Informations - Informationen - Informazioni - Notes

STUDIORUM PROGRESSUS

The Integumental Pigment of *Asellus*

By A. E. NEEDHAM and P. C. J. BRUNET*

The body-pigment of the isopod crustacean *Asellus aquaticus* Linn., usually considered to be a melanin, is known to turn red after death[1]. This colour-change also occurs after certain chemical treatments[2]. We have noticed, further, that the post-mortem red colour becomes brown once more on exposure to air, the colour-change being a reversible oxidation-reduction reaction. This change resembles the behaviour of xanthommatin[3], the best known of the 'ommochromes'—shown to be a group of phenoxazone-pigments. This resemblance has been further investigated.

Experimental.—Specimens of *Asellus* were obtained in quantity from local waters, and after washing were finely ground. The carotenoids[2] were removed by grinding with repeated changes of acetone. Material prepared in this way was used immediately for extraction of the body-pigment, which deteriorates in stored material. It was most readily extracted with methanolic HCl (methanol containing 1% HCl weight/volume); but 1% (W/V) aqueous HCl was generally used, since it permitted easier precipitation of the reduced form of the pigment, subsequently, for purposes of purification. The calcium carbonate of the exoskeleton was dissolved out in the process, neutralising some of the acid, and the pigment could therefore be obtained in a very weakly acid solution. Proteins were removed by shaking the extract with a 9:1 mixture of chloroform and amyl alcohol.

A sample of xanthommatin, kindly supplied by Prof. BUTENANDT, was available for comparison.

Results.—Chemical Properties.—The solution of the pigment, in 1% aqueous or methanolic HCl, golden in colour, turned pink on reduction with sodium dithionite, sulphur dioxide and other reducing agents, provided the pH was adjusted to the range 4–6, by dilution or partial neutralization. A fine precipitate formed, aggregating slowly to a brilliant crimson flock, and leaving a colourless supernatant. In more concentrated acid the colour remained a deep tangerine and no precipitate was formed. The crimson precipitate was resoluble in methanolic HCl and in phosphate buffers of pH 7·0 or higher. The pigment reoxidized in the process, and in alkaline phosphate it bleached rather rapidly to a pale yellow pigment, which no longer had redox properties. These properties compare closely with those of xanthommatin[3].

Some of the pigment was extracted by water alone, from homogenized material, and considerable amounts by 10% (W/V) aqueous urea, and by ethylene chlorhydrin[4].

Crystals formed, on standing, in solutions of the pigment, redissolved in methanolic HCl after precipitation

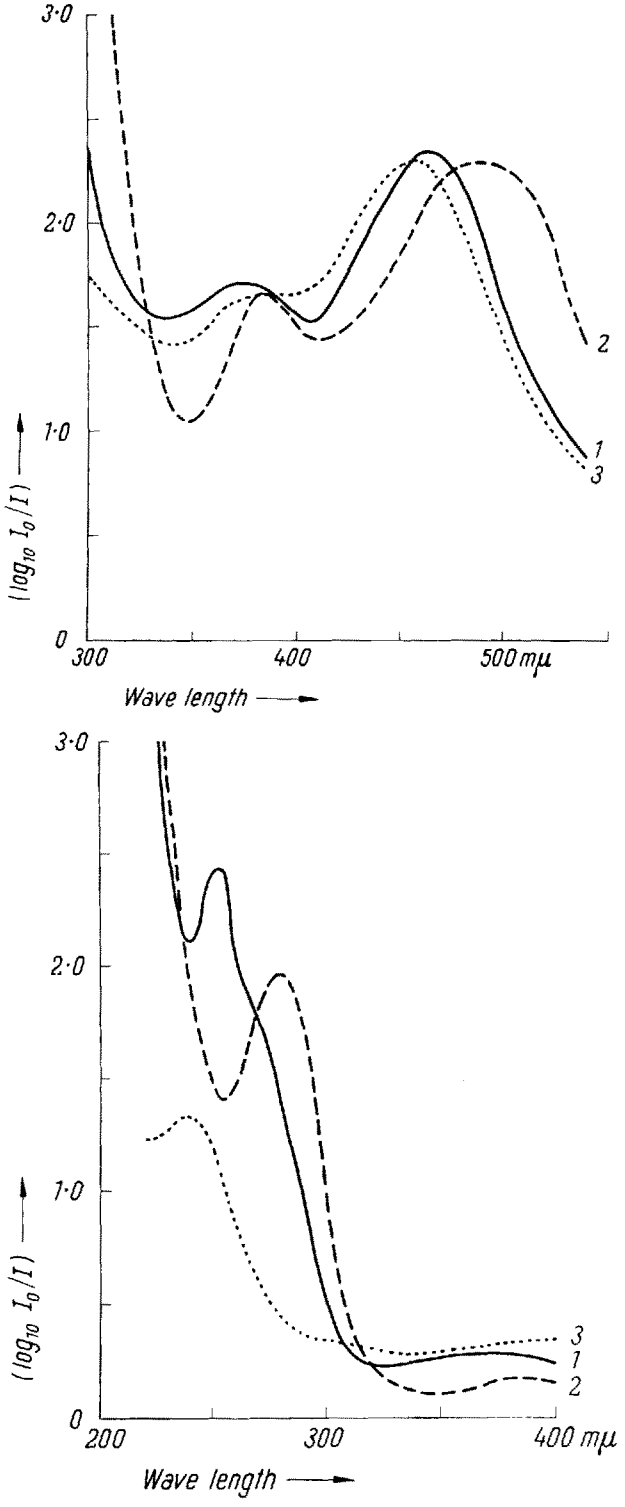

Fig. 1.—Absorption spectra of (*1*) oxidized form in N-HCl and (*2*) reduced form in amyl alcohol, of the ommochrome of *Asellus*-integument, and (*3*) of xanthommatin (BUTENANDT) in 0·1N-HCl.

A-Range: 300–500 mμ; *B*-Range: 200–400 mμ; solutions diluted 6 × with the solvent.

* Department of Zoology and Comparative Anatomy, University of Oxford.

[1] C. J. VON KAULBERSZ, Zool. Jb., Abt. allg. Zool. Physiol. *33*, 287 (1913).

[2] E. BALDWIN and R. A. BEATTY, J. exp. Biol. *18*, 136 (1941).

[3] A. BUTENANDT, U. SCHIEDT, E. BIEKERT, and P. KORNMANN, Liebigs Ann. Chem. *586*, *217* (1954a).

[4] A. J. LEA, Nature *156*, 478 (1945).

in the reduced form. These were birefringent, berry-like clusters of sherry-brown crystals, similar to those described by BUTENANDT *et al.*[3], and were resoluble on addition of more methanolic HCl.

Absorption spectra.—The absorption-spectra of the oxidized and reduced pigment, in normal aqueous HCl and in amyl alcohol respectively, are recorded in Figure 1. The oxidized form had absorption-maxima at 250, 380, and 465 mμ, and the reduced form at 280, 385, and 490 mμ. The relative magnitudes of the three peaks and their approximate positions compare with those of xanthommatin (Fig. 1)[3] and of other pigments with similar chemical properties[5]. The precise positions vary somewhat in different ommochromes[6], and they also vary with the nature of the medium.

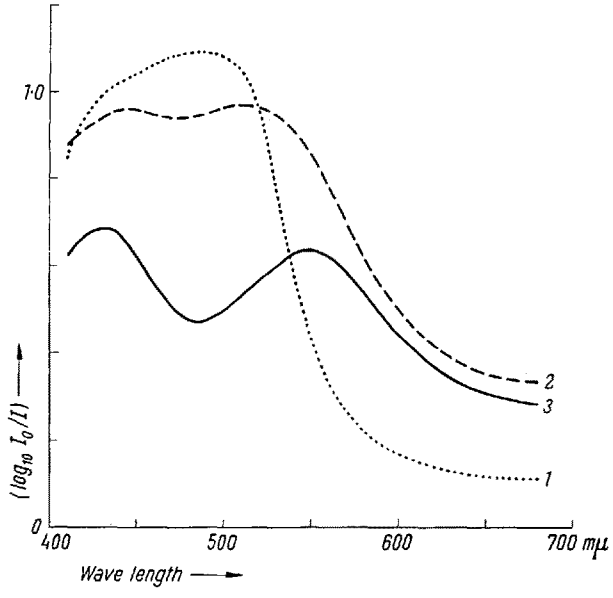

Fig. 2.—Absorption spectra, in the visible range, of *Asellus*-ommochrome in different concentrations of H_2SO_4: (1) 10%, (2) 55%, (3) 77·5%.

Thus the position of the absorption-maximum of the oxidized *Asellus*-pigment, in the visible region of the spectrum, was moved to a progressively longer wavelength by increasing concentrations of HCl, and the colour of the solution became more orange. On the contrary the presence of methanol in the solvent caused a shift of the maximum towards a shorter wave-length, and the solution became more lemon-yellow.

The pigment was also extracted by dilute sulphuric acid of 4% initial concentration. With increasing concentration of this acid the colour of the solution showed the same reddening, and shift in spectral absorption, as that in HCl. In concentrations of sulphuric acid greater than 40%, a violet colour was detectable (the 'halochromie' of BECKER[7], and this completely replaced the orange-red colour at a concentration of 70% H_2SO_4. Figure 2 shows the absorption spectra, in the visible range, of the pigment in increasing concentrations of H_2SO_4. The single peak at 465 mμ was replaced by two peaks, respectively at a shorter and at a longer wave-length[8], and these moved progressively farther apart as the concentration of acid was increased. Solutions in concentrated sulphuric acid changed, in one to two days to a dark brown colour, and the absorption spectrum then showed no peaks in the visible range.

Chromatography.—No completely satisfactory solvent-mixture for partition paper chromatography was devised. Collidine saturated with $M/2$ KH_2PO_4[3], was tried, but in it both the *Asellus*-pigment and xanthommatin faded badly. Aqueous solutions of urea, and of sodium dihydrogen phosphate, either alone, or mixed, were found to be fairly satisfactory. The best results were obtained using Whatman 3MM paper with 0·05 M NaH_2PO_4, in which the R_F value of xanthommatin was 0·23, and with a 0·05 M NaH_2PO_4 solution containing 5 M urea, in which the R_F value was 0·61. The *Asellus*-pigment moved chromatographically very like xanthommatin, and the main yellow spot of both chromatogramms was reversibly reduced by SO_2-vapour to the crimson form.

Alkaline Hydrolysis.—The *Asellus*-pigment showed the same reactions as xanthommatin when hydrolyzed with alkali. As it dissolved in N–NaOH, it was rapidly bleached but, after heating for 2 h on the water-bath, had become a deeper yellow. The solution was then acidified with HCl to pH 3, and shaken with *n*-butanol. The upper phase was washed with 0·1N–HCl, and applied, with concentration, to Whatman No. 1 paper for partition-chromatography, using as solvent the upper phase of a mixture of *n*-butanol, acetic acid and water, in ratio 4:1:5. After chromatography, three areas, fluorescent in U.V., were apparent on the paper: a streak of R_F value 0·05 to 0·35, a well marked spot, R_F 0·56, which fluoresced blue, and a larger spot, R_F 0·90. Such a pattern of spots was obtained by BUTENANDT *et al.*[9] by chromatography of hydrolyzed xanthommatin. The two faster-moving spots were common also to alkaline hydrolysates of rhodommatin and of 3-hydroxykynurenin, a precursor of the ommochromes. The spot of R_F value 0·56 was considered to represent xanthurenic acid, a degradation-product of xanthommatin and other phenoxazones[10].

Discussion.—*Xanthommatin* has been isolated from two sources[11], fully characterized and synthesized. Certain pigments in other animals[12] are very similar though possibly not identical[6]. The integumental pigment of *Asellus* clearly belongs to this group.

Acid methanol eventually extracts all the colour from the integument of *Asellus*, and there is no reason to believe that, when fresh, the solution contains significant amounts of other pigments. GOODWIN and SRISUKH[8], and SCHWINK[13], also believed that they were dealing with a single native pigment, the latter notwithstanding the variety of colour among the chromatophores of

[5] T. W. GOODWIN and S. SRISUKH, Biochem. J. *47*, 549 (1950). – L. SCHWINCK, Naturwissenschaften *40*, 365 (1953); Zool. Anz. *19*, Supplementband, 71 (1956).

[6] I. SCHWINCK, Zool. Anz. *19*, Supplementband, 71 (1956).

[7] E. BECKER, Naturwissenschaften *29*, 237 (1941).

[8] T. W. GOODWIN and S. SRISUKH, Biochem. J. *47*, 549 (1950).

[9] A. BUTENANDT, U. SCHIEDT, and E. BIEKERT, Liebigs Ann. Chem. *586*, 229 (1954b).

[10] A. BUTENANDT, U. SCHIEDT, and E. BIEKERT, Liebigs Ann. Chem. *586*, 229 (1954b). – A. BUTENANDT, U. SCHIEDT, E. BIEKERT, and R. J. T. CROMARTIE, Liebigs Ann. Chem. *590*, 75 (1954c).

[11] A. BUTENANDT, U. SCHIEDT, E. BIEKERT, and P. KORNMANN, Liebigs Ann. Chem. *586*, 217 (1954a). – A. BUTENANDT, U. SCHIEDT, and E. BIEKERT, Liebigs Ann. Chem. *586*, 229 (1954b). – A. BUTENANDT and G. NEUBERT, Z. physiol. Chem. *301*, 109 (1955).

[12] B. EPHRUSSI and J. L. HEROLD, Genetics *29*, 148 (1944). – G. WALD and G. ALLEN, J. gen. Physiol. *30*, 41 (1946). – W. K. MAAS, Genetics *33*, 177 (1948). – T. W. GOODWIN and S. SRISUKH, Biochem. J. *47*, 549 (1950). – I. SCHWINCK, Naturwissenschaften *40*, 365 (1953).

[13] I. SCHWINCK, Naturwissenschaften *40*, 365 (1953).

Sepia. However two other pigments appeared in aged extracts of the *Sepia* pigment; one was identified[6] with rhodommatin, a second ommochrome extracted by BUTENANDT *et al.*[3] from the excreta of *Vanessa*. GOODWIN and SRISUKH[8] produced a pigment with similar properties on extraction of locust tissues with N–NaOH. On standing a similar material appears also, in solutions or suspensions of both reduced and oxidized forms of the *Asellus*-pigment. This artefact is a dark red-brown substance, stable in alkali (thus unlike xanthommatin), soluble in phosphate buffers of pH 7·0 or higher, and precipitable by HCl at pH 2·0. It does not show the easily reversible redox colour-change of xanthommatin, but is oxidized to a yellow solution by such strong oxidizing agents as hydrogen peroxide and sodium nitrite[6]. A further artefact, of a purple to violet colour, was regularly produced. It became firmly bound to the 'flesh' of *Asellus* during extraction of the pigment from the chromatophores, and to filter papers during filtration. It had certain of the properties of ommatin C[3].

Although in *Asellus* there appears to be only the one native pigment, in the terrestrial isopod, *Porcellio scaber*, there is, in addition to the xanthommatin-like pigment, another which is also extracted as a yellow solution by methanolic HCl, but has no redox properties. *Porcellio* has both brown and golden chromatophores but these do not appear to correspond respectively to the two pigments extracted, since both cells are reduced to the same crimson colour, just as the pigments of the three chromatophores of *Sepia*[13]. HOWARD[14] concluded that the two differently coloured chromatophores of another woodlouse, *Armadillidium vulgare*, are due to distinct pigments, and both this and the polychromatism of *Jaera* (BOCQUET[15]) merit further investigation. The overall colour of woodlice is not entirely due to their integumentary pigment. Although the overlying exoskeleton is quite colourless, notwithstanding the statements of KAULBERSZ[6], it is probably responsible for the bluish tinge (Tyndall effect) of thick shelled forms such as *Porcellio* and *Armadillidium*. Again, the red of *Androniscus* is due mainly to a pigment in the body-fluid; hydrogen peroxide does not oxidize this pigment to a yellow colour, so that it may not be an ommochrome[6].

BECKER[7] had already given some indication of the widespread occurrence of ommochromes in the integument and eyes of arthropods, and the known range of distribution has since been considerably extended. We have found the xanthommatin-like pigment also in the integument of the marine isopod, *Ligia* and the myriapod *Julus scandinavius*.

Previously the body-pigment of *Asellus*[16] and of *Sepia*[13] and other ommochrome pigments[17] have been included among the 'melanins', golden to black pigments, occurring usually as intracellular granules, relatively insoluble in organic solvents, and in acids, capable of reducing silver nitrate, and being bleached by strong oxidizing agents[18]. Such properties could apply to substances differing widely in chemical composition, and of different metabolic origin; and, in fact, they did not distinguish between the ommochromes and the melanins of vertebrates, which have different chemical properties and are probably polymerized oxidation products of dopa[19]. Mason has given a biochemical definition of melanins which could include both: '... pigments of high molecular weight formed by enzymic oxidation of phenols.' This may be taken to include, as well as 'dopa'-melanins, pigments derived from 3-hydroxykynurenin (the ommochromes), and perhaps such pigments as the 'phaeo-melanin' of FOSTER[20]. It seems possible, indeed, that the phenolase complex which produces dopa-melanin[21] may actually catalyse the production of ommochrome pigments: for instance, melanin is deposited from solutions of dopa in developing chromatophores of *Asellus*[22], and insect-tyrosinase catalyses the production of pigment in albino woodlice[23]. Arthropods, including those which produce ommochromes, also have more inert dopa-type melanins in the blood[24], digestive fluids and elsewhere. The ink of *Sepia* also is more inert[6]. Indeed, recently BUTENANDT *et al.*[25] have shown not only that tyrosinase will catalyse the synthesis of xanthommatin, *in vitro*, but also that dopa and other intermediaries of the dopa-melanin series promote the reaction. Dopa-melanin also is formed in the reaction mixture. INAGAMI[26] suspects that the two pigments may be mixed in various proportions *in vivo*, and it is possible that such properties of natural melanins as solubility in ethylene chlorhydrin[4] may be due to ommochrome components.

Our great thanks are due to Prof. A. BUTENANDT for the gift of a sample of xanthommatin, and to Prof. H. A. KREBS and members of his staff, for facilities and help in the use of ultraviolet spectrophotometers.

Résumé

La mélanine intégumentaire du crustacé isopode, *Asellus*, est un pigment ommochrome, avec des propriétés très semblables à celles de la xanthommatine. Ce pigment existe aussi dans l'intégument de *Porcellio* et d'autres isopodes, ainsi que du myriapode *Iulus*.

[14] H. W. HOWARD, J. Genetics *51*, 259 (1952).
[15] C. BOCQUET, Arch. Zool. exp. gen. *90*, 187 (1953).
[16] E. BALDWIN and R. A. BEATTY, J. exp. Biol. *18*, 136 (1941). – A. E. NEEDHAM, Nature *164*, 177 (1949).
[17] A. BUTENANDT, U. SCHIEDT, E. BIEKERT, and P. KORNMANN, Liebigs Ann. Chem. *586*, 217 (1954a). – E. BECKER, Naturwissenschaften *29*, 237 (1941).
[18] L. LISON, *Histochimie et Cytochimie Animales* (Gauthier-Villars, Paris 1953).
[19] H. S. MASON, in *Pigment-Cell Growth* (Ed. M. GORDON, N. Y., Academic Press 1953), p. 277.
[20] M. FOSTER, J. exp. Zool. *117*, 211 (1951).
[21] H. S. MASON, Adv. Enzymol. *16*, 105 (1955).
[22] A. E. NEEDHAM, Nature *164*, 177 (1949).
[23] J. DE LATTIN, Zool. Anz. *125*, 309 (1939).
[24] K. G. PINHEY, J. exp. Biol. *7*, 19 (1930).
[25] A. BUTENANDT, E. BIEKERT, and B. LINZEN, Z. physiol. Chem. *305*, 284 (1956).
[26] K. INAGAMI, Nature *174*, 1105 (1954).