THE PIGMENTATION OF CAVERNICOLOUS ANIMALS

I. THE PIGMENTS OF SOME ISOPOD CRUSTACEA

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INTRODUCTION

The colonization of caves, and the evolution of the peculiar characteristics of most members of the cave fauna, have interested many workers. The cave environment is of a specialized nature, its most obvious characteristics being absence of light, lack of food, uniformly low temperature and, in subterranean waters, low oxygen content. Cave animals are likewise specialized and commonly exhibit loss of pigmentation, development of specialized taste and tactile organs, together with degeneration of the eyes (*vide* the accounts in Spandl (1926) and in Chappuis (1927)). We felt that a comparison of the pigments of cave animals and of nearly related above-ground epigean species might throw some light on the reduction of pigmentation so commonly met with in cave animals. We decided to examine some isopod and amphipod Crustacea in particular, since these families have many subterranean and closely related epigean species and genera, and the literature includes experimental work on the inheritance of the reduced pigmentation in a typical subterranean (hypogean) isopod, *Asellus aquaticus covernicolus* Rac., together with observations on the development of pigmentation in cavernicolous Amphipoda when they are exposed to light, and on the loss of pigmentation in epigean Amphipoda kept in darkness.

Several lines of investigation suggested themselves. In the first place it seemed desirable to study the action of light on the pigmentation of cavernicolous forms and of its absence upon that of closely related epigean species. Secondly, a survey of as many epigean forms as are available would help to determine whether any given group of species or genera normally contains any characteristic pigment or pigments. The comparative study of hypogean forms might then reveal the persistence or the loss of particular types of pigmentation. Further, it is now known that many Crustacea depend upon external sources for the development of their own carotenoid pigmentation, and a third line of approach is therefore the investigation of the sources of carotenoid material in the food of cave animals. The present paper is mainly concerned with work along the first and second of these lines.

As material we had at our disposal large numbers of *A. aquaticus*, all of which were collected from a small area on Coe Fen, Cambridge, and identified as

A. aquaticus Linn. in the strict sense of Racovitza (1919). This is the dominant species in northern Europe generally. Smaller numbers of *A. meridianus* Rac., the dominant species in southern Europe, were obtained from a stream at Vrhnika, Slovenia, and the cave-living form *A. aquaticus cavernicolus* Rac. from the Postumia Grotte, northern Italy.

Most of the work reported here is concerned with pigments of the melanin group and with carotenoid substances. It will be most convenient if these are discussed separately.

Although the term 'melanin' is familiar to biologists interested in many different branches of the subject, it is not possible to give any satisfactory definition of the term on chemical lines. The well-known work of Raper (Raper, 1928; Raper & Wormall, 1923) on melanin formation and the more recent observations of Pryor (1939) are valuable contributions to our knowledge of these pigments, but for present purposes we must be content to use the term in the sense of Verne (1926): 'On entend par mélanines des pigments apparaissant dans l'organisme des Vertébrés et des Invertébrés, dans des conditions normales ou pathologiques caractérisés par leur couleur noire ou brune, leur existence sous forme de grains, leur grande résistance à divers solvants et réactifs chimiques.' On account of their chemical inertia these substances do not lend themselves well to chemical investigation.

In the carotenoids, however, we have a group of substances which have been much studied in recent years and about the chemistry of which a great deal is becoming known. They are, moreover, pigments to whose study the elegant methods of partition between organic solvents and chromatographic adsorption can be applied with particular convenience and success.

We have in the main followed the standard procedures described by Zechmeister (1934), and Zechmeister & Cholnoky (1937). The pigments were extracted with suitable solvents, transferred to petrol ether (b.p. 50–60° C.) and submitted to partition between this solvent and 90 % methanol. After saponification the pigments present in the petrol phase were recovered in petrol ether, and a second partition against 90 % methanol was carried out. We thus obtained three main fractions, each of which was subsequently recovered in pure petrol ether. These fractions comprised (*a*) xanthophylls originally present in the free state, extracted by methanol at the first partition, (*b*) xanthophylls originally present in the esterified state, extracted by methanol at the second partition, and (*c*) carotenes, remaining throughout in the petrol ether phase. After purification and recovery in petrol ether, these fractions were further examined chromatographically, the arsenic-free magnesium oxide, of British Drug Houses, being the adsorbent most frequently employed. Our columns usually measured about 10 × 1 cm. The pigments were subsequently eluted and examined spectroscopically in suitable solvents with the aid of a Hartridge reversion spectrometer fitted with a symmetrical slit. The greatest probable error of this instrument was estimated at $\pm 2\mu\mu$ approx.

As is well known, considerable discrepancies appear between the positions of the absorption maxima as determined by different instruments, and Smith (1936) in particular points out that the best instrument for making these measurements is

a good spectrophotometer. We, however, did not have a suitable machine at our disposal, and preferred to use the less laborious Hartridge apparatus and to compare our readings with those obtained using standard specimens of known pigments prepared from other sources. The standard pigments most generally used in the present work were cryptoxanthine and zeaxanthine, prepared from maize by an adaptation of the technique of Strain (1938), and β-carotene prepared from tomato pulp by the general procedure outlined by Zechmeister (1934).

Use was also made of the technique of mixed chromatography (cf. Lederer, 1938), and our standard preparations were of great value here also. Preliminary experiments using these standard preparations were carried out and it was found that, if the chromatograms were developed by means of 2% methanol in petrol ether, the bands appeared very rapidly, were very narrow and deep in colour, and could easily be recognized by their characteristic behaviour. Thus β-carotene gave a characteristic brown, very narrow band, cryptoxanthine an orange-yellow band, somewhat more diffuse, and zeaxanthine a yellow and relatively very diffuse band. Development with petrol ether alone or with petrol ether containing a small proportion of benzene is a more usual procedure, and is, indeed, most suitable when it is desired to separate pigments from each other for subsequent spectroscopic examination, but it has the disadvantage of being a somewhat lengthy process which uses considerable amounts of solvents. Our method is economic of solvents, is rapid and very convenient, and in our experience is very reliable when standard pigments are available for comparison with the unknowns.

MELANIN IN THE HYPODERMIS OF *ASELLUS* SPP.

The hypodermal pigment of *A. aquaticus* is responsible for the yellow-brown external colour of this species and appears to be a melanin, as stated by Kosswig (personal communication). The following observations support this view. After the animal has been ground to a fine paste and all carotenoid material extracted, a purplish paste remains, and from this no further pigment can be extracted with ethanol, methanol, ether, petrol ether, benzene, chloroform, carbon disulphide, pyridine or trichloracetic acid. Nor is the hypodermal pigment extracted from intact specimens after several years' preservation in alcohol or formalin. The pigment, whether in the intact animal or in the extracted paste, is bleached by hydrogen peroxide in acid solutions, and can be extracted by treating the paste with boiling alcoholic potash to give a deep red solution. Microscopic examination shows that the pigment is present in the form of fine granules within branched chromatocytes and we are indebted to Dr Mark Pryor for informing us that these granules reduce silver nitrate. In all these respects, therefore, the hypodermal pigment of *A. aquaticus* behaves like a typical melanin (see Verne, 1926).

A. meridianus is as deeply pigmented as *A. aquaticus*, but the body colour of *A. aquaticus cavernicolus* is very variable. The hypodermal pigment of all three species is present in the form of a granular deposit enclosed within branched chromatocytes and is not dissolved by toluene, cresol or alcohol over a period of years. It is highly probable therefore that melanin is present in all three species.

EFFECT OF LIGHT ON THE MELANIN OF *ASELLUS* SPP.

If the characteristic paleness of cavernicolous forms of *Asellus* were due to a phenotypic suppression of pigmentation associated with the absence of light, we should expect to find a uniform rather than a variable degree of paleness within a given population. The inheritance of the hypodermal pigmentation has been studied by Kosswig (1935) using *A. aquaticus cavernicolus* from northern Italy. He finds that in this species the pigmentation varies in different populations from a depth of colour equal to that of normal epigean specimens of *A. aquaticus* to an almost complete lack of pigment. His breeding experiments demonstrate segregation of characters determining pigmentation; thus a considerable range of pigmentation was observed in the offspring from a cross of two almost unpigmented individuals.

The same author has also stated that light is without influence on pigmentation, and this appears to be the case from our own observations also. Five very darkly pigmented specimens of *A. aquaticus* were kept in total darkness for a period of $7\frac{1}{2}$ months, but no evident reduction in colour took place during this time. During the sixth month, moreover, one specimen was found to be bearing eggs, and at the end of the period the young derived from these eggs appeared darker than most young *A. aquaticus* of similar age, and might well have attained as deep a colour as the parent had they reached adult size. Large numbers of this species have been kept in the dark for periods of several weeks or months on a number of occasions, and in every case the young produced appeared to be normal in colour while that of the adults underwent no evident diminution. It appears therefore that the absence of light for periods of many months has no effect on the persistence of the external coloration of adult specimens, nor on the origin of the colour in specimens kept in darkness throughout their embryonic development and for the early part of their subsequent free life.

In view of these results we must conclude that it is very doubtful whether the paleness observed in many populations of *A. aquaticus cavernicolus* can be ascribed to the phenotypic effect of the darkness which characterizes the cave environment. The reduced pigmentation of the cave variety must probably be due to another factor and is more probably a permanent element in its genetic constitution (see Kosswig, 1935, 1937; Kosswig & Kosswig, 1936).

The value of Kosswig's work lies in the establishment of the occurrence of segregation of characters in *Asellus* but, as he himself states, the kind of segregation is too complex for Mendelian analysis at the present time. We have obtained evidence which suggests that an extension of Kosswig's genetical work would be of considerable value, for it was found possible to cross *A. aquaticus* with *A. aquaticus cavernicolus* with the production of live offspring. The male in the cross was an almost colourless specimen of the cave variety, and was collected from the Postumia Grotte and brought back to England in a thermos bottle with the addition of ice during the journey. The female was an English specimen of *A. aquaticus* Linn. It appeared that the possibility that the female was already fertilized was excluded for the following reasons. Eight females, including the one eventually used in the cross,

were placed in a dish in October 1938, and by the following January five specimens had died without having produced eggs. In late February two specimens were bearing eggs the development of which had progressed considerably, and the remaining two were not. Of the two remaining specimens one was used for crossing at the end of March and the other was found still to be without eggs in the middle of July. When the male and female were placed together pairing took place at once, and it may be pointed out that, according to Unwin (1919), pairing does not take place between a male *A. aquaticus* and a female of the same species if she has already been fertilized. We may therefore reasonably regard the instant pairing which took place in our experiment as some confirmation of the unfertilized state of the female. In July 1938, one young specimen, pale in colour, was found in the jar, and this died at the age of 40 days, by which time it had attained a fair degree of pigmentation.

Kosswig's theory of the origin of the lack of pigmentation in cave forms involves the spread of loss mutations in the population and may be briefly stated as follows. In the darkness of a cave colour has no apparent significance and may be regarded as of neutral survival value. Since loss mutations are more frequent than positive ones it may be assumed that random mutations for loss of colour will gradually spread through a population, unchecked by any counter-selection. In the initial stages of this process we should expect to find a variable colour in the population and in the final stages a uniform absence of pigment. Kosswig himself has observed both variable populations of *A. aquaticus cavernicolus*, as in the Postumia Grotte, and uniformly unpigmented populations, as in the Grotta di Trebiciano. Our observation that viable young can be produced by crossing the cave variety with the closely related *A. aquaticus*, which is epigean, shows that it may be possible to evaluate the characters determining colour in the epigean species by suitable genetical studies, and to state which specific colour factors have been lost in the cavernicolous form in order to gain some insight into the course of evolution of the cave type.

CAROTENOID PIGMENTS IN *ASELLUS AQUATICUS*

In order to clear the guts of extraneous carotenoid materials, which a preliminary examination of the faeces showed to be present in considerable quantities,[1] the animals were fed on grains of soluble starch, a carotenoid-free diet which was readily taken. This diet was given for at least a week, after which the guts of all specimens examined appeared to contain nothing but starch. The animals were kept in the dark during this preliminary clearance period in order to preclude algal growth, since we found that the green algae which develop in river water exposed to sunlight for several days in the laboratory are relatively rich in carotenoid substances. After this preliminary treatment, some 1500 specimens of *A. aquaticus*, weighing 48 g., were extracted with successive small quantities, first of methanol and then of petrol ether. The pulverized residues were dark purplish red in colour (see p. 138), and this

[1] Chlorophylls *a* and *b*, at least three xanthophylls and a carotene could be detected by use of the micro-procedure of Kuhn & Brockmann (1932).

coloration is apparently due to a melanin. Kaulbersz (1913) had already noticed that this animal develops a pink colour after death, and we have examined a number of recently dead, pink specimens separately and found that the nature of their carotenoids is the same as that of fresh specimens but that the reticulated melanin deposits appeared to have disintegrated. The reddish colour is evidently due to melanin rather than to the production of any new carotenoid.

The extracts were combined and the pigments quantitatively transferred to petrol ether by the addition of water. The resulting deep yellow solution of carotenoid pigments in petrol ether was submitted to partition against 90% methanol. The hypophasic layer contained very little pigment indeed; after transference to dichloroethane and adsorption on magnesium oxide two very small bands were obtained, indicating (probably) the presence of traces of free xanthophylls in the original material. This fraction was too small for further study.

The main epiphasic fraction was saponified in the usual way and, after recovery in petrol ether, a second partition against 90% methanol was carried out. The pigments remained entirely epiphasic, however. Generally speaking, such a result would be taken as showing that no xanthophyll esters were present in the original material since, after saponification, the xanthophylls would pass into the alcoholic layer at this second partition. As a rule such a conclusion is perhaps justified, since the most commonly occurring xanthophylls are those containing at least two alcoholic groups and these are wholly hypophasic in the partition test. But the majority of workers appear to have neglected the fact that monohydric xanthophylls such as rubixanthine and cryptoxanthine are wholly epiphasic under these conditions and thus resemble the esterified xanthophylls and the carotenes proper. They may, however, be distinguished from the carotenes by the fact that they are in part hypophasic with respect to 95% methanol; thus Zechmeister (1934) has written of cryptoxanthine and β-carotene: 'bei der Verteilung zwischen Benzin und 90 proz Methylalkohol suchen beide Farbstoffe die Oberschicht auf; verwendet man jedoch 95 proz Methanol, so wandert das Kryptoxanthin deutlich nach unten. (Abweichung von β-carotene.)' In the present case a part of the saponified pigment passed into 95% methanol from petrol ether, indicating the presence of a monohydric xanthophyll or of some material behaving in the same manner.

The pigments of the main saponified epiphasic fraction were accordingly separated chromatographically on magnesium oxide, the column yielding three well-defined bands on development with pure petrol ether; these were (1) an orange region 15 mm. deep at the top of the column, (2) a very pale yellow band 3 mm. deep, and (3) a second orange region 10 mm. deep below. These were eluted and examined further.

Band 3. The pigment of this band was wholly epiphasic to 90% and to 95% methanol and therefore appeared to be a carotene. The spectroscopic data of Table 1 were obtained. Rechromatographed on magnesium oxide a single brownish band was obtained; a precisely similar band was obtained with our standard sample of β-carotene and another with a mixture of this with the *Asellus* sample. It may therefore be concluded that band 3 corresponds to β-carotene.

Table 1. *Values for β-carotene*

(All values in μμ)

Solvent	Maxima observed	Kuhn & Brockmann (1932)	Smith (1936)	Standard sample
Petrol ether	452; 486	452; 484		452; 485
Carbon disulphide	486; 516		486; 517	

Band 2. The pigment of this band was wholly epiphasic to 90% and 95% methanol but too small in amount for further examination.

Band 1. The pigment of the uppermost band was almost wholly epiphasic to 90% methanol but a part passed readily into 95% methanol. This behaviour strongly suggested that we had here to deal with a monohydric xanthophyll. The epi- and the hypophasic portions gave the same absorption maxima, indicating that we were in all probability concerned with a single substance rather than with a mixture of two substances which happened to behave in a similar manner on the column. Rechromatographed on magnesium oxide and separated from a faint band which appeared below it (? neocryptoxanthine—cf. Zechmeister & Tuzson, 1938) the pigment again gave the same absorption maxima, which agreed well with those of cryptoxanthine.

Since, however, the maxima for cryptoxanthine, and β-carotene are very similar, and since the differences in the phase test alone are not very convincing evidence, we compared the behaviour of our material on a magnesia column with that of standard samples of cryptoxanthine, zeaxanthine and β-carotene. The *Asellus* material gave a band which differed sharply from those of zeaxanthine and β-carotene but was indistinguishable from that given by cryptoxanthine. Mixed chromatograms were also prepared and whereas mixtures of the *Asellus* pigment with β-carotene and zeaxanthine gave rise to a pair of bands in each case, mixtures with cryptoxanthine gave a single band only.

Table 2. *Values for cryptoxanthine*

(All values in μμ)

Solvent	Maxima observed	Kuhn & Grundmann (1933)	Standard sample
Petrol ether	451; 485	452; 485·5	451; 485·5
Carbon disulphide + trace ethanol	482; 518	483; 519	486; 516·5
Chloroform	463; 497	463; 497	
Ethanol	454; 487	452; 486	

Finally, the remainder of the *Asellus* pigment was dissolved in methanol and freed from a quantity of white, crystalline material by the addition of enough water to bring the concentration of methanol to 85%. After removal of the precipitate by centrifugation, the pigment was transferred to petrol ether and re-extracted with successive small quantities of 95% methanol until the petrol layer was nearly colourless. The relatively pure pigment present in the alcoholic extracts now gave

the spectroscopic data of Table 2.[1] It may therefore be concluded that band 1 corresponds to cryptoxanthine.

It is still not clear, however, whether this pigment, which so far as we know has not hitherto been reported from animal materials, was originally present in the free or in the esterified condition, since the behaviour of its esters in the phase test and on adsorption columns has not been investigated. It is likely that esters would resemble the free xanthophyll rather closely.

SUMMARY

1. The widespread reduction of pigmentation found in cavernicolous animals is discussed.

2. The externally visible coloration of *Asellus aquaticus* Linn., *A. meridianus* Rac., and *A. aquaticus cavernicolus* Rac., appears to be of the melanin type.

3. The melanin content of *A. aquaticus* is not appreciably affected if this animal is kept in the dark for periods of several months. Offspring produced during this period are normal in colour.

4. Fertile young resulted from a cross between a colourless female *A. aquaticus cavernicolus* and a normally pigmented male *A. aquaticus*. This observation is probably of significance for the genetical analysis of the colour types found among the hypogean asellids.

5. The predominant pigments of *A. aquaticus*, apart from melanin, are β-carotene and cryptoxanthine. Other carotenoid pigments are present only in traces.

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[1] In ethereal solution our product gave no blue coloration with concentrated hydrochloric acid.