95. *Colouring Matters of the Aphididæ. Part III. Colouring Matters from* Tuberolachnus salignus.

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The four aphin colouring matters *protoaphin*-sl, *xanthoaphin*-sl, *chrysoaphin*-sl, and *erythroaphin*-sl have been isolated from the willow-tree aphid, *Tuberolachnus salignus*. Although in most respects the aphins-*sl* are very similar in physical and chemical properties to the aphins-*fb*, the differences between them are sufficiently marked to warrant the conclusion that the pigments of the two series are not identical. A method for the purification of erythroaphins, depending on partition between chloroform and sulphuric acid of varying strengths, has been developed. Erythroaphin-*sl* has been converted into a *diacetyl* and a *tetra-acetyldihydro*-derivative, and from the products of its oxidation with nitric acid mellitic acid has been isolated.

Parallel with the investigation of the colouring matters of *A. fabæ* already described (Human, Johnson, MacDonald, and Todd, preceding paper) a study has been made of the large brown aphid, *Tuberolachnus salignus* (Gmelin). This insect parasitises willow trees, especially the white willow, *Salix alba*, on which it forms large colonies on the underside of the branches from late summer until the first severe frosts. It is a very suitable aphid for chemical study as its colonies are usually free of foreign insects, its pigment content (up to 2% of the live weight) is very high, and it is large enough to be easily dislodged from the willow branches by gentle tapping, so that it is readily obtained alive and undamaged without resort to the washing processes necessary with *A. fabæ* and other species. From *T. salignus* we have obtained a series of colouring matters exactly analogous to those from *A. fabæ*. Following the system of nomenclature which has been laid down elsewhere (Duewell, Human, Johnson, MacDonald, and Todd, *Nature*, 1948, **162**, 759), the *T. salignus* pigments isolated have been named *protoaphin*-sl, *xanthoaphin*-sl, *chrysoaphin*-sl, and *erythroaphin*-sl. Although very similar in many of their properties to the corresponding *A. fabæ* pigments, they are apparently not identical with them.

As in the case of *A. fabæ*, the living insects contain protoaphin-*sl* which can be obtained only if the insects are killed by a method (*e.g.*, heating) which simultaneously inactivates or destroys the enzyme system which brings about the protoaphin–xanthoaphin conversion. Preliminary fermentation of dead insects gives material from which xanthoaphin-*sl*, chrysoaphin-*sl*, and erythroaphin-*sl* in variable amount can be obtained. The pure aphins-*sl* undergo the same interconversions as do the aphins-*fb*, and the methods of extraction and isolation used for them

are generally similar to those used for the aphins-*fb*. The virtual absence of any green pigment similar to that which occurs in extracts of *A. fabæ* makes it easier to observe colour changes in the earlier stages of the extractions and differences in detail arise from the greater stability of xanthoaphin-*sl* as compared with xanthoaphin-*fb*. Thus, although specimens of *T. salignus* which have been long in storage or have been improperly fermented require extraction with acetone to obtain the mixture of fluorescent aphins, fresh insects which have been carefully fermented at pH 6·5 yield xanthoaphin-*sl* almost exclusively, and this compound can be directly extracted with ether. The isolation of all the erythroaphin-*sl* present in insect extracts from which the xanthoaphin and chrysoaphin have been largely removed presents difficulty owing to the accompanying fatty material and other impurities. This difficulty has been overcome by the discovery that the erythroaphin can be quantitatively extracted from chloroform by 72% sulphuric acid, and from 64% sulphuric acid by chloroform. Provided that the chloroform used is free from alcohols and chlorine this method gives excellent results and it can be applied not merely to erythroaphin-*sl* but also to the erythroaphins of *A. fabæ* and other species. It cannot, of course, be applied to either xanthoaphins or chrysoaphins, since these are converted at once into erythroaphins under such acid conditions. Partition between organic solvents and strong acids has been employed in the purification of several natural colouring matters, *e.g.*, porphyrins (Willstätter and Stoll, "Untersuchungen über Chlorophyll," Berlin, 1913, p. 262), stercobilin (Watson, *Z. physiol. Chem.*, 1935, **233**, 39), and azulenes (Plattner, Heilbronner, and Weber, *Helv. Chim. Acta*, 1949, **32**, 574) and might well find more general application; we have found, for example, that it is possible to separate mixtures of hydroxyanthraquinones, *e.g.*, catenarin and cynodontin, by partition between chloroform and 80% sulphuric acid.

In colour and general reactions the four aphins-*sl* are very similar to the corresponding aphins-*fb* (preceding paper). In visible and ultra-violet absorption and in fluorescence they are virtually indistinguishable. Despite these facts, the two series do not appear to be identical. There are differences in solubility, behaviour on heating, and crystalline form, and *X*-ray diffraction photographs of the aphins-*sl* show definite differences from those of the corresponding aphins-*fb*. According to observations made by Dr. N. Sheppard, differences also occur in the infra-red absorption spectra. The most striking difference however is found in the xanthoaphins. While the chrysoaphins and erythroaphins of the two series give analytical values indicating that they might well have the same molecular formulæ, the two xanthoaphins are markedly different in their carbon content; moreover, in the course of experimental work xanthoaphin-*sl* has consistently seemed more stable than its counterpart from *A. fabæ*.

As in the *A. fabæ* series, most of the chemical work so far carried out has been on erythroaphin-*sl*. This pigment we believe has a formula $C_{30}H_{24}O_8$, the evidence being analogous to that adduced in the case of erythroaphin-*fb* (preceding paper). Molecular-weight determinations by ebullioscopic methods gave variable results between 252 and 615 and *X*-ray crystallographic examination gave a value of 534 $\pm$ 5%, assuming 12 molecules per unit cell. Erythroaphin-*sl* is evidently quinonoid in nature and undergoes reversible reduction with sodium dithionite or with hydrogen in presence of a platinum catalyst, 1 mole of hydrogen being absorbed on the basis of a C_{30} formula for the pigment. Acetylation yields *diacetylerythroaphin*-sl and reductive acetylation *tetra-acetyldihydroerythroaphin*-sl.

Of the oxygen atoms in the erythroaphin-*sl* molecule, two are present in a quinone grouping and two in hydroxy-groups, which are probably phenolic in nature since the pigment can be extracted from organic solvents by aqueous sodium hydroxide giving a bright green precipitate. The function of the remaining four oxygen atoms is unknown. There is no evidence of carboxy-groups since neither diacetylerythroaphin-*sl* nor tetra-acetyldihydroerythroaphin-*sl* can be extracted from chloroform solution with dilute alkali. On the other hand, certain observations suggest the possibility that one or more lactonic or other latent acidic groups may be present. When a little sodium hydroxide is added to a solution of diacetylerythroaphin-*sl* in aqueous acetone, the colour changes from brownish-orange to green; this change does not involve deacetylation, since, if the green solution is quickly acidified, the original diacetylerythroaphin-*sl* is recovered unchanged. Similar treatment of tetra-acetyldihydroerythroaphin-*sl* with alkali under conditions which do not remove the acetyl groups gives a colour change yellow–red. Again, treatment of erythroaphin-*sl* in ethanol–dioxan solution with anhydrous hydrogen chloride gives a deep-green product, and addition of ethanol to the red solution of erythroaphin-*sl* in concentrated sulphuric acid causes a colour change to green.

In its behaviour towards bases erythroaphin-*sl* shows properties analogous to those described by Scholl (*Ber.*, 1941, **74**, 1129, 1171, 1182) in the hydroxyanthraquinone group. In

dry pyridine it dissolves to a red solution, but in aqueous pyridine its solutions are green. Addition of excess of dry pyridine to the aqueous solution brings about a change from green to red. In general, erythroaphin-*sl* dissolves in tertiary bases to give red solutions which become green on addition of water, whereas in many primary and secondary amines (aniline is an exception) the solutions are green and are unaffected by water. We have also observed that hypericin behaves in a similar manner with bases.

As to the nature of the nucleus in erythroaphin-*sl* little is known. It evidently contains a fully substituted aromatic ring, since oxidation with nitric acid gives mellitic acid, just as it does with erythroaphin-*fb*. Other methods of oxidation did not yield identifiable products in preliminary experiments and zinc-dust fusions or distillations gave fluorescent oils, evidently mixtures of hydrocarbons, from which no solid products were isolated.

Very little work has yet been done on the other aphins from *T. salignus* and it is not yet possible to assign molecular formulæ to them with any certainty. On the assumptions that erythroaphin-*sl* has a molecular weight of 512 and that 1 mol. of xanthoaphin gives rise to 1 mol. of chrysoaphin and this in turn to 1 mol. of erythroaphin, the yields of the last-named pigment obtained in conversion reactions suggest that chrysoaphin-*sl* should have a molecular weight of $\nearrow 584$, which would not conflict with a formula such as $C_{30}H_{26}O_9$. On the same basis, xanthoaphin-*sl* should have a molecular weight of $\nearrow 615$ but it must be admitted that the surprisingly low hydrogen content (4·5%) found on elementary analysis makes it difficult to advance a formula bearing a simple relation to those of the others, as was possible in the case of the *A. fabæ* series (Human *et al.*, *loc. cit.*). Further examination of xanthoaphin-*sl* is clearly needed before any conclusions can be drawn. Since protoaphin-*sl* has been more superficially examined than the other *T. salignus* pigments, it is difficult to say whether the slightly lower carbon content observed as compared with protoaphin-*fb* is significant. It is at any rate clear that the pigments of the two series are very closely related to one another.

Experimental.

Unless otherwise stated, light petroleum refers to the fraction, b. p. 40—60°.

Collection of Insects.—Willow branches infected with *T. salignus* were carefully sawn into convenient lengths for handling and the insects dislodged from the undersides by gentle tapping and collected on Cellophane sheets. If not worked up immediately, the aphids so obtained were stored below −10° in glass bottles. Stored insects could not be used for the preparation of the protoaphin and were also unsatisfactory as raw material for the pure xanthoaphin. In the 1947 season some 960 g. of insects were collected and in 1948 *ca.* 450 g.; although by the final extraction methods up to 2% of mixed pigments can be isolated, losses were heavy in the earlier stages of our investigations and the total quantity of mixed aphins isolated was 11·9 g. Further losses were involved in separating the individual aphins from the mixture.

Protoaphin-sl.—The aphids (5 g.) were killed by plunging them into water at 80° for one minute. The dead insects were collected, washed with a little aqueous acetone (60%), and then extracted with the same solvent by grinding in a Waring Blendor for a few minutes. The mass was filtered and the filtrate (50 c.c.) thrice extracted with light petroleum, the volume being brought up to 50 c.c. after each extraction by addition of acetone. The filtered extract was concentrated until it began to foam, whereupon it was cooled and shaken with active charcoal (Darco G. 60; 0·25 g. and 0·5 g. in successive lots). Elution of the second lot of charcoal (0·5 g.) with aqueous acetone (75%), followed by concentration to small bulk and prolonged cooling, yielded *protoaphin*-sl (30 mg.) as small tan-coloured needles (Found: C, 54·5; 54·7; H, 5·8, 6·2%). No crystalline material was obtained from the elution of the first lot of charcoal. Protoaphin-*sl*, which has not been very closely studied as yet, is similar to protoaphin-*fb* (preceding paper) in all its properties. Its solutions are yellow in neutral or acidic conditions and purple-red in alkaline. Addition of fresh crushed aphids to an aqueous solution of protoaphin-*sl* buffered to pH 6·5 causes rapid conversion into xanthoaphin-*sl*.

Xanthoaphin-sl.—*Method* 1. Freshly collected *T. salignus* (100 g.) suspended in phosphate buffer (250 c.c.; pH 6·5) were pulped in a Waring Blendor and the insect sludge kept for 3 hours at room temperature before removal of fat by extraction with cold carbon tetrachloride (2 × 100 c.c.). The mixture of fluorescent aphins was then extracted with ether (5 × 100 c.c.), and the solvent was slowly distilled from the combined ethereal extracts. After the volume had been reduced to 150 c.c. it was maintained by slow addition of carbon tetrachloride (90 c.c.). The distillation of the resulting mixture was continued until the temperature of the vapour was 72°, whereupon the solution was set aside at room temperature for several hours. The *xanthoaphin*-sl (774 mg., 0·8%) which separated was purified by extraction from a thimble with acid-free ether, concentration to small bulk, and initiation of crystallisation by addition of pure carbon tetrachloride. It then formed bright yellow plates which had a yellow fluorescence in ultra-violet light and on heating darkened from *ca.* 170° and finally melted at 203—204° (Found, in material dried at room temperature: C, 59·9, 59·4, 59·6, 59·3; H, 4·4, 4·6, 4·3, 4·4. $C_{30}H_{26}O_{14}$ requires C, 59·1; H, 4·3%).

Because of the ease of conversion into chrysoaphin-*sl*, it is preferable to recrystallise not more than *ca.* 400 mg. at one time, and to store the solid pigment in the dark. Xanthoaphin-*sl* is very soluble in pyridine, acetone, ethanol, or ethyl acetate, moderately so in chloroform or ether, and sparingly soluble in cold benzene; it is very sparingly soluble in light petroleum or carbon tetrachloride. Solutions in

organic solvents are yellow with a strong bluish-green fluorescence which is intensified in ultra-violet light. Alkaline solutions are pink with a green fluorescence when freshly prepared but, when kept, quickly change through crimson to green owing to progressive change to chrysoaphin-*sl* and erythroaphin-*sl*. Neutral solutions are more stable but the same changes occur on prolonged heating or if they are made acid. Light absorption in chloroform: Maxima at 2590, 2815, 3600, 3800, 4070, 4310, and 4590 A.; $E^{1\%}_{1\,cm.}$ = 821, 880, 196, 394, 118, 189, and 205 respectively. The bands at 4310 and 4590 A. are easily detected with a hand spectroscope.

Method 2. Fresh *T. salignus* (80 g.) were suspended in phosphate buffer (200 c.c.; pH 6·5) and pulped in a Waring Blendor, giving a deep purplish-red sludge, which was left at room temperature for 1 hour, the colour changing to greenish-yellow. The sludge was extracted with light petroleum (200 c.c.) which removed most of the fat, together with a little of the pigment. Acetone (200 c.c.) was added to the insect sludge, the whole thoroughly stirred, and solid material removed by centrifugation; this solid was re-extracted in the same way with aqueous acetone (5 × 200 c.c. of 80%). The combined acetone extracts were then repeatedly shaken with light petroleum (11 × 200 c.c.), and the combined petroleum layers evaporated under reduced pressure (temp. $\not>20°$). The residue was diluted with light petroleum and again concentrated to small bulk (40 c.c.). The mixed aphins separated as a light-orange granular precipitate (1·4—1·7 g.) which was collected, washed with light petroleum, and dried at room temperature. The colouring matter remaining in the mother-liquor was later converted into erythroaphin-*sl* by treatment with formic acid (see below). The crude solid pigments (400 mg. at one time) were dissolved in acid-free ether by extraction from a thimble and the solution slowly distilled, carbon tetrachloride being added continuously until crystallisation set in. On cooling xanthoaphin-*sl* separated and was recrystallised from ether–carbon tetrachloride. In favourable cases the yield was about 40% of the weight of the crude pigments.

Chrysoaphin-sl.—This pigment was usually isolated from the mother-liquors of the xanthoaphin-*sl* prepared by Method 1 or 2 above, by further concentration and replacement of ether by carbon tetrachloride. The crude chrysoaphin so obtained (*e.g.*, 493 mg., 0·5% from 100 g. of insects by Method 1) was dissolved in chloroform and shaken with aqueous formic acid (10%) until the 4310-A. band of xanthoaphin-*sl* was no longer visible with the hand-spectroscope. The chloroform solution was then washed with water, dried, and evaporated. The residue was triturated with light petroleum and the granular *chrysoaphin*-sl collected and recrystallised from ether by extraction from a thimble followed by concentration of the solution. It formed small orange needles which fluoresce orange in ultra-violet light, m. p. 238—239° (decomp. with previous darkening) (Found, in material dried at 60°/0·01 mm. for 6 hours: C, 68·3, 68·2, 68·4; H, 5·0, 4·7, 5·0. $C_{30}H_{36}O_9$ requires C, 67·9; H, 4·9. $C_{34}H_{28}O_{10}$ requires C, 68·4; H, 5·0%). Light absorption in chloroform: Maxima at 3210, 3820, 4040, 4565, and 4850 A.; $E^{1\%}_{1\,cm.}$ = 195, 419, 553, 206, and 253 respectively. The bands at 4850 and 4565 A. are readily detected with a hand-spectroscope. Chrysoaphin-*sl* is very souble in pyridine, soluble in chloroform, acetone, ethyl acetate, or benzene, sparingly soluble in carbon tetrachloride, ether, or ethanol and virtually insoluble in light petroleum. Solutions in organic solvents are yellow with a green fluorescence which in ultra-violet light becomes intense yellowish-green. Alkaline solutions are crimson but become green as conversion into a salt of erythroaphin-*sl* proceeds.

Erythroaphin-sl.—(i) *From chrysoaphin*-sl *mother-liquors*. The mother-liquors from the above chrysoaphin-*sl* preparation were evaporated, and the residue was dissolved in ethyl acetate (120 c.c.). Formic acid (20 c.c. of 85%) was added and the solution heated on the steam-bath for 5 minutes. On cooling, *erythroaphin*-sl (691 mg., 0·7% from 100 g. of insects) separated as dark red needles, m. p. 250—252° (decomp. with previous darkening), and was recrystallised from ether (extraction in a thimble) or from chloroform–ethanol (Found, in material dried at 60°/0·01 mm. for 12 hours: C, 70·2, 70·1, 70·1; H, 4·3, 4·3, 4·5. $C_{30}H_{24}O_8$ requires C, 70·3; H, 4·7. $C_{30}H_{22}O_8$ requires C, 70·6; H, 4·4%). Light absorption: (1) *In chloroform.* Maxima at 2555, 3195, 3335, 4215, 4470, 4855, 5200, 5600, and 5860 A.; log $\varepsilon_{max.}$ 4·57, 3·65, 3·65, 4·49, 4·61, 3·77, 4·11, 4·30, and 3·90 respectively. (2) *In concentrated sulphuric acid.* Maxima at 2330, 2730, 2900, 4620, 5260, and 5680 A.; log $\varepsilon_{max.}$ 4·66, 4·35, 4·27, 4·46, 4·28, and 4·55 respectively. (3) *In dioxan–*N*/100-sodium hydroxide* (1 : 1). Maxima at 2880, 4220, 4480, 5750, and 6215 A.; log $\varepsilon_{max.}$ 4·70, 4·47, 4·56, 4·18, and 4·45 respectively.

Erythroaphin-*sl* is very soluble in pyridine or chloroform, soluble in carbon tetrachloride, acetone, or benzene, and sparingly soluble in ethyl acetate, ethanol, or light petroleum. Solutions in organic solvents are deep red with an orange fluorescence which is intense when viewed in ultra-violet light. The solid pigment has a dull red fluorescence in ultra-violet light. Solutions in sulphuric acid are red and show no fluorescence. With acetic anhydride containing a drop of sulphuric acid the solution passes through green to pure blue with an intense red fluorescence. Extraction of chloroform solutions of erythroaphin-*sl* with aqueous sodium hydroxide yields the insoluble green sodium salt at the interface.

Erythroaphin-*sl* is reversibly reduced by sodium dithionite or by catalytic hydrogenation, the reduced form being rapidly reoxidised in air. Quantitative hydrogenation with a platinum catalyst gave values of 487 and 495 for the molecular weight, assuming one quinone grouping per molecule. Ebullioscopic methods gave variable results (from 252—615) but *X*-ray crystallographic examination gave *M* 534 ± 5%, assuming 12 mols. per unit cell ($C_{30}H_{24}O_8$ requires *M*, 512).

(ii) *From crystalline chrysoaphin*-sl *or xanthoaphin*-sl. Chrysoaphin-*sl* (50 mg.) was dissolved in ethanol (50 c.c.) and concentrated sulphuric acid (0·3 c.c.) was added with shaking. The solution was set aside for 5½ hours and the erythroaphin-*sl* which separated was collected, washed with ethanol and light petroleum, and dried (yield, 43·7 mg.). Xanthoaphin-*sl* (50·1 mg.) treated in the same way also yielded erythroaphin-*sl* (41·7 mg.). The conversion of either pigment into erythroaphin-*sl* could also be brought about by heating it with ethyl acetate containing formic acid.

On the assumptions that the conversions are unimolecular and that erythroaphin-*sl* has a molecular weight of 512, the yields obtained in the conversions fix an upper limit for the molecular weight of chrysoaphin-*sl* at 584 and for xanthoaphin-*sl* at 615.

Purification of Erythroaphin-sl *by Partition.*—Crude erythroaphin-*sl* (1·018 g.), dissolved in alcohol-

free chloroform (70 c.c.), was shaken with sulphuric acid (125 c.c.; 74%), whereupon the pigment passed into the acid layer. The acid solution was washed with chloroform (50 c.c.), then diluted with sulphuric acid (400 c.c. of 62%), and extracted with chloroform (6 × 50 c.c.); the red pigment passed into the chloroform, leaving a brownish aqueous-acid layer. The chloroform extracts were repeatedly washed with water, dried, and concentrated to small bulk under reduced pressure. Ethanol was added until a turbidity appeared and the mixture was warmed to bring all into solution and set aside overnight, whereupon pure erythroaphin-*sl* separated.

Diacetylerythroaphin-sl.—Erythroaphin-*sl* (398 mg.) was dissolved in cold dry pyridine (50 c.c.), the solution cooled in ice, and acetyl chloride (8 c.c.) added dropwise with vigorous shaking, the temperature being kept below 10°. After the addition of acetyl chloride cooling was no longer necessary and the mixture was kept at room temperature for 15 minutes, by which time the initially red solution had changed to yellow. The solution was poured, with stirring, into ice-water, and the flocculent brown precipitate collected, washed with water, and dried; it then formed a brown amorphous powder (439 mg.). The crude product was extracted from a thimble with hot light petroleum–ether (1 : 1) during 2 hours and the separated material collected; a further quantity was recovered by evaporation of the solution. Further purification was effected by chromatography on a 1 : 1 talc–precipitated silica column (10 g.; 12 × 1·6 cm.). The diacetyl compound (218 mg.) in 50% benzene–light petroleum was brought on to the column and the solution forced through by application of pressure. The column was washed with more solvent until the eluate was colourless and then most of the solvent was removed from the combined eluate. *Diacetylerythroaphin*-sl was then precipitated by the addition of excess of light petroleum, being obtained as a light orange powder. It has been obtained crystalline only on one occasion so far and the analyses and determinations of spectra, etc., have mostly been carried out on amorphous material [Found: C, 68·8, 68·4; H, 4·6, 4·5; *O*-acetyl, 15·2, 13·5, 13·4%; *M* (ebullioscopic in chloroform), 572; (hydrogenation in dioxan over Pt), 593, 598. $C_{30}H_{22}O_8(CO{\cdot}CH_3)_2$ requires C, 68·4; H, 4·7; *O*-acetyl, 14·4%; *M*, 596). Diacetylerythroaphin-*sl* is very soluble in acetone, ethyl acetate, benzene, or pyridine, soluble in ether, alcohol, or methanol, sparingly soluble in light petroleum, and insoluble in water. Light absorption in chloroform (amorphous material): Maxima at 5870, 5360, 4920, 4280, 3490, 3330, and 2560 A.; log $\varepsilon_{max.}$ = 2·80, 3·34, 3·85, 4·55, 3·95, 4·07, and 4·42 respectively.

Tetra-acetyldihydroerythroaphin-sl.—(i) *Reductive acetylation of erythroaphin*-sl. Erythroaphin-*sl* (123 mg.), pure zinc dust (246 mg.), and anhydrous sodium acetate (25 mg.) were suspended in acetic anhydride (5 c.c.), and glacial acetic acid (1 c.c.) was added. The mixture was warmed and gently shaken until solution had been effected, and then heated under gentle reflux for 15 minutes. The reddish solution then showed a green fluorescence, and the completion of the reaction could be conveniently determined by adding a drop of the mixture to chloroform (1 c.c.) and shaking with water. When the chloroform solution stayed yellow, showing absorption bands at 4990 and 4670 A. (hand-spectroscope), and no re-oxidation took place in air, the solid material was separated from the hot reaction mixture, and the residue washed with hot acetic acid. The filtrate and washings were diluted with water, excess of acetic anhydride was hydrolysed, and the pale brown precipitate (151 mg.) separated, washed with water, and dried *in vacuo*. The crude acetyl derivative was dissolved in benzene (20 c.c.) and light petroleum (10 c.c.), and brought on to a 1 : 1 talc–precipitated silica column (10 g.; 11 × 1·6 cm.), and the solution forced through by application of pressure. The column was washed with light petroleum until the eluate was no longer coloured. The benzene addition *compound* of tetra-acetyldihydroerythroaphin-*sl* began to separate from the combined eluates after a few minutes (addition of more light petroleum if necessary), and after 12 hours the golden-yellow crystals of the benzene adduct were collected (123 mg.), m. p. 325° (decomp.). For analysis a sample was re-chromatographed and dried *in vacuo* at 60° (Found: C, 69·8; H, 5·1. $C_{38}H_{34}O_{12},C_6H_6$ requires C, 69·5; H, 5·3%). The benzene adduct was dissolved in chloroform (2 c.c.), and hot ethanol (25 c.c.) added. After 24 hours the orange platelets of *tetra-acetyldihydroerythroaphin*-sl were separated, washed with ethanol, and dried as above. The product darkened but did not melt below 320° (Found: C, 66·9; H, 5·1. $C_{38}H_{34}O_{12}$ requires C, 66·9; H, 5·0%). The tetra-acetyl compound is very soluble in pyridine and acetone, soluble in ethyl acetate, chloroform, or benzene, sparingly soluble in ether or ethanol, and insoluble in light petroleum. Light absorption in chloroform: Maxima at 2785, 4365, 4650, and 4980 A.; log $\varepsilon_{max.}$ = 4·56, 3·94, 4·30, and 4·43 respectively. Samples of the tetra-acetyl compound tend to deteriorate on storage, with the production of amorphous impurities, which, however, can be conveniently removed by chromatography on talc–precipitated silica columns as outlined above.

(ii) *Catalytic reductive acetylation of erythroaphin*-sl. Erythroaphin-*sl* (200 mg.), acetic anhydride (20 c.c.), anhydrous sodium acetate (50 mg.), and Adams's platinum catalyst (50 mg.) were hydrogenated at atmospheric pressure until no more hydrogen was absorbed. The catalyst was separated from the yellow solution, excess of ethanol added, and the mixture kept overnight. The solvent was removed under reduced pressure, the residue treated with water, and the crude acetyl compound separated, washed with water, and dried (250 mg.). The further purification was similar to that described in method (i). The acetyl compound was dissolved in 50% benzene–light petroleum and twice chromatographed on 1 : 1 talc–precipitated silica to yield the benzene addition product (175 mg.) (Found: C, 69·3; H, 4·9%). Recrystallisation from ethanol–chloroform gave pure tetra-acetyldihydroerythroaphin-*sl* [Found: C, 66·9; H, 4·7; *O*-acetyl, 24·8. Calc. for $C_{30}H_{22}O_8(CO{\cdot}CH_3)_4$: C, 66·9; H, 5·0; *O*-acetyl, 25·2%]. The absorption spectrum of the product was identical with that of the tetra-acetyl compound obtained by method (i). The catalytic reductive acetylation method is best for preparative purposes. In the absence of sodium acetate acetylation was incomplete.

(iii) *Catalytic reductive acetylation of diacetylerythroaphin*-sl. Diacetylerythroaphin-*sl* (216 mg.), acetic anhydride (15 c.c.), anhydrous sodium acetate (30 mg.), and Adams's platinum catalyst (50 mg.) were hydrogenated at atmospheric pressure until equilibrium was attained. The catalyst was separated, ethanol (45 c.c.) added, and the mixture kept overnight. The light-brown crystals were removed and a further crop (135 mg.) was obtained by concentration of the mother-liquors and addition of more ethanol. The crude material was purified by chromatography and crystallisation as described above

(Found : C, 66·7; H, 4·8; *O*-acetyl, 25·5%). The identity of the product with the previous preparations was also shown by comparison of the absorption spectra which revealed no differences.

Oxidation of Erythroaphin-sl *with Nitric Acid.*—Erythroaphin-*sl* (98 mg.) was warmed on the steam-bath with nitric acid (3 c.c.), a vigorous reaction setting in with copious evolution of nitrous fumes. The reaction soon subsided and the solution was heated for a further 2 hours. The reaction solution was transferred to a small flask and evaporated at 100° in a stream of air, leaving a pale yellow solid. Nitric acid (2 c.c.) was added, the solution again evaporated to dryness, and the process then repeated twice with water (2 c.c.) in order to remove any excess of nitric acid. The crude oxidation product was treated with excess of ethereal diazomethane, and the methylated material dissolved in benzene and put on a column of neutral alumina (5·4 g.). The chromatogram was washed with benzene, and then with benzene containing increasing amounts of chloroform. Those fractions which were obtained from the washings, up to benzene–40% chloroform, were crystalline (76·7 mg. in all), but the later runnings obtained with higher concentrations of chloroform and with chloroform–methanol mixtures were oily (70·6 mg.) and their nature has not been established. The combined crystalline fractions were re-chromatographed on neutral alumina (2·3 g.) with benzene–light petroleum (1 : 1) as solvent and elution with benzene–light petroleum (3 : 1). Recrystallised from aqueous methanol the product formed silky needles, m. p. 187—188° (Found : C, 50·9, 50·8; H, 4·7, 4·5. Calc. for $C_{18}H_{18}O_{12}$: C, 50·7; H, 4·3%). A mixed m. p. with authentic hexamethyl mellitate (m. p. 187—188°), kindly supplied by Dr. H. C. Howard of the Coal Research Laboratory, Pittsburgh, Pa., showed no depression.

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