects (100 g.) and methanol (150 c.c.) was ground in a Waring Blendor for 2 minutes. Hyflo Supercel (5 g.) was stirred into the suspension which was filtered through a thin layer of Supercel. The filter residue was again extracted with aqueous methanol (260 c.c. of 60%) in the Waring Blendor and filtered as before, and the combined extracts were diluted with an equal volume of methanol and extracted with light petroleum (b. p. 40—60°; 2 × 250 c.c.) to remove any traces of fluorescent aphins. The aqueous-methanolic layer was then concentrated under reduced pressure to 150 c.c., treated with active charcoal (6 g.; Darco G. 60) with stirring, and filtered. The filtrate at this point had pH *ca.* 5 and no longer became red at pH >7. The charcoal containing the adsorbed protoaphin-*fb* was thoroughly washed with water and eluted by being stirred with aqueous acetone (75%; 6 × 25 c.c.). On removal of the acetone from the eluate under reduced pressure, *protoaphin*-fb (0·25—0·5 g.) separated as small, flat, golden needles which were washed with water and dried. For further purification the product was dissolved in aqueous sodium hydroxide (N./100) and filtered, and the filtrate acidified to pH 4 with hydrochloric acid (N./100). The purified pigment had no m. p. and on heating gradually darkened and decomposed above 210° [Found, in material dried at 56°/0·1 mm.: C, 55·2; 55·8, 55·9; H, 5·8, 5·6, 5·6; active H, 1·41; *C*-CH_3 (Kuhn–Roth), 7·9. $C_{36}H_{44}O_{19}$ requires C, 55·4; H, 5·7%]. Protoaphin-*fb* contains neither nitrogen, sulphur, halogens, phosphorus, nor methoxyl. It is readily soluble in dilute sodium hydroxide solution, 70—80% acetone, or pyridine, moderately so in 80% ethanol, 80% acetic acid, or aqueous dioxan, and very sparingly soluble in water, ether, light petroleum, chloroform, ethanol, acetone, or acetic acid. Solutions in water and organic solvents, including anhydrous pyridine, are yellow and those in sodium hydroxide and aqueous pyridine purplish-red. In concentrated sulphuric acid the pigment dissolved with decomposition, the solution darkening rapidly. Light absorption: (i) In KH_2PO_4 solution: Maxima at 2730, 3530, and 4420—4450 A.; $E^{1\%}_{1cm.}$ = 220, 73, and 62 with inflections at 2920—2960, 3000—3060, and 3450—3470 A.; $E^{1\%}_{1cm.}$ = 175, 140, and 70 respectively. (ii) In Na_2HPO_4 solution: Maxima at 2340, 2960, 3560, and 5180 A.; $E^{1\%}_{1cm.}$ = 725, 250, 75, and 70.

Xanthoaphin-fb.—The live insects were washed off infected bean plants as described under the preparation of protoaphin-*fb* (above), except that cold water was used throughout. The fresh wet aphids (150 g.) were crushed with Sörensen buffer (150 c.c.; pH 6·2) in a large mortar and left at room temperature for 1 hour. Acetone (600 c.c.) was then added and the mixture ground in a Waring Blendor for 2 minutes. After being stirred with Hyflo Supercel (5 g.) and filtered through Supercel, the residue was re-extracted with aqueous acetone (80%; 3 × 25 c.c.) until the extract was only faintly coloured. The combined greenish-brown filtrate and extracts were concentrated under reduced pressure, a " boiling stick " being used to promote even evaporation, until separation of solid on the surface set in, whereupon the mixture was cooled to −10° by addition of solid carbon dioxide. The precipitated fat was separated (Supercel) and the clarified solution extracted with light petroleum (b. p. 40—60°; 3 × 500 c.c.), emulsions formed during the extraction being readily broken by addition of small amounts of acetone. The combined petroleum extracts normally had a light-yellow colour and showed strong bluish-green fluorescence; they contained mainly xanthoaphin-*fb* with small amounts of chrysoaphin-*fb* and erythroaphin-*fb*. Solvent was removed at ⩾35° (bath-temp. ⩾50°) under slightly reduced pressure, giving a pale brown residue of crude colouring matter (0·6—0·9 g.) which was normally free from all but traces of fat. The powder was washed with light petroleum, dried, and boiled with ether (80 c.c.). The ethereal extract was concentrated to smaller bulk (30 c.c.), acid-free carbon tetrachloride (30 c.c.) added, and the solution concentrated until crystallisation set in, whereafter it was cooled to 0°. The *xanthoaphin*-fb was collected and recrystallised from ether; it formed bright yellow needles (0·4 — 0·5 g.) which on heating did not melt but gradually decomposed above 210° (Found, in material dried at room temperature/0·1 mm. for 12 hours: C, 63·7, 63·5, 64·4, 64·3; H, 5·9, 5·5, 5·7, 5·9. $C_{30}H_{30}O_{11}$ requires C, 63·6; H, 5·3%). Qualitative tests for nitrogen, phosphorus, sulphur, and halogens were negative.

Xanthoaphin-*fb* is readily soluble in acetone, ethanol, pyridine, or ethyl acetate, moderately so in chloroform, ether, or benzene, and very sparingly in carbon tetrachloride or light petroleum; it is insoluble in water. Solutions in organic solvents or aqueous organic solvents at neutral or acid pH are bright yellow and show a bluish-green fluorescence even in daylight; in ultra-violet light the fluorescence is intense even for the solid substance. It dissolved readily in aqueous sodium hydroxide, giving a pink solution with a powerful yellowish-green fluorescence in ultra-violet light. Light absorption in chloroform: Maxima at 2820, 3580, 3780, 4050, 4290, and 4590 A.; $E^{1\%}_{1cm.}$ = 992, 198, 400, 116, 205, and 228 respectively.

It is difficult to work with xanthoaphin-*fb* or at times even to purify it because of its great lability, particularly to traces of acid or to heat, under the influence of which it undergoes irreversible conversion into chrysoaphin-*fb* (see below). This conversion has frequently occurred during recrystallisation, presumably owing to the presence of unsuspected traces of acid. In the same way, crystalline xanthoaphin-*fb* is liable to undergo slow conversion on being kept for long periods in specimen tubes. Under favourable conditions extractions carried out as described above give xanthoaphin-*fb* in amounts greater than half of the crude aphin mixture. On the other hand, if preserved insects are used it is very difficult to isolate pure xanthoaphin-*fb* and in such cases it was usually better to employ a modified procedure, as described later, and to proceed directly to the isolation of the chrysoaphin and erythroaphin.

Chrysoaphin-fb.—The combined ether–carbon tetrachloride mother-liquors (40—60 c.c.) from the separation and crystallisation of the xanthoaphin-*fb* in the above experiment were heated under reflux with chloroform (20 c.c.; not specially purified and therefore slightly acidic) until the characteristic 4300-A. line in the visible-light absorption spectrum of the xanthoaphin could no longer be detected with the hand-spectroscope and was replaced by the 4860-A. band of the chrysoaphin. The solution was now concentrated until crystallisation began and then set aside for a short time. *Chrysoaphin*-fb (0·2—0·3 g.)

separated as minute orange crystals. Recrystallised from an acid-free mixture of chloroform and carbon tetrachloride, the substance had no definite m. p. but decomposed with darkening when heated above 250° (Found, in material dried at 60°/0·01 mm. for 14 hours: C, 68·0, 68·4, 68·2; H, 5·2, 4·8, 4·8; active H, 0·57. $C_{30}H_{26}O_9$ requires C, 67·9; H, 5·0%). Qualitative tests for nitrogen, phosphorus, sulphur, halogens, and methoxyl were negative. Light absorption in chloroform: Maxima at 2680, (3260), 3800, 4020, (4300), and 4860 A.; $E^{1\%}_{1\,cm.}$ = 685, (101), 350, 454, (102), 190, and 213 respectively.

Chrysoaphin-*fb* is readily soluble in chloroform, acetone, dioxan, ethyl acetate, or pyridine, moderately so in ether, benzene, ethanol, or carbon tetrachloride, and sparingly in light petroleum; it is insoluble in water. Neutral or acidic solutions in organic solvents are yellow and have a blue-green fluorescence which is particularly brilliant when viewed in ultra-violet light. The substance dissolves in aqueous alkali to give a crimson solution with an intense orange fluorescence in ultra-violet light. The solid pigment itself has a fine orange fluorescence in ultra-violet light. Chrysoaphin-*fb* is converted into erythroaphin-*fb* on being heated with acids.

Erythroaphin-fb.—The combined mother-liquors from the chrysoaphin-*fb* preparation described above were evaporated under reduced pressure, the residue taken up in chloroform (50 c.c.), and formic acid (5 c.c.; anhydrous) added. The mixture was heated under reflux for 20 minutes, by which time examination of the solution with a hand-spectroscope revealed only the characteristic visible-light absorption bands of erythroaphin-*fb*. Most of the solvent was removed by distillation, warm ethanol (30 c.c.) was added, and the solution concentrated until crystallisation set in. Set aside overnight, *erythroaphin*-fb had separated as small deep-carmine-red needles (0·1 — 0·15 g.); for analysis it was recrystallised several times from chloroform–ethanol [Found, in material dried at 60°/0·01 mm. for 12 hours: C, 70·5, 70·6; H, 4·8, 4·6; active H, 0·84; *C*-CH_3 (Kuhn–Roth), 12·4. $C_{30}H_{22}O_8$ requires C, 70·6; H, 4·4. $C_{30}H_{24}O_8$ requires C, 70·3; H, 4·7%]. Erythroaphin-*fb* contains no halogens, nitrogen, phosphorus, sulphur, or methoxyl, and it has no definite m. p., charring and decomposing above 250°. Light absorption in chloroform: Maxima at 2670, 4210, 4465, 4850, 5205, 5600, and 5860 A.; $E^{1\%}_{1\,cm.}$ = 586, 550, 721, 115, 244, 375, and 165 respectively.

Erythroaphin-*fb* is moderately soluble in chloroform or carbon tetrachloride, somewhat less so in pyridine, acetone, benzene, dioxan, ether, acetic acid, or acetic anhydride; it is very sparingly soluble in ethanol or ethyl acetate and quite insoluble in water. Neutral or acid solutions in organic solvents are red with an orange-red fluorescence which is intense in ultra-violet light. With sodium hydroxide it gives deep-green solutions with a ruby-red fluorescence visible in ultra-violet light. Although neither sodium carbonate nor hydrogen carbonate will extract erythroaphin-*fb* from its solutions in benzene or chloroform, sodium carbonate, but not hydrogen carbonate, will extract it from ether; again, if the pigment is first dissolved in a little acetone, ethanol, or dioxan, addition of either sodium carbonate or hydrogen carbonate will give the characteristic green solutions of the alkali salt, although neither of them in aqueous solution will dissolve the solid pigment. Erythroaphin-*fb* gives a red solution in concentrated sulphuric acid and with acetic anhydride containing a drop of sulphuric acid the initially red solution passes rapidly through green to blue and acquires a strong red fluorescence (cf. Blount, *loc. cit.*, on the same reaction with strobinin). Hydrogenation of the pigment (22·8 mg.) in dioxan solution with Adams's catalyst proceeded with uptake of 1·12 mols. of hydrogen (1·19 c.c. at 15°/754 mm.) calculated on a formula $C_{30}H_{24}O_8$, giving a pale brown solution with an intense green fluorescence. On exposure of the reduced solution to air it was rapidly re-oxidised, yielding the original erythroaphin-*fb*. A similar reversible reduction occurred with sodium dithionite.

Modified Extraction Procedure for Preserved Insects.—Since insects which have been preserved in cold storage are useless for preparation of protoaphin and since the isolation of the xanthoaphin was difficult, at any rate in the case of *A. fabæ*, a modified method of extraction has usually been employed in which only chrysoaphin-*fb* and erythroaphin-*fb* were isolated; the yields obtained varied considerably from batch to batch, as indeed they also did with different batches of fresh insects.

The preserved aphids (100 g.) were thoroughly extracted with acetone by the method described above in the preparation of xanthoaphin-*fb*, but the removal of fat by freezing was usually omitted. Any insoluble material was carefully removed before proceeding further, however, in order to avoid undue emulsification in the extraction of the aqueous-acetone solution with light petroleum (b. p. 40—60°; 3 × 250 c.c.). Evaporation of the orange-red petroleum extract left a viscous red oil which was freed from residual acetone under reduced pressure at room temperature. On boiling this oil with a small amount of light petroleum (b. p. 40—60°; 50 c.c.) for 10 minutes most of the fat was removed with little loss of colouring matter. The residue (0·4—0·5 g.), now a granular red powder, was washed with light petroleum and dissolved in chloroform (20 c.c.), carbon tetrachloride (50 c.c.) was added, and the solution concentrated until crystallisation set in. On cooling chrysoaphin-*fb* (75—150 mg.) separated. Erythroaphin-*fb* (50—100 mg.) was obtained from the chrysoaphin-*fb* mother-liquors by treatment with formic acid and working up as described above.

Enzymic Conversion of Protoaphin-fb *into the Fluorescent Derived Aphins.*—In the absence of a purified enzyme preparation, crushed insects were employed to bring about the conversion. A control experiment was first carried out in which preserved insects (10 g.) were crushed with water (2 c.c.), and the mixture set aside at room temperature for 18 hours and then ground with Hyflo Supercel (0·5 g.) and acetone (20 c.c.). The mixture was filtered and the residue thrice re-extracted in the same way with aqueous acetone (80%; 3 × 20 c.c.). The combined filtrates were extracted with light petroleum (b. p. 40—60°; 3 × 40 c.c.), and the extract was filtered and evaporated under reduced pressure. Fat was removed from the residue by digestion with light petroleum (25 c.c.), and the insoluble mixture of aphins was collected on a sintered-glass funnel, dried, and weighed. The result obtained gave an estimate of the weight of fluorescent aphins derived from the insects used. In subsequent experiments using varying amounts of insects, a weighed amount (*ca.* 60 mg.) of pure protoaphin-*fb* was dissolved in aqueous sodium hydroxide (N./100), the pH was adjusted to 5·8—6·2 by hydrochloric acid (*ca.* 5·6 c.c. of N./100), and the solution added to the insects about to be crushed; subsequent operations were carried out as above. It was found that the results were not materially affected by varying the amount of water used in the first

crushing, but there was some variation according to the length of time the crushed insects were kept before extraction with acetone. For this reason, 18 hours was used as a standard time in the experiments recorded in the accompanying table. It is evident that one aphid contains enough enzyme to convert many times its own content of protoaphin-*fb* into the fluorescent aphins.

Wt. of insects (g.).	Wt. of added protoaphin-*fb* (mg.).	Total yield of aphins (mg.).	Wt. of aphins derived from insects (mg.).	Wt. of aphins derived from protoaphin-*fb* (mg.).
10	—	40·8	40·8	—
0·508	60·1	41·0	2·0	39·0
0·35	60·4	33·6	1·4	32·2
0·25	60·5	32·7	1·0	31·7

Diacetylerythroaphin-fb.—(*a*) *From erythroaphin*-fb. Acetylpyridinium chloride was prepared as a white semi-solid mass by dropwise addition of acetyl chloride (2 c.c.) to pure pyridine (5 c.c.) with shaking and external cooling. Erythroaphin-*fb* (200 mg.) dissolved in ice-cold pyridine (100 c.c.) was now added rapidly and the mixture stirred for 15 minutes and then allowed to come to room temperature; the initially red solution changed during this operation to a bright yellow semi-solid suspension. An equal volume of benzene was added, the mixture poured on ice, and the benzene layer washed thoroughly with water, hydrochloric acid, and again water, dried, and evaporated under reduced pressure. The resinous residue was dissolved in hot ethanol, and the solution concentrated to incipient crystallisation. On cooling, *diacetylerythroaphin*-fb separated as mustard-yellow needles. After two further recrystallisations from ethanol by a similar procedure the pure substance (190 mg.) melted with decomposition at 245—250° after previous darkening [Found: C, 68·5; 68·3; H, 4·5, 4·5; *O*-acetyl, 13·8, 14·2. $C_{30}H_{20}O_8(CO\cdot CH_3)_2$ requires C, 68·7; H, 4·4; *O*-acetyl, 14·5. $C_{30}H_{22}O_8(CO\cdot CH_3)_2$ requires C, 68·6; H, 4·7; *O*-acetyl, 14·4%). Light absorption in ethanol: Maxima at 2560, 3300, 3470, and 4245 A.; $E^{1\%}_{1\,cm.}$ = 428, 217, 167, and 566 respectively. Molecular weight by the freezing-point method in ethylene dibromide, 534 ($C_{34}H_{28}O_{10}$ requires *M*, 596).

Diacetylerythroaphin-*fb* appears to be dimorphous, since it was obtained on occasion as red prisms which were not closely examined but changed to the normal yellow form on recrystallisation. It is soluble in most of the common organic solvents with the exception of light petroleum, in which it is very sparingly soluble. Its yellowish-orange solutions in organic solvents fluoresce orange in ultra-violet light, and it is not extracted from such solutions by aqueous sodium hydroxide. Hydrolysis with ethanolic alkali yields erythroaphin-*fb*. Reduction with sodium dithionite or with hydrogen in presence of a platinum catalyst yields (in solution) a yellow product, rapidly re-oxidised to the original compound on exposure to air. Quantitative hydrogenation showed that the uptake of hydrogen during this reduction corresponds to 1 mol.

(*b*) *From chrysoaphin*-fb. Chrysoaphin-*fb* (108 mg.) was dissolved in cold dry pyridine (4 c.c.), acetic anhydride (4 c.c.) added, and the mixture kept in a stoppered flask for 12 hours at room temperature with occasional shaking. Benzene (100 c.c.) was added and the mixture washed with water (3 × 100 c.c.), dilute hydrochloric acid, and again with water. Dried and evaporated, the benzene layer yielded a red gum, which on dissolution in ethanol followed by concentration of the solution furnished yellow needles of diacetylerythroaphin-*fb* (87 mg.), identical with the product obtained by method (*a*) above (Found: C, 68·7, 68·7; H, 4·8, 4·6; *O*-acetyl, 14·6%).

Tetra-acetyldihydroerythroaphin-fb.—Acetic anhydride (2 c.c.) and anhydrous sodium acetate (20 mg.) were added to a solution of erythroaphin-*fb* (28 mg.) in dioxan (2 c.c.), and the whole was shaken with hydrogen at room temperature and atmospheric pressure in presence of Adams's platinum catalyst; hydrogen uptake (1·35 c.c., 1·1 mols.) was complete in 18 minutes. The catalyst was separated, an equal volume of chloroform added, and the solution washed with dilute aqueous sodium carbonate. The chloroform layer was dried and concentrated to small bulk (5 c.c.), and hot ethanol (20 c.c.) added. The filtered solution was then concentrated until crystallisation set in and was set aside to cool. Recrystallisation of the product in the same way gave *tetra-acetyldihydroerythroaphin*-fb (28 mg.) as orange needles which charred but did not melt at <320°. The same product was obtained from diacetylerythroaphin-*fb* by hydrogenation of a solution in acetic anhydride using Adams's platinum catalyst followed by removal of the solvent, treatment with ethanol, evaporation, and crystallisation of the product as above described [Found: C, 66·6, 66·6, 66·8; H, 4·7, 4·9, 4·9; *O*-acetyl, 24·4, 25·3. $C_{30}H_{20}O_8(CO\cdot CH_3)_4$ requires C, 67·0; H, 4·7; *O*-acetyl, 25·3. $C_{30}H_{22}O_8(CO\cdot CH_3)_4$ requires C, 66·8; H, 5·0; *O*-acetyl, 25·2%]. Molecular-weight determination in ethylene dibromide by the cryoscopic method gave a value 740 ($C_{38}H_{34}O_{12}$ requires *M*, 682); the difficulty encountered throughout the aphin series in obtaining consistent values for molecular weights by the common methods leads us to regard this figure as of qualitative rather than quantitative value. The compound is soluble in chloroform (although solutions appear to decompose on storage), moderately soluble in benzene, and sparingly so in ethanol. The yellow solutions in organic solvents exhibit an intense green fluorescence in ultra-violet light. Tetra-acetyldihydroerythroaphin-*fb* is not extracted from such solutions by aqueous sodium hydroxide, nor is it reducible with sodium dithionite. Light absorption in ethanol containing 5% of chloroform: Maxima at 2520, 2785, (3400), 3560, (3865), 4110, 4350, and 4955 A.; $E^{1\%}_{1\,cm.}$ = 221, 547, (19), 21, (16), 43, 134, 322, and 443 respectively.

Dibenzoylerythroaphin-fb.—Erythroaphin-*fb* (23 mg.) was shaken with pyridine (4 c.c.), benzoyl chloride (0·2 c.c.), and a small crystal of benzoic acid for 15 hours, then warmed to 50° for a few minutes, and evaporated under reduced pressure. The solid residue was dissolved in ether–benzene (1 : 1), and the solution washed with hydrochloric acid, then with sodium hydrogen carbonate, and finally with water before drying (Na_2SO_4). The solution was evaporated, the residue dissolved in hot ethanol, and water added until a cloudiness appeared. On cooling, *dibenzoylerythroaphin*-fb separated as a light-brown amorphous powder (Found: C, 73·1; H, 4·4. $C_{44}H_{32}O_{10}$ requires C, 73·3; H, 4·5%). Molecular-weight determination by the cryoscopic method in ethylene dibromide gave a value 728, but ebullioscopic methods in chloroform gave inconsistent results ($C_{44}H_{32}O_{10}$ requires *M*, 720).

*Nitric Acid Oxidation of Erythroaphin-*fb.—Erythroaphin-*fb* (100·6 mg.) was heated on the steam-bath with concentrated nitric acid (4 c.c.) until evolution of brown fumes had almost ceased (2 hours), and the pale brownish solution was evaporated to dryness. The residue was again heated with nitric acid (3 c.c.) for 1 hour and then once more evaporated to dryness, and the residue boiled with water (3 c.c.) for 1 hour. Evaporation of the aqueous solution gave a semi-solid mass which was dissolved in methanol (5 c.c.) and treated with excess of ethereal diazomethane. The mixture was set aside overnight and then evaporated. The solid residue was dissolved in a mixture of benzene (2·5 c.c.) and *n*-pentane (2·5 c.c.) and put on a column of neutral alumina (3 g.). The column was washed with the same solvent and then eluted by washing with benzene containing increasing amounts of chloroform. The main bulk of product was located in the benzene–20% chloroform (30 c.c.; wt. of eluate 5·6 mg.; m. p. 183—184°) and benzene–40% chloroform (30 c.c.; wt. of eluate 9·2 mg.; m. p. 184—185°) washings. The material from these two fractions was combined, re-chromatographed in the same way, and furnished hexamethyl mellitate (13·8 mg.), m. p. 184—185°, unchanged by recrystallisation from aqueous methanol (Found: C, 51·1; H, 4·3. Calc. for $C_{18}H_{18}O_{12}$: C, 50·7; H, 4·3%). The m. p. of the ester was not depressed on admixture with authentic hexamethyl mellitate (m. p. 187°).

The benzene–75% chloroform washings (55 c.c.) of the first chromatogram above gave a crystalline material (5·6 mg.; m. p. 125—150°) which was probably impure hexamethyl mellitate, and washing the column finally with chloroform and methanol gave a resinous material (10 mg.) which was not identified.

Grateful acknowledgment is made to the Council of Scientific and Industrial Research, Australia, for a grant (to J. P. E. H.) and to the Wellcome Trustees for a Fellowship (held by S. F. M.). The participation of one of us (S. F. M.) in the early phases of the investigation was made possible by Prof. C. H. Best, F.R.S., who also gave facilities for carrying out work during 1947—1948 at the Department of Medical Research, University of Toronto. We are also indebted to the staff of the Cambridge University Farm for their co-operation, to Messrs. H. Duewell and R. Manning for their invaluable assistance in collecting insects, and to Dr. R. N. Haszeldine for certain of the absorption data. Finally, we record our thanks to Dr. B. K. Blount for a sample of strobinin and to Prof. H. Brockmann for one of hypericin.

UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, November 3rd,* 1949.]