Biochem. J. (1974) **143**, 207–217
Printed in Great Britain

Isolation of Oligomers of 5,6-Dihydroxyindole-2-carboxylic Acid from the Eye of the Catfish

By SHOSUKE ITO and J. A. COLIN NICOL
The University of Texas Marine Science Institute, Port Aransas, Tex. 78373, *U.S.A.*

(*Received* 29 *April* 1974)

The reflecting material of the tapetum lucidum of the sea catfish (*Arius felis*) was chromatographed on Sephadex LH-20 in methanol–dimethyl sulphoxide–formic acid. Two components were present: one, showing an absorption maximum at 330nm, was tapetal pigment; the other, at 257nm, was an associated nucleoside. The tapetal pigment was extracted in methanol–HCl and isolated by adsorption chromatography on Sephadex LH-20. It yielded a methoxy methyl ester on treatment with diazomethane, and permanganate oxidation gave pyrrole-2,3,5-tricarboxylic acid. From the information provided by u.v. and i.r. spectra of the pigment and its methoxy methyl ester, from elemental analyses and from the oxidation products, we suggest that the tapetal pigment is derived from oxidative coupling of 5,6-dihydroxyindole-2-carboxylic acid. A molecular-weight determination and chromatography of the methoxy methyl ester indicate that the pigment is a mixture of oligomers, among which the tetramers probably predominate. We consider that the monomers are joined mainly by C-C linkages at positions 4 and 7. A synthetic pigment having spectral properties nearly identical with those of the natural pigment was prepared by enzymic oxidation of 5,6-dihydroxyindole-2-carboxylic acid with mushroom tyrosinase. The identity of the tapetal pigment with the synthetic pigment was further confirmed by comparing u.v. and i.r. spectra of their methoxy methyl esters. Formation of the tapetal pigment from tyrosine and relationships of the tapetal pigment to melanin are discussed.

Eyeshine occurs in many animals owing to the presence of a tapetum lucidum that lies behind the retina (Walls, 1942; Pirie, 1966). Guanine has long been recognized as a tapetal reflecting material in some fishes (Walls, 1942) and a triglyceride and a pteridine have recently been identified in others (Arnott *et al.*, 1972; Zyznar & Nicol, 1973). Wunder (1925) mentioned the occurrence of a guanine-like material in the brown bullhead catfish (*Ictalurus nebulosus*). Our recent work has shown, however, that the tapeta lucida of catfishes and of gars contain light-tan or light-yellow reflecting materials that are not purines or pteridines. Their phenolic nature has been indicated mainly by histochemical and preliminary chemical investigations (Nicol & Arnott, 1973; Arnott *et al.*, 1974).

The tapetum lucidum of the catfish lies in the pigment epithelium, the cells of which contain reflecting (tapetal) pigment and melanin. The tapetal pigment occurs in the cell processes and the melanin is capable of migrating into and out of the processes, under light- and dark-adapted conditions respectively. In this paper, we describe the isolation of tapetal pigment from the sea catfish and its chemical and spectroscopic properties. A plausible and representative structure for the pigment is proposed on the basis of the evidence presented, the enzymic synthesis of a probably identical pigment is described, and the structural relationship between the tapetal pigment and melanin is discussed.

Materials and Methods

Animal material

Sea catfish (*Arius felis* L.) were collected in channels and bays near Port Aransas, Texas, U.S.A. Whole eyes for preparative-scale extractions were dissected out and stored on solid CO_2 until required.

Chemicals and reference standards

Bilirubin, glucagon, indole-2-carboxylic acid and mushroom tyrosinase [type III, 3690 units/mg (Sigma); EC 1.14.18.1] were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. 5-Hydroxyindole-2-carboxylic acid was purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., and Sephadex LH-20 was from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. Precoated plates (silica gel and cellulose) for t.l.c. were obtained from E. Merck, Darmstadt, West Germany. Organic solvents used were purified by distillation.

5,6-Dihydroxyindole-2-carboxylic acid and methyl 5,6-dimethoxyindole-2-carboxylate were prepared

from methyl 5,6-dihydroxyindole-2-carboxylate. The latter was obtained by the method of Omote *et al.* (1966), and was purified by sublimation at 180–210°C/0.05mmHg, m.p. 243°C (decomp.) [literature (Dukler *et al.*, 1971) m.p. 235–238°C].

A solution of methyl 5,6-dihydroxyindole-2-carboxylate (40mg) in 10% (w/w) NaOH (1.0ml) containing $Na_2S_2O_4$ (100mg) was stirred for 6h at 22°C. Acetic acid (0.5ml) was added and the mixture was extracted with ether (5×10ml). The extract was dried over Na_2SO_4, evaporated, and the residue was recrystallized from acetone–hexane to yield microcrystals (15.5mg) of 5,6-dihydroxyindole-2-carboxylic acid, m.p. 228°C (decomp.) [literature (Beer *et al.*, 1949) m.p. 234°C (decomp.)]. T.l.c. on cellulose showed single spots at R_F 0.42 in butan-1-ol–acetic acid–water (12:3:5, by vol.), and R_F 0.05 in 0.1M-HCl. The u.v. spectrum is shown in Fig. 5(*a*).

A solution of methyl 5,6-dihydroxyindole-2-carboxylate in methanol was treated with ethereal diazomethane for 24h at 22°C. The product was recrystallized from ethyl acetate–hexane to give pale-yellow crystals of methyl 5,6-dimethoxyindole-2-carboxylate, m.p. 168–170°C (Found: C, 61.3; H, 5.3; N, 5.8. $C_{12}H_{13}NO_4$ requires C, 61.3; H, 5.6; N, 6.0%). The u.v. spectrum is shown in Fig. 5(*b*).

Pyrrole-2,3,5-tricarboxylic acid was prepared by oxidation of 5-hydroxyindole-2-carboxylic acid with $KMnO_4$; m.p. above 290°C (decomp.) [literature (Nicolaus, 1953) m.p. 294°C (decomp.)].

Dopa melanin was prepared from DL-3,4-dihydroxyphenylalanine (DL-dopa) by autoxidation at pH8.0.

Analytical isolation of tapetal pigments from exposed tapeta

Reflecting material for analytical use was obtained from the pigment epithelia of two dark-adapted eyes (from a fish of 27.5cm total length) after removal of the retina. The epithelia were gently stroked with a camel's-hair brush, thereby releasing reflecting material and melanin. The resultant suspension in 0.25M-sucrose (1.5ml) was centrifuged at 1500*g* and 3°C for 10min. The precipitate was resuspended in 2.0M-sucrose (1.5ml) and centrifuged for 30min at 20000*g* and 3°C. The residue, after washing with water (1.0ml), was extracted with methanol–dimethyl sulphoxide–1M-HCl (8:1:1, by vol.; 2.0ml) at 3°C. The extract of the reflecting material from the exposed tapeta was chromatographed on a column of Sephadex LH-20, as shown in Fig. 1.

Preparative isolation of tapetal pigments from whole eyes

For this 260 whole eyes (wet wt. 94g) were homogenized in a prechilled Waring blender with 4vol. (v/w) of 0.25M-sucrose, the homogenate was filtered through a double layer of nylon netting (aperture 53μm), and the filtered mass was washed well with 0.25M-sucrose to make a 10% (w/v) suspension. The combined filtrates (940ml) were centrifuged at 7000*g* for 10min, yielding a brown–black precipitate, which was suspended in 0.25M-sucrose (200ml) and centrifuged again at 7000*g* for 10min. The precipitate was washed with water (100ml) by centrifugation at 7000*g* for 10min. The pellet was extracted with methanol–1M-HCl (19:1, v/v; 200ml) by stirring the mixture for 10min, followed by centrifugation, yielding a yellow extract of the tapetal pigments; the procedure was repeated twice. The combined extracts were cooled to −20°C and kept for at least 1h; some precipitate formed, which was removed by filtration through fine fritted glass. The methanol–1M-HCl extract (600ml) of tapetal pigments was chromatographed on a column of Sephadex LH-20, as described in Fig. 2.

Fractions 42–78 (fraction B) were combined and the methanol was evaporated in a vacuum at 30°C. The solution was then extracted with chloroform (4×260ml) to remove the dimethyl sulphoxide and

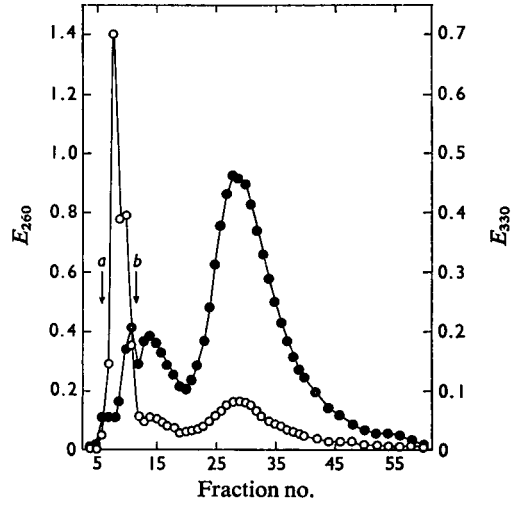


Fig. 1. *Analytical chromatography of an extract of tapetal pigments from exposed tapeta on a column of Sephadex LH-20*

A column (1.05cm×52cm) of Sephadex LH-20 was equilibrated with methanol–dimethyl sulphoxide–1M-formic acid (8:1:1, by vol.) at −3°C and the methanol–dimethyl sulphoxide–1M-HCl extract (2.0ml) was applied. The column was eluted with methanol–dimethyl sulphoxide–1M-formic acid at a flow rate of approx. 10ml/h. Fractions (3.5ml) were collected and monitored by their E_{330} (●) and E_{260} (○) values. The positions of elution of glucagon (mol.wt. 3500; *a*) and acetone (mol.wt. 58; *b*) are indicated by arrows.

the residual chloroform was expelled with a stream of N_2. The resulting aqueous suspension was diluted to 130ml with water, adjusted to pH2.0 with 90% (w/w) formic acid, and kept for 2 days at 4°C. A precipitate formed, which was collected by centrifugation, washed with 1M-formic acid (3×15ml) and then freeze-dried from 0.1M-formic acid. The tapetal pigment thus obtained from fraction B formed a grey-coloured powder and weighed 50.1mg. The results of elemental analyses are shown in Table 1. The u.v. spectrum is shown in Fig. 5(*a*) and the i.r. spectrum in Fig. 6(*a*).

Fractions 24–41 (fraction A) were treated in a similar manner and 10.8mg of a dark-grey-coloured powder was obtained (tapetal pigment of fraction A). The u.v. spectrum is shown in Fig. 5(*a*). Chromatographic analyses of the tapetal pigments of fractions A and B are shown in Fig. 3.

Preparation of methoxy methyl ester of tapetal pigment

Ethereal diazomethane (20ml) was added to a stirred suspension of the pigment of fraction B (10.0mg) in methanol (10ml). After 24h a few drops of acetic acid were added to destroy the excess of diazomethane and the solvents were evaporated to dryness. The product was purified by two successive t.l.c. on silica gel (20cm×20cm, 0.25mm thickness) in chloroform–methanol (49:1, v/v). Three bands formed that were intensely blue fluorescent under u.v. light (254 and 366nm); they were combined and eluted with chloroform–methanol (9:1, v/v). The product was further purified by precipitation from chloroform–hexane. A yellow powder (7.0 g) was obtained. T.l.c. on silica gel (chloroform–methanol, 49:1, v/v) showed the presence of three rather streaky spots, R_F 0.24, 0.38 and 0.52. The results of an elemental analysis, a methoxyl analysis and a molecular-weight determination are shown in Table 1, and a chromatographic analysis on Sephadex LH-20 in chloroform is shown in Fig. 7. The u.v. spectrum is shown in Fig. 5(*b*) and the i.r. spectrum in Fig. 6(*b*).

Ethereal diazomethane (20ml) alone was treated in exactly the same manner and only 0.1–0.2mg of material was found as a residue.

Permanganate oxidation

A procedure essentially the same as described by Piattelli *et al.* (1962) and by Hackman & Goldberg (1971) was used. Samples were oxidized in 1M-K_2CO_3 with 3% (w/w) $KMnO_4$ and the products

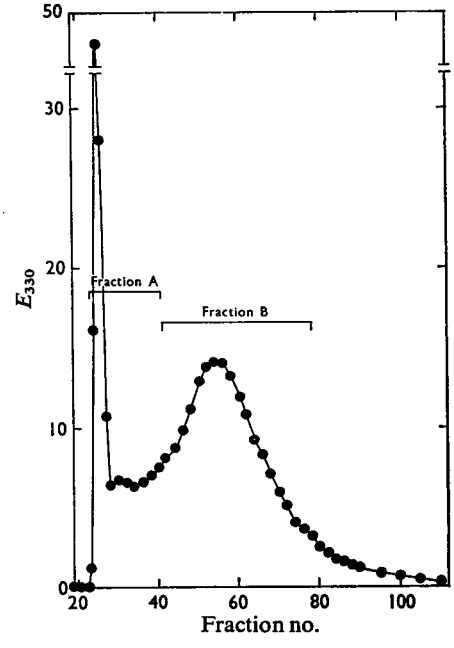


Fig. 2. *Preparative chromatography of an extract of tapetal pigments from whole eyes on a column of Sephadex LH-20*

Chromatography was carried out at 3°C. The methanol–1M-HCl extract (600ml) from 260 eyes was applied to a column (4.1cm×35cm) of Sephadex LH-20, which had been equilibrated with methanol–1M-HCl (19:1, v/v) and was washed with the same solvent (3400ml) at a flow rate of approx. 130ml/h. The tapetal pigments were then eluted with methanol–dimethyl sulphoxide–1M-formic acid (8:1:1, by vol.) at a flow rate of approx. 90ml/h. Fractions (14ml) were collected and monitored by their E_{330} values. Recovery of the pigments was almost quantitative.

Table 1. *Results of elemental and methoxyl analyses and a molecular-weight determination*

All analyses were carried out by the Alfred Bernhardt Microanalytical Laboratory (West Germany). Each sample was dried at 80°C and 0.01mmHg to constant weight: the pigment of fraction B showed a weight loss of 15.0%. Results of elemental analyses were expressed on an ash-free basis (ash content: the pigment of fraction B, 1.1, 0.5%; the methoxy methyl ester, 0.9%).

	C	H	N	O	S	OCH_3	Mol.wt.
Tapetal pigment of fraction B	55.7, 56.0	3.0, 3.2	7.7	32.9	0.0	0.0%	
Calc. for structure I*a*	56.4	2.9	7.3	33.4	0.0	0.0%	766
Methoxy methyl ester of tapetal pigment	61.4	5.3	5.7			38.4%	955 (osmometric)
Calc. for structure I*b*	61.7	5.0	6.0			39.8%	934

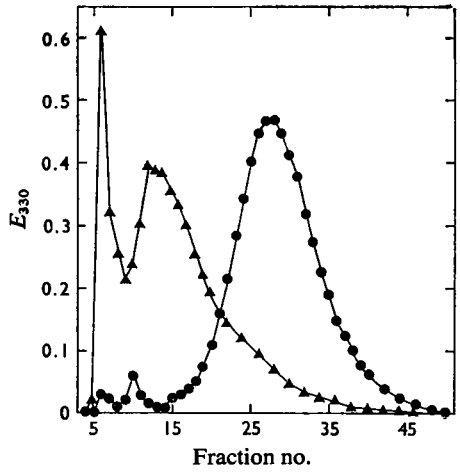


Fig. 3. *Analytical chromatography of tapetal pigments of fraction A and B on a column of Sephadex LH-20*

The pigment of fraction A (0.45mg; ▲) or fraction B (0.35mg; ●) was dissolved in methanol–dimethyl sulphoxide–1M-HCl (8:1:1, by vol.; 0.5ml) and chromatographed under the conditions described in Fig. 1. Recoveries of the pigments were approx. 85%.

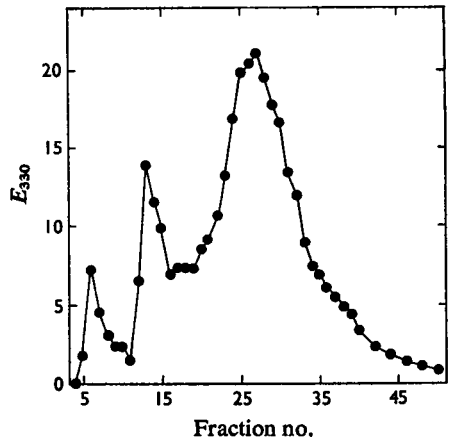


Fig. 4. *Preparative chromatography of synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid on a column of Sephadex LH-20*

The extract (3.0ml) was chromatographed under the conditions described in Fig. 1.

obtained were analysed by t.l.c. on cellulose (20cm×20cm, 0.10mm thickness). Plates were developed two-dimensionally, in propan-1-ol–conc. NH_3 (sp.gr. 0.90)–water (6:3:1, by vol.) (solvent A) in the first direction and butan-1-ol–acetic acid–water (12:3:5, by vol.) in the second (Binns *et al.*, 1970). The chromatograms were sprayed with sulphanilic acid reagent (Smith *et al.*, 1969). A few red spots were detected. The main one was identified as pyrrole-2,3,5-tricarboxylic acid by co-chromatography with the authentic sample on cellulose t.l.c. plates in both solvent A and ethanol–conc. NH_3 (sp.gr. 0.90)–water (20:1:4, by vol.). Two minor spots from the oxidation products of dopa melanin were used as standards of pyrrole-2,3-dicarboxylic acid and pyrrole-2,3,4,5-tetracarboxylic acid (Piattelli *et al.*, 1962).

The pigments of fractions A and B were decarboxylated at 200°C in a high vacuum (0.7Pa, or 0.005mmHg) for 24h (Swan & Waggott, 1970), and then oxidized. The products were analysed by t.l.c. as described above.

To determine yields of pyrrole-2,3,5-tricarboxylic acid, portions of the oxidation products, and 2, 4, 8 and 16μg of the authentic acid, were chromatographed on a cellulose t.l.c. plate in solvent A. After spraying with the sulphanilic acid reagent, coloured spots were eluted with water (1.2ml) and their extinctions at 400nm were measured.

Enzymic synthesis of oligomers of 5,6-dihydroxyindole-2-carboxylic acid with mushroom tyrosinase

To a solution of 5,6-dihydroxyindole-2-carboxylic acid (20.0mg) in 0.1M-sodium phosphate buffer, pH6.8 (9.4ml), was added mushroom tyrosinase (3000 units) in the same buffer (0.6ml), and O_2 was bubbled into the solution at a rate of approx. 10ml/min at 23°C. The reaction was monitored by measuring the u.v. spectrum of a solution of a sample (10μl) in 0.1M-formic acid (2.0ml) at intervals of 0.5h: 0h, $\lambda_{max.}$ 319nm ($E_{max.}$ 1.01), $\lambda_{min.}$ 267nm ($E_{min.}$ 0.13); 1.5h, $\lambda_{max.}$ 324nm ($E_{max.}$ 0.82), $\lambda_{min.}$ 269nm ($E_{min.}$ 0.21); 3h, $\lambda_{max.}$ 327nm ($E_{max.}$ 0.67), $\lambda_{min.}$ 271nm ($E_{min.}$ 0.24). After 3h the solution was adjusted to pH2.0 with 90% (w/w) formic acid and the resulting suspension was kept for 3h at 4°C. The precipitate formed was collected by centrifugation, washed with 0.1M-formic acid (3×12.5ml), and extracted with methanol–dimethyl sulphoxide–1M-HCl (8:1:1, by vol.; 3.0ml). The extract was chromatographed on a column of Sephadex LH-20, as shown in Fig. 4.

Fractions 22–39 were combined and synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid were isolated by a method similar to that used to isolate the tapetal pigments on a preparative scale (see above). A grey powder (4.5mg) was obtained. The u.v. spectrum is shown in Fig. 5(*a*) and the i.r. spectrum in Fig. 6(*a*).

Methoxy methyl ester of the synthetic pigment was prepared by a method similar to that described for the preparation of the methoxy methyl ester of the tapetal pigment (see above). The t.l.c. pattern on silica gel of the methoxy methyl ester of the synthetic pigment was identical with that of the methoxy methyl ester of the natural pigment. A chromatographic analysis on Sephadex LH-20 in chloroform is shown in Fig. 7. The u.v. spectrum is shown in Fig. 5(*b*) and the i.r. spectrum in Fig. 6(*b*).

Results and Discussion

Isolation of the tapetal pigments

The reflecting material from exposed pigment epithelia of the sea catfish contained two different components. One, which was insoluble in dilute acids, was easily extracted with methanol–1 M-HCl (19:1, v/v) to give a pale-yellow solution, the u.v. spectrum of which showed an absorption maximum at 330nm. This component is referred to as 'tapetal pigments'. The other, which was soluble in dilute acids, showed an absorption maximum at 257nm in 1 M-formic acid. The main constituent of this fraction is known to be an adenine nucleoside which is associated with the tapetal pigments (S. Ito & J. A. C. Nicol, unpublished work).

Attempts to purify the tapetal pigments by column chromatography on various adsorbents, including Sephadex G types, proved unsuccessful because the pigments were irreversibly adsorbed. Although they did not move on a column of Sephadex LH-20 when acidic methanol was used as a solvent, they could be eluted with acidic methanol containing dimethyl sulphoxide. The elution pattern of the extract of the reflecting material from the exposed tapeta (Fig. 1) revealed that the tapetal pigments were separated into a few components, of which the main one was strongly retarded and constituted 78% of extinctions at 330nm. The elution position of this component was temperature-dependent; at a lower temperature the peak appeared later. The 257nm-absorbing materials were eluted faster, without retardation.

The methanol–1 M-HCl extract of 260 whole eyes contained, besides the tapetal pigments, many kinds of contaminants. However, most of them were removed during the course of elution with a large volume (about 7 column vol.) of methanol–1 M-HCl before elution of the tapetal pigments with methanol–dimethyl sulphoxide–1 M-formic acid. The 257nm-absorbing materials were eluted in the 200–1000ml fraction of the methanol–1 M-HCl. The tapetal pigments were separated into two fractions as shown in Fig. 2. The extreme sharpness of the peak (fractions 24–27) in fraction A was caused by the pigments moving in the dimethyl sulphoxide front. About 80% of the tapetal pigment in fraction B precipitated from the aqueous solution at pH2.0, whereas only 50% of that in fraction A precipitated under the same condition.

On rechromatography of the tapetal pigment of fraction B, 97% of the 330nm-absorbing materials ran in the same position as before (Fig. 3). The relative amount of the fastest-moving component (peak fraction 6) of the pigment of fraction A was apparently higher than that found in the eluates of the extract from the exposed tapetum (Figs. 1 and 3). This suggests that the component might be an artifact formed during the isolation procedure, possibly by the action of acid.

The elution positions of the main components of the pigments from exposed tapeta (peak fractions 28 and 29 in Fig. 1) and of the pigment of fraction B from whole eyes (peak fractions 27 and 28 in Fig. 3) were almost identical and their u.v. spectra were coincident with each other. These facts demonstrate that the tapetal pigment of fraction B from whole eyes came from the reflecting material in the pigment epithelium.

The pigments of fractions A and B and the synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid were hydrolysed in 6M-HCl for 24h at 110°C and the amino acid contents of the hydrolysates were determined with the ninhydrin reagent (Moore, 1968). The numbers of amino acid residues found per 100 residues of 5,6-dihydroxyindole-2-carboxylic acid were: pigment of fraction A, 3.2; pigment of fraction B, 1.0; the synthetic oligomers, 0.9. Consequently, the tapetal pigment of fraction B may be considered to be free from proteins or amino acids. Sugars were not found in the 0.5M-H_2SO_4 hydrolysate (at 100°C for 3h) of the tapetal pigments.

Properties

The purified tapetal pigments were fairly soluble in dimethyl sulphoxide, methanol–1 M-HCl, pyridine and 0.05M-sodium phosphate buffers at pH6.8 and 8.0. They were positive to tests for phenols, such as $FeCl_3$–ferricyanide (Barton *et al.*, 1952), $AgNO_3$ in aqueous acetone (Burke *et al.*, 1950) and $FeCl_3$ in ethanol. They remained at the origin or streaked a short distance from the origin on cellulose plates in several solvent systems tried (Arnott *et al.*, 1974). Reducing reagents such as $NaBH_4$, H_2SO_3 and ascorbic acid did not affect the structure of the chromophore, judging from the u.v. spectra.

The pigments in sodium phosphate buffer, pH8.0, were rapidly oxidized by atmospheric O_2, and the absorption spectrum of the solution kept for 24h showed a general absorption similar to those of melanins (Mason, 1948; Van Woert *et al.*, 1967); the intensity of the end absorption in the visible region was diminished by addition of either $Na_2S_2O_4$ or

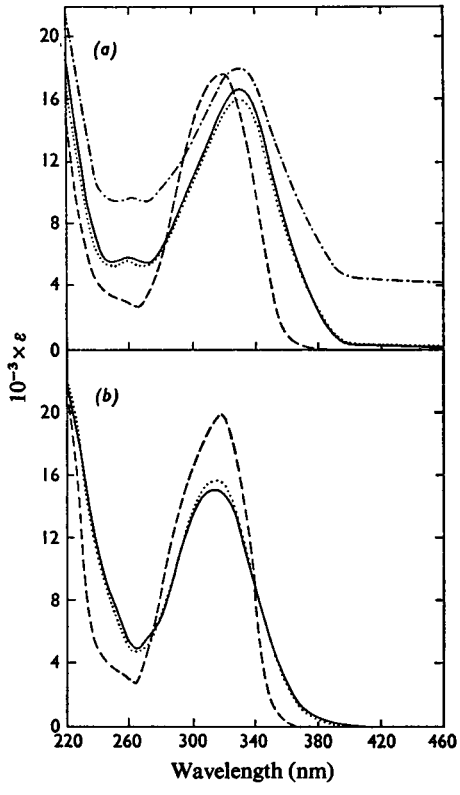


Fig. 5. *U.v.-absorption spectra of natural and synthetic pigments and their methoxy methyl esters*

(*a*) ——, Tapetal pigment of fraction B: λ_{max} 330 and 259nm (ε_{max} 16700 and 5900); -·-·, tapetal pigment of fraction A: λ_{max} 331 and 260nm (ε_{max} 14100 and 5700), the curve is displaced upwards by $4 \times 10^3 \varepsilon$ for clarity of presentation; ····, synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid: λ_{max} 330 and 258nm (ε_{max} 16000 and 5800); ----, 5,6-dihydroxyindole-2-carboxylic acid: λ_{max} 320nm (ε_{max} 17700) in methanol–1M-HCl (19:1, v/v). (*b*) ——, Methoxy methyl ester of the tapetal pigment: $\lambda_{max.}$ 314nm ($\varepsilon_{max.}$ 15100); ····, methoxy methyl ester of the synthetic pigment: $\lambda_{max.}$ 314nm ($\varepsilon_{max.}$ 15700); ----, methyl 5,6-dimethoxyindole-2-carboxylate: $\lambda_{max.}$ 318nm ($\varepsilon_{max.}$ 20100) in methanol. All the spectra were recorded on a Cary spectrophotometer model 118c. The term ε means here the molar extinction coefficient per monomer unit (mol.wt. 766/4 = 191.5 for the pigments and 934/4 = 233.5 for the methoxy methyl esters).

$NaBH_4$, but the peak that had originally been found at 326nm in the buffer at pH 8.0 could not be restored. Therefore the change brought about by autoxidation must be irreversible. The tapetal pigments were also destroyed slowly in acid: after 3 days at 22°C in methanol–dimethyl sulphoxide–1M-HCl (8:1:1, by vol.), 30% of the pigment of fraction B changed into faster-moving components on Sephadex LH-20. The degradation took place even in the presence of H_2SO_3, indicating that the change in acid was not due to oxidation.

T.l.c. and column chromatography (Fig. 7) of the methoxy methyl ester of the tapetal pigment indicated that it was a mixture of many compounds having closely related structures. It dissolved easily in many organic solvents and was negative to the $FeCl_3$–ferricyanide test.

The u.v. spectra of the tapetal pigments of fractions A and B (Fig. 5*a*) were similar to each other and showed absorption maxima at almost the same positions. The spectra in methanol and in methanol–1M-HCl (19:1, v/v) were almost identical. The absorption maximum at 330nm in either a neutral or an acidic solution displayed a bathochromic shift of 17nm in methanol–1M-NaOH (19:1, v/v). The i.r. spectra of the pigments of fractions A (not shown) and B (Fig. 6*a*), although not identical, closely resembled each other.

Permanganate oxidation

Permanganate oxidation of various samples gave pyrrole-2,3,5-tricarboxylic acid as the main product among the pyrrolecarboxylic acids obtained (Table 2). It was characteristic of the tapetal pigments that pyrrole-2,3-dicarboxylic acid could not be detected in their oxidation products. Oxidation of the pigments which had been decarboxylated by heating at 200°C afforded small amounts of pyrrole-2,3-dicarboxylic acid, although pyrrole-2,3,5-tricarboxylic acid still remained the main product. Heating at 220°C for 48h did not change the ratio of the products.

Structure and synthesis of tapetal pigment

The presence of carboxyl groups was indicated by a broad absorption band at 3600–2200cm^{-1} and a strong band at 1685cm^{-1} in the i.r. spectrum of the pigment of fraction B (Fig. 6*a*). This was confirmed by the presence of an intense band at 1710cm^{-1} and the lack of a broad band at 2800–2200cm^{-1} in the spectrum of the methoxy methyl ester (Fig. 6*b*). The phenolic nature of the pigments was suggested by the spot tests and their instability in alkaline solutions, and also by the bathochromic shift of the u.v. maximum in an alkaline medium.

The formation of pyrrole-2,3,5-tricarboxylic acid by permanganate oxidation of the pigments indicates that an indole-2-carboxylic acid having phenolic hydroxyl groups should be a principal constituent of the pigments. Although the yields of pyrrole-2,3,5-tricarboxylic acid from the pigments were far from quantitative, they were reasonably high when compared with those from 5-hydroxyindole-2-carboxylic

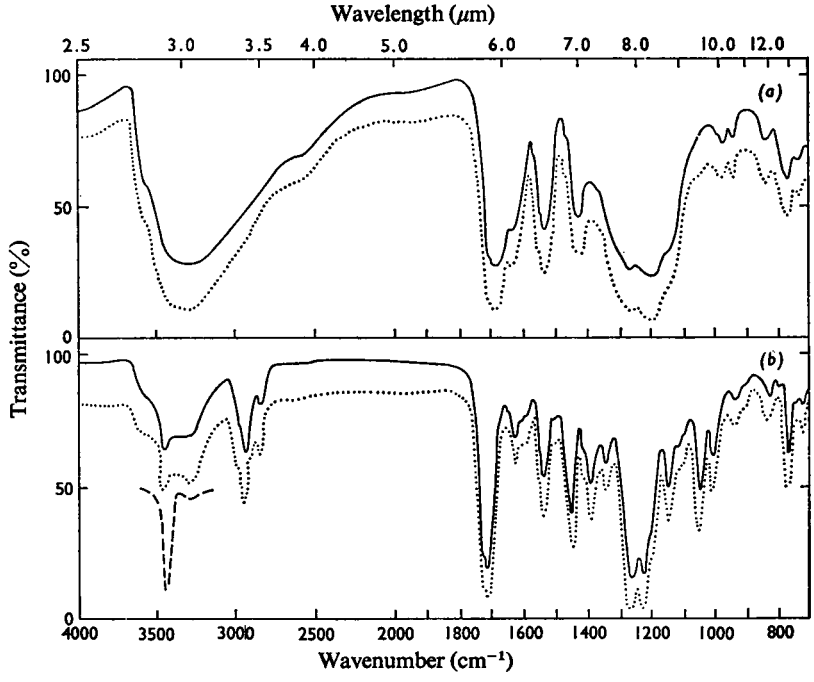


Fig. 6. *I.r.-absorption spectra of natural and synthetic pigments and their methoxy methyl esters*

(*a*) ——, Tapetal pigment of fraction B; ····, synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid. (*b*) ——, Methoxy methyl ester of the tapetal pigment; ····, methoxy methyl ester of the synthetic pigment. All the spectra were taken in KBr pellets on a Perkin–Elmer Grating IR Spectrophotometer model no. 237B. A partial spectrum (----; 3600–3100cm^{-1}) of the methoxy methyl ester of the tapetal pigment in chloroform is also included.

Table 2. *$KMnO_4$ oxidation products of pigments and reference compounds*

The pyrrole carboxylic acids obtained by $KMnO_4$ oxidation of the various samples were examined either qualitatively or quantitatively by t.l.c. on cellulose as described in the text. The intensity of spots is indicated by: ++++, very strong; +++, strong; ++, medium; +, light; ±, trace; −, nil.

	Products (yield in percentage)		
Sources	Pyrrole-2,3-dicarboxylic acid	Pyrrole-2,3,5-tricarboxylic acid	Pyrrole-2,3,5,6-tetra-carboxylic acid
Tapetal pigment of fraction A	−	++++ (6.1)	+
Tapetal pigment of fraction B	−	++++ (6.2)	+
Tapetal pigment of fraction A after heating at 200°C	+	+++ (1.9)	+
Tapetal pigment of fraction B after heating at 200°C	+	+++ (2.0)	+
Dopa melanin	+	++ (0.4)	+
5-Hydroxyindole-2-carboxylic acid	−	++++ (5.2)	+
5,6-Dihydroxyindole-2-carboxylic acid	−	++++ (5.4)	±

acid and 5,6-dihydroxyindole-2-carboxylic acid (Table 2). The yields of pyrrole-2,3,4,5-tetracarboxylic acid were quite low, and even 5-hydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole-2-carboxylic acid yielded the same pyrrole carboxylic acids.

Pyrrole-2,3-dicarboxylic acid was produced from the pigments only after heating at 200°C, also supporting the presence of the indole-2-carboxylic acid

derivatives in the pigments. Although the low yield of the dicarboxylic acid remained unexplained, it should be noted that yields of hydroxyindoles from the corresponding hydroxyindole-2-carboxylic acids by decarboxylation also are rather low (Beer *et al.*, 1948).

Interpretation of the i.r. spectra offered further evidence that the tapetal pigments consist of the indole-2-carboxylic acid derivative: a signal at 3430 cm^{-1} (in chloroform) in the spectrum of the methoxy methyl ester (Fig. 6*b*) can be assigned to an indolic NH group; the spectrum of methyl 5,6-dimethoxyindole-2-carboxylate shows the corresponding signal at 3440 cm^{-1} (in chloroform). A strong and complex absorption band at 1530 cm^{-1} is a characteristic feature of the spectra of the pigments; corresponding strong and multiple absorption bands were observed at almost the same positions in the spectra of indole-2-carboxylic acid, 5-hydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole-2-carboxylic acid.

It has long been known that 5,6-dihydroxyindole-2-carboxylic acid can be formed from tyrosine in the presence of tyrosinase (Raper, 1927; Mason, 1948). Cells of the pigment epithelium of the catfish contain both melanin and tapetal pigments (Arnott *et al.*, 1974). Further, we have observed that the albino catfish (*Corydoras*), which lacks melanin, also lacks the tapetal pigments in eyes, whereas normally pigmented *Corydoras* do contain them (S. Ito & J. A. C. Nicol, unpublished work).

These several lines of evidence suggest that 5,6-dihydroxyindole-2-carboxylic acid could be a monomer unit of the tapetal pigments. This view is also supported by the similarity between the u.v. spectra of the tapetal pigments ($\lambda_{max.}$ 330nm) and 5,6-dihydroxyindole-2-carboxylic acid ($\lambda_{max.}$ 320nm) and also between those of the methoxy methyl ester ($\lambda_{max.}$ 314nm) and methyl 5,6-dimethoxyindole-2-carboxylate ($\lambda_{max.}$ 318nm) (Fig. 5). The elemental analytical values for the pigment of fraction B agreed closely with the calculated values for oligomers formed by oxidative coupling of 5,6-dihydroxyindole-2-carboxylic acid (Table 1).

Although Piattelli *et al.* (1963) have stated that 5,6-dihydroxyindole-2-carboxylic acid can be oxidized by mushroom tyrosinase to yield a melanin, Mason & Peterson (1965) found it to be a very poor substrate. Therefore we examined the enzymic oxidation of 5,6-dihydroxyindole-2-carboxylic acid with mushroom tyrosinase. Products were formed having various degrees of oligomerization. They were purified by column chromatography on Sephadex LH-20 in methanol–dimethyl sulphoxide–1 M-formic acid (8:1:1, by vol.) as shown in Fig. 4. The methoxy methyl ester of the synthetic oligomers was also prepared for comparison with that of the tapetal pigment.

The u.v. spectra of the tapetal pigment of fraction B and its methoxy methyl ester were almost identical with those of the synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid and the synthetic methoxy methyl ester respectively (Fig. 5). Further, the i.r. spectra of the tapetal pigment of fraction B and its methoxy methyl ester coincided with those of the synthetic pigment and its methoxy methyl ester (Fig. 6). These results confirm that the tapetal pigments of the catfish are oligomers formed by oxidative coupling of 5,6-dihydroxyindole-2-carboxylic acid.

Although the degree of oligomerization is difficult to determine, the molecular weight (955) of the methoxy methyl ester obtained by vapour-pressure osmometry in chloroform indicates that the tapetal pigments are tetramers. However, the elution profiles of both the natural and synthetic methoxy methyl esters are very broad and not symmetrical (Fig. 7), indicating that the pigments are not homogeneous in degree of oligomerization. It is most likely that both the tapetal pigment of fraction B and the synthetic pigment consist mainly of trimers, tetramers and pentamers, among which tetramers are the main components in the tapetal pigment of fraction B.

Although there are numerous possible structures for the tetramers, they can be classified into three

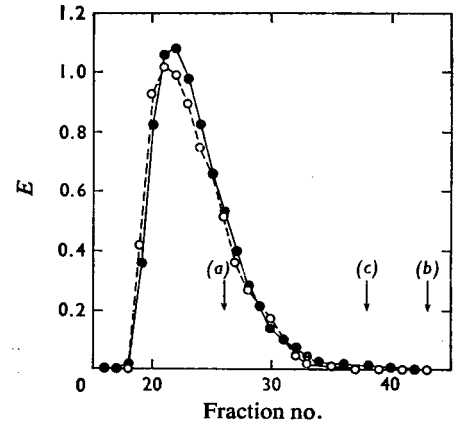
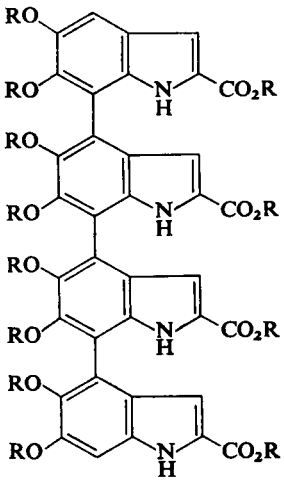


Fig. 7. *Analytical chromatography of methoxy methyl esters of natural and synthetic pigments*

A solution of the methoxy methyl ester of the tapetal pigment (0.21mg; ●) or that of the synthetic oligomers of 5,6-dihydroxyindole-2-carboxylic acid (0.19mg; ○) in chloroform (0.5ml) was applied to a column (1.45cm × 51.5cm) of Sephadex LH-20 and eluted with chloroform at a flow rate of approx. 40ml/h. Fractions (1.6ml) were collected and monitored by their E_{317} values. Recoveries of the esters were higher than 90%. The positions of elution of bilirubin (mol.wt. 585; *a*), methyl 5,6-dimethoxyindole-2-carboxylate (mol.wt. 235; *b*), and acetone (mol.wt. 58; *c*) are indicated by arrows.



Ia: R=H
Ib: R=CH_3

Fig. 8. *A plausible and representative structure for the tapetal pigment of the catfish (Ia), and for its methoxy methyl ester (Ib)*

The structure I*a* represents all the tetramers with C–C linkages between C-4 and C-7 in any combination (C-4 against C-4, C-4 against C-7, C-7 against C-7). Trimers and pentamers are also present in addition to tetramers in the tapetal pigment and oligomers having C–O linkages may also occur.

different types: those constructed by formation of C–C linkages at C-4 and C-7; those constructed by formation of C–O linkages at phenolic oxygen atoms and C-4 or C-7; and those constructed by formation of both C–C and C–O linkages in the same molecule. Formula I*a* (Fig. 8) may represent all structures of the first type. Calculated values of methoxyl contents of the methoxy methyl esters for the three above-mentioned structures are 39.8% (C–C linkages), 31.3% (C–O linkages) and 37.1 or 34.3% (C–C and C–O linkages). The methoxyl content (38.4%) of the methoxy methyl ester indicates that the structure I*b* (Fig. 8) may be the most likely one for the methoxy methyl ester of the tapetal pigment. However, co-existence of C–C and C–O linkages in the same molecule, as in valoneaic acid (Schmidt & Komarek, 1955), may also be possible.

The above discussion leads to the conclusion that the tapetal pigment of the sea catfish consists of a number of compounds derived from oxidative coupling of 5,6-dihydroxyindole-2-carboxylic acid. The monomer units of the pigments may be joined mainly by C–C linkages at C-4 and C-7. The pigments are oligomers, mainly tetramers, of closely related structures. The formula I*a* (Fig. 8) is a representative structure for the tapetal pigment.

Although some part of the pigments collected in fraction A may have been degradation products, some probably existed *in vivo*, because even the extract from exposed tapeta contained a considerable amount of faster-moving components (Fig. 1). The resemblance of the u.v. and i.r. spectra of the pigments of fraction A to those of fraction B shows that the same chromophore, 5,6-dihydroxyindole-2-carboxylic acid, is also included to a large extent in the pigment of fraction A.

Forsyth & Quesnel (1957) have examined the oxidation of catechol with mushroom tyrosinase and detected all the possible dimers, the three isomeric tetrahydroxydiphenols and diphenylene dioxide 2,3-quinone (Forsyth *et al.*, 1960). It is therefore very plausible that a variety of oligomers may also be formed by the enzymic oxidation of 5,6-dihydroxyindole-2-carboxylic acid.

Various natural products formed in micro-organisms and higher plants are considered to be derived from oxidative coupling of phenolic compounds involving C–C and C–O linkages (Scott, 1967). Whereas considerable numbers of them are formed by oxidative dimerization of single phenolic monomers, natural products with trimeric structure such as valoneaic acid (Schmidt & Komarek, 1955), pilocereine (Djerassi *et al.*, 1962) and trityrosine (Andersen, 1964, 1966) are rare. So far as we know, the tapetal pigment of the catfish is the first example in higher animals of an oligomer formed by phenolic oxidation of a single monomer.

Relationship to melanins

It may generally be said that melanins are insoluble pigments of higher molecular weight formed by the enzymic oxidation of phenols (Mason, 1959). Their u.v. and visible spectra are featureless and the absorption bands in the i.r. spectra are too broad to be well assigned (Thomson, 1962). Dopa melanins from natural sources are commonly associated with proteins by means of sulphur–carbon linkages (Piattelli *et al.*, 1963; Mason & Peterson, 1965), and therefore they cannot be obtained free from sulphur even after prolonged acid hydrolysis. It was first postulated by Raper (1927, 1938) and spectroscopically supported by Mason (1948) that dopa melanin (or tyrosine melanin) was formed by oxidative polymerization of 5,6-dihydroxyindole which had been derived from tyrosine via dopa, dopaquinone, leucodopachrome and dopachrome. Intermediates in melanin formation beyond 5,6-dihydroxyindole have been repeatedly studied (Thomson, 1962). Although some characterization of the dimers has been done by Bu'Lock (1960), their structures are yet to be determined.

The tapetal pigment is obviously different from animal melanins with respect to its physical properties such as solubilities, spectroscopic features, degree of polymerization and sulphur content. Some procedures, however, which are used to purify melanin, such as extraction in alkaline solution and hydrolysis with HCl, easily change the tapetal pigment to melanin-like material.

In addition to differences in physical properties, there is a striking difference between the structural features of the tapetal pigment and dopa melanin; the tapetal pigment does not contain units of 5,6-dihydroxyindole, but it is composed exclusively of 5,6-dihydroxyindole-2-carboxylic acid. There are two hypotheses by which the difference can be explained. First, the tapetal pigment may be formed from a tyrosine derivative having a substituted carboxyl group such as amide and ester so that decarboxylative rearrangement of dopachrome forming 5,6-dihydroxyindole can be prevented. This hypothesis would require a proteolytic cleavage. Second, there may be an enzyme which favours isomerization of dopachrome into 5,6-dihydroxyindole-2-carboxylic acid. The first hypothesis may be supported by the result by Yasunobu *et al.* (1959) that the formation of 5,6-dihydroxyindole-2-carboxylic acid derivatives was spectroscopically observed ($\lambda_{max.}$ 325nm) in the oxidation of *N*-terminal tyrosine peptides with mushroom tyrosinase.

The slow enzymic oxidation of 5,6-dihydroxyindole-2-carboxylic acid with tyrosinase is significant in two ways. First, it may be possible to isolate the dimers and analyse them, so that more detailed information on the structure of the tapetal pigment can be obtained. Secondly, as the reaction process of 5,6-dihydroxyindole-2-carboxylic acid to the oligomers may be better understood than that of 5,6-dihydroxyindole to the melanin, 5,6-dihydroxyindole-2-carboxylic acid may be a convenient model compound for studies of melanin synthesis.

Besides the sea catfish, a similar light-coloured tapetum has been found in many other catfishes (Nicol *et al.*, 1973; Arnott, *et al.*, 1974), and it is, perhaps, of general occurrence among the ubiquitous and numerous siluroid fishes. The tapetal pigment of the catfish is one of several different types of reflecting materials found in tapeta lucida of fishes. In common with several of them it has a high refractive index, it is enclosed in small spheres, and acts as an efficient back-scatterer, forming a very effective reflector (Arnott *et al.*, 1974). In the dark-adapted animal the tapetum reflects light which has already traversed the retina back into it, thus affording opportunity for further absorption and enhancing sensitivity.

We thank Dr. C. Van Baalen, Dr. P. L. Parker, and the Department of Chemistry, The University of Texas, for providing us with opportunities to use their instruments, and Miss M. Welch for her assistance. We are also indebted to Dr. H. S. Forrest for reading a draft of the manuscript. This work was supported in a large part by a grant from the National Institutes of Health, no. EY 00495.

References

Andersen, S. O. (1964) *Biochim. Biophys. Acta* **93**, 213–215
Andersen, S. O. (1966) *Acta Physiol. Scand.* **66**, *Suppl.* **263**, 1–81
Arnott, H. J., Nicol, J. A. C. & Querfeld, C. W. (1972) *Proc. Roy. Soc. Ser. B* **180**, 247–271
Arnott, H. J., Best, A. C. G., Ito, S. & Nicol, J. A. C. (1974) *Proc. Roy. Soc. Ser. B* **186**, 13–36
Barton, G. M., Evans, R. S. & Gardner, J. A. F. (1952) *Nature (London)* **170**, 249–250
Beer, R. J. S., Clarke, K., Khorana, H. G. & Robertson, A. (1948) *J. Chem. Soc. (London)* 1605–1609
Beer, R. J. S., McGrath, L., Robertson, A. & Woodier, A. B. (1949) *J. Chem. Soc. (London)* 2061–2066
Binns, F., Chapman, R. F., Robson, N. C., Swan, G. A. & Waggott, A. (1970) *J. Chem. Soc. C* 1128–1134
Bu'Lock, J. D. (1960) *Arch. Biochem. Biophys.* **71**, 189–193
Burke, W. J., Potter, A. D. & Parkhurst, R. M. (1960) *Anal. Chem.* **32**, 727–728
Djerassi, C., Brewer, H. W., Clarke, L. & Durham, L. J. (1962) *J. Amer. Chem. Soc.* **84**, 3210–3212
Dukler, S., Wilchek, M. & Lavie, D. (1971) *Tetrahedron* **27**, 607–614
Forsyth, W. G. C. & Quesnel, V. C. (1957) *Biochim. Biophys. Acta* **25**, 155–160
Forsyth, W. G. C., Quesnel, V. C. & Roberts, J. B. (1960) *Biochim. Biophys. Acta* **37**, 322–326
Hackman, R. H. & Goldberg, M. (1971) *Anal. Biochem.* **41**, 279–285
Mason, H. S. (1948) *J. Biol. Chem.* **172**, 83–89
Mason, H. S. (1959) in *Pigment Cell Biology* (Gordon, M., ed.), pp. 563–582, Academic Press, New York and London
Mason, H. S. & Peterson, E. W. (1965) *Biochim. Biophys. Acta* **111**, 134–146
Moore, S. (1968) *J. Biol. Chem.* **243**, 6281–6283
Nicol, J. A. C. & Arnott, H. J. (1973) *Can. J. Zool.* **51**, 501–508
Nicol, J. A. C., Arnott, H. J. & Best, A. C. G. (1973) *Can. J. Zool.* **51**, 69–81
Nicolaus, R. A. (1953) *Gazz. Chim. Ital.* **83**, 239–251
Omote, Y., Fujinuma, Y., Kuo, K.-T. & Sugiyama, N. (1966) *Nippon Kagaku Zasshi* **87**, 760–762
Piattelli, M., Fattorusso, E., Magno, S. & Nicolaus, R. A. (1962) *Tetrahedron* **18**, 941–949
Piattelli, M., Fattorusso, E., Magno, S. & Nicolaus, R. A. (1963) *Tetrahedron* **19**, 2061–2072
Pirie, A. (1966) in *Aspects of Comparative Ophthalmology* (Graham-Jones, O., ed.), pp. 57–68, Pergamon Press, New York and London
Raper, H. S. (1927) *Biochem. J.* **21**, 89–96
Raper, H. S. (1938) *J. Chem. Soc. (London)* 125–130
Schmidt, O. T. & Komarek, E. (1955) *Justus Liebigs Ann. Chem.* **591**, 156–176
Scott, A. I. (1967) in *Oxidative Coupling of Phenols* (Taylor, W. I. & Battersby, A. R., eds.), pp. 95–117, Marcel Dekker, New York

Smith, I., Seakins, J. W. T. & Dayman, J. (1969) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), 3rd edn., vol. 1, p. 370, Wiley–Interscience, New York

Swan, G. A. & Waggott, A. (1970) *J. Chem. Soc. C* 1609–1618

Thomson, R. H. (1962) in *Comparative Biochemistry: A Comprehensive Treatise* (Florkin, M. & Mason, H. S., eds.), vol. 3, pp. 727–753, Academic Press, New York and London

Van Woert, M. H., Prasad, K. N. & Borg, D. C. (1967) *J. Neurochem.* **14**, 707–716

Walls, G. L. (1942) *The Vertebrate Eye and Its Adaptive Radiation*, Bull. no. 19, pp. 228–246, Cranbrook Institute of Science, Bloomfield Hills, Michigan

Wunder, W. (1925) *Z. Vergl. Physiol.* **3**, 1–66

Yasunobu, K. T., Peterson, E. W. & Mason, H. S. (1959) *J. Biol. Chem.* **234**, 3291–3295

Zyznar, E. S. & Nicol, J. A. C. (1973) *Proc. Roy. Soc. Ser. B* **184**, 15–27