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THE COLOURS OF THE PLUMOSE ANEMONE *METRIDIUM SENILE* (L.)

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The anemone *Metridium senile* occurs in a number of striking colour varieties. These are to be found side by side in nature. The colours are inherited at least during asexual reproduction and do not depend upon special food. The colour varieties are founded on three systems of pigment, black granular melanin in the endoderm, brown diffuse melanin in the ectoderm and red to orange lipochrome in fat droplets in both endoderm and ectoderm. In any variety each system may be present or absent. If all are absent we have a white form, if one is present we get red, brown or grey forms, if two or more are present we get other varieties. In combinations each pigment retains the distribution it has in the corresponding simple form.

The black endodermal pigment is a true melanin. The brown ectodermal pigment is also a melanin but is less stable. Even the white variety can be shown to possess a complete tyrosinase system when the tissues are finely ground. It is perhaps comparable to a case of dominant albinism.

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The red and orange colours are due to a variety of lipochromes. The pigment of the red variety is chiefly the acidic carotenoid described by Heilbron, Jackson and Jones. It is related to astacene and we have named it 'metridene'. Yellow-orange anemones of the red series may contain considerable quantities of xanthophylls in addition to metridene esters. The only pigment of the white form is contained in the gonads and appears to be esters of astacene.

Many of the carotenoids are present in too small quantities to influence the appearance of the animal. The assortment of carotenoids present varies considerably from one lot of *Metridium* to another. There is evidence that the occurrence of a variety of xanthophylls and carotene is more common in varieties which develop melanin. But there is no obvious correlation between the presence of a particular assortment of carotenoids and a particular distribution of melanin pigments. Flavines are not present in any of the colour varieties.

Coloured purine compounds are not present. The presence of significant quantities of uric acid was demonstrated and of this purine alone. It is present in the mesenteric filaments, in the yellowish excreted mucus, and granules of uric acid form white bands in the endoderm of the tentacles. In this position they contribute their whiteness to the total colour effect. Haematins do not contribute to the colour. Those that are present are derived from the ordinary intracellular respiratory systems.

The colours of the varieties are not an adaptation to the external environment as in warning coloration etc. nor is there evidence as yet that the colour as such is of direct physiological importance to the animal. The varieties appear to illustrate Poulton's category of 'non-significant colours', in which colour is a by-product of biochemical processes utilized for other purposes.

The vivid colours of the sea anemones and their allies have always been placed among the wonders of nature, but the question why it is these animals possess them remains unanswered. From time to time during the last 60 years various pigments have been isolated from anemones, but the metabolic origin and function of the pigments remain obscure. Not only are the colours often intense, but they vary so greatly within the same species that even anemones living side by side may show a complete contrast of pigmentation. This variation is as important as the fact of pigmentation itself if we are to consider the function of the pigments. In order, therefore, to attack this problem we chose a species *Metridium senile* (L.) in which colour variations are striking and of many kinds. Its varieties depend not so much on complex patterns as upon large washes of a few pigments over well-defined regions of the body. This simplifies both description and biochemical analysis.

The colour varieties of *Metridium senile*

The animals were collected from Hunstanton, Lowestoft, Southwold and Tollesbury in East Anglia, and from Plymouth and Millport on Clyde. There was no essential difference in the colour varieties obtained from these places. Apart from its colour variation, *M. senile* shows some indications of varieties which differ slightly in size and in structure (Stephenson 1935). The majority, perhaps all, of our animals were the variety *dianthus*. Some of the smaller ones showed grey lines over the mesenteries near the tentacles as in the variety *pallidum*, but there were numerous intermediates.

Moreover, our specimens were generally over 2 cm. across and possessed the lobed capitulum typical of *dianthus*. All varieties of *Metridium* show similar colour variations (Stephenson 1935; Rawlinson 1934).

Gosse (1860) distinguished four main colour varieties, white, red, brown and a lemon yellow form described by Dalyell. Despite Gosse's wide experience he never saw the last-named variety, and this has been the experience of many naturalists with whom we have corresponded. Walton (1908) failed to find it in the North Sea, but mentions that it is abundant in places on the North Wales coast. We shall presently show that individuals occasionally contain xanthophylls in sufficient quantity to affect the colour, and though we have not yet found specimens in which the colour of the xanthophyll is unobscured by other pigments, such may perhaps occur.

Stephenson remarks that all varieties, including those of colour, are connected by innumerable intermediates. Colour may vary from a faint tint in an otherwise white specimen to a brilliant hue. The apparent colour depends on the state of expansion of the animal. A light brown form on expansion becomes both paler and yellower, though never lemon-yellow.

Though gradation of the colours makes classification of varieties difficult, the colour varieties stand in some definite relationship to each other. Stephenson detects three series; one centring about white, one about orange, and one of various shades of brown. Our analysis was aided by examination of paraffin sections and of frozen sections of material freshly fixed in 5 % formalin. The animals were anaesthetized with 50 % 0·4 M $MgCl_2$ + 50 % sea water before fixation. The following main types were found.

(1) *White*. The animal is most commonly pure white. It may be pale cream or there may be faint traces of the colours of other varieties. The stomodaeal ridges are sometimes very pale brown (Stephenson 1935, plate 16·1), but the siphonoglyph is dead white. The whiteness of the body is enhanced by the refractile cnidae, and locally by white endodermal granules. These granules are present in all colour varieties, and form a transverse band across the tentacles. When these are contracted in the white or the lightly pigmented varieties the whole oral disk becomes dead white owing to the closing together of the granules. Discussion of the chemical nature of the granules will be reserved till later, but they appear to be uric acid. They resemble those found in the reticular tract of the mesenteric filaments and in the stomodaeum, but they are smaller and the endodermal cells which contain them are more diffuse.

The granules in the mesenteric filaments do not affect the appearance of the animal even in the white specimens, but developing gonads in the mesenteries may give a pink tint through the translucent body wall.

(2) *Simple red*. All the tissues, both ectoderm and endoderm, partake of an orange, red, or salmon pink hue. The intensity varies enormously. The hue varies to an extent which suggests that several pigments in varying proportions may sometimes be present. Just occasionally a family of 'simple red' anemones is found in which the

orange colour is nearer yellow than red. But in the majority of reds, the colour is near no. 81 (orange red) of Klincksieck and Valette's *Code des couleurs* (1908). The colour is rather concentrated in the scapus and intensely so in the ridges of the stomodaeum. It continues down into the mesenteric filaments. The rim of the siphonoglyph is intensely red, but its inner portion is much paler and may be white. The relative colouring of the parts is not always the same. Occasionally an anemone will have an unusually pale disk and tentacles and a very red scapus; and sometimes these intensities will be reversed. But these variants are less frequent than a fairly even colouring (Stephenson 1935, plate 16·2).

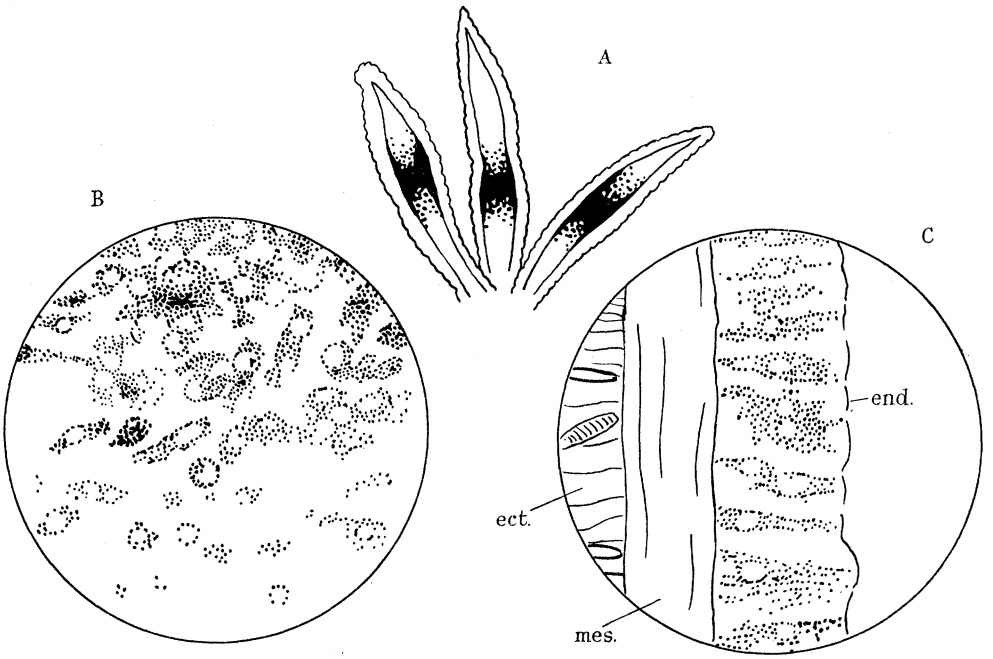

FIGURE 1. Uric acid granules in the endoderm of the tentacles of *Metridium*. *A*. Showing the transverse band (shown black) of cells laden with granules in three tentacles. Length of tentacles 3 mm. *B*. Granule-laden cells of the endoderm viewed horizontally from within the tentacle surface. Diameter of circle 100 μ. *C*. Optical longitudinal section of tentacle showing granule-laden endodermal cells. Diameter of circle 70 μ. *ect.* = ectoderm; *end.* = endoderm; *mes.* = mesogloea.

The pigment is evenly distributed through most of the endoderm. In the ectoderm it is denser, but confined to the base of the cells near the mesogloea. It is similarly localized in the stomodaeum, being particularly rich at the base of the stomodaeal ridges corresponding to the complete mesenteries.

When fresh, the pigment consists of clear orange droplets the largest of which reach a diameter of 3–4 μ. Their distribution corresponds exactly with that of fat in the cells as shown by frozen sections stained with sudan IV. They fade slowly after fixation in formalin and disappear completely with alcoholic dehydration and mounting in balsam. They behave like fat droplets containing lipochromes.

(3) *Simple brown.* The 'brown' series covers a number of complex varieties, of which this is the simplest. It is similar to the white variety except that the ectoderm is suffused with brown (*Code des couleurs* 157–182). The pigment is evenly distributed through the thickness of the ectoderm. It appears diffuse under the highest magnification, though with strongly coloured specimens brown granules are also to be seen against the homogeneous background near the outer border of the ectoderm (figure 3). The brown pigment withstands fixation in formalin and mounting in balsam. It is very stable and appears to be related to melanin.

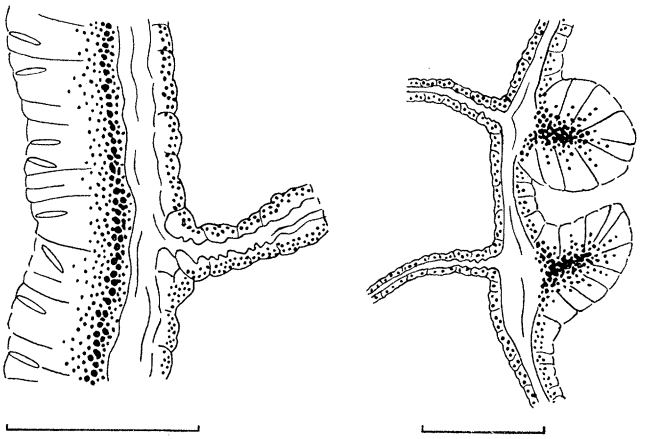

FIGURE 2. Left: Transverse section of body wall of simple red *Metridium* showing base of a mesentery. Ectoderm contains nematocysts and red fat droplets containing carotenoid at base of cells. Endoderm contains red fat droplets evenly distributed or nearer free surface of cells. Right: Transverse section of stomodaeal wall of simple red *Metridium* showing red fat droplets in endoderm. Note aggregation of red droplets at base of stomodaeal ridges opposite insertion of mesenteries. Bars = 100 μ each.

The colour may be restricted to the scapus except for a faint tint in the stomodaeum. The siphonoglyph remains dead white. The colour may extend to the capitulum, to the tentacles, or to the pedal disk as well. These parts may differ in intensity. The most striking form of this variety is that in which a brown scapus is sharply demarcated from a white capitulum and tentacles with a white or faintly brown stomodaeum and white siphonoglyph.

(4) *Brown with grey.* The scapus is olive brown at the base, acquiring a smoky tint as the parapet is approached (ranging round *Code des couleurs* nos. 187–198, orange yellow diluted with black). The brown colour decreases greatly or disappears at the parapet. A grey colour begins at the base of the capitulum rising in intensity towards the base of the tentacles. The tentacles may be almost black at the base, with the usual white band in the middle, and may be light brown distally. The disk is grey and the stomodaeum pale grey or brownish grey, and the siphonoglyph dead white.

The colour of this variety is due to two distinct pigmentations. The brown component is precisely the same as that of the simple brown. The hue is the same and it is

confined to the ectoderm in the same way. The apparent difference in tone of the animal seen as a whole is due to the existence in the endoderm of dark granules of melanin up to 1 μ across. Highly magnified, the larger granules appear black and the smaller ones various shades of brown (figure 3). In the column region these granules alter the tone due to the overlying ectodermal brown to a dusky hue. In the capitulum and tentacles, the relative absence of brown and the intensity of melanic deposition gives the grey and black appearance. This variety corresponds in fact to the superposition of two varieties, a simple brown and a simple grey described next.

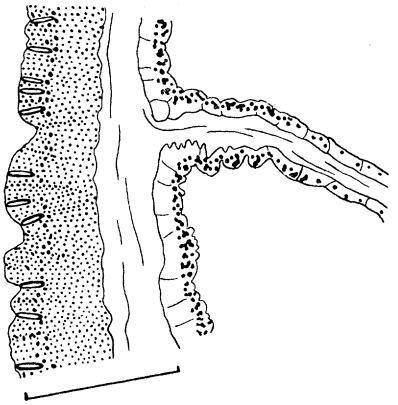

FIGURE 3. Transverse section of wall of brown with grey *Metridium* showing base of a mesentery (right). Ectoderm contains nematocysts and diffuse brown pigment represented by stippling. Endoderm contains black granular melanin especially at base of mesentery. Bar = 100 μ.

(5) *Simple grey*. This form is rarely well developed, but is fairly often seen in 'dirty whites'. In contraction it resembles a white except for a greenish grey hue. When expanded, however, it is seen that there is a diffuse distribution of grey pigment within the animal, rising in intensity in the capitulum to near the bases of the tentacles. The disk is pale grey. The stomodaeum is usually pale brownish grey with dead white siphonoglyph. The grey is due to melanin granules in the endoderm. The whole appearance corresponds to the brown-with-grey variety with the ectodermal brown left out. Indeed there is often some trace of brown, in the column or the tips of the tentacles or in the stomodaeum. But in a few cases no pigment other than endodermal melanin is present. In all varieties which contain it the endodermal melanin is most intense along the upper parts of mesenteries near their insertion on the body wall. In small animals these dark insertions show through the body as grey stripes like those ascribed to the variety *pallida*.

(6) *Red with grey*. 'Simple grey' is very occasionally superimposed on 'simple red'; endodermal melanin superimposed on ectodermal red with no other pigment. The resulting variety is a very handsome animal with a decidedly red column and a striking grey flush over the red tentacles, capitulum and disk.

(7) *Red with brown*. This is the commonest form of the brown variety. The brown scapus ranges round *Code des couleurs* nos. 107–132 (orange diluted with grey), but

the tint varies enormously with the proportion of red to brown in the tissues. The capitulum, disk and tentacles are brown, usually lighter than the scapus. The stomodaeum is almost always a deep red orange or orange brown. This variety seems to be a simple combination of the red and brown varieties. The ectoderm contains an even deposition of brown pigment together with red globules in their usual situation at the base of the ectoderm cells. The endoderm contains evenly distributed red globules only.

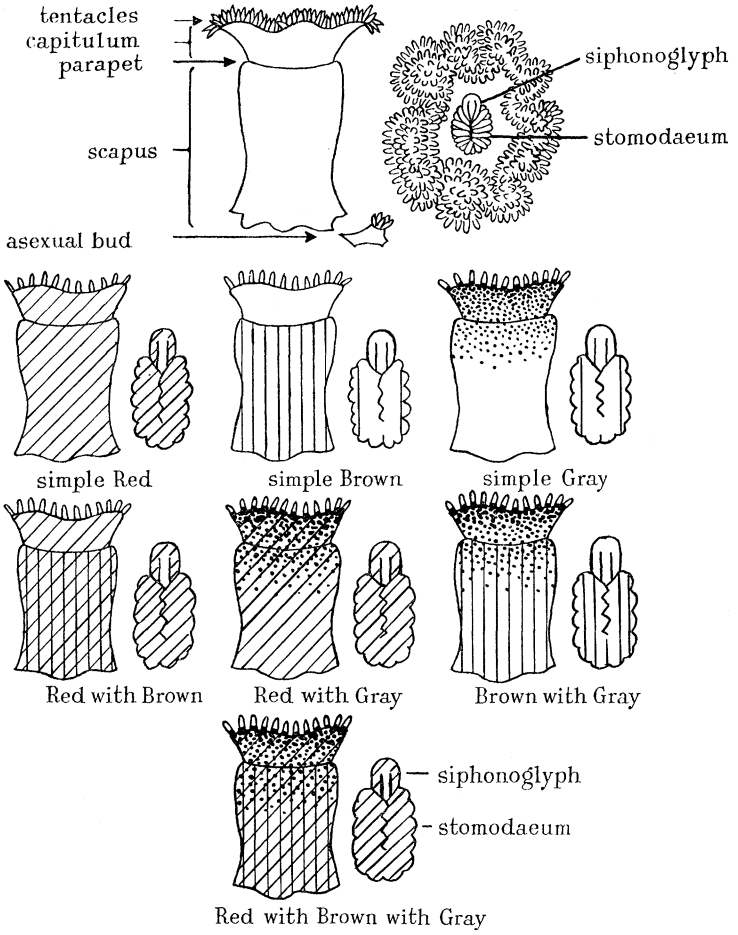

FIGURE 4. Chart showing principal colour varieties of *M. senile*. The diffuse black melanic pigmentation of the grey forms is diagrammatically represented by stippled dots. The brown melanic pigmentation is represented by vertical lines. The red lipochrome is represented by oblique lines.

(8) *Red with brown with grey*. This not very common variety is very striking in appearance. The scapus is usually a deep chocolate brown near *Code des couleurs* 108 (orange diluted with much black) or even 83 (orange red diluted with much black). The capitulum disk and tentacles are brownish grey or even black. The stomodaeum is often a rich orange. The distribution of the pigments corresponds to a triple superposition of red on ectodermal brown and endodermal black, each pigment occurring

as in the simple colour type. There is, however, some evidence that where the red pigment develops best the brown pigment develops least, as in the stomodaeum.

These colour types are connected by innumerable intermediates in which the intensity of each pigment and its extent over the body vary. Nevertheless, in all these it appears that the pattern is founded on the presence or absence of three systems of pigment, red to orange lipochrome, brown melanic pigment in the ectoderm, and black granular melanin in the endoderm. If all are absent we have the white form. If only one is present we get red, brown or grey forms. If two or more are present we get the other varieties. In combinations, each pigment retains the distribution it has in the corresponding simple form.

These facts suggest that we have here a genetical phenomenon in which the presence or absence of each pigment is an independent heritable quality. There is no doubt that the colour of a variety is inherited, at least during the common asexual reproduction, and that under natural conditions it is not due to accidental environmental influences such as the consumption of a particular food. *Metridium* appears to feed on coarse plankton (Stephenson 1935). The different colour varieties occur side by side in identical situations, and all varieties accept the same kinds of animal food when it is offered to them. Moreover, Gosse (1860) himself was struck by the occurrence of the animal in asexually produced colonies in each of which the animals maintain the characteristic colour. The constancy of the colour both in the parent and in the asexually produced young can be seen in the aquarium under a variety of conditions of feeding and starvation.

Proportions of the varieties

It is not possible to estimate numerically the proportions of the colour varieties in any locality. The proportions vary greatly at different points because of the overwhelming effect of asexual reproduction. Over a large area, however, it is evident that certain varieties are very much commoner than others so that rough estimates of their relative abundance can be made. Table 1 shows the proportions at several British localities. The various brown varieties are for convenience divided among light brown and dark brown only. We wish to thank Dr R. Elmhirst of Millport, Dr H. O. Bull of Cullercoats, and Professor F. W. R. Brambell of Bangor for valuable information concerning the local distribution of *Metridium*.

The local variation is great, but in general there is a striking preponderance of the white variety and next to it of the red. The light brown varieties together are not on the whole common, while the dark browns are decidedly rare. It is, however, noticeable that between tidemarks in some localities there is often a predominance of reds. These are often small individuals found on piles together with *Mytilus edulis*, and may correspond to the variety described by Rawlinson (1934) from rather similar situations.

If the colour varieties are of genetical origin the ascendancy of the white variety is remarkable. It suggests that this may correspond to a dominant rather than to a recessive white.

TABLE 1

	Plymouth deep water	Plymouth inshore	Southwold old pier (mussel-beds)	Southwold harbour	Lowestoft	Millport below low water	Millport inshore	Bangor	Cullercoats deep water	Cullercoats inshore
White	+++	+++	++	+++	+++	+++	⊕	+++	+++	⊕
Red	++	+	+++	++	++	+	+++	⊕	++	+++
Light brown	++	++	+	+	+	+	0	+	⊕	⊕
Dark brown	⊕	0?	0	⊕	+			0	0 to ⊕	
Lemon yellow	Not recorded from any station									

Abundant +++ Common ++ Moderate numbers + Rare ⊕ Absent 0

THE NATURE OF THE PIGMENTS

We will now consider the relation of the colour varieties to the pigments which they contain. A number of pigments have been described from Actinians, but the literature is scattered and confused, chiefly because of the changes in specific nomenclature. We adhere to the nomenclature of Stephenson's monograph on *British sea anemones* (1928, 1935), and place any synonym used by other authors after the correct name. It is convenient to classify the pigments in the following groups:

(1) Miscellaneous pigments, often imperfectly described.
(2) The haematin series.
(3) Purines.
(4) Melanins.
(5) Carotenoids.

Miscellaneous pigments

A number of pigments about which little is known have been obtained from anemones. One of these has been recorded from a number of species. Moseley (1873) found a pigment which he called 'Actiniochrome' in the red tips of the tentacles of *Tealia felina* (*Bunodes crassicornis*), though not in the red body of the animal. It was insoluble in all the solvents he used. He did not find it in *Actinia equina* (*A. mesembryanthemum*) even in the variety *rosea*. MacMunn (1885) confirmed this and found that the same pigment, which he showed to be slightly soluble in glycerine, occurred in the tentacles of several other anemones, notably *Anthopleura ballii* (*Bunodes ballii*) and the violet tentacle tips of *Anemonia sulcata* (*Anthea cereus*). Fulton (1922) concludes that it also occurs in *Actinia bermudensis* and *Condylactis passiflora*. But he bases his con-

clusion on its similarity of chemical behaviour to that of a pigment clearly described by MacMunn as actiniohaematin and not actiniochrome as Fulton supposed. Despite its wide distribution in anemones, actiniochrome is not found in *Metridium senile*.

Symbiotic algae are common among Actinozoa. Some recorded pigments originate in these plants rather than in the animal host (MacMunn 1887), as in the 'Antheagrün' of Krukenberg (1881) obtained from *Anemonia sulcata* (*Anthea cereus*). *Metridium* possesses no algae.

The haematin series

The comprehensive work of MacMunn (1885, 1890) showed that many Actiniarians possessed haematins and their derivatives. The occurrence of these well-known substances in such simple animals made a great impression upon him. He showed that in many colour variants of *Actinia equina* (*A. mesembryanthemum*), in *Tealia felina* (*Bunodes crassicornis*) and in other species, there existed a pigment which he called actiniohaematin. This pigment yielded a haemochromogen and a haematoporphyrin indistinguishable from those of haemoglobin. Because of this, and because of the capacity of one of the decomposition products of actiniohaematin to undergo reversible oxidation and reduction, MacMunn concluded that the new pigment possessed a respiratory function.

Roche (1932, 1936) brings strong evidence that actiniohaematin is not in fact a pure substance, but a mixture derived from the intracellular respiratory enzyme systems such as occur in all animals. He relates it to the cytochromes and to Keilin's 'free intracellular haematins'. He shows that the spectrum corresponds to a mixture mainly of cytochrome (*b*) and of parahaematin similar to Keilin's protohaematin. It is much stronger in the muscles than in other tissues, and it is best developed in those species of Actiniarians which have the best developed musculature, such as *Tealia felina*, *Hormathia coronata* (*Bunodes coronata*), and *Cereus pedunculatus* (*Heliactis bellis*), while it is more feebly developed in *Anemonia sulcata*, *Actinia equina*, *Adamsia palliata* and in the Ceriantharian, *Cerianthus membranaceus*, which have a weaker musculature. This agrees with MacMunn's (1890) observations that in *Calliactis* (*Sagartia*) *parasitica* actiniohaematin is situated in the endoderm, which contains most of the muscle. MacMunn showed that even the white variety of *Metridium senile* (*Sagartia dianthus*) possessed actiniohaematin. The pigment is therefore of no importance for coloration in spite of its great functional significance.

MacMunn found various derivatives of haematin occurring naturally in Actinians. He found biliverdin beneath the ectoderm in *Actinia equina* (*A. mesembryanthemum*) and in the green parts of *Tealia felina* (*Bunodes crassicornis*). This is not to be confused with the ectodermal green of the green variety of *Actinia equina*. Moseley (1877) had recorded a pigment 'polyperythrin' from a number of Madreporarian corals, a *Discosoma* sp., an *Actinia* sp., and also from a *Cassiopeia* and a *Rhizostoma* among the Scyphozoa. This pigment was shown by MacMunn (1886) in Moseley's own specimens

to be a haematoporphyrin. Such pigments have not been recorded from *Metridium senile*.

*Purines**

Although Chrometzka (1937) points to the wide distribution in nature of complex purines such as the pterines, they have not been recorded from Coelenterates. Strictly, the simpler purines are not pigments since they are white in pure crystalline form. But we have seen that white granules in the tentacles contribute to the colour effect in *Metridium*, and this is our problem. We have mentioned that these granules resemble those of the mesenteric filaments. Their general properties indicate that they are purines. Both sets of granules blacken on exposure to hydroquinone after treatment of washed tissue with 1 % silver nitrate in 20 % ammonium hydroxide (Klein 1928). The solubility of the granules is the same as that of purines. Experiments were performed in which pieces of tentacle and mesentery fixed with formalin were exposed to various reagents side by side with minute quantities of uric acid and of xanthine. The behaviour was in all cases the same except that the granules dissolved more rapidly than did the free substances, probably owing to their very small quantity and size.

The granules are completely insoluble for an indefinite period in alcohol, ether, acetone and chloroform. They are insoluble in picric acid and very slowly soluble in glycerine. In distilled water they dissolve rather slowly. In 5 % hydrochloric, acetic, lactic and citric acids the granules were at first insoluble, but dissolved after about 15 min. in great excess of the solvent. The granules dissolve in half a minute in 5 % piperazine and in 2 % ammonia. They dissolve slowly in excess of 5 % ammonium carbonate. They are insoluble in 2 % ammonium hydroxide when saturated with ammonium chloride. They dissolve instantly in 5 % potash. None of these reactions is specific, but together they support the view that the granules are purines.

Mouchet (1929*a*, 1929*b*), working on the granules in the mesenteric filaments of various anemones, claimed that these were xanthine. Among the species examined was *M. marginatum*, and since he worked upon the European species this is a synonym for our *M. senile*. He based his conclusions on the solubility of the granules in various reagents such as we have used. But he considered that their solubility in ammonia and piperazine indicated the presence of xanthine and not uric acid. This conclusion is not justified, for uric acid as well as xanthine is readily soluble in both these reagents. The histochemical behaviour of the granules thus suggests that they are purines, but does not serve to distinguish between uric acid and xanthine. There is, however, no doubt that they are not guanine on account of their solubility in ammonia and relative insolubility in acids.

Despite Mouchet's conclusion there is evidence which suggests that the granules are uric acid. It is noteworthy that Sulima (1913) recorded uric acid in *Anemonia*

* In collaboration with Mr R. Markham of the Biochemical Laboratory, Cambridge.

sulcata, though Przylecki (1926) was doubtful of its presence in the same species. The metabolism of this species is complicated by the presence of abundant symbiotic algae.

Mr R. Markham kindly carried out for us a thorough investigation of the purine bodies present in a number of our specimens. There remains little doubt that any purine granules must be uric acid and not xanthine. The tissues of a whole anemone were ground up in a mortar with sand to a fine paste, then suspended in 0·01 normal hydrochloric acid and digested overnight with pepsin. This resulted in the dissolution of all the material except for a fine sludge. The material was boiled, neutralized, and a sample placed in a Thunberg tube with 1 c.c. of a xanthine oxidase solution (Green 1939, unpublished) and 0·2 c.c. of 1/2000 methylene blue.

There was insufficient xanthine present to discharge the colour of the methylene blue. Subsequent addition of hypoxanthine to the tube resulted in the decolorization of the dye, showing that the enzyme was active. Similar experiments were carried out on freshly ground material, undigested by pepsin, but boiled to inactivate the tissue enzymes. The same results were obtained.

Fresh aqueous extracts of anemone tissues were tested for xanthine oxidase, again by the Thunberg technique, but the enzyme was not found in detectable quantities. It was ascertained, in addition, that such extracts possessed no inhibitors for ordinary milk xanthine oxidase. The extracts were tested also for the presence of adenine by addition of picric acid, but no detectable traces of the highly insoluble adenine picrate were found. This may be a specific peculiarity, since Ackerman, Holtz and Reinwein (1924) obtained a substance which they concluded was adenine from *Actinia equina*.

In *Metridium*, guanine was found in very small amounts, i.e. about 0·01 mg. per 200 mg. dry tissue, equivalent to about 1 : 100,000 wet tissue. *Metridium* thus contains no measurable amount of hypoxanthine, xanthine or adenine, very minute amounts of guanine (presumably from cellular nucleic acids), and no xanthine oxidase.

By the use of uricase, and by the application of Newton's (1937) lithium arsenotungstate modification of Folin's uric acid reagent, considerable quantities of uric acid were demonstrated; both in the tissues of anemones and in the yellow sheets of mucous material at times excreted from the mouth and ectoderm of the column by the animals (even during starvation). The yellow colouring matter shows none of the characteristics of carotenoids. Estimates of the uric acid content were made. An anemone was hydrolysed with 2·5 normal hydrochloric acid for 3 hr. to break up the tissue proteins, etc., and a part of the resulting suspension was distilled to dryness in vacuo in a weighed flask. The residue was resuspended in alcohol and again distilled to dryness, thereby removing most of the residual hydrochloric acid. After drying in a vacuum over concentrated sulphuric acid overnight the dry weight of the material was found to be 0·324 g. The residue was suspended in 5 c.c. 0·1 normal

sodium hydroxide, and 1 c.c. of this suspension was withdrawn and made up to 10 c.c. with saturated lithium carbonate solution. The uric acid in this sample, both by the direct method of Newton and by an unpublished method involving precipitation as a nickel compound (cf. Curtman and Lehrman 1918) and subsequent colorimetry, was found to be 0·025 % wet weight of anemone tissue.

A similar sample was incubated with 1 c.c. of xanthine oxidase solution, and the total uric acid then estimated as before gave a figure of 0·15 % of the dry weight for the original uric acid plus the oxypurines converted to uric acid. This is a further indication that the quantities of hypoxanthine and xanthine originally present are very small, and probably not significant.

It is clear that uric acid and not xanthine is present in considerable quantities in the tissues and also that it is excreted in mucus. The nearness of the granules in the reticular tract to the excretory (intermediate) tracts of the mesenteric filaments may be important (Stephenson 1935). The presence of uric acid is apparently primarily related to the function of excretion, and the existence of such a 'uricotelic' mechanism among the simplest aquatic animals is noteworthy. Mouchet (1929*a*) suggested that xanthine was stored up by anemones as a food reserve. While such a system would itself be unusual, it is scarcely possible to conceive of uric acid being used in such a manner.

Whether the uric acid granules which form bands in the tentacles are simply part of the excretory machinery, or whether their effect on the appearance of the animal indicates some additional function related to colour pattern, remains to be seen.

Melanins

It is remarkable that despite their wide occurrence in the animal kingdom true melanins have not been recorded from Coelenterates (Verne 1926). The yellow 'uranidines' obtained by Krukenberg (1882) from a number of Coelenterates were found by him to blacken on the death of the animal, and Verne has suggested that these may be precursors of melanin, though little is known about them. Nevertheless, in *Metridium* melanins are not only present but dominate the darker colour varieties. In these the dark granules of the endoderm and the brown pigment of the ectoderm are both remarkably stable pigments. They survive fixation in most fixatives and undergo paraffin embedding unchanged. The colours do not lose their intensity in aqueous or glycerine mounts from frozen sections or mounted in balsam, even after several months. The pigments are not dissolved or destroyed by prolonged immersion in alcohol, ether, acetone, or chloroform. They even survive concentrated hydrochloric acid for several days. They are excellently preserved in formalin. Strong oxidizing agents, on the other hand, rapidly destroy the pigments. If formalin-fixed pieces of tissue are put in concentrated nitric acid the brown ectodermal pigment disappears almost at once, though the yellow reaction of the proteins obscures the effect. The black endodermal granules disappear in less than 10 min. In hydrogen

peroxide (20 vol.) the brown pigment is bleached in 2 hr. and the black granules in about 10 or 12 hr. Bromine water bleaches the brown pigment in less than $\frac{1}{2}$ min. and the black granules in 20–30 min. Neither pigment shows characteristic absorption bands.

These reactions indicate the presence of either melanin or of the so-called 'chromolipoids' (Verne 1926, 1930). The pigments are not, however, chromolipoids, for they do not take up typical fat stains (Lison 1936). Thus if formalin-fixed frozen sections are stained with sudan IV, the fat in the ectoderm is seen as bright red droplets at the base of the cells, while the homogeneous brown pigmentation over the cell body is entirely unaffected. Similarly, the dark granules of the endoderm show no trace of red when the tissue is stained, even in the smaller granules which are brown rather than black.

The endodermal granules appear to be typical melanin. They are unaffected even by prolonged immersion in 5 % potash. Boiling for a few minutes in 5 % potash has no effect on the granules. In addition to this the granules show a strong argentaffine reaction when stained by Masson's ammoniacal silver nitrate method (Lison 1936). The granules stain as large densely black bodies.

Although the ectodermal homogeneous brown pigment resembles the melanin granules of the endoderm in its general behaviour, it is more sensitive to reagents. We have seen that it is attacked much more quickly by oxidizing agents. Glacial acetic acid decolorizes it almost completely in the course of 1 hr., while this reagent has no effect on the endodermal granules. Fresh brown ectoderm exposed to 5 % potash acquires a greenish hue and then swells to a viscous mass through which the pigment is homogeneously diffused. The pigment does not pass into the external solution. 0·2 % potash dissolves the pigment along with other products of the cytolysed protoplasm. It is noteworthy that fixation with formalin renders the pigment insoluble in alkalis, though these still produce the greenish hue; it also delays the destruction by glacial acetic acid.

The brown pigment of the ectoderm does not give an obvious argentaffine reaction, though sections exposed to the silver reagent sufficiently long (4 days) to begin to darken the whole tissue show a distinctly darker tint in the ectoderm than elsewhere. It is possible, however, to show by experiments on a larger scale that brown ectoderm truly contains melanins and their derivatives. The ectoderm with its pigment can easily be scraped from the column of a brown animal. We subjected a quantity of such ectoderm to digestion with trypsin in phosphate buffer pH 8·0 overnight. During this period, the system became far blacker than did the control to which no trypsin had been added. Boiling of the system gave no protein coagulum, while in the control the turbidity was markedly increased in boiling. The boiled system, treated with strong hot potash, cleared considerably, becoming greenish, then yellow to brown; neutralization with acid decolorized the system and rendered it turbid. On standing, a brown to black precipitate slowly formed. This procedure could be

repeated. The brown pigment centrifuged from acidic systems was found to dissolve in concentrated sulphuric acid and to give a brown homogeneous solution, just as in concentrated potash.

Ferric chloride solution added to acidified suspension of the brown pigment showed a persistent green-yellow colour, quite distinct in tint and intensity from the colour due to the reagent alone. This contrasted with the colour reactions of phenols such as resorcinol which gives a purple, or catechol which yields a green colour turning deep violet on subsequent treatment with sodium bicarbonate. The latter reagent added to our acidic suspension of the brown pigment did not change the green-yellow to violet, but only to an orange red colour which might have been due, at least in part, to incipient formation of colloidal ferric hydroxide. These reactions are characteristic of melanins.

On one occasion a large brown anemone was covered with acetone preparatory to grinding it and extracting its carotenoids with succeeding washes of that solvent. The ectoderm peeled off, leaving the pale blueish mesogloea and endoderm beneath. After grinding the ectoderm to a state of fine division with sand in a manner to be described later, it was found that the final aqueous acetone solution retained a pale greenish yellow colour which it failed to impart to petroleum ether washes, after complete removal of carotenoids with that solvent. Brown animals always gave these results while pure red or pure white animals did not. The aqueous acetone solution was reduced in volume by distillation to concentrate the pigment, which was now light yellow-brown. At this stage, petroleum ether removed no pigment, whether the system were rendered acid or alkaline; strong potash produced a deepening of colour, which faded on acidification with strong hydrochloric acid or even on restoring to pH 7·2 with phosphate buffer. Acidification, with fading of the colour, was always accompanied by a turbid appearance. Acidic solutions gave the green-yellow reaction already encountered, on the addition of ferric chloride. The clear alkaline solution showed no fluorescence or spectral bands; it gave no test for flavines with sodium hydrosulphite.*

It may be concluded from these observations that the yellow-brown pigment is not a carotenoid (insolubility in fat solvents; solubility in water, acid-base change in colour, etc.); it is not a haematin (giving no spectral bands) nor a purine (solubility in aqueous acetone, etc.). Its behaviour confirms its relation to melanin. It is, however, distinct from and more reactive than the granular melanin of the endoderm. That pigment resembles true or 'eumelanin' in its behaviour (Schereschewsky 1929); while the brown ectodermal pigment behaves like a melanin precursor and resembles 'phaeomelanin' or the 'melanoprotein' of Gortner (1911 *b*).

Not only are substances related to melanin to be obtained from brown *Metridium*, but the tissues can be shown to possess a system analogous to tyrosinase. Moreover, it is remarkable that this same system can be obtained from pure red and pure white

* We wish to thank Dr D. E. Green of the Biochemical Laboratory, for conducting this test.

animals, neither of which deposits melanins in its tissues. Aqueous filtrates of finely ground melanin-free anemones consistently darkened in a few hours, passing through a brown reversible stage to a black irreversible one. During the preparation of extracts for purine investigation Mr R. Markham carried out the following experiments for us. Extracts of white specimens of *Metridium*, prepared by grinding the whole animal thoroughly in a mortar with pure, acid-washed sand and 0·2 M phosphate buffer (pH 7·4), together with a few drops of capryl alcohol added as a preservative, were placed in a refrigerator. They became brown in a few hours, and black after a few days. In test-tubes the system was consistently observed to darken progressively from the top downward. It was suspected that this darkening was due to the formation of melanin by the tyrosinase reaction (Raper and Wormall 1923; Raper 1928), and it was possible to demonstrate experimentally that *Metridium* possesses an enzyme system of this kind. Extracts prepared as above were allowed to stand in tubes under various conditions (table 2).

TABLE 2

Tube	Contents	Temp. ° C	Results
1	3 c.c. extract	18	Darkened in 12 hr.
2	3 c.c. extract neutralized NaCN	18	No darkening after several days
3	3 c.c. extract, boiled	18	No darkening after several days
4	3 c.c. extract in evacuated Thunberg tube with mercury seal	18	No darkening after several days
5	3 c.c. extract in open tube with air bubbling through system	37	Darkened in 2 hr.

This series was repeated many times with the same results. Potato juice showed the same behaviour (cf. Raper and Wormall). It was also observed repeatedly that the brown intermediate phase of the *Metridium* extracts was, like the similar phase of potato juices, readily reversible by the introduction of an active xanthine oxidase-xanthine system, when the whole system was placed in vacuo in Thunberg tubes. In the more advanced, black stage, the system was no longer reversible. These facts are in agreement with other tyrosinase systems.

We are not yet able to state the reasons for the failure of white and red varieties of *Metridium* to develop melanin in nature, and for its appearance in the dark forms. Since the red and white varieties possess all the substances required for melanin formation, the facts support the idea that we are dealing with an instance of 'dominant albinism' in a genetical sense (Gortner 1911 *a*). The brown and the grey colour systems differ from the white in that each permits deposition of melanin products in its special way. We have already seen that these systems behave as though they were genetically independent.

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Carotenoids

MacMunn (1890) pointed out the wide distribution of the lipochromes in both the plant and animal kingdoms. The most important of these pigments are the carotenoids, many of which have been obtained from Coelenterates (Lönnberg 1931; Payne 1931). Among the Actiniaria probably most of the red, orange and yellow colours are due to them. Only in recent work are accurate descriptions of the pigments given. Fabre and Lederer (1934) found in a red variety of *Actinia equina* a new and unstable carotenoid ester which they named 'actinioerythrin'. From the green variety they obtained a red orange pigment with the properties of a xanthophyll. Most of this new carotenoid exists as an ester bound to protein: in which condition it is green. Both varieties contained α- and β-carotenes. The blue pigment of the 'marginal tentacles' (the acrorhagi?) is not a lipochrome. Heilbron, Jackson and Jones (1935) confirmed the presence of actinioerythrin in this species. They also obtained 'violerythrin', an 'acid' obtained by hydrolysis of actinioerythrin. They found a trace of carotene and of a xanthophyll with a spectrum which appears to resemble that obtained by Fabre and Lederer from the green variety. Heilbron *et al.* do not state what varieties they used.

Fox and Moe (1938) investigated *Epiactis prolifera*, a small orange red anemone. They found an ester of a red acidic carotenoid together with a trace of carotene and possibly a xanthophyll. The ester and its free acid resembles in spectrum similar pigments obtained by Heilbron and his colleagues in *Tealia felina*. *Tealia*, however, also contains another carotenoid resembling actinioerythrin, though of lower melting-point, and a trace of carotene.

The work of Heilbron *et al.* is the most thorough that has been done. They worked with large quantities of material and investigated several species of anemone. From *Anemonia sulcata* they obtained a new xanthophyll, sulcatoxanthin, in addition to pigments derived from the symbiotic algae. They also worked on *Metridium senile* (*Actinoloba dianthus*), in which they found a red ester and a small amount of another carotenoid. Dr Jackson kindly informs us that the red variety was used.

It is evident that each species of anemone may contain a variety of carotenoids, many of which are chemically unique. Moreover, the work of Fabre and Lederer on the green and the red Actinia shows that different colour varieties may differ in their carotenoids. In *Metridium senile* the variation of hue even in the red variety is great enough to suggest that more than one pigment may be concerned in different cases, and our observations will show that this is indeed the case. This fact raised for us a great practical difficulty. If individual varieties vary in their carotenoids, one can no longer be content with results obtained from large masses of material. Single individuals or collections restricted to the same asexual stock must be used, and the quantity of pigment available for accurate investigation is correspondingly diminished.

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(1) *Methods.*

The living animals were made to contract maximally and superficial sea water was removed with cloth or filter paper. They were then weighed, and finally ground under acetone with pure sand to a very fine state of division in an electrically driven earthenware 'End-runner Mill'. Under these conditions, the acetone originally added together with a few succeeding washes extracted all carotenoids and much other lipoid matter. By diluting the acetone with much water and shaking the system with repeated washes of petroleum ether, all of the carotenoid pigments were recovered in the latter solvent. This was finally freed from acetone by repeated water washes.

TABLE 3. ANALYTICAL SCHEME FOR CAROTENOIDS

Acetone extract of tissues

Add water, extract all pigment with petroleum ether

Petroleum-ether solution of mixed carotenoids

Treat with repeated washes of 90 % CH_3OH (partition test)

Soluble in petroleum ether (epiphase): carotenes; xanthophyll esters; certain esters of acidic carotenoids	Soluble in 90 % CH_3OH (hypophase); free xanthophylls, certain esters of acidic carotenoids
Hydrolyse in 90 % CH_3OH plus KOH	Hydrolyse in 90 % CH_3OH plus KOH
Unchanged carotenes, free xanthophylls, K-salts of carotenoid acids	Unchanged xanthophylls; K-salts of carotenoid acids
Dilute; extract with petroleum ether	Dilute; extract with petroleum ether
Petroleum ether: carotenes, xanthophylls:	*Interface:* / *Petroleum ether:*
Apply partition test with 90 % CH_3OH	K-salts of carotenoid acids. Wash with petroleum ether; dissolve in more, containing trace of glacial acetic acid; remove acid, transfer to CS_2, and determine absorption spectrum
Epiphase:	*Hypophase:*
Carotenes Apply partition test with 96 % CH_3OH	Xanthophylls Dilute, transfer to petroleum ether, and chromatograph on $CaCO_3$; separate, elute, transfer to CS_2, and determine absorption spectrum
Epiphase:	*Hypophase:*
Carotenes Chromatograph on MgO or $Ca(OH)_2$, separate, elute, transfer to CS_2, determine absorption spectrum	Monohydroxy-xanthophylls Treat as in above hypophase

Interface:	Petroleum ether:
K-salts of carotenoid acids. Carry through separately, but exactly as outlined opposite	*Xanthophylls:* Carry through separately, but exactly as outlined opposite

The resulting petroleum-ether solutions were subjected to the partition test by shaking with approximately equal volumes of 90 % methanol. *Hypophasic* carotenoids (i.e. those taken into the lower, aqueous alcohol phase and not appreciably removable therefrom by fresh petroleum ether) were thereafter kept separately from *epiphasic* carotenoids (remaining in the upper, petroleum-ether layer). Typically epiphasic carotenoids are the various carotenes, esterified xanthophylls, and certain esters of some of the carotenoid acids (e.g. astacene), while carotenoids which pass into the hypophase are free xanthophylls and certain esters of carotenoid acids. Treatment of the epiphasic fraction with 95 % methanol removes certain rather uncommon monohydroxy-xanthophylls such as cryptoxanthin or rubixanthin, none of which was ever detected with certainty in our studies. Preliminary shaking of petroleum-ether solutions containing epiphasic carotenoids with cold methanolic solutions of potassium hydroxide removes any unesterified carotenoid acids, but these were never encountered in the investigations.

The petroleum ether was driven off in a stream of nitrogen and the system maintained under this inert gas to prevent oxidative destruction of carotenoids. The mixed epiphasic carotenoids were heated for a few hours or stored overnight at room temperature, in the presence of methanolic potash. This hydrolysed the esters of xanthophylls and carotenoid acids while the carotenes remained unaffected. Treatment of the system with petroleum ether following the hydrolysis resulted in the removal of carotenes only, after which, moderate dilution of the methanolic potash phase forced any xanthophylls now set free into the petroleum-ether phase, while potassium soaps of any carotenoid acids gathered at the interface as red- or orange-coloured muffs. These could be separated, washed with petroleum ether, and finally taken up in that solvent by adding a little glacial acetic acid. Similarly, the total hypophasic carotenoids were treated with methanolic potash under nitrogen, the systems diluted, xanthophylls extracted, and potassium soaps of acidic carotenoids recovered in the manner described.

Carotenes can be resolved into their component isomers if more than one be present, by passing their dry alcohol-free petroleum-ether solutions through Tswett chromatographic columns of magnesium oxide or calcium hydroxide, and then removing mechanically the resulting coloured zones and eluting each separate fraction with petroleum ether containing traces of methanol. Xanthophylls from the original epiphase are separated in a similar way by chromatography on calcium carbonate followed by elution and recovery. Acidic carotenoids were not subjected to further purification by chromatography because they tended to form very stable associations, which were perhaps actual magnesium or calcium salts. In such condition they could not be eluted, and attempts to recover them by decomposition, even with dilute acid, usually resulted in great loss of pigment. A small quantity of the unhydrolysed material was therefore taken in the beginning for chromatographic separation of the esters of the acidic compounds. These gave discrete coloured zones readily elutable

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and recoverable in a relatively pure state. The final products were dissolved in carbon disulphide for spectroscopic examination of the absorption bands by methods described in the next section. Pyridine was often employed for comparison in studies of some of the acidic carotenoids. The accompanying analytical scheme summarizes the general procedure followed in separating and studying the carotenoid components in the various extracts (table 3). Further details concerning the chemical procedure may be found in Zechmeister (1934), Zechmeister and Cholnoky (1939), and Strain (1938).

(2) *Astacene and 'metridene'*.

A number of different carotenoids were obtained from different lots of *Metridium*. It will be shown that among these appears to be the 'acidic' carotenoid astacene. It is acidic only by enolization. Kuhn and Sörensen (1938), working on crustacean eggs and exoskeletons, concluded that astacene itself, tetraketo-β-carotene, is an artifact. It is produced by oxidation during the hydrolysis of esters of the naturally occurring alcoholic carotenoid, astaxanthin (diketo-dihydroxy-β-carotene). Astaxanthin can be esterified to give epiphasic compounds, such as the dipalmitate, which has a maximum absorption at about 490 mμ in pyridine; or hypophasic compounds, such as the diacetate, which shows three absorption maxima, at 500, 485 and 475 mμ in pyridine. Free astaxanthin shows a chief maximum at about 493 mμ and a lesser one at about 477 mμ in pyridine. These values are taken from Kuhn and Sörensen's curves. Possibly our astacene pigments may likewise have been derived from esters of astaxanthin existing naturally in the anemones, and the presence of such compounds might explain why some of the diffuse and indistinct maxima seen through the Hartridge spectroscope appear to be composed of two close, poorly defined bands, rather than a single one. Mixtures of astaxanthin and astacene might also produce such effects.

But in addition to astacene there is another acidic carotenoid, which occurs as an ester often in large quantities. We shall show that it is found in a number of varieties, notably in red specimens. It is this carotenoid which was reported by Heilbron *et al.* (1935) from red *Metridium*; and doubtless the pigment from which it is obtained is that recorded by MacMunn (1890) as having an absorption band between 535 and 565 mμ in the living red tissue of *M. senile* (*Sagartia dianthus*). Its constitution is still unknown, but it closely resembles astacene in its chemical behaviour. Heilbron *et al.* found the natural red pigment to be an ester of low melting-point. It is non-crystallizable and yields a red insoluble sodium salt on saponification. After hydrolysis the free carotenoid acid crystallizes from pyridine as deep violet-red prisms. The carotenoid acid departs consistently from astacene in showing a lower absorption maximum. This occurs at 495 mμ in both carbon disulphide and pyridine, whereas astacene shows a maximum at 510 mμ in carbon disulphide, and close to 500 in pyridine. Heilbron reports the melting point of this new carotenoid as 195–196° C, whereas astaxanthin melts at 215·5–216° C (Kuhn and Sörensen). Astacene itself is stated to

melt at 240–243° C. For convenience we shall refer to this new carotenoid as 'metridene'.

When examining the spectra of anemone pigments we used a Hartridge Reversion Spectroscope and a Hilger visual spectrophotometer. The spectra of astacene and metridene in carbon disulphide each show a diffuse asymmetrical absorption maximum, and this makes measurement of the position of the maximum somewhat difficult with the Hartridge spectroscope (cf. Emerson and Fox 1940). The reading varies with slit width, concentration and the use of coloured glass filters to a much greater extent than in the case of sharply defined bands. We therefore standardized our method by consistently employing moderate concentrations of pigment, by using the maximal slit width consistent with satisfactory perception of the broad absorption band, and by taking readings by matching the two spectra at the region of maximum density. We tested our method by observations on crystalline astacene prepared by Messrs J. S. Bacon and R. Markham of the Biochemical Laboratory, Cambridge. The astacene was prepared in May 1938 and stored in vacuo in a sealed glass tube. By matching the darkest portion of the absorption band in both dilute and concentrated solutions, both with and without a light filter on the spectroscope, we obtained values in carbon disulphide which ranged from 509·6 to 512·6 mμ with an average of 511·2 mμ for all readings. If, however, the whole asymmetrical absorption band was matched edge to edge, a value as high as 517 mμ was obtained even in a dilute solution. Matching the darkest portion of the band in pyridine solutions gave values from 494 to 501·5 mμ with an average of 497·6 mμ. The values obtained are close to those accepted for astacene; 510 mμ in carbon disulphide and 500 mμ in pyridine.

Astacene gave readings in the Hartridge instrument agreeing well with those taken by the Hilger spectrophotometer. But metridene, when dissolved in carbon disulphide, gave readings some 5–10 mμ higher in the former; that is, 500–505 mμ, against 495 mμ in the Hilger spectrophotometer. Pyridine solutions, however, gave much closer agreement between the two instruments. Acidic carotenoids obtained from the anemones which showed with the Hartridge instrument an absorption band within 507–512 mμ in carbon disulphide, or 498–500 mμ in pyridine, were concluded to be astacene: while acidic carotenoids, similar to astacene in other respects but giving readings when dissolved in carbon disulphide at 495 mμ with the spectrophotometer, or 500–505 mμ with the Hartridge instrument, and readings at 494–496 mμ in pyridine, were concluded to be metridene.

The Hilger spectrophotometer was installed in another building from that in which the chemical work was done, and owing to the small quantities of pigment often available and to the convenience of the instrument the Hartridge spectroscope was usually employed. For convenience in the following account wave-lengths measured by the Hartridge spectroscope will be signified by the symbol (*H*); by the Hartridge spectroscope with a colour filter by (*HF*); and by the Hilger spectrophotometer by (*S*).

(3) *Carotenoids of the white variety.*

As may be expected, animals of this variety yielded little pigment. The gonads in both sexes, however, are usually pale pink and are responsible for such pigment as there is. In no case did we certainly detect the presence of carotenes, xanthophylls or xanthophyll esters. An epiphasic acid ester was obtained which was poorly adsorbed from petroleum ether by calcium carbonate, but strongly so by magnesium oxide as a diffuse purple band. The red to orange solutions in carbon disulphide showed a single absorption maximum which gave values from 498 (*HF*) to 504 (*H*) mμ.

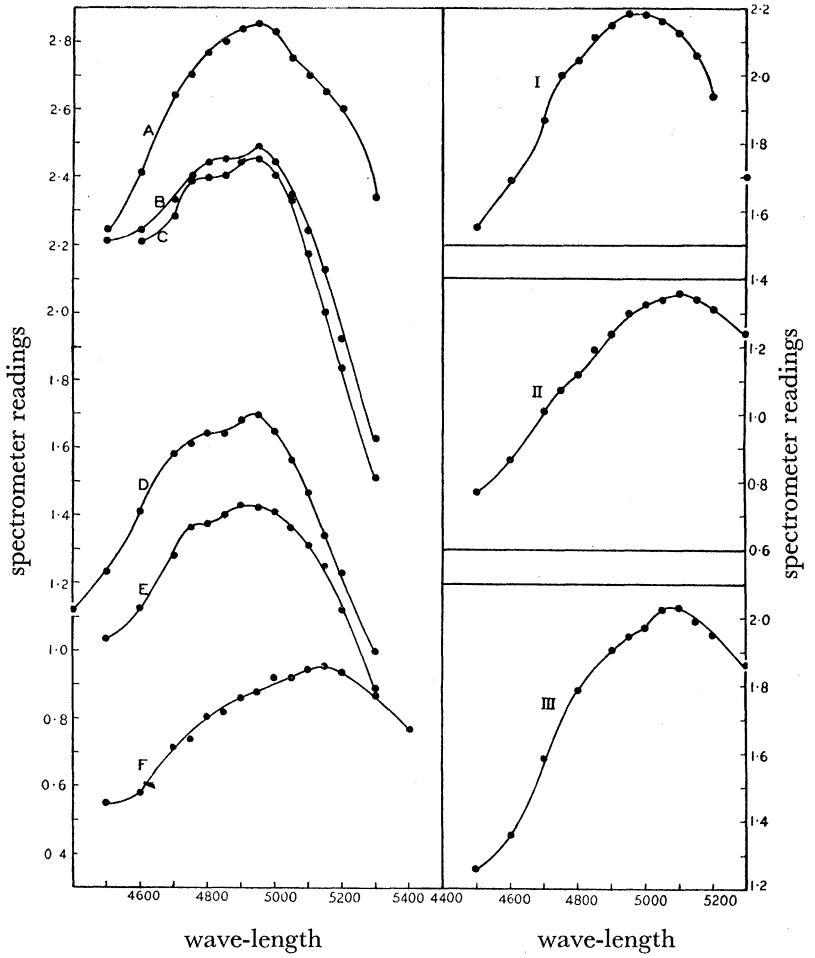

FIGURE 5. Acidic carotenoids and their esters from colour varieties of *Metridium senile*. *A*. Acid of epiphasic ester from orange-red form of 'simple red' variety; solvent, carbon disulphide. *B*. Same as *A*; solvent, pyridine. *C*. Acid of hypophasic ester from yellow-pink form of 'simple red' variety; solvent, pyridine. *D*. Whole pigment (mixed epiphasic and hypophasic esters) from orange-red anemone; solvent, pyridine. *E*. Epiphasic ester from orange-red anemone; solvent, pyridine. *F*. Acid of hypophasic ester from an orange-red anemone; solvent, carbon disulphide. I. Epiphasic ester from white anemone; solvent, carbon disulphide. II. Free acid of epiphasic ester from white anemone; solvent, carbon disulphide. III. Free acid of hypophasic ester of white anemone; solvent, carbon disulphide.

Curve I, figure 5, shows spectrophotometer measurements on this pigment from two large immature animals. The free acid from this ester gave red to magenta red solutions in carbon disulphide with a single absorption maximum at 510 mμ (*S*) (curve II, figure 5). In pyridine the maximum was at 499 mμ (*H*).

A hypophasic acid ester was found, though only occasionally in sufficient concentration for examination. In one batch of animals it gave a long diffuse pink zone on calcium carbonate in the chromatograph, and a magenta red solution in carbon disulphide with an absorption maximum at 513 (*H*) to 509 mμ (*HF*) (506 mμ before chromatographing). In another case, in which there was too little pigment to chromatograph, an orange solution was obtained with absorption maximum 502 (*HF*) to 503 (*H*) mμ. The free acid from the hypophasic ester gave absorption maxima at 508 (*S*) to 510 mμ (*H*) in carbon disulphide. Curve III, figure 5, shows the spectrophotometer values for this acid in carbon disulphide from the same individuals that gave curves I and II. In pyridine a maximum was found at 499 mμ (*H*).

The free acid from the hypophasic ester is similar to that of the epiphasic ester. In one batch of animals the acid from both sources was added together and gave an absorption maximum at 511 (*H*) to 510 mμ (*HF*) in carbon disulphide and 502 (*H*) to 499 mμ (*HF*) in pyridine. Its spectrum corresponds to that of astacene.

Astacene esters are the only carotenoids present in the white anemones and they are apparently restricted to the gonads. We have not however analysed a sufficiently large number of batches of white *Metridium* to state that other carotenoids are never found, though the amounts must necessarily always be very small, particularly in sexually immature individuals. Our results on this and other varieties do not lead us to suppose that the sexes store different carotenoids in their gonads. It is interesting to note that Mr B. T. Scheer, a graduate student of one of us (D. L. F.), working at the Scripps Institution of Oceanography at La Jolla, California, finds that female and male California mussels (*Mytilus californianus*) carry most of their carotenoids to the developing gonads, and that, while the female may contain about twice as much carotenoid as the male, the various constituent pigments are identical in both (personal communication).

(4) *Carotenoids of the red variety.*

Red animals contain a great deal of pigment, which is mostly epiphasic. This proved to be chiefly esters of metridene, and in most animals carotenes, xanthophylls and xanthophyll esters were not found. The total pigment dissolves in pyridine to form an orange solution with an absorption maximum at 495 mμ (curve *D*, figure 5). Most of the pigment is epiphasic and forms a red solution in carbon disulphide with a band at 505 (*H*) to 501 mμ (*HF*). It consists chiefly of metridene esters, and there were often no other pigments. Curve *E*, figure 5 (maximum at 490–495 mμ), was taken from the orange-red pyridine solution of the whole epiphasic acid ester fraction in such a case. In the chromatograph a diffuse red-violet zone was formed in calcium

carbonate, while a sharp purple zone was formed at the top of the magnesium oxide column beneath it. No pigment passed through the column. In carbon disulphide the sorbate from the calcium carbonate gave a red-orange solution with a band at 505 mμ (*S*), and the magnesium oxide sorbate gave a red-orange solution with a band at 490 mμ (*S*).

These acidic carotenoid esters adsorbed on the magnesium oxide could be eluted with petroleum ether. They then formed an orange solution which gave red microcrystals with some lighter coloured impurity, on evaporation under nitrogen. The crystals dissolved in cold methanol with considerable difficulty but were readily soluble in it when hot. Hydrolysis in methanolic potash gave a red potassium salt which in turn gave the free carotenoid acid on addition of glacial acetic acid.

The free acid from the epiphase was taken up by magnesium oxide in the chromatograph so strongly that it could not be removed by any of the usual solvents. Dissolved in either carbon disulphide or pyridine it gave a red solution with a band at 495 mμ (*S*) (curves *A* and *B*, figure 5). Its properties show that it is metridene derived from its esters present in the living animal.

Hypophasic esters of metridene were also found, but in some cases the free acid derived from these showed a band in carbon disulphide decidedly nearer the red at 507 (*H* and *HF*) to 515 mμ (curve *F*, figure 5), and we concluded that this was astacene. The quantity of astacene esters even when present is far less than that of metridene esters. We do not know whether the astacene esters are restricted to the gonads as in the white variety.

Astacene esters are not the only carotenoid sometimes encountered among red animals in addition to metridene esters. In one batch of typically red animals a small amount of a carotene was found in the epiphasic fraction with absorption maxima in carbon disulphide at 517 and 485 mμ (*HF*). These values agree with those of β-carotene. This same fraction yielded faint traces of xanthophyll derived from original esters. The hydrolysed hypophasic fraction also yielded xanthophyll with two absorption maxima at 512 mμ (*H* and *HF*) and between 479 (*HF*) and 480 mμ (*H*) (fucoxanthin ?).

We have mentioned that in some asexual stocks the hue of the 'red' variety may approach a decidedly yellow orange; though the pigment is contained in lipoid droplets in the cells in just the same way as in typical reds. In these yellower forms carotenes and xanthophylls which we have just seen to occur at times in typically red animals are to be found in decidedly larger amounts. Epiphasic and smaller quantities of hypophasic esters of metridene are still the chief pigments present, though the quantity is less than in typical reds. Curve *C*, figure 5, shows the spectrophotometer values for the free acid dissolved in pyridine, obtained from the epiphasic carotenoid ester. Two batches of the somewhat uncommon yellow-orange forms were investigated. In both cases carotene was present as a non-saponifiable constituent which remained epiphasic against 96 % methanol even after treatment with methanolic potash. The

solution from this pigment in carbon disulphide gave bands in one batch at 516 and at 482 mμ (*H* and *HF*), which agree closely with those of β-carotene. In a second batch bands were found at 519 and 487 mμ (*H*) which do not correspond closely with those of any known carotene, though close to the bands of the monoketonic carotenoid echinenone (Lederer 1938).

In addition to carotene traces of xanthophylls derived from their esters were found in the epiphasic fraction. The hypophase yielded comparatively large quantities of xanthophyllic carotenoids. In one case relatively large quantities of crystals separated when the total carotenoid pigments were being transferred from acetone to petroleum ether, preparatory to partition between petroleum ether and 90 % methanol. The crystals were insoluble in petroleum ether, but very soluble in methanol and gave an orange solution in carbon disulphide with two absorption bands at 516 (*H*) to 514 mμ (*HF*) and at 483 (*H*) to 481 mμ (*S*). These values are close to those for a number of xanthophyll pigments such as sulcatoxanthin, zeaxanthin, petaloxanthin and cynthiaxanthin. The fact that the pigment is entirely hypophasic in 90 % methanol against petroleum ether differentiates it from cryptoxanthin. In one case a second xanthophyllic pigment was obtained which gave an orange solution in carbon disulphide with bands at 509–510 and 478–481 mμ (*HF*) which are close to the bands of fucoxanthin or lutein.

These observations show that the red animals store large quantities of the esters of the acidic carotenoid metridene, and in some cases small amounts of astacene, carotenes and xanthophylls with their esters: while among the yellower members of this series carotenes and various xanthophyllic carotenoids may attain much larger quantity.

(5) *Relative amount of pigment in red and white anemones.*

The characteristic feature of the red variety is the abundance of metridene esters. In contrast, the white variety contains astacene and that only in small amounts in the gonads. To estimate the relative quantity of pigment in each variety the total pigment was extracted from weighed specimens. The colour intensities of the extracts were measured by a photoelectric photometer. A blue-violet glass filter was interposed in the light beam, screening out nearly all light except in the region of wave-lengths from about 405 mμ to about 540 mμ which covers the spectral region absorbed by both astacene and metridene.

The petroleum-ether solution of pigments from a white anemone was adjusted to give 2 c.c. of extract per gram of original wet tissue. A similar extract of an orange-red animal had to be diluted to a final volume representing a ratio of 17 c.c. of extract per gram of original wet weight of tissue before it yielded the same photometric reading as did the pigment extract from the white animal. Judged by the colour there was thus 8·5 times as much pigment in the red as in the white animal.

The carotenoid from white animals is apparently astacene itself and the metridene

of the red animals has a spectrum similar to it except that the absorption maximum is rather nearer the red. The two extracts could therefore be assigned 'astacene equivalents' in terms of milligrams of pigment per gram of live tissue by comparing the photometric readings with those for a series of solutions of crystalline astacene. This had been prepared from lobster eggs, and was dissolved in petroleum ether containing a little glacial acetic acid to render it sufficiently soluble. Nine different concentrations of astacene lying between 0·75 mg. % and 1·5 mg. % yielded a set of points falling on a smooth curve. The pigment solutions obtained from our experimental material corresponded to a value of 0·88 mg. %. On this basis the white animal contained $0{\cdot}88 \times 2 = 1{\cdot}76$ mg. per 100 g. wet weight, while the metridene of the red animal was equivalent to $0{\cdot}88 \times 17 = 14{\cdot}96$ mg. of astacene per 100 g. wet weight. Numerous animals in the light brown variety yielded extracts certainly far lower in concentration of carotenoid pigments than even the white animals, and the same is probably true of immature whites without pale pink gonads.

(6) *Test for a possible 'metridene oxidase'.*

The white animals differ from the red in the absence of metridene. It was thought possible that this might be due to the presence of a metridene oxidase. Two small red anemones were ground finely with pure sand in phosphate buffer at pH 7·2. One small white anemone was ground separately in exactly the same way. The resulting extracts were filtered separately through cotton. Each extract was divided into four portions of a few ml. each. Two of the tubes of extracts of the white animal were boiled, while the other two, and all four tubes of red animal extract were left unboiled. Four tubes were then set up as follows: two containing normal red animal extract plus normal white animal extract; two containing normal red animal extract plus boiled white animal extract. Each of the four systems was preserved against bacterial decomposition by the addition of a drop of toluene.

All four systems showed progressively increasing melanin formation from the meniscus downward which was more rapid in the tubes containing unboiled extract of white animals than in the other set, which contained active enzymes from only the red specimens. Nevertheless, there was no evidence for the presence of an oxidase destroying red anemone carotenoids. Equal quantities of such pigments were still extracted from each system at the end of 3 days.

Whereas this experiment indicates the absence of oxidative enzymes in white specimens for the destruction of the carotenoids already present in the orange-red variant of *Metridium*, it does not exclude the possible presence of oxidases in white animals for the destruction of other specific carotenoids which may be precursors of the pigment characterizing the red animals.

(7) *Carotenoids of the brown and the grey series.*

We have already described the complex variations due to the intensity and distribution of melanin pigments. The presence of these in the tissues necessarily makes

it more difficult to discern the first traces of carotenoid pigmentation than in the simple red series. Nevertheless, we showed that certain varieties such as simple brown, brown with grey and the rest appeared to resemble white animals on which various melanic pigmentations were superposed. It might therefore be expected that these varieties would resemble the white in possessing no carotenoid other than astacene in small quantities. In certain cases this is so.

A batch of simple brown anemones, with pale brown body, and white mouth, tentacles and basal plate, together with pink gonads was found to contain very little pigment. Acidic carotenoid esters alone were present and were almost entirely hypophasic. The free acid from these showed an absorption maximum at 506 mμ (*HF*) in carbon disulphide and at 500 mμ (*HF*) in pyridine. It was probably astacene. A very large brown with grey specimen showed rather similar effects. As in white specimens, the presence of astacene in simple brown or brown with grey animals is correlated with the possession of pink gonads. When the gonads were not pink, acidic carotenoid esters were not found even though other carotenoids were present in fair amount. It is noteworthy that animals of the red with brown series did not contain astacene even though the gonads were red, as is often the case with simple red animals. We shall discuss the red with brown series later.

In many brown animals there was less pigment than we found even in the white ones. Some simple brown anemones with pale brown bodies and tentacles, and with a pale mouth and basal plate yielded no more than traces of any carotenoid, though xanthophyll esters were certainly present. These animals contained much sterol matter.

Very similar results were obtained from a series of anemones with dark brown body and tentacles, pale brown mouth and dark basal plate. Only very small amounts of pigments were present, which included traces of carotene, of xanthophyll and its esters, and, in three cases, of an acidic carotenoid.

While therefore all of these browns possessed very little carotenoid, only some resemble the white in possessing astacene esters alone. Small amounts of very different carotenoids are found in others, and almost every batch of anemones in this variety possesses a different collection of this series of pigments. Thus two batches of simple browns possessing little total pigment showed traces not only of acidic carotenoid esters but also of xanthophylls and of a carotene. A spectrum in carbon disulphide of this carotene gave bands at 514 and 482 mμ (*H*) in one case and at 517 and 482 mμ (*H*) in the other. In another batch of brown with grey animals with pink gonads there was little pigment. The epiphasic and hypophasic fractions were about equal, and esters of astacene were present in both. The free acid from hydrolysis of the epiphase gave a magenta red solution in carbon disulphide with a band at 514 (*HF*) to 517 mμ (*H*). In pyridine there was a band at 501 (*H*) to 496 mμ (*HF*). The free acid from the hypophase gave readings at 509 (*H*) to 507 mμ (*HF*) in carbon disulphide; and readings at 499 (*H*) to 498 mμ (*HF*) in pyridine. These values indicate astacene.

This batch of animals possessed no carotenes. But from the hydrolysed epiphasic fraction there was obtained a trace of xanthophyll derived from esters, together with another pigment. This new pigment obtained by hydrolysis of the epiphase was itself hypophasic. It was not absorbed by calcium carbonate but readily by magnesium oxide to form a pink complex. It possessed a single narrow band in carbon disulphide at 490 (*H*) or 489 mμ (*HF*). The spectrum is like that of myoxanthin, but that pigment is epiphasic.

Another brown with grey individual yielded only faint traces of acidic carotenoid esters. Chromatography of the epiphasic fraction gave a yellow orange band which, travelling slowly through the calcium carbonate, settled on top of the magnesium oxide layer as a distinct pink zone. This pigment possessed two bands, at 517 and at 485 mμ (*HF*). Hydrolysis had no effect on this carotene which remained epiphasic to 95 % methanol. This suggests β-carotene.

In the hypophase from this individual, a xanthophyllic pigment crystallized from dry petroleum-ether solution in long thin rectangular platlets like scales of lacquer. These were brick red with faint purplish lustre. The crystals were insoluble in water and slightly soluble in petroleum ether, pure methanol, carbon disulphide and ethyl-ether. They were very soluble in pyridine, chloroform and acetone. An ethereal solution with 25 % hydrochloric acid gave no blue colour, but a blue colour was developed with antimony trichloride in chloroform. Prolonged treatment with methanolic potash did not change the chemical behaviour. Chromatography of the crude petroleum-ether solution showed a single yellow band which travelled slowly through calcium carbonate and settled on top of the magnesium oxide as an orange layer contaminated with white solid. In carbon disulphide solutions of this orange layer spectral bands were found at 507 and 472 mμ (*HF*). Crystals of the pigment dissolved in carbon disulphide gave similar or identical bands at 509 and 474 mμ (*HF*). Crystals dissolved in pyridine gave bands at 494 and 471 mμ (*HF*).

A solution of the crystals was made in petroleum ether and chromatographed. All the pigment was adsorbed on the calcium carbonate as a lemon yellow band near the top of the column. In carbon disulphide bands were found at 512 mμ, at 474 mμ, and with a faint band at 441 mμ (*HF*). The average spectral readings for the pure material approach those for pentaxanthin.

Our results show that though simple brown and brown with grey animals possess little lipochrome, there is a great variation in the carotenoids present. As in white animals, astacene esters in the gonads may sometimes be the only carotenoids. Astacene however is only present if the gonads are pink, and there may be traces of a variety of xanthophyll esters and carotene. The carotenoids thus vary with almost every batch of animals.

(8) *Carotenoids of the red with brown series.*

Melanic animals visibly containing red carotenoids also varied in the kinds of carotenoid present. Thus a typical batch of red with brown animals yielded much

carotenoid. This was epiphasic rather than hypophasic and contained no carotenes or free xanthophylls. Metridene esters were present in both the epiphase and hypophase: carbon disulphide solutions of the free acids giving bands at 504 mμ (*H*) from the epiphase and 506 mμ (*H*) from the hypophase while in pyridine the free acid from the hypophase gave a band at 496 mμ (*HF*) and that from the epiphase a band at 495 mμ (*H*).

The hypophase yielded only traces of other pigments but the epiphase contained xanthophyll esters. The crude epiphasic pigment, when dissolved in carbon disulphide, gave a blood-red solution with a band at 511 mμ (*H*). In pyridine the red-orange solution had a band at 500 mμ (*H*). On chromatography an orange band travelled slowly through the calcium carbonate and settled on top of the magnesium oxide layer as a single sharp magenta violet zone. Traces of a pigment forming a pale pink zone in the calcium carbonate could not be recovered in sufficient amounts for analysis. The main fraction of the pigment from the chromogram, when dissolved in carbon disulphide, gave a band at 504 mμ (*HF*). On hydrolysis no carotenes were found, but there were two other pigments. One was metridene, the other a xanthophyll hydrolysed from its esters. The spectrum of this showed two bands, at 516–517 and at 486 mμ (*H*). The spectrum thus resembled that of pectenoxanthin.

In contradistinction to this batch of animals another large red with brown containing much pigment gave very different results. Nearly all the pigment was epiphasic. No carotenes, xanthophylls or xanthophyll esters were detected, and on hydrolysis of the epiphasic pigment the free acid of an acidic carotenoid was obtained as the sole pigment. It was more soluble in 90 % methanol than in petroleum ether; adsorbed only slightly by calcium carbonate, but very strongly and permanently by magnesium oxide. In carbon disulphide the cherry-red solution showed bands at 513 (*H*) or 512 mμ (*HF*). In pyridine the band was at 501 (*H*) and 498 mμ (*HF*). It is remarkable that the pigment in this case would therefore appear from its spectrum to be astacene. Only small traces of free acidic carotenoid were obtained from the hypophase.

Like simple red animals, the red with brown variety is rich in metridene esters. Small amounts of xanthophyll esters have however sometimes been found, and in one case the metridene was accompanied, or perhaps replaced, by astacene.

(9) *Summary of the carotenoids.*

Considering the various brown series it is self-evident that but little carotenoid is to be obtained from those varieties with no visible pink pigmentation. Yet some trace is always present and whether there is much or little pigment, the carotenoids which compose it vary in a quite remarkable way from one batch of animals to another, even when these are of the same apparent colour variety as seen with the naked eye. There is evidently no correlation between any particular set of carotenoids and any particular development of brown or black melanic pigmentation. The various carotenoids may or may not be superposed upon any of the melanic backgrounds just as ectodermal

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brown melanin and endodermal black melanin may occur together or exist independently. At the same time when we compare the variation in the kinds of carotenoid found in the white or red varieties with those found in the various brown ones, there appears to be no question that there is far greater carotenoid variety in the latter. Reds and whites are fairly consistent from one batch to another; browns vary almost individually. This suggests that there may be some correlation between those biochemical mechanisms responsible for melanin formation and those that result in the formation of various carotenoids. This must however only be taken as a suggestion because the number of varieties is so great and their availability in the field often very limited, so that we cannot say for certain that investigation of fresh batches would not bring to light fresh carotenoid pigments even among the white and simple red varieties.

The carotenoids of the varieties are summarized in the following table. The chief pigments are in italics.

TABLE 4

Colour	Relative quantity of carotenoid	Kind of carotenoid
White	Very little	*Astacene esters*
Red	Much	*Metridene esters* sometimes accompanied by astacene esters, xanthophylls and carotenes
Yellow-orange	Considerable	*Metridene esters*, carotenes, xanthophylls, *xanthophyll esters*
Brown (various shades)	Very little	Astacene esters, carotenes, xanthophylls, and xanthophyll esters in varying alternatives and varying relative quantities
Red with brown	Much	*Metridene esters*, or astacene esters

Other lipoids in Metridium senile

During the recovery of carotenoids from anemone tissues, considerable quantities of sterols and other lipoids were encountered in the extracts. The presence of sterols in the non-saponifiable fraction of pigment extracts often interfered to some extent with studies on the pigments themselves, especially when the latter occurred in small quantities.

The anemone sterols were readily crystallized from hydrolysates, and gave positive colour reactions with the Liebermann-Burchard reagent (acetic anhydride and concentrated sulphuric acid). Our sterol material differed from cholesterol in not readily yielding a precipitate with digitonin, and in exhibiting a saturated character in its failure to absorb bromine from acetic acid solution.

We were constantly impressed with the far greater degree of contamination by sterols in saponified pigment extracts from carotenoid-poor variants, such as browns and whites, than in similar preparations from the carotenoid-rich forms. Because of this, it seemed desirable to make some quantitative biochemical comparisons between

a number of representative colour variants by determining water content, crude lipoid content and by resolving the latter into proportionate quantities of sterols (non-saponifiable matter) and mixed organic acids.

Water content was determined by first stimulating the animals to assume a state of contraction, then wiping away the water adhering to exposed surfaces, weighing, and finally drying to constant weight in an oven at a temperature of 110° C. Crude lipoids (chiefly organic acids, sterols and perhaps resistant esters of the latter) were determined in the following way. The tissues, whether fresh or dried for water-content determination, were covered with concentrated potash solution and heated on a steam bath for from 6 to 8 hr. to assure complete digestion of all solid material. The digest was then diluted, neutralized or slightly acidified with concentrated hydrochloric acid, and all material soluble in diethyl ether was extracted by repeated washings. It was necessary, after this, to filter the ethereal extract through anhydrous sodium sulphate to remove small amounts of black insoluble polymerized material from suspension and to effect partial dehydration of the solvent. The extract was finally transferred to a clean, weighed flask, the ether distilled off, and the residue rendered free of remaining traces of water by drying in an oven at about 100° C. Such flasks with their final oily, water-free residues were allowed to cool to room temperature in a desiccator, and were then weighed to 1 mg.

In order to fractionate the lipoids into organic acids and sterols (total non-saponifiable matter), the crude residues were heated in a steam bath for 8 hr. in the presence of excess concentrated potash in alcohol to hydrolyse any resistant sterol esters of fatty acids which might have been present. After dilution of the hydrolysate, the sterols were extracted from the alkaline liquor by repeated washing with ether. Following the completion of this operation, the aqueous alkaline liquor was acidified with hydrochloric acid; the free organic acids were then removed by repeated ether extractions. Each of the ethereal extracts was washed a few times with water (carefully, to avoid formation of emulsions). The solutions were transferred to tared flasks, the solvent distilled off and the respective residues were dried and weighed. At the final stage, sterol residues contained crystals in the form of glistening flattened rosettes in a pale yellow waxy solid film of amorphous substance; the organic acid residues, on the other hand, were dark, red-brown semi-solid or viscous fluid masses.

Table 4 giving the results of the analyses, shows a close agreement in water content and relatively little variation in crude lipoid values throughout a whole series of colour varieties. Sterol contents show a rather striking constancy, while the same applies to total organic acids with the exception of two analyses of animals which were rather rich in carotenoids (H_1; R). Considering differences in age, size and possible variations in sex and maturity in the material with which we were dealing, we cannot attempt any correlation between carotenoid content and organic acid content. Furthermore, another set of carotenoid-rich animals (K_1) identical with those of the R analysis, showed a value above the general average in organic acid content.

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This investigation shows that the chief lipoid constituents are surprisingly uniform in the different varieties, despite their great differences in carotenoid content. It must be remembered that the absolute concentration of carotenoids in the tissues is very small even in highly coloured animals. The constancy of the sterol content shows that the persistent sterol contamination of white and of brown varieties is not due simply to excessive amounts of sterols in these as opposed to the red variety.

TABLE 5

Serial no.	Colour variant	No. of animals	Wet wt. g.	Dry wt. g.	Water %	Crude lipoids Wt. g.	Crude lipoids %	Sterols Wt. g.	Sterols %	Organic acids Wt. g.	Organic acids %
1 (B_1)	Olive-brown column, brown capitulum and tentacles, with a little black; white stomodaeum; very little carotenoid	2	105·2	20·7	80·35	2·007	1·91	0·394	0·375	1·402	1·33
2 (C_1)	Warm brown column, brown tentacles; pale red stomodaeum; considerable carotenoid	1	87·9	14·0	84·1	1·452	1·65	0·274	0·31	1·085	1·24
3 (F_1)	Yellowish pink, and some pale brown; pink gonads (?). Much carotenoid	1	126·3	20·9	83·45	2·016	1·60	0·438	0·35	1·401	1·11
4 (G_1)	Pink scapus, white capitulum and tentacles; pink stomodaeum. Much carotenoid	2	56·5	9·7	82·85	0·920	1·63	0·171	0·30	0·647	1·15
5 (H_1)	Very pale pink column; strongly pink tentacles; considerable carotenoid	2	96·6	17·8	81·6	1·377	1·42	0·314	0·325	0·912	0·945
6 (K_1)	Red-orange throughout. Very much carotenoid	2	105·9	18·9	82·15	1·727	1·63	0·379	0·36	1·357	1·28
7 (W)	White; pinkish gonads (?). Very little carotenoid	3	315	—	—	5·791	1·84	1·322	0·42	4·157	1·32
8 (R)	Red-orange throughout. Very much carotenoid	4	100	—	—	1·505	1·505	0·382	0·38	0·97	0·97
	Average %				82·31		1·65		0·35		1·17

THE SIGNIFICANCE OF THE COLOURS IN *METRIDIUM*

As distinguished by the eye, the colour varieties of *Metridium* depend on the presence, absence or superposition of three pigment main systems; ectodermal brown melanin, endodermal black melanin, and red to yellow lipochrome. Our investigations have shown that the lipochrome includes several extractable carotenoids. Some of these have little influence on the appearance of the animal, and the assortment of carotenoids present varies considerably from one lot of *Metridium* to another. There is some evidence that the appearance of a variety of xanthophylls and carotene is more common in varieties which develop melanin, but there is no obvious correlation between the presence of a particular assortment of carotenoids and a particular distribution of melanin pigments. Indeed each melanin or carotenoid pigment appears to be able to occur independently of the others in one or other variety, and the distribution of the pigment systems indicates that they can be inherited independently.

The colour of anemones does not depend directly upon food, though M. and R. Abeloos-Parize (1926) showed in *Actinia equina* that the food could influence the colour. They found in this species a red or orange pigment in fine granular form in the ectoderm of the body and tentacles, and a green pigment similarly dispersed in the endodermal tissues. From the brown and the green varieties they recovered an orange carotenoid, and a red one from the red animals (cf. Fabre and Lederer 1934). The Abeloos-Parizes found that animals raised from the eggs upon a diet free of carotenoids lacked carotenoid pigmentation when grown. Also, starved animals regenerated the pharynx and tentacular cycles with only a very feeble carotenoid colouring. Such specimens with carotenoids reduced or absent rapidly recovered the normal pigmentation when fed on a carotenoid-rich diet of shrimps' eggs; furthermore, the regenerating 'browns' and 'greens' reformed their orange pigment, while former 'reds' fed on identical diet recovered their red colour. The work of these investigators indicates two things. First, that in this species the carotenoids are derived ultimately from carotenoids in the diet, and secondly that there is an important specificity either in the primary selection of a given pigment, or in the metabolism of the pigments selected by each variety.

In *Metridium senile* we find, in agreement with Elmhirst and Sharpe (1920, 1923) that the colours of the varieties are much more stable than those of *Actinia equina* seem to be. Though controlled feeding experiments have not been completed, the lipochrome colour of the *Metridium* varieties does not appear to change over long periods under various conditions of feeding and starvation. Whether this indicates ability in the varieties of *Metridium senile* to construct their carotenoids from simpler substances remains to be seen. There is no doubt that the melanic colours are stable and that the animal constructs them itself. Thus it is clear that though the colours of anemones may vary under certain conditions, the colour varieties correspond to underlying physiological differences and are not simply the result of differences of food.

If the pigmentary systems of *Metridium* depend upon the genetical constitution, what function, if any, can be applied to them? Walton (1911) classified the colouring of anemones under warning, aggressive, protective and physiologically significant colours; though any particular case might partake of more than one of these classes. It is hard not to believe that the colour and pattern of some anemones such as *Cereus pedunculatus* is of protective or aggressive value, and Walton cites observations which seem to support this. Nevertheless it must be remembered that we judge these supposed adaptations by our own senses and there is yet little experimental evidence to show that any potential enemy or prey appreciates them as we do; and, as M'Intosh (1901) has pointed out, there are difficulties in supposing the coloration of many anemones and other marine animals to be protective.

Walton's classification assumes that colour is always a functional adaptation to external or internal environment. But there can be no doubt of the essential soundness of Poulton's (1890) original division of animal colours into (1) non-significant colours

and (2) significant colours. The latter he divides again into colours of direct physiological value and a series of categories, concerned with adaptation to the external environment: protective and aggressive resemblance, mimicry, warning colours, and colours displayed in courtship. It is hard to include the colour varieties of *Metridium* in these last categories, especially when it is remembered that all the varieties may occur side by side.

Metridium certainly possesses 'colours' of direct physiological value in Poulton's sense. The haematins are directly concerned in respiration, and perhaps the uric acid of the tentacles is concerned in excretion. But the effect of these pigments on coloration is negligible or slight. It has, however, been suggested that the chief pigments of anemones may serve to screen the tissues from excess light (Stephenson 1935). Elmhirst and Sharpe (1920, 1923) investigated the correlation between the light intensity of the environment and the degree of pigmentation in anemones under both natural and artificial conditions. They concluded that *Actinia equina*, *Anemonia sulcata* and *Tealia felina* increase their lipochrome and haematin content when in a region of greater light, and vice versa. Walton (1911) had noted that *Actinia equina* from well-illuminated situations were dark red while those in the shade were lighter, or of the green variety. In the case of *Anemonia sulcata* Elmhirst and Sharpe's results were perhaps the result of the known variation of the population of symbiotic algae with the light intensity. But this cannot be the case in *Actinia equina* which possesses no algae.

In *Metridium* (*Actinoloba*) Elmhirst and Sharpe found no changes of pigmentation under varied light conditions. But it is unquestionably sensitive to illumination, which causes the column to shorten (Parker 1919). Whether the sensitivity differs among the colour varieties has not yet been tested. Fleure and Walton (1907) state that in *Tealia* those specimens with white tentacles tend to be more sensitive to light than those with dark red ones. Even if some parallel behaviour to this should emerge in *Metridium*, it does not affect the distribution of the animals. If anything, the white animals are more abundant at the higher and more illuminated levels of the shore.

This brings us to the possibility that the colours of *Metridium* are non-significant in Poulton's sense. As Sumner (1937) says, much of the colouring of animals may be a mere by-product of their metabolism, having no relation to ecological needs. The colours may be incidental properties of molecules concerned with other biochemical events. It is significant that in the colour patterns of anemones the pigments tend to accentuate morphological axes of differentiation (Stephenson 1928). The processes which produce the one may be those that produce the other.

On this view the colour varieties of *Metridium* are comparable to random mutations in systems serving other biological ends than coloration. In some anemones the resulting colours may be utilized as an adaptation, though the idea of adaptation must be qualified when it concerns simple physiological processes (Pantin 1932). Whether or not this proves to be true the fact remains that the Actinozoa are the most

brilliantly coloured of all animals, and it seems at first sight against probability if this occurrence of bright colours in so many individual species and varieties is not correlated with some unique metabolic characters of the group. But let it be remembered that it is the brilliance of the colours and not their presence which is the feature of these animals. Some tissues of almost all animals contain bright pigments, and colourless animals are the exception. Among the higher animals, however, the exterior coloration is usually arranged in a drab protective pattern in adaptation to the environment; though the component pigments may be bright. Many physiologically important substances produced by animals happen to be brightly coloured. Natural selection usually arranges those which contribute to the external appearance in a drab pattern, though it sometimes does otherwise as in warning coloration.

If selection is weak, as it appears to be between the colour varieties of *Metridium*, we have no right to suppose that this will entail absence of colour. It is rather that the natural production of various coloured substances along various morphological axes will not be checked and many bright varieties will appear. The vivid colours of the anemones may signify freedom from certain environmental restraints, rather than any functional adaptation.

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