10. *Colouring Matters of the Aphididæ. Part XVII.*[1] *The Structure and Absolute Stereochemistry of the Protoaphins.*

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Structures (XII) and (XIII) are proposed for protoaphins-*fb* and -*sl*, respectively. On mild reduction, a process involving fission of an activated 1,1'-binaphthyl system, each aphin yields a mixture of a 5,7-dihydroxy-1,4-naphthaquinone and the glucoside of a 1,3,8-naphthalenetriol. The quinone A obtained from the -*fb* isomer differs from that (A') of the -*sl* isomer, but the same glucoside B, which can be converted into quinone A, is obtained from each. Oxidation of quinones A and A' yields the same DD(+)-dilactic acid and this determines both structure and absolute configuration of the non-aromatic portions of the molecules. The two protoaphins differ from one another in the configuration at one centre of asymmetry only.

EARLIER papers of this series [2-4] have described the isolation of protoaphins from the hæmolymph of dark species of *Aphididæ*. To date, two protoaphins have been studied, which are isomeric with one another and are, respectively, the parent substances of the *fb* and *sl* series of aphin pigments. The protoaphins are yellow, water-soluble, acidic substances which, when the insects are crushed, are converted sequentially into the fat-soluble, highly fluorescent pigments, xanthoaphin, chrysoaphin, and erythroaphin by enzymic action. Protoaphin-*fb* is most conveniently prepared from the common bean aphid (*Aphis fabae*), and protoaphin-*sl* from the willow aphid (*Tuberolachnus salignus*). The present paper deals with the structure and stereochemistry of these two substances; we shall deal first with the *fb* isomer and then define the difference between it and protoaphin-*sl*.

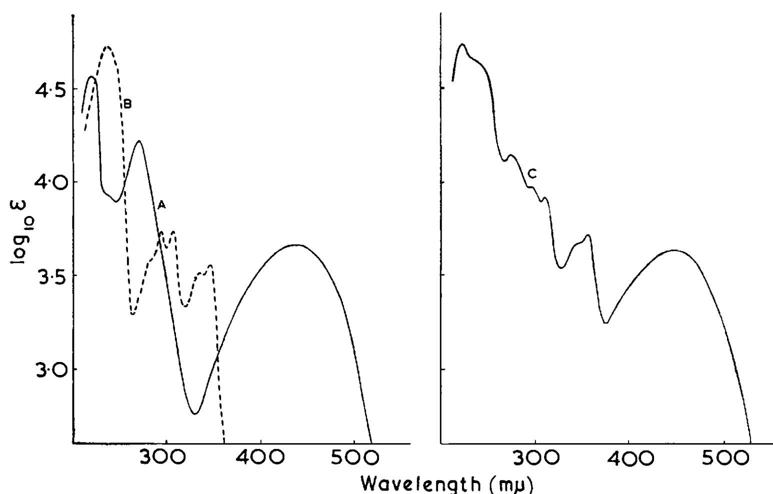
In previous work, difficulty was experienced in purifying the protoaphins. This arose largely from the extremely hygroscopic nature of the anhydrous pigments and from their slow (presumably oxidative) decomposition in solution. Milder methods of purification have now been developed, a suitable solvent system for countercurrent distribution has been devised, and homogeneous material suitable for degradative studies has consistently been obtained in overall yield of 0·3% from wet *A. fabae* and 1% from dry *T. salignus*. On the basis of our work with this material, the molecular formula ($C_{36}H_{44-46}O_{19}$) tentatively assigned to the protoaphins in previous publications [2] must now be amended to $C_{36}H_{38}O_{16}$. The glucosidic nature of the protoaphins has been confirmed; on mild acid hydrolysis [4] they both yield D-glucose and an intractable amorphous "aglucone." Only traces of fluorescent aphins are produced by this means, in contrast to treatment with pigment-free enzyme extracts from crushed insects,[4] which remains the only practicable route from the protoaphins to the other members of the series; the significance of this will be discussed in a subsequent paper. The quinonoid system in the protoaphins undergoes ready catalytic hydrogenation (1·1 mol.), to give yellow leuco-compounds which although readily reoxidised in air yield stable, pale yellow dodeca-acetates. The latter have absorption spectra which are unmistakably naphthalenic, in sharp contrast to the corresponding erythroaphin derivatives which have perylenic spectra.

Prolonged reduction of a neutral aqueous solution of protoaphin-*fb* with sodium dithionite, followed by aerial oxidation, yielded two products, an orange acidic quinone A, $C_{15}H_{14}O_6$, and a colourless glucoside B, $C_{21}H_{26}O_{10}$, in isolated yields of 55 and 60%,

[1] Part XVI, Cameron, Cromartie, and Todd, preceding paper.
[2] Human, Johnson, MacDonald, and Todd, *J.*, 1950, 477.
[3] Duewell, Johnson, MacDonald, and Todd, *J.*, 1950, 485.
[4] Brown, Ekstrand, Johnson, MacDonald, and Todd, *J.*, 1952, 4925.

respectively. The formation of these substances from protoaphin involves the effective addition of two atoms of hydrogen, and a summation of their ultraviolet and visible spectra gives a curve remarkably similar to that of the starting material (the Figure).

The light absorption of the quinone A, in both neutral and basic media, indicated that it was almost certainly a derivative of 5,7-dihydroxy-1,4-naphthaquinone.[5] In accordance with this view it formed a boroacetate (*peri*-OH) and, although it was insufficiently acidic to contain a 2- or 3-hydroxyl in a 1,4-naphthaquinone system, its more acidic hydroxyl group had pK_a 6·5, similar to that in 5,7-dihydroxy-1,4-naphthaquinone (pK_a 6·2). Moreover, it showed two infrared bands [$\nu_{max.}$ 1655 (infl.) and 1635 cm.$^{-1}$] corresponding to non-bonded and hydrogen-bonded carbonyl groups (although in some derivatives these were not clearly resolved). As would be expected from such a chromophoric system, quinone A yielded with diazomethane a yellow monomethyl ether, with methyl iodide–silver oxide a yellow dimethyl ether, and by the action of dimethyl sulphate on the leuco-compound a pale yellow leuco-tetramethyl ether. In solution, the monoanion of quinone A is reddish-violet and the dianion deep violet, as is also the case with the protoaphins;[2,3] the colour of alkaline solutions fades gradually as a result of atmospheric oxidation.



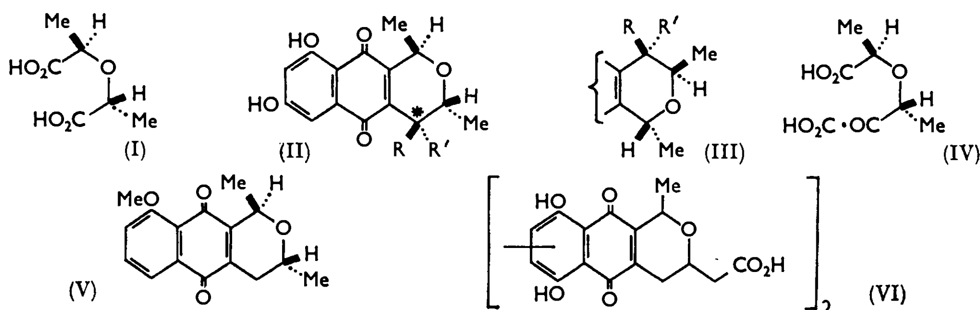
Ultraviolet absorption spectra of (A) quinone A, (B) quinone A′, and (C) protoaphin-*sl* in 75% aqueous ethanol.

These facts are compatible only with the view that the chromophoric system of quinone A is that of 5,7-dihydroxy-1,4-naphthaquinone, and so ten carbon and four oxygen atoms in the molecule are accounted for. The remaining non-aromatic portion of quinone A must therefore contain five carbon and two oxygen atoms; this is in agreement with previous work on the erythroaphins [6] in which two such non-aromatic residues were shown to be present, and it suggests a direct relation between this portion of quinone A and the erythroaphins. Indeed, the only obvious chemical difference in this context is that, whereas in quinone A one of the oxygen atoms is present as an alcoholic hydroxyl group ($\nu_{max.}$ 3440 cm.$^{-1}$) and the other as ethereal oxygen, in the erythroaphins both oxygen atoms are ethereal. The presence of an alcoholic hydroxyl group in quinone A was confirmed by preparation of a monoacetate both from its dimethyl and its leuco-tetramethyl ether; toluene-*p*-sulphonylation of the hydroxyl group with a view to subsequent reductive removal failed, presumably because of ready elimination. From its molecular

[5] Garden and Thomson, *J.*, 1957, 2483.
[6] Brown, Calderbank, Johnson, Joshi, Quayle, and Todd, *J.*, 1955, 959.

formula, quinone A must contain outside the chromophore either an additional ring or a double bond. The latter can almost certainly be excluded since only the quinonoid system in substance A can be reduced; so a ring is preferred. Since quinone A contains no alkoxy-group its ethereal oxygen must be part of this extra ring. The most likely points of attachment of this heterocyclic system would be C-2 and C-3 in the naphthaquinone residue and the fact that no nitrogen-containing products are obtained on methylation with diazomethane supports the view that the unsaturated linkage between C-2 and C-3 is doubly substituted.[7] Moreover, the ultraviolet and visible spectra of quinone A dimethyl ether were significantly closer to those of 5,7-dimethoxy-1,4-naphthaquinone [8] than to those of 2,5,7- and 3,5,7-trimethoxy-1,4-naphthaquinone,[9] which showed extra maxima at 295 (log ε 4·03) and 293 mμ (log ε 4·23), respectively. This finding strongly suggests that the heterocycle in quinone A is attached at positions 2 and 3 by two carbon atoms, and not by one carbon and one oxygen atom.

A consideration of the proton magnetic resonance (p.m.r.) spectra of several of the derivatives of quinone A mentioned above resulted in a substantial clarification of the remaining structural details, and provided valuable evidence regarding their relation to those found in the erythroaphins. A full discussion of this aspect of the investigation is reserved for a subsequent paper.[10] Chemical clarification was obtained by mild chromic acid oxidation of quinone A: DD(+)-dilactic acid [11] (I) was produced. Only two structures (II and III; R, R′ = H, OH) for quinone A are consistent with this finding, and with the evidence already presented. Structure (III) can, in fact, be eliminated by taking into account the chemistry of the erythroaphins which contain no aliphatic



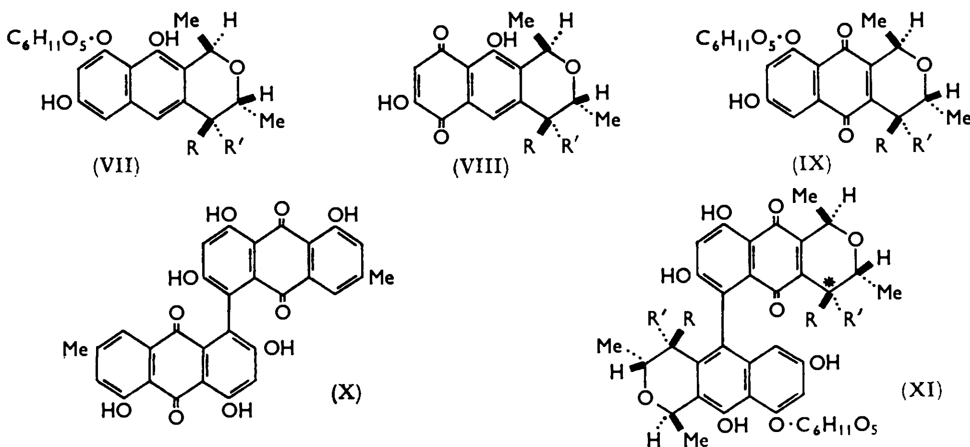
hydroxyl group and by making the assumption, justified by spectroscopic and chemical similarities, that there is no significant difference between the non-aromatic portions of quinone A and the erythroaphins. It is not possible on the basis of formula (III) to devise a structure for the latter which accords with their properties, whereas this can easily be done on the basis of (II). We shall return to this point in the following paper.[12] The only structural feature in quinone A which is not established by the results obtained above is the stereochemical configuration at the carbon atom bearing the alcoholic hydroxyl group; this point will be dealt with later in discussing the degradation of protoaphin-*sl*. Quinone A (II) is structurally and stereochemically analogous to isoeleutherin (V), isolated from tubers of *Eleutherine bulbosa* [13] and biogenetically related to the mould metabolite actinorhodin (VI).[14]

Glucoside B, the second product obtained on treating protoaphin-*fb* with dithionite,

[7] Brockmann and van der Merve, *Naturwiss.*, 1962, **49**, 130.
[8] Smith and Thomson, *J.*, 1961, 1008.
[9] Davies, King, and Roberts, *J.*, 1955, 2782.
[10] Part XXII, Cameron, Kingston, Sheppard, and Todd, *J.*, 1964, 98.
[11] Cf. Fredga, *Tetrahedron*, 1960, **8**, 126.
[12] Cameron, Cromartie, Hamied, Scott, and Todd, following paper.
[13] Cf. Schmid and Ebnöther, *Helv. Chim. Acta*, 1951, **34**, 1041.
[14] Brockmann, Müller, and van der Merve, *Naturwiss.*, 1962, **49**, 131.

was very hygroscopic when anhydrous and darkened slowly in air. Its ultraviolet and infrared absorption spectra suggested that it contained a hydroxylated naphthalene nucleus,[15] and, when treated with acid or with pigment-free enzyme extracts from fresh insects,[4] it was converted into an ether-soluble aglycone the spectrum of which was very similar to that of the orginal material. The aglycone was extremely susceptible to atmospheric oxidation [16] and could not be obtained pure. The structure of glucoside B was, however, shown to be (VII; R, R′ = H, OH) by oxidation with potassium nitrosodisulphonate (Fremy's salt) at pH 6. This yielded a sugar-free orange quinone (VIII; R, R′ = H, OH) and a sugar-containing quinone (IX; R, R′ = H, OH); the latter was not isolated as such but on treatment with pigment-free enzyme extracts from *A. fabae* [4] gave quinone A (II), identical with the substance obtained directly by dithionite reduction of protoaphin-*fb*. The sugar-free quinone (VIII) was strongly acidic (pK_a 3·8), and its monoanion was orange, both features characteristic of 2-hydroxy-1,4-naphthaquinones; boroacetate formation indicated a *peri*-hydroxyl group. Further, a comparison of visible ultraviolet and infrared spectra showed that in this respect it resembled 2,5-dihydroxy-1,4-naphthaquinone much more closely than the 3,5-isomer; details of these comparisons are given in the Experimental section. The production of compounds (VIII) and (IX) by oxidation determines the structure of glucoside B (VII) unambiguously.

It is of interest that in our studies on materials A and B we treated the former with sodium hypoiodite and obtained iodoform (0·4 mol.). That this was due to oxidative breakdown of the chromophoric system and not to the presence of a CH_3·CH(OH) group was shown by the fact that similar treatment of products (VII) and (VIII), where the heterocyclic system is fused to a benzenoid ring which is therefore less prone to disruption, gave no iodoform. The production of iodoform on hypoiodite oxidation of some simple quinones (*e.g.*, benzoquinone) which contain no "iodoform-yielding" groups is well known [17] (although its formation has been used on at least one occasion [18] successfully to diagnose the presence of an acetyl group even though the experiment involved the breakdown of a quinonoid system). Our results emphasise the need for caution in interpreting the results of such oxidations.



The structures of quinone A (II) and glucoside B (VII) being now known, it is possible to deduce a structure for protoaphin-*fb*. The facts that a combination of their spectra

[15] Cf. Daglish, *J. Amer. Chem. Soc.*, 1950, **72**, 4859.
[16] Part XX, Calderbank, Cameron, Cromartie, Hamied, Haslam, Kingston, Todd, and Watkins, *J.*, 1964, 80.
[17] Booth and Saunders, *Chem. and Ind.*, 1950, 824.
[18] Arnstein and Cook, *J.*, 1947, 1021.

and chemical properties agree so well with the corresponding features of protoaphin-*fb*, and that the dithionite reduction leading to them was carried out under mild conditions, strongly suggest absence of rearrangement during their formation. Between them, compounds A and B account for all the carbon and oxygen atoms in protoaphin-*fb*, the structure of which must accordingly rest on a simple combination of them. Dithionite is known to cleave fungal dianthraquinonyls such as skyrin (X) to anthraquinones provided that the former are suitably activated by oxygen substituents.[19,20] We regard the reduction of protoaphin-*fb* as being an analogous process, and the most probable structure for this pigment (and the only one that can account for the production of erythroaphin from it [12]) is that given by (XI; R, R′ = H, OH).

The two naphthalenic systems in (XI) cannot achieve coplanarity, either as written or in the alternative form in which one of them is rotated through 180° about the connecting linkage. Hence little resonance interaction between them is possible and the properties of compound (XI) are essentially the sum of those of (II) and (VII). Thus the reddish-violet colour of protoaphin solutions at neutral pH and also in the living insect is doubtless due to ionisation of the 7-hydroxyl group in the quinone portion. In the ultraviolet–visible spectrum of the anion, only the peaks corresponding to the naphthaquinone undergo a bathochromic shift relative to that of un-ionised protoaphin, those due to the glucosidic portion remaining substantially unchanged. Similarly the carbonyl absorption bands in the infrared spectrum of protoaphin (1673, 1645 $cm.^{-1}$), far higher than in any other aphin, can be interpreted as due to simple non-bonded and hydrogen-bonded naphthaquinone carbonyl groups. The production of a leuco-protoaphin-*fb* dodeca-acetate, the spectrum of which is similar to that of naphthalene apart from a small bathochromic shift, accords well with its formulation as an acetoxylated 1,1′-binaphthyl derivative, since acetoxy-groups have little effect on the spectrum of an aromatic system [21] and the spectrum of 1,1′-binaphthyl closely resembles that of naphthalene.[22] Finally, on the basis of structure (XI) the spectrum of protoaphin-*fb* deca-acetate should resemble a combination of the spectra of naphthalene and 1,4-naphthaquinone with a small bathochromic shift affecting each. Its spectrum does indeed contain obvious naphthalene bands of the same type and at the same wavelengths as those shown by the leuco-acetate, but these swamp the absorption due to the naphthaquinone. However, if the spectrum of the leuco-acetate, its intensity reduced to half as a rough measure of the contribution due to one naphthalene unit, is subtracted from that of the deca-acetate the resulting curve ($\lambda_{max.}$ 255—260, 355—360 mμ; log ε 4·1—4·2 and 3·3—3·4) is very similar to the expected bathochromically shifted 1,4-naphthaquinone spectrum ($\lambda_{max.}$ 246, 256, and 334 mμ; log ε 4·28, 4·13, and 3·44).

All evidence from earlier work indicates that the aphins of the *fb* and *sl* series are stereoisomeric. Hence it can reasonably be assumed that structure (XI) must, stereochemical considerations apart, represent both protoaphins-*fb* and -*sl*. It remains, therefore, to determine the extent of the difference between the two substances. When protoaphin-*sl* is treated with dithionite as described for the *fb*-isomer, it yields in the same way two products. One of these is identical with glucoside B (VII); the other, a quinone A′, although isomeric with and very similar to quinone A (II) obtained from protoaphin-*fb*, is not identical with it and gives a different dimethyl ether. However, mild oxidation of this quinone by chromic acid yields the same DD-(+)-dilactic acid as does the *fb*-quinone. In the experiments carried out by us the dilactic acid could not be crystallised directly, even after chromatography—freshly isolated protoaphin-*sl* was not available at the time and the sample used had probably undergone some decomposition during storage. The optical rotation of the appropriate fraction of the oxidation product was 80% of that

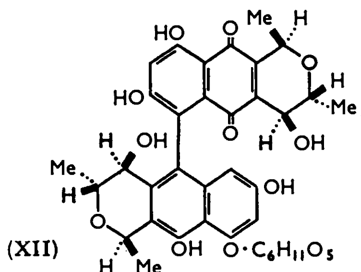
[19] Shibata, Tanaka, and Kitagawa, *Pharm. Bull.* (*Japan*), 1955, **3**, 278.
[20] Howard and Raistrick, *Biochem. J.*, 1954, **56**, 56.
[21] See, *e.g.*, Brockmann, *Proc. Chem. Soc.*, 1957, 304.
[22] Friedel, Orchin, and Reggel, *J. Amer. Chem. Soc.*, 1948, **70**, 199.

for pure DD-(+)-dilactic acid; it was finally isolated as the readily crystallisable racemate by adding the calculated amount of the pure LL-isomer.

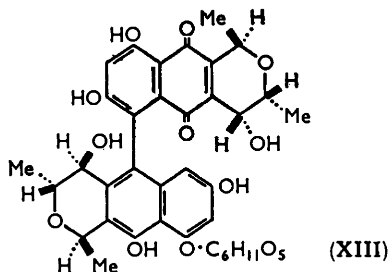
It follows from these experiments that the only difference between the quinones A and A′, and consequently between the two protoaphins,* lies in the configuration at the carbon atom marked with an asterisk in (II) and (XI), the only centre of asymmetry in quinone (II) destroyed during the oxidation to dilactic acid. The configuration at this centre in the two isomers can be determined by a consideration of the proton magnetic resonance spectra (40 Mc./sec.) of the dimethyl ethers of the isomeric quinones (II). The proton attached to C* appears as a doublet in both spectra because of spin–spin interaction with the proton on the adjacent carbon atom and is centred at τ 5·57 (*fb*) and 5·55 (*sl*). However, the coupling constants J, 8·0 c./sec. (*fb*) and 2·0 c./sec. (*sl*) differ substantially; the magnitude of the former is characteristic of axial–axial coupling, indicating that the two protons are *trans* to one another in the product from protoaphin-*fb*, and it follows that in the *sl*-product they must be *cis*. These conclusions are strongly supported by studies on the erythroaphins which we will describe in detail in a succeeding paper; these show that the erythroaphins-*fb* and -*sl* differ solely at the carbon atom corresponding to C* in (XI) and that the *fb*-isomer is thermodynamically the more stable.

The structural and stereochemical conclusions reached in this paper are in complete accord with the results of proton magnetic resonance studies to be described later. The structure of protoaphin-*fb* is accordingly (XII) and that of protoaphin-*sl* (XIII).

In each of these formulæ restricted rotation of the two naphthalenic moieties about their connecting bond, besides influencing spectra as described above, also introduces a further element of asymmetry into the molecule. A formal determination of configuration about this bond would be very difficult and has not been attempted, although useful



(XII)



(XIII)

observations regarding it may be made on other grounds. These favour one particular configuration and will be discussed fully in Part XX [16] where they can more appropriately be introduced. The only feature still unresolved is the configuration at the glucosidic centre (which must be identical in both *fb* and *sl* isomers); this is now under investigation.

EXPERIMENTAL

Unless otherwise stated, ultraviolet and visible spectra were measured for ethanolic solutions and infrared spectra for Nujol and/or hexachlorobutadiene mulls. pK_a measurements were spectroscopic.

Protoaphin-fb.—Crude protoaphin-*fb* [4] (100 mg.) was dissolved in phosphate–citrate buffer (pH 7·3; 10 ml.) and extracted with 1 : 1 v/v butan-1-ol–ether (8 × 6 ml.) until the extracts were nearly colourless. The combined organic extracts were washed with small amounts of water, then diluted with ether, and extracted with aqueous sodium hydrogen carbonate, and the red-violet aqueous extracts, after being washed repeatedly with ether, were acidified to pH 4.

* The possibility of further differences in the protoaphins, due to hindered rotation around the binaphthyl bond or to the glucosidic centre, is discussed below.

Protoaphin-*fb* separated on refrigeration as small, yellow prisms (Found: C, 58·7; H, 5·7; O, 34·7%; Equiv., by titration in 50% aqueous methanol, 710. $C_{36}H_{38}O_{16}$ requires C, 59·5; H, 5·2; O, 35·3%; Equiv., 726). It had: $\lambda_{max.}$ (*a*) in 70% aqueous ethanol, 223, 273, 296, 310, 343, 357, and 449 mμ (log ε 4·82, 4·19, 4·06, 3·97, 3·73, 3·78, and 3·69), and (*b*) in aqueous 0·2M-Na_2HPO_4, 234, 298, 342, 356, and 512 mμ (log ε, 4·75, 4·26, 3·73, 3·75, and 3·72); $\nu_{max.}$ 3430 (OH), 2930, 1673, 1645 (quinone C=O), 1613, 1590 (C=C), 1450, 1419, 1382, 1374, 1340, 1320, 1280, 1240, 1192, 1155, 1087, 1050, 1027, 958, 894, 869, 850, 832, 798, 780, and 770 cm.$^{-1}$; pK_a 6·2. Hydrogenation of protoaphin-*fb* in butan-1-ol over palladium oxide proceeded with uptake of 1·10 mol. of hydrogen, to yield a yellowish solution of the leuco-compound, which on exposure to air was reoxidised rapidly to protoaphin-*fb*.

Crude protoaphin-*fb* [4] (0·82 g.) was subjected to countercurrent distribution between 1 : 1 butan-1-ol–di-isopropyl ether and 8 : 1 aqueous 0·2M-Na_2HPO_4–aqueous 0·1M-citric acid, (20 tubes; 100-ml. phases). After 19 transfers, carried out as rapidly as possible, the contents of tubes 6—15 were combined, the phases were separated, and the aqueous phase was extracted with butan-1-ol. These extracts were combined with the organic phase, which was then diluted with ether and extracted with aqueous sodium hydrogen carbonate. The resultant aqueous solution was washed with ether and acidified to pH 4; protoaphin-*fb* separated as above.

Protoaphin-sl.—Freshly collected willow aphids (*T. salignus*; 400 g.), killed by immersion in water at 70° for 5 min., were macerated with acetone (400 ml.) in a Waring blender. The extract was separated by centrifugation and the process repeated successively with 90%, 80%, and 75% aqueous acetone. The combined extracts were filtered (Hyflo), stirred for 1 hr. with acid-washed charcoal (Darco G.60; 40 g.), again filtered, acidified to pH 3—4, and extracted with light petroleum (b. p. 40—60°; 3 × 500 ml.). Residual acetone was removed by evaporation and final ether-extraction (3 × 200 ml.). The aqueous solution was then extracted with butan-1-ol (10 × 20 ml.), and the extracts were diluted two-fold with ether and extracted with aqueous sodium hydrogen carbonate. This aqueous-alkaline extract was acidified to pH 3—4 and set aside at 0° overnight; crude protoaphin-*sl* (4 g.) separated. (Attempts to remove residual butan-1-ol before acidification led to supersaturated solutions of the protoaphin and made crystallisation very difficult.) The crude aphin was collected, washed with a little butan-1-ol, then with ether, dried, and stored under nitrogen. Recrystallised from butan-1-ol protoaphin-*sl* formed a yellow microcrystalline powder rather darker than the *fb*-isomer; as it decomposed on being heated, the light absorption at 449 mμ was taken as a criterion of purity. Before analysis it was dried over P_2O_5 at 50° *in vacuo* for 4 hr. and analysed immediately (Found: C, 59·4; H, 5·7. $C_{36}H_{38}O_{16}$ requires C, 59·5; H, 5·2%). It had (*a*) in 75% ethanol, $\lambda_{max.}$ 223, 274, 297, 311, 343, 356, and 450 mμ (log ε, 4·73, 4·14, 3·98, 3·91, 3·67, 3·72, and 3·64), $\lambda_{infl.}$ 234 mμ; (*b*) in aqueous 0·2M-Na_2HPO_4, $\lambda_{max.}$ 233, 299, 340, 354, and 516 mμ (log ε, 4·74, 4·23, 3·59, 3·63, and 3·71), $\lambda_{infl.}$ 310 mμ; $\nu_{max.}$ 3540, 3440 (OH), 1651 (quinone C=O), 1615, 1593 (C=C), 1517, 1422, 1280, 1255, 1203, 1184, 1171, 1143, 1105, 1085, 1062, 1045, 1030, 1000, 972, 957, 910, 875, 863, 850, 830, 805, 782, 745, and 717 cm.$^{-1}$; pK_a 6·5.

Deca-acetylprotoaphin-fb.—A solution of protoaphin-*fb* (430 mg.) in acetic anhydride (5 ml.) and pyridine (5 ml.) was set aside for 5 days at room temperature, then evaporated *in vacuo*. The dark resinous product was dissolved in benzene and chromatographed on silica gel. Ethyl acetate–benzene (6 : 4) yielded the *deca-acetate*, a clear orange resin which crystallised as a yellow powder from carbon tetrachloride–cyclohexane (Found: C, 58·9; H, 5·4. $C_{56}H_{58}O_{16}$ requires C, 58·6; H, 5·3%); $\lambda_{max.}$ 240, 294, 306, and 338 mμ (log ε 4·86, 4·03, 4·04, and 3·84); $\nu_{max.}$ 2930, 1758 (acetate C=O), 1668 (quinone C=O), 1627, 1577 (C=C), 1430, 1370, 1330, 1230, 1195, 1160, 1064, 1030, 900, 830, and 728 cm.$^{-1}$.

When acetylated in the same way, protoaphin-*sl* gave a similar but amorphous product, with $\lambda_{max.}$ 239, 293, 306, and 337 mμ, and $\nu_{max.}$ 2980, 2940, 1752, 1668, 1627, 1582, 1433, 1372, 1325, 1225, 1198, 1063, 1036, 960, 903, 827, 786, and 757 cm.$^{-1}$.

Dodeca-acetyldihydroprotoaphin-fb.—A suspension of protoaphin-*fb* (330 mg.) in dioxan (10 ml.)–acetic anhydride (5 ml.) was shaken for 6 hr. with zinc dust (500 mg.) and triethylamine (1·5 ml.). The protoaphin passed into solution and the colour faded to yellow with a green fluorescence. After filtration and evaporation, the resulting resin was dissolved in 1 : 1 acetic anhydride–pyridine (6 ml.) and set aside for 3 days. Evaporation yielded a resin which was chromatographed, in benzene, on silica gel. Elution with ethyl acetate–benzene (4 : 1) yielded a clear yellow resin from which the last traces of acetic anhydride were removed under reduced

pressure. Crystallisation from carbon tetrachloride–cyclohexane gave the *dihydro dodeca-acetate* as a pale yellow powder (Found: C, 58·7; H, 5·5. $C_{60}H_{64}O_{28}$ requires C, 58·4; H, 5·2%); $\lambda_{max.}$ 238, 309, and 335 mμ (log ε, 5·06, 4·21, and 3·99); $\nu_{max.}$ 2978, 2934, 1760 (acetate C=O), 1628, 1600, 1570 (C=C), 1350, 1190, 1060, 1035, 1020, 975, 935, 898, 847, 783, 755, and 717 cm.$^{-1}$.

Reductive acetylation of protoaphin-*sl* as above gave a similar but amorphous product, $\lambda_{max.}$ 239, 293, 307, and 335 mμ; $\nu_{max.}$ 2970, 2930, 1757, 1625, 1600, 1578, 1503, 1435, 1372, 1348, 1195, 1062, 1035, 939, 900, 785, and 763 cm.$^{-1}$.

Reduction of Protoaphin-fb.—Protoaphin-*fb* (3 g.) was added to stirred phosphate–citrate buffer (pH 7·3; 2 l.), and solid sodium dithionite (30 g.) was added. Immediate reduction to the yellow leuco-compound occurred and the solution was stirred for an hour, then treated with further portions of dithionite (5 × 10 g.) at half-hourly intervals, more dithionite being added if re-oxidation occurred during this time. The mixture was kept for a further 2 hr., then reoxidised (colour change) by a stream of air. Citric acid (17·0 g.) was now added, the final pH being 4·3, and the solution was extracted with ether (10 × 200 ml.). The aqueous layer was worked up separately as described below. The orange ethereal extract was evaporated, redissolved in a small volume of ether to remove insoluble matter, percolated through a column of silica gel in ether, and evaporated to yield *quinone A* (II) (636 mg., 55%). Recrystallisation from benzene yielded orange-brown crystals, m. p. 200° (decomp.), a small sample of which was sublimed (110°/2 × 10^{-4} mm.) for analysis (Found: C, 61·6; H, 4·7%; Equiv., by titration, 290. $C_{15}H_{14}O_6$ requires C, 62·0; H, 4·9%; Equiv., 290); (*a*) in ethanol, $\lambda_{max.}$ 218, 271, and 437 mμ (log ε 4·53, 4·16, and 3·63); (*b*) in aqueous 0·2M-Na_2HPO_4; $\lambda_{max.}$ 230, 251, 292, and 513mμ (log ε 4·38, 4·12, 4·18, and 3·66); $\nu_{max.}$ (*a*) mull in Nujol, 3440, 3275 (OH), 1635 (quinone C=O), 1610, 1557 (C=C) cm.$^{-1}$; (*b*) mull in hexachlorobutadiene, an additional band at 3580 cm.$^{-1}$ (OH). (*c*) in $CHCl_3$ solution, an additional inflexion at 1655 cm.$^{-1}$ (non-bonded quinone C=O); pK_a 6·5.

[Light absorption of 5,7-dihydroxy-1,4-naphthaquinone: (*a*) in ethanol, $\lambda_{max.}$ 217, 263, and 432 mμ (log ε 4·80, 4·12, and 3·62); (*b*) in aqueous 0·2M-Na_2HPO_4, $\lambda_{max.}$ 230, 290, and 510 mμ; infrared absorption in chloroform solution [5] at 1675 and 1642 cm.$^{-1}$; pK_a 6·2.]

Quinone A was very soluble in ether, less so in chloroform or benzene. It showed similar redox behaviour to protoaphin, could be extracted from ether with aqueous sodium hydrogen carbonate to give a red-violet solution and with aqueous sodium hydroxide to give a deep violet, the colour of which faded on storage overnight. With boroacetic anhydride it gave a red colour and, like 5,7-dihydroxy-1,4-naphthaquinone,[5] it gave with concentrated sulphuric acid a red colour changing to green.

The ether-extracted aqueous mother-liquors from the above reduction were extracted with butan-1-ol (6 × 125 ml.), and the extracts were washed with a small volume of water and evaporated to yield *glucoside B* (VII) (1·1 g., 60%), which crystallised from water as colourless needles m. p. 180—182° (decomp.). The material was dried and analysed immediately (Found: C, 56·9; H, 6·0. $C_{21}H_{26}O_{11}$ requires C, 57·5; H, 6·0%); in 50% aqueous ethanol, $\lambda_{max.}$ 236, 293, 306, 334, and 347 mμ (log ε 4·73, 3·72, 3·72, 3·54, and 3·55), $\lambda_{infl.}$ 282 mμ (log ε 3·58); $\nu_{max.}$ 3400 (OH), 1637, 1623, 1592 (C=C) cm.$^{-1}$; $[\alpha]_D^{20}$ +60·7° ± 4·0° (*c* ·78 in water).

The glucoside (2 mg.) was warmed with N-sulphuric acid (2 ml.) on the water-bath for 1 hr., cooled, and extracted with ether to give a pale greenish solution, which yielded an amorphous product, $\lambda_{max.}$ 292, 305, 339, and 345 mμ. Treatment of the glucoside in buffer (pH 6·3) with a pigment-free enzyme extract [4] from *A. fabae* proceeded with uptake of 0·94 mol. of oxygen and was complete overnight. Extraction with ether then gave a product having an ultraviolet spectrum identical with that of the product of acid treatment.

Reduction By-product.—On one occasion reduction of protoaphin-*fb* (3 g.) as above gave, in the ether-soluble fraction, a further substance. This was separated from quinone A on silica gel, being eluted with benzene–ether (19 : 1) before that quinone, and formed reddish-violet crystals (65 mg.), which after recrystallisation from benzene had m. p. 196° (decomp.). It was very soluble in ethanol and chloroform, giving brilliant red solutions and sparingly soluble in carbon tetrachloride and carbon disulphide. It could not be extracted from ether by sodium hydrogen carbonate, but with carbonate gave a purple solution. With boroacetic anhydride it gave in the cold a blue-green colour (Found: C, 66·1; H, 4·7. $C_{15}H_{12}O_5$ requires C, 66·1; H, 4·4%); $\lambda_{max.}$ 216, 282, 361, and 516 mμ (log ε 4·43, 4·13, 3·67, and 3·67); $\nu_{max.}$ 3380, 3050, 1657, 1637, 1608, and 1564 cm.$^{-1}$.

Reductive acetylation with zinc, acetic anhydride, and sodium acetate in the usual way

gave a very pale yellow solution which after dilution with ether, repeated washing with aqueous sodium hydrogen carbonate, and evaporation had $\lambda_{max.}$ in ether 216, 236, 262, 272, 284, 312, and 324 mμ.

Reduction of Protoaphin-sl.—Protoaphin-*sl* (3 g.) was reduced with sodium dithionite as for protoaphin-*fb*, to yield *quinone A′* (II), m. p. 236° (benzene), depressed in admixture with quinone A (II) (Found: C, 61·8; H, 5·1%); (*a*) in ethanol, $\lambda_{max.}$ 218, 271, and 435 mμ (log ε 4·57, 4·19, and 3·66), $\lambda_{infl.}$ 237 mμ (log ε 3·93); (*b*) in aqueous 0·2M-Na_2HPO_4, $\lambda_{max.}$ 229, 250, 291, and 514 mμ (log ε, 4·43, 4·16, 4·21, and 3·67); $\nu_{max.}$ 3430, 3220, 1645, 1620, 1598, 1498 cm.$^{-1}$; pK_a 6·55.

The aqueous solution left after removal of quinone A′ from the reduction mixture was worked up as described under protoaphin-*fb* and yielded glucoside B (VII), identical in m. p., mixed m. p., and ultraviolet and infrared spectra with glucoside B from protoaphin-*fb*, and having $[\alpha]_D^{20}$ +58·6° ± 4·3° (*c* 0·77 in water).

Monomethylation of Quinone A.—The quinone (120 mg.) was treated with an excess of ethereal diazomethane for 40 min., and the solution washed with aqueous sodium hydrogen carbonate, dried, and evaporated to yield a partially crystalline product, which was dissolved in benzene, adsorbed on silica gel, and eluted with benzene–ether (49 : 1). The eluate, when evaporated, gave an orange product (50 mg.) which crystallised on trituration with ether. Recrystallisation from ether gave yellow, well-defined crystals of *quinone A monomethyl ether*, m. p. 141°. This could be extracted from ether with aqueous sodium carbonate to give a red solution and it gave a red colour with boroacetic anhydride (Found: C, 63·3; H, 5·5. $C_{16}H_{16}O_6$ requires C, 63·2; H, 5·3%) $\lambda_{max.}$ 219, 269, and 429 mμ (log ε 4·59, 4·19, and 3·68); $\nu_{max.}$ 3495 (OH), 1640(C=O), 1615, and 1567 (C=C) cm.$^{-1}$.

On one occasion this compound was contaminated by a by-product which could be separated from it on silica gel by virtue of stronger adsorption. It formed orange-red crystals (from ether), m. p. 263°. It gave a positive boroacetate test, did not contain nitrogen (Lassaigne), and had $\lambda_{max.}$ in ether 275 and 453 mμ and $\nu_{max.}$ 3470, 1638, 1610, and 1565 (infl.) cm.$^{-1}$.

Dimethylation of Quinone A.—A solution of the quinone (200 mg.) in chloroform (15 ml.) was shaken with freshly prepared silver oxide (1·5 g.) and redistilled methyl iodide (1 ml.) for 1 hr. The mixture was filtered, diluted with ether, washed with aqueous sodium hydroxide till extracts were colourless, then with water, dried, and evaporated to yield a yellow-brown oil (150 mg.). This was treated with a small volume of ether and refrigerated overnight, to yield a yellow solid (50 mg.) which on recrystallisation from ether gave the *dimethyl ether* as golden-yellow needles, m. p. 172·5—174° (Found: C, 64·0; H, 5·7. $C_{17}H_{18}O_6$ requires C, 64·1; H, 5·7%); $\lambda_{max.}$ 216, 267, and 412 mμ (log ε 4·66, 4·36, and 3·69); $\nu_{max.}$ 3550 (OH), 1653 (C=O), 1632, 1600, and 1567 (C=C) cm.$^{-1}$; $[\alpha]_D^{20}$ −54° ± 1° (*c* 0·36 in chloroform).

[5,7-Dimethoxy-1,4-naphthaquinone [8] has $\lambda_{max.}$ 216, 258, and 412 mμ (log ε, 4·52, 4·17, and 3·55), $\lambda_{infl.}$ 360 mμ (log ε 3·38).

2,5,7-Trimethoxy-1,4-naphthaquinone [9] has $\lambda_{max.}$ 215, 260, 295, and 412 mμ (log ε 4·49, 4·18, 4·03, and 3·49).

3,5,7-Trimethoxy-1,4-naphthaquinone [9] has $\lambda_{max.}$ 213, 267, 293, and 402 mμ (log ε 4·54, 4·18, 4·23, and 3·56.]

Dimethylation of Quinone A′.—Quinone A′ (380 mg.) from protoaphin-*sl*, treated as above, gave a *dimethyl ether* as yellow needles, m. p. 201° (from ether), depressed in admixture with the quinone A derivative (Found: C, 64·1; H, 5·7%), $\lambda_{max.}$ 216, 268, and 412 mμ (log ε 4·59, 4·25, and 3·63), $\nu_{max.}$ 3521, 1652, 1606, and 1570 cm.$^{-1}$, $[\alpha]_D^{20}$ +158° ± 9° (*c* 0·06 in chloroform).

Acetylation of Quinone A Dimethyl Ether.—The dimethyl ether (50 mg.) was left in acetic anhydride (0·5 ml.) and pyridine (2 ml.) overnight at room temperature. The solution was then evaporated, the yellow solid residue was extracted with successive portions of boiling ether, and the extracts were concentrated to yield lemon-yellow crystals (30 mg.) of the *acetate*, m. p. 180·5—181°, depressed in admixture with starting material (Found: C, 63·1; H, 5·7. $C_{19}H_{20}O_7$ requires C, 63·3; H, 5·6%), $\lambda_{max.}$ 217, 269, and 416 mμ (log ε, 4·53, 4·19, and 3·58), $\nu_{max.}$ 1743 (acetate C=O), 1665, 1655 (quinonoid C=O), 1638, 1602, and 1566 (C=C) cm.$^{-1}$.

Reductive Methylation of Quinone A.—Quinone A (200 mg.) in ethylene glycol dimethyl ether (10 ml.) was hydrogenated over commercial palladium oxide. To the yellow, reduced solution 10% aqueous potassium hydroxide (9 ml.) and dimethyl sulphate (7·5 ml.) were added, and the mixture was shaken for 2 hr. and then left overnight at room temperature. A further portion of alkali (50 ml.) was then added, the mixture shaken for 45 min. and extracted with

ether, and the extract washed with water and evaporated. Chromatography in benzene–ether (1 : 1) on silica gel yielded a yellow oil (102 mg.) which was sublimed (140°/7 mm.) to yield the pale yellow *leuco tetramethyl ether*, m. p. 40°. This was difficult to handle and tended to re-form a viscous oil which could not be induced to crystallise other than by re-sublimation. For analysis a sample was " crystallised " from light petroleum at low temperature (Found: C, 66·1; H, 7·0. $C_{19}H_{24}O_6$ requires C, 65·5; H, 6·9%), and had $\lambda_{max.}$ 243, 291, 303, and 344 mμ (log ε 4·80, 3·78, 3·82, and 3·56), $\lambda_{infl.}$ 327 mμ (log ε 3·53), $\nu_{max.}$ 3490, 3415 (OH), 1623, 1597, and 1585 (C=C) cm.$^{-1}$.

Treatment of the leuco-ether with acetic anhydride–pyridine at room temperature gave an acetate which after percolation through silica gel had $\lambda_{max.}$ (in ether) 240, 291, 303, 331, and 347 mμ and $\nu_{max.}$ 1740 (acetate C=O), 1625, and 1595 (C=C) cm.$^{-1}$.

Oxidation of Quinone A.—Quinone A (700 mg.) was dissolved in purified acetone (50 ml.), and 8N-chromic acid (25 ml.) was added. After an initial vigorous reaction, the mixture was left overnight, then a green solid was filtered off and washed with acetone, and the combined filtrate and washings were diluted with water (20 ml.). The acetone was then removed under reduced pressure, the residual aqueous liquors were extracted with ether (6 × 40 ml.), and the ethereal solution was re-extracted with a small volume of aqueous sodium hydrogen carbonate. The aqueous layer was acidified, extracted with ether, dried, and evaporated to constant weight *in vacuo* (yield of mixed acids 91 mg.). Paper-chromatographic results were: (*a*) in ethanol–water–ammonia [23] (80 : 17 : 3) (system A), R_F 0·32, 0·38, 0·42, 0·50, 0·58; for racemic dilactic acid, R_F 0·32; (*b*) in eucalyptol–formic acid–propanol [24] (5 : 2 : 5) (system B), R_F 0·79; for racemic dilactic acid, R_F 0·79. The mixed acids were then chromatographed on silicic acid (Mallinckrodt 2847) (100 g.) containing water (33 ml.), with chloroform–butan-1-ol (9 : 1 v/v) as mobile phase.[25] The column was 3 cm. in diameter and 26 cm. in height after pressure had been applied. Fractions of 50 ml. were collected. Fractions 3 and 4 yielded 26 mg. of an acid which readily formed colourless crystals, m. p. 95—96°, from benzene and had $\nu_{max.}$ 2700 and 1710 cm.$^{-1}$, $[\alpha]_D$ −17° (*c* 0·2 water), and R_F in system A 0·51. It gave an orange precipitate on treatment with 2,4-dinitrophenylhydrazine in dilute hydrochloric acid. It was left overnight in aqueous solution containing an excess of hydrogen peroxide, then treated with ammonia. The solution on paper chromatography (system A) showed three spots, R_F 0·53 (starting material), 0·36 (dilactic acid), and 0·25 (weak spot). The acid was therefore assumed to be the keto-acid (IV).

Fractions 7 and 8 (41 mg.) contained DD-(+)-dilactic acid, which after two crystallisations from benzene had m. p. 85—88·5°, $\nu_{max.}$ 3200, 1750—1715 (broad) cm.$^{-1}$, R_F (system A) 0·35, and $[\alpha]_D$ +89° ± 3° (*c* 0·18 in water). Synthetic LL-(−)-dilactic acid had m. p. 89—91°, and $[\alpha]_D$ −105° ± 3° (*c* 1·3 in water), and its infrared spectrum and paper-chromatographic behaviour were indistinguishable from that of the DD-acid above. Addition of an equal amount of synthetic LL-acid to the oxidation product, followed by recrystallisation from benzene, yielded racemic dilactic acid, m. p. 110—111·5°, undepressed in admixture with an authentic sample.

Synthetic Dilactic Acids.—The racemic acid, prepared by hydrolysis of the diamide,[26] had m. p. 112°. Its infrared spectrum which had bands at 3600—2600 (very broad) and 1750—1715 (broad) cm.$^{-1}$ was distinguishable from that of the enantiomers in the fingerprint region. Resolution [26] by crystallisation of the diamide yielded LL-(−)-dilactic acid, m. p. 89—91°, $[\alpha]_D$ −105° ± 3° (*c* 1·3 in water). The synthetic DD-acid was not isolated.

Oxidation of Protoaphin-sl.—Similar oxidation of a sample of quinone A′ (430 mg.), from protoaphin-*sl* which had been stored 9 months, yielded a product which was twice chromatographed,[25] to give an acid (6 mg.), having $[\alpha]_D$ +60° (benzene) {LL-(−)-dilactic acid has $[\alpha]_D$ −75° (*c* 0·1 in benzene)}. This acid could not be crystallised directly. To a portion (1·5 mg.), the LL-acid (0·9 g.) was added and recrystallisation of the mixture from benzene then yielded racemic dilactic acid, m. p. 110—111°, undepressed in admixture with authentic material. Paper chromatography in system A gave R_F 0·34.

Oxidation of Glucoside B.—A crude sample of glucoside B (1·5 g.), dissolved in water (130 ml.), was treated with aqueous potassium nitrosodisulphonate (2·0 g., 15% excess, in 100 ml.).

[23] Cheftel, Munier, and Macheboeuf, *Bull. Soc. Chim. biol.*, 1952, **34**, 380.
[24] Cheftel, Munier, and Macheboeuf, *Bull. Soc. Chim. biol.*, 1951, **33**, 840.
[25] Vandenheuvel and Hayes, *Analyt. Chem.*, 1952, **24**, 960.
[26] Cf. Vièles, *Ann. Chim.* (*France*), 1935, **3**, 213.

The pH was adjusted to 6, and the mixture left overnight and then extracted with ether. The pH was then readjusted and the process repeated with an additional quantity (2 g.) of oxidant. The combined ether extracts were washed, dried, and evaporated to yield orange crystals of *quinone* (VIII) (280 mg.), which was recrystallised from chloroform and sublimed for analysis; it had m. p. 214° (Found: C, 62·3; H, 5·2. $C_{15}H_{14}O_6$ requires C, 62·1; H, 4·9%), $\lambda_{max.}$ (*a*) in ethanol, 253, 279, and 410 mμ (log ε 4·14, 3·98, and 3·64), (*b*) in aqueous 0·2M-Na_2HPO_4, 227, 263, 303, 399, and 471 mμ (log ε 4·28, 4·31, 3·95, 3·65, and 3·64), $\nu_{max.}$ 3280 (OH), 1669, 1649 (C=O), and 1608 (C=C) cm.$^{-1}$, and pK_a 3·8.

The same product was obtained whether glucoside B from protoaphin-*fb* or -*sl* was used.

The ether-extracted aqueous liquors from the oxidation were brought to pH 6·5 and set aside overnight with a pigment-free enzyme extract [4] from *A. fabae*. The solution was then acidified, extracted with ether, washed, dried, and evaporated to yield quinone A (200 mg.), m. p. 200° (benzene), undepressed in admixture with an authentic specimen and identical with the latter in absorption spectrum.

[2,5-Dihydroxy-1,4-naphthaquinone [27,28] had m. p. 217—218°, $\lambda_{max.}$ (*a*) in ethanol, 240, 283, and 410 mμ (log ε 4·07, 4·04, and 3·55), (*b*) in aqueous 0·2M-Na_2HPO_4, 226, 258, 290, 394, and 466 mμ (log ε, 4·22, 4·23, 3·92, 3·50, and 3·56), $\nu_{max.}$ 3200, 1678, 1662, and 1620 cm.$^{-1}$, and pK_a 4·15.

3,5-Dihydroxy-1,4-naphthaquinone [28,29] had m. p. 217—218° (decomp.), $\lambda_{max.}$ (*a*) in ethanol, 284 and 411 mμ (log ε 4·11 and 3·63), (*b*) in aqueous 0·2M-Na_2HPO_4, 227, 263, and 407 mμ (log ε 4·14, 4·07, and 3·56), $\lambda_{infl.}$ 285 mμ (log ε 4·01), $\nu_{max.}$ 3260, 1628, and 1580 cm.$^{-1}$, and pK_a 3·9.]

Hypoiodite Oxidation.—The following substances were oxidised to completion with iodine in sodium hydroxide at 60° in the usual way.

(*a*) Quinones A and A′ yielded iodoform in 40 and 34% yield, respectively.

(*b*) Glucoside B and quinone (VIII) yielded no iodoform.

(*c*) Eleutherin [13] [cf. (V)] gave no iodoform, though difficulty was encountered in view of its sparing solubility in aqueous media.

(*d*) Dihydroxy-, monohydroxy-, and dibromodihydroxy-erythroaphin-*fb* yielded no iodoform.

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27 Mylius, *Ber.*, 1885, **18**, 463.
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