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MODE OF INCORPORATION OF PRECURSORS INTO ALIZARIN (1,2-DIHYDROXY-9,10-ANTHRAQUINONE)*

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Key Word Index—*Rubia tinctorum*; Rubiaceae; biosynthesis; quinones; alizarin.

Abstract—The biosynthesis of alizarin (1,2-dihydroxy-9,10-anthraquinone) in *Rubia tinctorum* L. has been studied by tracer techniques. Specific incorporation of label from carboxyl-^{14}C-D-shikimic acid, 2-^{14}C-DL-glutamic acid and 5-^{14}C-DL-mevalonic acid suggests that these compounds provide the carbon skeleton of alizarin. Nonsymmetrical incorporation of label from carboxyl-^{14}C-D-shikimic acid and 2-^{14}C-DL-glutamic acid into alizarin indicates that the symmetrical 1,4-naphthoquinone is probably not an intermediate. Activity from *o*-(succinyl-2,3^{14}C)-benzoic acid was found in the substituted benzene ring of alizarin. These data indicate that α-ketoglutaric acid or a derivative thereof combines with shikimic acid, chorismic acid or phrephenic acid to give *o*-succinylbenzoic acid which is then transformed to a nonsymmetrical intermediate γ,γ-Dimethylallylpyrophosphate is then attached, ring closure and further modification leading to alizarin.

INTRODUCTION

QUINONES, from a biogenetic point of view, are a heterogeneous group of naturally occurring compounds; acetic acid phenylpropanoids, mevalonic acid or shikimic acid may serve as the starting material for quinones in different organisms.[2–4] There is, however, a striking similarity in the biogenesis of certain naphthoquinones and certain anthraquinones: the carbon skeleton of shikimic acid is incorporated intact into ring A of the naphthoquinones vitamin K (I),[5,6] juglone (II),[7] lawsone (III)[8] as well as the anthraquinones alizarin (IV) and purpurin[9] (Scheme 1). Moreover, a radioactively labelled sample of naphthol, derivatives of which have been isolated from anthraquinone containing plants,[10,11] has been incorporated into the naphthoquinone vitamin K.[5,12,13] This finding suggested that naphthol is hydroxylated in the plant to give 1,4-naphthoquinol which is then an intermediate in the biosynthesis of compounds I–VI. Indeed, 1,4-^{14}C-naphthoquinone, which is likely to be in equilibrium with 1,4-^{14}C-naphthoquinol in the organism, was incorporated into juglone (II) in *Juglans regia* L., lawsone (III) in *Impatiens balsamina* L.[7] and alizarin (IV) in *Rubia tinctorum* L.[14]

* A preliminary account of part of this work has been published.[1]

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[2] M. H. ZENK and E. LEISTNER, *Lloydia* **31**, 275 (1968).
[3] M. H. ZENK, *Hoppe-Seylers Z. Physiol. Chem.* **353**, 123 (1972).
[4] E. LEISTNER, *Hoppe-Seylers Z. Physiol. Chem.* **353**, 123 (1972).
[5] E. LEISTNER, J. H. SCHMITT and M. H. ZENK, *Biochem. Biophys. Res. Commun.* **28**, 845 (1967).
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[7] E. LEISTNER and M. H. ZENK, *Z. Naturforsch.* **23b**, 259 (1968).
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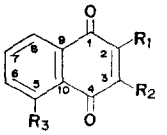
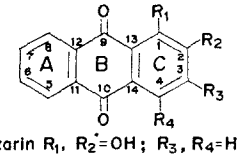
Visual dissection of structural formulae and the discovery of possible intermediates led to the assumption that ring C of alizarin (IV), purpurin carboxylic acid (V) and related anthraquinones are derived from mevalonate.[10,11] This hypothesis was confirmed experimentally[15,16] although it needed further confirmation in the case of alizarin. These results did not account for the origin of the so-called 'three missing C-atoms' (in the case of alizarin C-atoms 10, 13 and 14, Scheme 1). Campbell[17] and Robins *et al.*[18] showed, however, that glutamic acid is incorporated into lawsone and vitamin K which suggested that α-ketoglutaric acid and the proposed 1,4-dihydroxy-2-naphthoic acid[18] may also be involved in anthraquinone biosynthesis. The result of Dansette and Azerad that *o*-succinylbenzoic acid is incorporated into *Rubia* anthraquinones[19] as well as the results reported herein are in good agreement with this suggestion.



(I) Vitamin K R_1 = Me ; R_2 = n isoprene units ; R_3=H
(II) Juglone R_1, R_2 = H; R_3 = OH
(III) Lawsone R_1 = OH ; R_2, R_3=H



(IV) Alizarin R_1, R_2=OH ; R_3, R_4=H
(V) Purpurin carboxylic acid R_1, R_2, R_4=OH; R_3 = COOH
(VI) Tectoquinone R_1, R_3, R_4=H ; R_2=Me

SCHEME 1. QUINONES KNOWN (I–V) OR PRESUMED (VI) TO BE DERIVED FROM SHIKIMIC ACID.

RESULTS

Labelled precursors were fed through the cut end to *Rubia tinctorum* roots. After feeding, alizarin was isolated and purified to constant specific activity. Feeding of carboxyl-^{14}C-D-shikimic acid[20] led to labelled alizarin (IV), thus confirming our previous results.[9] The distribution of radioactivity in the alizarin molecule was determined by degradation of the alizarin dimethylether which yielded benzoic (VII, ring A plus C-atoms 9) and veratric acid[21] (VIII, ring C plus C-atom 10) (Scheme 2).* The result of this degradation showed that the carboxyl group of shikimic acid is exclusively incorporated into C-atom 9 of alizarin; phthalic acid (IX), which was obtained by oxidation of alizarin with $KMnO_4$, contained 106·5% of the activity of alizarin (Table 2).

Decarboxylation of phthalic acid showed that all the activity was confined to the carboxyl groups of phthalic acid which correspond to the keto-C-atoms of alizarin. The conclusion that the carboxyl-C-atom of shikimic acid is incorporated into C-atom 9 can be drawn because veratric acid (the carboxyl group of which corresponds to C-atom 10 of alizarin) contains no activity, while benzoic acid formed by the degradation procedure of

* The degradation procedure of Swan[22] which had been modified and employed previously[1] yielded veratric acid and benzoic acid in small yield, the carboxyl group of benzoic acid being equally derived from C-atoms 9 and 10.

[15] E. LEISTNER and M. H. ZENK, *Tetrahedron Letters* 1395 (1968).
[16] A. R. BURNETT and R. H. THOMSON, *Chem. Commun.* 1125 (1967).
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[18] D. J. ROBINS, I. M. CAMPBELL and R. BENTLEY, *Biochem. Biophys. Res. Commun.* **40**, 1081, 1970).
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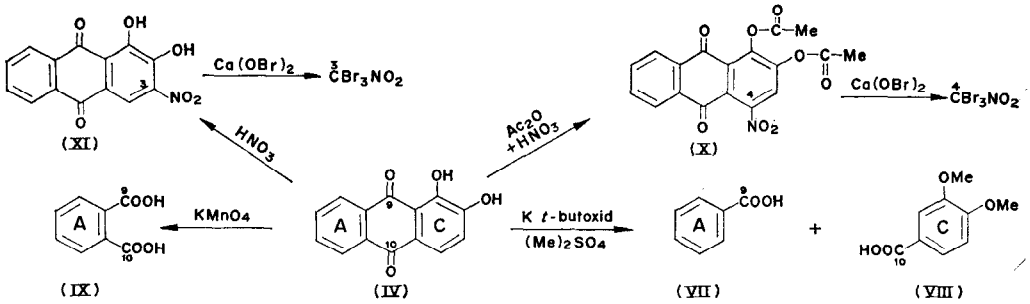
[21] D. G. DAVIES and P. HODGE, *J. Chem. Soc.* **C**, 3158 (1971).
[22] G. A. SWAN, *J. Chem. Soc.* 1408 (1948).



SCHEME 2. DEGRADATION OF ALIZARIN.

Davies and Hodge[21] contains all the radioactivity. Therefore, all the radioactivity must be located in the carboxyl group of benzoic acid (98·4%) (Table 2). Thus, it follows that shikimic acid is incorporated into alizarin nonsymmetrically. Furthermore, the specific incorporation of activity from 5-^{14}C mevalonic acid was also demonstrated. Different mechanisms of incorporation are plausible: the $\gamma\gamma$-dimethylallylpyrophosphate is attached either to the C-atom of the hypothetical naphthalenic precursor which in turn corresponds to either C-atom 13 or 14 of the resulting alizarin, or in the case where the naphthalenic precursor is a symmetrical compound (e.g. naphthoquinol) an attachment to both C-atoms is expected to an equal extent. Degradation of alizarin after 5-^{14}C-mevalonic acid feeding was carried out as follows: acetylation of alizarin yielded 1,2-diacetylalizarin, which was then nitrated in the 4-position[23] and the resulting α-nitrodiacetylalizarin (X) submitted to bromopicrin-cleavage.[24] It was established by IR spectroscopy that the bromopicrin was not contaminated by bromoform or tetrabromomethane.[25] The bromopicrin was combusted[7] and the resulting CO_2 trapped as $BaCO_3$,[9] which contained 83·3 or 89·5% for two different experiments. A control experiment which established the reliability of the methods employed was carried out. Direct nitration of alizarin[26] labelled from 5-^{14}C-mevalonic acid yielded β-nitroalizarin (XI) which was submitted to the same degradation procedure as α-nitrodiacetylalizarin. As expected, the bromopicrin did not contain any radioactivity. Thus, activity from 5-^{14}C-mevalonic acid enters position 4 of alizarin specifically. C-atom 3 is inactive (Table 3). These results account for the origin of all but three C-atoms (C-10, -13, -14) of alizarin. Feeding experiments with compounds closely related to the TCA cycle (Table 1) indicated that the incorporation of U-^{14}C-aspartic-acid into alizarin was much better than that from 1-^{14}C- or 4-^{14}C-aspartic acid. Incorporation was also observed with 2,4^{14}C-citric acid as well as 2-^{14}C-glutamic acid. Degradation of alizarin after 2-^{14} C glutamic acid feeding showed that C-2 of glutamic acid gives rise specifically to C-10 of alizarin. After degradation of the alizarin to benzoic acid the latter was shown to be completely inactive. Since the specific activity of phthalic acid is almost identical to that of the alizarin molecule, activity from 2-^{14}C-glutamic acid only entered C-10 of alizarin. This was confirmed by the observation that veratric acid, derived from ring C and C-10 of alizarin, had almost the same specific activity as the alizarin dimethyl ether (Table 2). Participation of the TCA-cycle in alizarin biosynthesis is also indicated by the specific incorporation of 2-^{14}C-acetate into carbon-atoms 13 and/or 14 of alizarin (see Discussion).

[23] R. BRASCH, *Chem. Ber.* **24,** 1610 (1891).
[24] R. BADDILEY, G. EHRENSVÄRD, E. KLEIN, L. REIO, and E. SALUSTE, *J. Biol. Chem.* **183,** 777 (1950).
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[26] E. SCHUNK and H. ROEMER, *Chem. Ber.* **12,** 583 (1879).

The highest incorporation was, however, obtained with *o*-(succinyl-2,3-^{14}C)-benzoic acid (Table 1). Degradation of the alizarin showed that activity was restricted to ring C; whereas veratric acid was radioactive, only negligible activity was found in phthalic acid (Table 2).

TABLE 1. RESULTS OF FEEDING ^{14}C-LABELLED COMPOUNDS TO *Rubia tinctorum*

Labelled compound administered	Total and specific activity fed (μCi)	(mCi/mM)	Alizarin (purified and diluted) spec. act. total act. incorporation (dpm/mM)	(dpm)	(%)
1-^{14}C-Acetate	200·0	28·8	1 100	172	0·000
2-^{14}C-Acetate	200·0	38·0	74 800	12 650	0·003
2-^{14}C-DL-Mevalonic acid	32·5	5·1	2 000	320	0·001*
5-^{14}C-DL-Mevalonic acid	90·0	11·8	2 400	3 300	0·003*
1-^{14}C-DL-Aspartic acid	25·0	2·9	4 500	1 016	0·004*
4-^{14}C-DL-Aspartic acid	25·0	9·9	0	0	0·000*
U-^{14}C-L-Aspartic acid	10·0	6·1	33 700	8 610	0·039
2,4-^{14}C-Citric acid	5·0	3·2	16 900	3 360	0·030
2-^{14}C-DL-Glutamic acid	35·0	4·3	40 000	8 000	0·021*
Carboxyl-^{14}C-D-shikimic acid	8·0	25·9	356 000	71 300	0·402
o-(Succinyl-2,3-^{14}C)-benzoic acid	2·0	0·5	214 000	57 600	1·295
2-($\gamma\gamma$-Dimethylallyl)-1,4-^{14}C-1,4-naphthoquinone (desoxylapachol)	1·6	1·0	0	0	0·000
2-($\gamma\gamma$-Dimethylallyl-1,4-^{14}C-1,4-naphthoquinol	0·7	1·0	8 700	1 950	0·125

* Calculated on the assumption that only one enantiomer is incorporated.

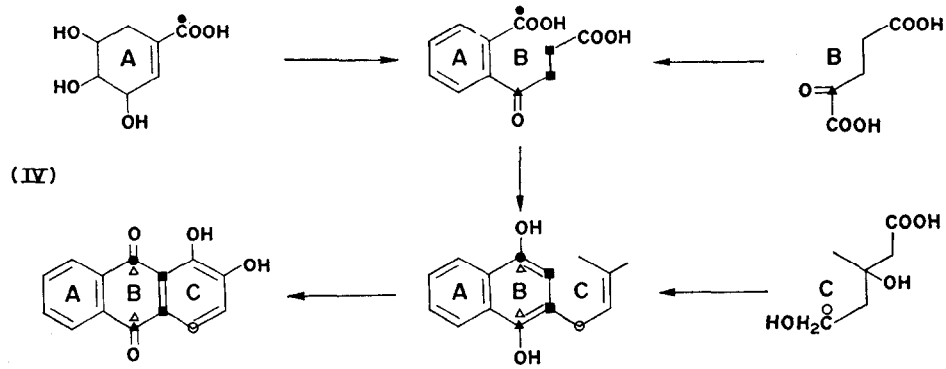
Incorporation of 1,4^{14}C-desoxylapachol (2-[$\gamma\gamma$-dimethylallyl]-1,4-^{14}C-1,4-naphthoquinone) was not observed. The reduced form of desoxylapachol (2-[$\gamma\gamma$-dimethylallyl]-1,4-^{14}C-1,4-naphthoquinol) was, however, incorporated (Table 1) into alizarin. The alizarin isolated after this feeding (Table 1) was recrystallized from ethanol–water (7:3). The diacetylalizarin was prepared and recrystallized from dioxane-water. The specific activity was shown to be constant.

TABLE 2. DEGRADATION OF ALIZARIN DIMETHYL ETHER WITH K *t*-BUTOXIDE

Precursor	Alizarin (IV) dpm/mmol (%)	Alizarin dimethyl ether dpm/mmol (%)	Phthalic acid (IX) ring A plus C-9 and -10 dpm/mmol (%)	Benzoic acid (VII) ring A plus C-9 dpm/mmol (%)	Veratric acid (VIII) ring C plus C-10 dpm/mmol (%)
2-^{14}C-Acetate	74 800 (100)	79 300 (106)	10 200 (13·6)	—	74 800 (100)
2,4-^{14}C-Citric acid	11 700 (100)	12 300 (105·1)	—	1300 (11·1)	11 400 (97·4)
2-^{14}C-DL-Glutamic acid	33 600 (100)	30 000 (92·2)	32 100 (98·4)	0 (0)	29 600 (90·8)
Carboxyl-^{14}C-D-shikimic acid	379 000 (100)	356 000 (94)	403 000 (106·5)	373 000 (98·4)	0 (0)
o-(Succinyl-2,3-^{14}C) benzoic acid	214 000 (100)	203 000 (94·8)	12 550 (5·9)	0 (0)	187 000 (87·4)

DISCUSSION

Chorismic acid has been postulated as an intermediate in quinone biosynthesis.[19,27] Although the incorporation of ^{14}C-chorismic acid into any quinone has not yet been observed, studies with bacterial mutants indicate that chorismic acid rather than shikimic acid may be the compound which enters directly into the pathway leading to vitamin K.[19] The observed incorporation of shikimic acid (carboxyl-^{14}C) (Table 1, Scheme 3) confirms our previous results which showed that U-^{14}C- and 1,2-^{14}C-shikimic acid is incorporated into alizarin[7] either directly or by way of chorismic acid. Feeding of carboxyl-^{14}C-D-shikimic acid and then degradation of alizarin (Table 2) supplied conclusive evidence that the carboxyl group of shikimic acid only gives rise to C-9 of alizarin. Thus, ring A and C-9 of alizarin are known to be derived from shikimic acid.



SCHEME 3. MIGRATION OF RADIOACTIVITY FROM DIFFERENT PRECURSORS TO ALIZARIN.
●—Shikimic acid; ▲—α-Ketoglutaric acid; ■—*o*-Succinylbenzoic acid; ○—Mevalonic acid; △—2-(γγ-Dimethylallyl)-naphthoquinol.

Earlier investigations on the biosynthesis of juglone gave a hint as to the origin of the so-called three missing C-atoms (C-2, -3 and -4 of juglone or 10, 13 and 14 of alizarin); experiments carried out by Leistner and Zenk[7] suggested that these C-atoms may be derived from the citric acid cycle. Later, it was shown that glutamic acid is specifically incorporated into lawsone[17] and vitamin K,[18] respectively. Campbell[17] proposed that glutamic acid is deaminated to α-ketoglutaric acid, which in turn is incorporated into quinones in the form of the succinyl-semialdehyde-thiaminpyrophosphate-carbanion. Since naphthoquinones are derived from shikimic acid and α-ketoglutaric acid, the latter was also likely to be a precursor of anthroquinones such as alizarin. As a matter of fact, activity from 2-^{14}C-glutamic acid is specifically incorporated into C-10 of alizarin (Table 2). Involvement of α-ketoglutaric acid was further suggested after degradation of alizarin labelled from 2-^{14}C acetate. If 2-^{14}C-acetate is channelled into the TCA-cycle and incorporation of activity via α-ketoglutarate is to be expected, C-atom 13 of alizarin should be specifically labelled. Table 2 shows that 2-^{14}C-acetate is primarily incorporated into ring C which consists of C-atoms 1–4, 13 and 14. Since C-3 and -4 which are derived from mevalonate (*vide infra*) are only slightly labelled (Table 3), the main portion of activity should be situated at C-13 and/or 14 of the alizarin molecule.

The nonsymmetric incorporation of activity from 2-^{14}C glutamate and carboxyl-^{14}C-D-shikimate (Scheme 3) into alizarin suggests that no symmetrical intermediates exist

[27] G. B. COX and F. GIBSON, *Biochem. J.* **100**, 1 (1966).

between shikimate (or glutamate) and alizarin. A pre-requisite of this assumption is that symmetrical intermediates cannot be incorporated nonsymmetrically into any product. As a result naphthoquinone, a symmetrical compound, which has been incorporated into alizarin[14] cannot be a true intermediate in alizarin biosynthesis. The same conclusion has been drawn in the case of lawsone (III).[28]

TABLE 3. RESULTS OF DEGRADATION OF ALIZARIN DERIVATIVES TO BROMOPICRIN

Precursor	β-Nitroalizarin (XI) dpm/mmol	(%)	CBr_3NO_2(C − 3) ex β-Nitroalizarin(XI) dpm/mmol	(%)	α-Nitrodiacetyl-alizarin (X) dpm/mmol	(%)	CBr_3NO_2(C-atom 4 α-Nitrodiacetyl-alizarin (X) dpm/mmol	(%)
2-^{14}C-Acetate	4750	(100)	189	(4·0)	4750	(100)	324	(6·8)
5-^{14}C-DL-Mevalonic acid	1996	(100)	0·0	(0·0)	2005	(100·5)	1660	(83·3)

However, both these findings are at variance with results obtained in the course of investigation of juglone (II) biosynthesis; the carboxyl group of shikimic acid is equally incorporated into the keto-C-atoms.[7] Moreover, a detailed degradation of ring A of the juglone molecule labelled from 1,2-^{14}C-shikimic acid showed that the activity is symmetrically distributed in the molecule[7] a finding which has been confirmed by Azerad[29] using a different degradation procedure. These data indicate that a symmetrical compound, possibly 1,4-naphthoquinone (or 1,4-naphthoquinol) is an intermediate in juglone (II) biosynthesis, but not in lawsone (III) and alizarin (IV) biosynthesis.

A hypothesis which accounts for the mechanism of incorporation of α-ketoglutaric acid and shikimic acid (or chorismic acid) into quinones has been proposed leading to the assumption that *o*-succinylbenzoic acid may be an intermediate in quinone biosynthesis.[19] This hypothesis has been shown to be valid in the case of several quinones. We confirmed these results. Our data indicate that a specific incorporation of activity from *o*-(succinyl-2,3-^{14}C)-benzoic acid into ring C of alizarin occurs (Table2).

The further metabolism of *o*-succinylbenzoic acid is still unknown. Sandermann and Simatupang[11] as well as Burnett and Thomson[10] proposed a reaction sequence which was based on the finding that substituted naphthols co-occur with anthraquinones. They suggested that there is a biogenetic relation between naphthols and anthraquinones. While several authors found α-^{14}C-naphthol to be incorporated into vitamin K,[3,8,9] others failed to observe this.[30–32]*

However, the fact that *o*-succinylbenzoic acid is incorporated into anthraquinones makes it unlikely that naphthol is an intermediate in quinone biosynthesis. Another naphthalenic precursor being discussed is 1,4-dihydroxy-2-naphthoic acid.[18] This compound

* We thank Drs. I. M. Campbell and R. Bently, Pittsburgh, U.S.A., for purifying a sample of vitamin K_2 which was obtained after feeding α-^{14}C naphthol to *Bacillus subtilis*.[5] The vitamin K_2 they isolated was found to be radioactive and had about the same specific activity (1262 dpm/μmol) when separated either on a Sephadex LH20 column[30] ('the method of choice') or by reversed-phase TLC.[5]

[28] E. GROTZINGER and I. M. CAMPBELL, *Phytochem.* **11**, 675 (1972).
[29] M. M. LEDUC, P. M. DANSETTE, and R. G. AZERAD, *Europ. J. Biochem.* **15**, 428 (1970).
[30] I. M. CAMPBELL, D. J. ROBINS, M. KELSEY and R. BENTLEY, *Biochemistry* **10**, 3069 (1971).
[31] J. R. S. ELLIS and J. GLOVER, *Biochem. J.* **110**, 22p (1968).
[32] B. S. BROWN, G. R. WHISTANCE, and D. R. THRELFALL, *FEBS Letters* **1**, 323 (1968).

fulfils the requirements of an intermediate in alizarin biosynthesis because, (i) it is a non-symmetrical compound, and (ii) the $\gamma\gamma$-dimethylallylpyrophosphate is likely to be attached in the *meta* position to C-9 of alizarin which is derived from the carboxyl group of shikimic acid (Scheme 3).

The latter observation emerges from the fact that activity from 5-^{14}C-mevalonic acid is specifically incorporated into C-4 of alizarin (Scheme 3) suggesting that ring C-1 to -4 are derived from mevalonic acid by way of $\gamma\gamma$-dimethylallylpyrophosphate. This finding is not surprising since the substituted ring of the anthraquinone, purpurin carboxylic acid, is already known to be derived from mevalonic acid.[15,16] Purpurin carboxylic acid (V) is associated with alizarin in *Rubia tinctorum*.

Another quinoid constituent is desoxylapachol. Although postulated as an intermediate in anthraquinone biosynthesis[10,11] incorporation of this compound labelled at C_1 and C_4 of the naphthoquinone moiety was not observed (Table 1). However, when 1,4-^{14}C-desoxylapachol was administered to a *Rubia* plant as the hydroquinone, incorporation was obtained (Table 1, Scheme 3). This may be due to the fact that the hydroquinone is more water soluble. One should take into account, however, that this compound might have been incorporated nonspecifically, since non-specific incorporation of naphthoquinone has been observed (*vide supra*) suggesting that late steps in alizarin biosynthesis may be catalyzed by nonspecific enzymes. If reduced desoxylapachol is an intermediate its cyclisation product would be tectoquinone (VI), a compound known to occur in plants belonging to the Rubiaceae.[33] Further biosynthetic reactions leading to alizarin have been discussed.[2] A biosynthetic scheme for the overall reaction from shikimic acid to alizarin has been proposed.[1]

EXPERIMENTAL

Feeding and extraction of plant material. Rubia tinctorum plants were grown in the green house. 1 plant (6 to 8 months old), was used for each feeding experiment. The aerial part of the plant was cut off and the root (4–5 g) dipped with the cut end into the tracer solution (0·5–1·0 ml). The root was kept in a container which was closed after all tracer-solution had been taken up. After 30 hr the root was cut to small pieces and extracted 6× with boil. EtOH (80%).

Purification of alizarin. The extract was concentrated under reduced pressure, the aq. residue extracted with benzene (6×), and the benzene phase containing free alizarin was washed, dried, evaporated and applied to preparative TLC plates. An aqueous solution of tartaric acid (37 g/l.) was used to prepare a slurry with silica gel.[34] The TLC plates were developed in $CHCl_3$–MeOH (100:1) (alizarin R_f 0·55). The alizarin was eluted with $CHCl_3$ and the $CHCl_3$ washed with H_2O and dried. Carrier alizarin (60 mg) was dissolved in the solution, which in turn was evaporated and the alizarin recrystallized from HOAc. Yield *ca.* 50 mg, (*ca.* 83%); m.p. 288–289°. The aq. phase containing alizarin as a glycoside (ruberythric acid) was filtered, evaporated and applied to chromatography paper. Ruberythric acid was purified as described previously.[9] The glycoside was hydrolyzed in 0·5 M HCl for 6 hr at 96°. The alizarin was extracted from the solution with Et_2O. The Et_2O was washed, dried and carrier-alizarin (60 mg) added. The solution was evaporated and the residue recrystallized from HOAc. Yield *ca.* 55 mg (*ca.* 90%), m.p. 288–289°. In all cases the radioactivity detected in alizarin derived from the glycoside was higher than the radioactivity in the free alizarin. Radioactivity in alizarin from the glycoside was used in the calculation of incorporation rates (Table 1).

Derivatives of alizarin. Alizarin dimethylether (see Briggs and Dacre[35]). Alizarin (50 mg) was dissolved in dry acetone (50 ml). Me_2SO_4 (0·54 ml) and dry K_2CO_3 (2·2 g) were then added. The slurry was stirred and refluxed for 2 hr, after which the same amounts of Me_2SO_4 and K_2CO_3 were again added and heating and stirring continued. After 2 days, Et_2O (30 ml) and an excess of H_2O was added, and the mixture acidified with 2 M HCl. The upper phase containing methylated alizarins was separated. The aqueous phase was reextracted with Et_2O, the combined ether extracts were washed with H_2O, dried, evaporated and the residue dissolved in dry acetone; Me_2SO_4 and K_2CO_3 were added and stirring and heating continued for 8 hr. The alizarin dimethylether was isolated (*vide supra*) and recrystallized from HOAc; yield 27 mg, (49%); m.p. 216–218°. The material was chromatographically homogeneous.

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α-Nitrodiacetylalizarin (X) (see Brasch[23]). Diacetyl alizarin (200 mg) was cooled (−20°) and HNO_3 (0·4 ml, 96%) added dropwise. The mixture was stirred and after 10 min transferred into icewater. The red precipitate was collected, washed and dissolved in warm acetone. The solution was applied to two chromatography-columns (1·5 × 4 cm) packed with Al_2O_3 (Woelm, acidic, Brockmann III). The 4-nitrodiacetylalizarin was eluted with the organic phase of tetrahydrofuran–EtOAc–H_2O (35:6:47). The eluate was evaporated. Yield 92 mg (41%), m.p. 194–195·5°.

β-Nitroalizarin (XI) (see Schunck and Roemer[26]). HNO_3 (0·06 ml, 64%) was added slowly to a stirred suspension of alizarin (100 mg) in HOAc (1 ml). After 15 min the collected product was washed with H_2O and recrystallized from 1M KOH. The β-nitroalizarin was regenerated from the K salt by the addition of conc. HCl. The product was washed with H_2O, dried and recrystallized from HOAc. Yield 72 mg (61%), m.p. 244°.

Degradation of alizarin derivatives (Scheme 3). *Degradation of alizarin to phthalic acid* has been described previously.[9] *Degradation of alizarin dimethylether to veratric and benzoic acid* (see Davies and Hodge[21]). A suspension of K *t*-butoxide (201 mg) and dimethoxyethane (2·5 ml) was stirred vigorously, H_2O (10 μl) added and the flask flushed with N_2 after which the alizarin dimethylether (18 mg) was added. The suspension was stirred and refluxed in N_2. After 2 hr the mixture was cooled, H_2O added, acidified (2M HCl), and the organic acids extracted into Et_2O. The dried ethereal solution was evaporated and applied to activated silica gel plates. The plates were developed in C_6H_6–HCO_2Et–HCO_2H (80:20:0·5). (Veratric acid, R_f 0·26; benzoic acid, R_f 0·41) Both acids were eluted with MeOH. Further purifications were carried out on paper (Schleicher und Schüll 2045 b) in *iso*-PrOH–NH_4OH–H_2O (8:1:1); (veratric acid, R_f 0·45; benzoic acid, R_f 0·55); 2% HCO_2H; (veratric acid, R_f 0·63; benzoic acid, R_f 0·77); EtOH–*iso*–AmoH–HOAc (1M) (2:1:1); (veratric acid R_f 0·86; benzoic acid, R_f 0·91). The concentration of the acids was determined spectrophptometrically; benzoic acid E_{272} 0·90 × 10^6 cm²/mol, yield 25 μM, (38%); veratric acid E_{255} 9·00 × 10^6 cm²/mol, yield 30 μM; (45%).

Bromopicrin-cleavage of substitute dalizarin (see Baddiley *et al*[24]). The nitro-derivatives of alizarin (amounts *vide supra*) were dissolved in KOH (6 ml, 0·5 M), chilled to 0° and an ice-cold paste (21·5 ml) of $Ca(OH)_2$ (7·5 g), H_2O (30 ml) and Br_2 (2·5 ml) was added. The mixture was shaken at 40° for 1 min and the flask attached to a steam distillation apparatus. The resulting bromopicrin was collected by centrifugation and washed. The bromopicrin was checked for contaminants. IR spectroscopy did not indicate the presence of tetrabromomethane or bromoform.[25] The bromopicrin was combusted according to Van Slyke. The CO_2 formed was trapped as $BaCO_3$. Yield 8 mg (17%), (α-nitrodiacetylalizarin) or 5 mg (10%), (β-nitroalizarin) respectively.

Synthesis of radioactively labelled precursors. Synthesis of 1,4^{14}C-*desoxylapachol* (see Sandermann and Simatupang[36]). 1,4-^{14}C-1,4-Naphthoquinone was prepared according to Teuber and Götz[37] following the experimental details of Leistner and Zenk.[38] This (50 μCi, spec. act. 1 mCi/mM) was dissolved in Et_2O by shaking with a saturated (24·4 g/100 ml at 20°) solution (1·5 ml) of sodium dithionite. The Et_2O phase was washed with H_2O, evaporated under N_2 and the residue dried over P_2O_5. To the 1,4-^{14}C-1,4-naphthohydroquinone a solution (0·11 ml) of borotrifluordietherate (0·1 ml) in redistilled dioxane (0·1 ml) and a solution (0·042 ml) of γγ-dimethylallyl alcohol (0·95 ml) in dioxane (0·05 ml) and finally 0·058 ml dioxane were added. The reaction mixture was kept for 1·5 hr at 50°. Et_2O (1 ml) was added and the solution shaken with H_2O (2 × 0·6 ml). The Et_2O solution was evaporated in N_2. The residue was dried and a part of the 2-(γγ-dimethylallyl)-1,4-^{14}C-1,4-naphthohydroquinone was oxidized to desoxylapachol; the hydroquinone was dissolved in dry Et_2O and a small amount of $MgSO_4$ and AgO (24 mg) added. The suspension was shaken for 15 min and the supernatant evaporated. Desoxylapachol and 2-(γγ-dimethylallyl)-1,4-^{14}C-1,4-naphthohydroquinone were chromatographed on silica gel H-plates in C_6H_6–light petrol (b.p. 30–50°) (3:1). R_f of both compounds: 0·5. The labelled compounds were eluted with CH_2Cl_2, evaporated in N_2 and dissolved in Tween 20 (0·5%). Total yield: 30%.

Synthesis of o-(*succinyl*-2,3^{14}C)-*benzoic acid* (see Roser[39]). 2,3^{14}C-Succinic acid (11·8 mg) (50 μCi), phthalic anhydride (11·8 mg) and NaOAc (dry, 3·5 mg) were mixed, a thick glass rod was placed above the mixture which was heated for 2 min at 240°. The mixture was allowed to cool and extracted 2× for 20 min with H_2O (5 ml) on a steam bath. The extract was filtered through glass wool and the dilactone formed was hydrolyzed with a small amount of Na_2CO_3 on a boiling H_2O bath (45 min). The cooled solution was extracted with Et_2O. The Et_2O was discarded and the *o*-succinylbenzoic acid was extracted from the acidified aq. solution into Et_2O. The Et_2O was evaporated and applied to thin layer plates (silica gel GF). The plates were developed in C_6H_6–HOAc (4:1), *o*-succinylbenzoic acid R_f 0·26. The acid was sublimed (120°, 0·003 mm Hg) and rechromatographed on paper in *iso*PrOH–NH_4OH–H_2O (8:1:1), *o*-succinylbenzoic acid R_f 0·1. Yield 14%.

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Measurement of radioactivity. Radioactivity was measured with a Unilux I (nuclear chicago) or a BF 5001 (Berthold-Frieseke) scintillation counter. Samples were prepared as follows: coloured material was combusted[9] and parallel to this another sample was dissolved in MeOH and counted after addition of 'liquifluor'. Radioactivity of $BaCO_3$ was counted after liberating the CO_2 from $BaCO_3$ by the addition of $HClO_4$.[9] Methanolic solutions containing phthalic, benzoic or veratric acid were directly counted. M.p.s are uncorrected.

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