3. An increase in the rate of decomposition of hydrogen peroxide was observed on adding small amounts of manganous sulphate to horse-radish extracts. This effect was attributed to manganese oxidation.

4. The manganese oxidizing system in horse-radish extract consists of a thermolabile and a thermostable factor together with hydrogen peroxide. Partially purified peroxidase preparations, in the presence of certain peroxidase substrates and hydrogen peroxide, oxidize Mn^{++} . It is suggested that the thermolabile and thermostable factors in

the extract are peroxidase and peroxidase substrate respectively.

5. The hypothesis is advanced that the manganese reduces the oxidized peroxidase substrate and thereby is itself oxidized. This involves the substrate in a cycle of oxidation and reduction. At low hydrogen peroxide concentrations, the manganese oxidation product may react with plant metabolites other than hydrogen peroxide; this would involve the manganese in an oxidation-reduction cycle, which may explain its effect on plant respiration.

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The Biochemistry of Locusts

1. THE CAROTENOIDS OF THE INTEGUMENT OF TWO LOCUST SPECIES (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F. AND *SCHISTOCERCA GREGARIA* FORSK.)

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A research programme on the biochemistry of locust pigmentation, with special reference to the problem of swarming, has recently been initiated. An essential preliminary to the study of carotenoid metabolism in these insects (Goodwin, 1949) was the identification of the pigments concerned. Lederer (1935), quoting unpublished work carried out by Volkonsky and himself, reported the presence of a mixture of xanthophylls and carotene in adult *Schistocerca gregaria* Forsk.; Chauvin (1941) noted an unidentified pink pigment in hoppers and a mixture of α - and β -carotenes in adult insects of the same species. No data on *Locusta* species have been recorded.

We have found that hoppers and adult insects of both *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* yielded, on extraction with acetone after removal of the alimentary tract, a mixture of two carotenoids. These pigments have been identified as β -carotene and astaxanthin. Small amounts of lutein (xanthophyll) and chlorophyll were occasionally encountered, but they probably originated in small amounts of unremoved alimentary tract or in small pieces of grass which can become tightly lodged in the buccal cavity and the claws of the forelegs.

The pigments were identified by their chromatographic and spectrographic properties; insufficient

material was available to make crystallization feasible. The most rigid chromatographic criterion applied was that of the mixed chromatogram. When the suspected β -carotene or astaxanthin fraction was admixed with an authentic sample of the corresponding pigment (crystalline β -carotene or astaxanthin isolated from hypodermis of lobster (*Homarus vulgaris* H. M. Edwards) and prawn (*Nephrops norvegicus* L.), and the mixture chromatographed on an appropriate adsorbent, no separation was obtained. The absorption spectra of the purified fractions were compared with those of the authentic pigments over the range 550–400 m μ . in a variety of solvents; in all cases the spectra were almost exactly superposable. In investigations in the carotenoid series, in which the absorption maximum of different components can differ only slightly, it should be emphasized that it is only when the complete absorption curves obtained in different solvents are compared that identification becomes unequivocal; mere recording of maxima is often insufficient.

Astaxanthin was further characterized by its reaction with potassium butoxide *in vacuo* to form a bluish purple enolic salt which on the admission of air is almost instantaneously converted into the orange-coloured astacin (3:4:3':4'-tetraketo- β -carotene; Kuhn & Sørensen, 1938). Further, treatment of the locust astaxanthin with alkali yielded a product which was chromatographically and spectroscopically identical with astacin produced in a similar way from lobster astaxanthin (Kuhn & Lederer, 1933). Almost all the astaxanthin isolated from locusts was unesterified.

A large number of eyes and wings of *Locusta* and *Schistocerca* were examined separately for carotenoids. All were found to contain β -carotene and free (unesterified) astaxanthin. The astaxanthin in

the wings occurs in combination with a protein, but in all probability β -carotene does not.

Examination of the fatty tissues, blood and eggs of *Schistocerca* and *Locusta*, revealed the presence of β -carotene only; neither α -carotene nor any xanthophylls could be detected.

Finally, advantage was taken of the availability of a large number of live locusts to determine whether or not they produce vitamin A. Using the most rigid tests for detecting vitamin A, it was never possible to demonstrate the presence of the vitamin in either species.

EXPERIMENTAL

Extraction, separation and identification of the carotenoids of Locusta and Schistocerca

The locusts were killed by cutting off the head just behind the pronotum; the thorax and abdomen were then opened ventrally and the fatty tissues and internal organs removed and discarded. The remaining tissues were thoroughly ground up with acid-washed silver sand and a small amount of anhydrous Na_2SO_4 and extracted 3–4 times with acetone. The combined acetone extracts were concentrated at the pump at a temperature not exceeding 35°; the final traces of acetone were removed at a slightly higher temperature in a stream of N_2 . The residue was then dissolved in light petroleum (b.p. 40–60°) and chromatographed using defatted bone meal as adsorbent (Mann, 1943; Goodwin & Morton, 1946; Glover, Goodwin & Morton, 1947). Elution with light petroleum removed the hydrocarbon carotenoids (fraction A) and the xanthophylls were subsequently removed from the column with acetone (fraction B).

Fraction A was found to have an absorption spectrum identical with that of β -carotene. Its identity with this pigment was confirmed by admixing a portion with a solution containing an approximately equal amount of crystalline β -carotene and chromatographing on activated alumina (Grade O, Peter Spence and Co.); even on slow development down a long column (20–30 cm.) no separation into two fractions could be obtained.

Fraction B was examined in one of three ways:

(a) The fraction was dissolved in light petroleum and partitioned between this solvent and 90% (v/v) aqueous methanol. The epiphase contained very small amounts of a yellow pigment whilst the main fraction was hypophasic and coloured orange red.

The epiphasic layer was examined spectroscopically and chromatographically; from this examination the presence of small amounts of lutein (xanthophyll) esters was indicated. The identification was not pursued further.

The orange-red methanol solution was diluted with 3 vol. of water and the pigment extracted with light petroleum. The extract, when washed free from acetone with water and dried over anhydrous Na_2SO_4 , was chromatographed either on a column of deactivated alumina or CaCO_3 . (Deactivated alumina was prepared by mixing activated alumina (Peter Spence and Co.) with 10% of its weight of methanol, allowing to stand for 1 hr., filtering the methanol off and drying at room temperature.) Using either adsorbent the pigment was adsorbed as a red zone at the top of the column; elution with ethanol removed a very small amount of brown material which was probably esterified astaxanthin. The

main pigment was eluted with a 2% (v/v) solution of glacial acetic acid in ethanol; this is a characteristic property of astaxanthin (Kuhn & Sørensen, 1938). A very small amount of pink material, which remained on the column, could be eluted with ethanol containing 2.5% (w/v) of KOH; this, according to Kuhn & Sørensen, is a characteristic property of astacin, and its presence in our extracts was probably caused by oxidation of astaxanthin during the working-up process, for astacin does not occur in nature (Kuhn, Stene & Sørensen, 1939).

The glacial acetic acid solution of astaxanthin was diluted with an equal volume of water, and the pigment extracted with ether; this extract was then identified as described below.

Fig. 1. The absorption spectra of astaxanthin obtained from locusts (—) and from lobsters (—x—x—x—); solvent, CS_2 . Scales for the extinctions (E) have been chosen so that the ordinates representing E_{max} for the two samples are equal.

(b) The extract was dissolved in light petroleum and chromatographed directly, using deactivated alumina as adsorbent. Small amounts of lutein (xanthophyll) were eluted with light petroleum containing 4% (v/v) of acetone; the astaxanthin was then separated and eluted as described under (a) above.

(c) The light petroleum solution was extracted with a 1% (w/v) solution of Na_2CO_3 . This removed astaxanthin from the petroleum phase and at the same time converted it into astacin (Kuhn & Sørensen, 1938). The Na_2CO_3 solution was acidified with acetic acid and the astacin extracted with ether. After removing the ether, the residue was dissolved in light petroleum containing a small amount of ether and chromatographed on deactivated alumina. The pigment was purified by eluting with ethanol containing 2.5% (w/v) of KOH.

The pigment isolated by procedures (a) and (b) was considered to be astaxanthin and that by procedure (c) astacin produced from astaxanthin. Further proof of their identity

was obtained: (i) by comparing their absorption spectra with those of authentic samples of astaxanthin and astacin obtained from marine Crustacea (Figs. 1 and 2); (ii) by failure to separate chromatographically a mixture of 'locust' and 'lobster' astaxanthin or astacin into two components; and (iii) by the conversion of astaxanthin into a purple potassium (enol) salt which was rapidly oxidized to astacin in the presence of O_2 . To prepare the purple K salt a solution of the pigment in light petroleum was placed in a filter flask closed with a rubber bung from which was suspended by thread a small test tube containing K butoxide. The flask was evacuated to about 1 mm. Hg; the light petroleum was evaporated and the pigment remained as a thin smear on the

Fig. 2. The absorption spectra of astacin obtained from locusts (—) and from lobsters (—x—x—x—); solvent, CS_2 . Scales for the extinctions (E) have been chosen so that the ordinates representing E_{max} for the two samples are equal.

bottom of the flask. The flask was then tilted so that the K butoxide poured on to the pigment; immediately a deep purple coloration was produced. On admitting air this immediately turned orange; this orange pigment possessed all the chromatographic and spectroscopic properties of astacin prepared from lobster astaxanthin.

When free and esterified astaxanthin are partitioned between light petroleum and 90% (v/v) aqueous methanol the free form accumulates in the methanol layer (hypophase) and the esterified pigment in the light petroleum layer (epiphase; Kuhn, Lederer & Deutsch, 1933). All the astaxanthin fractions isolated in this investigation were almost completely hypophasic in this test, indicating that the free pigment predominated.

When locust eyes were examined they were treated in the same way as the integuments; the wings, however, were subjected to different treatment.

Examination of locust wings. Various samples of wings from *Locusta*, *Schistocerca gregaria* and one sample of wings

of red locust (*Nomadacris septemfasciata* Serville) were examined in the following way. The wings were placed in a Waring mixer and extracted for 10 min. with light petroleum (b.p. 40–60°); the light petroleum was filtered at the pump through a G 4 sintered-glass filter and examined chromatographically and spectroscopically. The fraction contained only one pigment, β -carotene. The wing residue was divided into two portions, one of which was extracted with acetone in the Waring mixer. The acetone fraction was dark orange in colour and when tested was shown to contain only unesterified astaxanthin. This was good presumptive evidence that the astaxanthin was held attached to a protein.

Consequently, the second portion of the wings was extracted with about 150 ml. water containing two drops of NH_3 solution (sp.gr. 0.88) in the Waring mixer. The resulting mush was centrifuged and the dark brown supernatant liquid decanted off. Addition of solid $(\text{NH}_4)_2\text{SO}_4$ to saturation threw down a brown protein, leaving the supernatant liquid a pale yellow colour. The protein was centrifuged off and redispersed in a small volume of water. Shaking this solution with light petroleum extracted a small amount of β -carotene. After the protein pigment complex was broken down by the addition of an equal volume of ethanol the pigment was easily extractable with light petroleum. It showed all the properties of free astaxanthin. The wing tissues remaining after extraction with water were extracted with acetone; a small amount of astaxanthin was obtained, probably owing to the presence of unextracted protein. It is important to note that in similar tests with locust integuments it has not yet been possible to detect such an astaxanthin complex.

Body fat and eggs. Samples of body fat and eggs were ground up with anhydrous Na_2SO_4 and the pigment extracted with acetone. On transferring the pigment to light petroleum and chromatographing on activated alumina only one pigment was detected. Spectroscopic data and a 'mixed' chromatogram indicated that the pigment was β -carotene.

Extraction of astaxanthin from lobsters. It was necessary to obtain authentic specimens of astaxanthin with which to compare the locust pigment; lobsters were considered the best source. The methods of extraction are described in a subsequent communication (Goodwin & Srisukh, 1949).

Blood. About 1 ml. of blood was obtained from about fifty immature adult *Locusta*. It was examined according to the method of Glover *et al.* (1947). The only pigment obtainable was β -carotene which was identified in the usual way. The concentration of β -carotene in the blood was approx. 600 $\mu\text{g.}/100\text{ ml.}$ A similar result was obtained with *Schistocerca* blood, but the β -carotene content was considerably higher, approx. 3000 $\mu\text{g.}/100\text{ ml.}$

Tests for the presence of vitamin A in Locusta and Schistocerca

A number of acetone extracts of whole *Locusta* and *Schistocerca* were prepared as previously described. After removal of the acetone the extracts were dissolved in light petroleum and chromatographed on defatted bone meal according to the method of Glover *et al.* (1947). Two fractions were obtained, one of which was eluted with light petroleum and which should have contained any esterified vitamin A, and one which was eluted with acetone and should have contained any free vitamin A. The solvents were removed from the two fractions and the residues made up in cyclohexane and examined in two ways: (a) the absorption

spectrum of the colour produced by reaction of the extract with a CHCl_3 solution of SbCl_5 was examined in the Hilger-Nutting visual spectrophotometer to determine the wave length of the absorption band, and in the Beckman quartz photoelectric spectrophotometer for the measurement of the extinction at 617 $\text{m}\mu$. (i.e. at the λ_{max} for the SbCl_5 -vitamin A colour test). In no case was an absorption band noted at 617 $\text{m}\mu$. nor was any absorption of a transitory nature observed at 617 $\text{m}\mu$.; in fact, it always increased considerably on standing. Thus, using the SbCl_5 test, no positive evidence of the presence of vitamin A, either in the free or esterified form, could be obtained.

(b) The direct absorption spectra of the cyclohexane solutions were recorded using the Beckman instrument. Considerable absorption existed at 328 $\text{m}\mu$., but when corrected for absorption not due to vitamin A, most of the curves were 'uncorrectable'; that is, in most cases the absorption calculated as due to vitamin A possessed a small negative value. Thus again no evidence for the presence of vitamin A could be obtained.

DISCUSSION

The present investigation has demonstrated the presence of β -carotene and astaxanthin in two species of locusts, *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.; β -carotene exists in the integument, wings, fatty tissues, eggs and eyes, and astaxanthin in the integument, wings and eyes.

Almost all the astaxanthin occurring in the integument is unesterified; it has not yet been possible to demonstrate, as in the case of the wings and lobster cuticle (Kuhn & Lederer, 1933), the presence of astaxanthin as a protein complex in the locust integument.

It is hoped to present in a future paper data concerning the quantitative distribution of β -carotene and astaxanthin in locusts during the various stages of development. As this survey will not include separate data on eyes and wings it is interesting to note here that the mean concentrations in the eyes and wings are, respectively, 13.9 and 5.1 $\mu\text{g.}/\text{g.}$ of β -carotene and 7.45 and 10.4 $\mu\text{g.}/\text{g.}$ of astaxanthin. Both the flying wings and the elytra have the same concentration of astaxanthin. Whilst the relative amounts of the two pigments in the eyes are very similar to those found in the integument of adults (Goodwin, 1949) astaxanthin tends to preponderate in the wings. From the figures just quoted it can be calculated that one pair of locust eyes contain 0.130 $\mu\text{g.}$ of β -carotene and 0.048 $\mu\text{g.}$ of astaxanthin; the corresponding values for one set of wings are 0.0255 and 0.356 $\mu\text{g.}$

The wing astaxanthin occurs in combination with a protein, but in all probability β -carotene does not, for when the ground tissue is extracted with light petroleum (b.p. 40–60°), a solvent which does not liberate carotenoids from their protein complexes, β -carotene, but not astaxanthin, is extracted. When

the residue is extracted in the water containing about 0.05 % of ammonia (sp.gr. 0.88) a brown solution is obtained from which a brown protein is precipitated at full saturation with ammonium sulphate. When the brown protein is dissolved in water and denatured by the addition of ethanol, astaxanthin, but not β -carotene, appears in a light petroleum extract. The attachment of astaxanthin to a protein of the wings of the red locust, *Nomadacris septemfasciata* Serville, has also been demonstrated. All these results confirm and extend the claims of Junge (1941) and Okay (1947) that a component of the colour complex of insects, in particular the wings, is an (unspecified) caroteno-protein; that Okay noted such a pigment in the acridid, *Acrida turrata* L., is of special interest. More recently, and following our preliminary notice of the presence of astaxanthin in locusts (Goodwin & Srisukh, 1948), Okay (1948) has stated that the carotenoid of the protein complex in a number of insects is astaxanthin.

The demonstration of the presence of β -carotene only in the fatty tissues and eggs of *Locusta* and *Schistocerca* confirms in general the previous statement of Chauvin (1941) that β -carotene exists in the eggs of *Schistocerca*; however, he also claimed to have detected small amounts of α -carotene. It is interesting to note that investigations on the body fat and gonads of another acridid, the grasshopper (*Melanoplus bivattus* Say), have produced similar results (Grayson, 1942; Grayson & Tauber, 1942).

The carotenoids in locust blood are interesting in two respects. (i) The carotene levels are extremely high compared with those usually obtained for mammals whose blood contains carotene; however, in the only other investigation of the carotene levels in insect blood, Palmer & Knight (1924) found the astonishingly high value of 13,600 $\mu\text{g.}/100\text{ ml.}$ in the lymph of the potato beetle, *Leptinotarsa decemlineata* Say. (ii) As the blood is bright green and carotene is the only pigment present it follows that it is highly likely that the carotene occurs as a protein complex.

Using the most rigid tests for detecting vitamin A, the antimony trichloride test and the correction procedure of Morton & Stubbs (1946), both carried out using a photoelectric spectrophotometer, it was never possible to demonstrate the presence of vitamin A in either species examined.

Brodskis (1944) and Brodskis & Rungs (1944) recently reported 'traces' of vitamin A in *Schistocerca*; it is suggested that, owing to the somewhat inadequate apparatus at their disposal, these investigators have been unable to distinguish between the colour produced by carotenoids with antimony trichloride and that produced by vitamin A.

The absence of vitamin A from locusts is not unexpected, for up to now the vitamin has never been unequivocally detected and is absent from *Taeniopoda auricornis* Walker (Giral, Giral & Giral, 1943); further, a number of insects can develop normally on diets from which vitamin A or carotene or both are completely absent (McCay, 1938; Bowers & McCay, 1940). The absence from locusts of vitamin A, coupled with the presence of astaxanthin, which is present in their eyes, strongly suggests that the pigment is the photoreceptor in locusts, for Wald (1943) has pointed out that astaxanthin is an almost universal photoreceptor in organisms which do not manufacture vitamin A.

SUMMARY

1. The carotenoids of the integument of the migratory locust (*Locusta migratoria migratorioides* R. & F.) and desert locust (*Schistocerca gregaria* Forsk.) are β -carotene and astaxanthin; the latter occurs in the unesterified form.

2. Both pigments also occur in the eyes and the wings. Astaxanthin exists in the wings as a protein complex.

3. The presence of an astaxanthin protein complex has also been demonstrated in the wings of the red locust *Nomadacris septemfasciata* Serville.

4. Only β -carotene occurs in the fatty tissues, blood and eggs of both species. In the blood it is probably attached to a protein.

5. Preformed vitamin A does not occur in either *Locusta* or *Schistocerca*.

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Some Observations on Astaxanthin Distribution in Marine Crustacea

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During an investigation into the carotenoids of two locust species (Goodwin & Srisukh, 1949) it became necessary to obtain an authentic specimen of astaxanthin for comparative purposes; such a sample was obtained from the lobster (*Homarus vulgaris* H. M. Edwards). As the isolation of this material proceeded it became apparent that the experience of Kuhn & Lederer (1933) on the distribution of free and esterified astaxanthin was not being reproduced; consequently the distribution was reinvestigated in some detail. A similar investigation was carried out on the prawn (*Nephrops norvegicus* L.). At the time the lobsters and prawns were being examined an opportunity arose to examine the pigment of the small red copepod, *Tigriopus fulvus* Fisch. (Fraser, 1936).

EXPERIMENTAL

The first sample of astaxanthin used was obtained from old dry carapaces of lobster with which was mixed a small amount of carapaces of crab (*Cancer pagurus*). The carapaces were softened by placing in approx. 0.2N-HCl for 24 hr. to dissolve the CaCO_3 , filtered off, washed with water until free from acid, ground up in a mortar with acid-washed silver sand and anhydrous Na_2SO_4 and extracted 3 times with acetone at a temperature below 30° . The combined acetone extracts were concentrated at the pump, diluted with 2 vol. of water and extracted with light petroleum. When a small sample of the light petroleum extract was shaken with 90% (v/v) aqueous methanol the pigment was almost completely hypophasic; this indicated that the pigment was unesterified, for esterified astaxanthin is epiphasic in this test (Kuhn, Lederer & Deutsch, 1933). A small yellow residue in the epiphase showed no selective absorption in the region 400–450 m μ .

The light petroleum extract was then chromatographed using CaCO_3 (A.R.) as adsorbent. Development with light petroleum containing 4% (v/v) of ethanol resulted in the separation of three bands: (1) orange red—most strongly adsorbed, (2) reddish pink, and (3) yellow. The yellow fraction, which was only small, was discarded; it was the

same pigment as that found in the epiphase in the partition test. Absorption spectrum measurements in four solvents indicated that the orange-red pigment was astacin and the reddish pink pigment astaxanthin. Although the astaxanthin thus obtained was pure and was identical with the locust pigment, it was considered advisable to compare locust astaxanthin with astaxanthin prepared from fresh material. It was also important to examine fresh material because all our tests indicated that in the carapaces astaxanthin was present in the free form, although Kuhn & Lederer (1933) had claimed that the carapace pigment was esterified. In this experiment the presence of astacin in considerable amounts indicated that oxidative changes must have taken place in the stored carapaces and there was always the possibility that hydrolysis of the original ester (if ester were present) had also occurred.

Three fresh lobsters were killed by immersion in fresh water and the hypodermis removed carefully and as completely as possible from the carapace; the eggs of the two females and the hepatopancreases were also removed for examination.

The hypodermis on extraction with acetone in the manner just described yielded a pigment which was chromatographically homogeneous and epiphasic in a methanol-light petroleum partition. It was the astaxanthin ester previously described by Kuhn and his co-workers; its spectral characteristics were recorded using a number of solvents.

The carapace was divided into two portions. One portion was extracted with acetone after it had first been softened by standing overnight in 2N-HCl. The pigment extracted was not homogeneous; it consisted of two components, one epiphasic and the other, the major component, hypophasic. Neither component could be further fractionated by chromatography on weakened alumina (Goodwin & Srisukh, 1949). This experiment strongly indicated that the hypophasic fraction was obtained from the carapace itself and that the presence of a small amount of epiphasic pigment was due to contamination with small amounts of hypodermis. In order to confirm this, the second portion of carapace was allowed to stand for 48 hr. in 0.02N-HCl. This treatment softened the carapace and extracted a small proportion of the astaxanthin-protein complex but not the free pigment. The resulting clear blue solution was filtered off and some