4. At least 20–21 % of a dose of benzonitrile is excreted as ethereal sulphate. *o*- And *m*-cyanophenols were detected by paper chromatography in ether extracts of hydrolysed benzonitrile urine. In some experiments the administration of benzonitrile gave rise to the excretion of very large amounts of reducing material.

5. Qualitative examination of the excretion products obtained after administration of some possible metabolites of the tolunitriles has been made. *m*-Cyanobenzoic acid was excreted unchanged (66 % of dose). No glycine conjugation could be detected. *p*-Cyanobenzoic acid was excreted mainly un-

changed, but was conjugated to a small extent with glycine (50 and 2 % of the dose isolated, respectively). *p*-Cyanohippuric acid was excreted mainly as *p*-cyanobenzoic acid (50 % of the dose isolated); no glycine conjugate was detected. Phthalic anhydride and phthalic acid (7 and 86 % of the dose, respectively) were isolated after administration of phthalamic acid. Only unchanged terephthalamic acid (58 % of the dose) could be isolated after giving terephthalamic acid.

The micro-analyses were carried out by Drs Weiler and Strauss, Oxford, and by Miss B. G. Humphris.

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Biochemistry of Locusts

5. THE GREEN PIGMENT OF THE HAEMOLYMPH AND INTEGUMENT OF SOLITARY LOCUSTS (*LOCUSTA MIGRATORIA MIGRATORIOIDES*, R. & F., AND *SCHISTOCERCA GREGARIA*, FORSK.)

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In the solitary phase locusts are often green; this is not an essential characteristic of solitary insects because other colours, such as buff, are often encountered, but it is probably true to say that the green pigment does not occur in the integument of gregarious insects. Similarly, the haemolymph from solitary locusts is almost invariably bright green whilst that from gregarious locusts is golden-yellow; a light-green haemolymph is, however, very occasionally encountered in the latter. The present paper reports an investigation into the green pigment.

Green pigments are widespread in insects, but not a great deal is known about them. As early as 1882, Krukenberg considered that the green pigment of *Locusta* (*Tettigonia*) *viridissima* was different from chlorophyll although, later, Podiapolsky (1907) stated that the absorption spectrum of the pigment was somewhat similar to that of chlorophyll. Rearing experiments on a number of insect species showed, however, that the formation of the green pigment was independent of the amount of chlorophyll in the diet (Toumanoff, 1927; Giersberg, 1928); in particular, Faure (1932) noted that the produc-

tion of the green pigment in locusts depended on succulent green grass, but that the pigment was not chlorophyll. The first suggestion that the green pigments of insects, which in different species appear to be very similar, were heterogeneous was put forward by Przibram & Lederer (1933) who separated the green pigment of the haemolymph of *Dixippus morosus* into blue and yellow components; this has recently been confirmed by Junge (1941) and Okay (1945) in a number of insect species. Junge (1941), who gave the name 'insectoverdin' to the green complex, found that all the pigments he examined consisted of two chromoproteins, the prosthetic group of the blue component being a bile pigment and that of the yellow component either β -carotene or lutein. He considered the bile pigment to be an isomer of biliverdin and called it glaucobilin; Lemberg & Legge (1949) consider that this pigment is mesobiliverdin. Okay (1945), examining *Mantis religiosa* and many other Orthoptera, came to similar conclusions, but in some cases considered the yellow component to be a yellow water-soluble non-protein compound. He termed the bile pigment orthoptero-cyanobilin, which Lemberg & Legge (1949) suggest may be identical with mesobiliviolin.

Having reared solitary locusts (*Schistocerca*) to the fifth instar on a chlorophyll-free diet, Chauvin (1939) decided that the green pigmentation that he observed was either a relic of the chlorophyll present in the egg or a pigment synthesized *de novo*. Later, Chauvin (1941) considered that the green coloration was due to flavone (yellow) (responsible for the yellow coloration of adult gregarious *Schistocerca*) being so distributed on the chitin as to appear green. It has now been shown that the yellow coloration of gregarious *Schistocerca* is due to β -carotene and not to a flavone (Goodwin & Srisukh, 1950a).

EXPERIMENTAL

Collection and examination of haemolymph. The locusts were obtained from the Anti-Locust Research Centre where, in order to obtain them in phase *solitaria*, each was reared separately in a 1 l. cage. When received in this laboratory they were kept in a warm room with plenty of food (succulent grass) for some hours before the haemolymph was collected. Collection was carried out by piercing with a pin a small hole in the thin region of the integument just above the connexion between the hind-leg and the integument and then squeezing the insect gently but firmly until the lymph (about 2-3 drops/insect) dripped into a small test tube containing either distilled water or a phosphate buffer of pH 7; the insects quickly recover from this treatment. Invariably during the collection a small amount of bright-yellow coagulum was formed on the side of the test tube; the diluted haemolymph was either yellow or green.

The coagulum and solution were examined in the usual way for carotenoproteins (Goodwin & Srisukh, 1949); the solution was also examined for bile pigments by adding an equal volume of ethanol, removing the carotenoids by ex-

traction with light petroleum, acidifying with HCl, and extracting with ethyl acetate. Insectorubin was then tested for in the aqueous phase (Goodwin & Srisukh, 1950b).

The green pigment of the integument. The insects were killed, the fat bodies removed and the green portions of the integument separated from the rest. The green pigment was extracted from the tissues by grinding with sand and a very small volume of water. The mixture was then centrifuged and the residue, which contained free carotenoids and insectorubin, was discarded. The supernatant liquid was examined as follows: addition of $(\text{NH}_4)_2\text{SO}_4$ to half saturation yielded a brown precipitate which was filtered off and discarded. If the filtrate was found to contain combined carotenoids these were removed and then one-tenth the volume of conc. HCl was added and the solution (A) exhaustively extracted with ethyl acetate. The ethyl acetate was washed with a little water and then distilled off *in vacuo*; the residue was a small volume of a deep-blue aqueous-HCl solution from which the pigment only slightly soluble in pure ether was extracted with a mixture (1:1) of ether and methanol. The pigment was then purified by chromatography on acid-washed talc (Goodwin & Srisukh, 1950b); it was strongly adsorbed near the top of the column as a bright-blue zone and elution with water followed by methanol removed traces of a yellow impurity; the purified pigment was then eluted with methanol containing 5% (v/v) of conc. HCl and its properties examined in as much detail as was possible with the very small amounts available. The residual aqueous solution (B) was concentrated *in vacuo* and the resulting material chromatographed on talc. A very small bluish-grey zone was obtained, obviously due to traces of the pigment just discussed, but elution with water separated a very small quantity of a thermostable pigment.

Most of the experiments were carried out on solitary *Locusta*, but a few experiments with *Schistocerca* gave identical results.

RESULTS

Haemolymph. The absorption spectra of typical samples of haemolymph from solitary and gregarious locusts are recorded in Fig. 1. It will be seen that they differ quite strikingly in that the green *solitaria* haemolymph exhibits a very sharp band at 673 m μ ., which almost completely disappears on standing. On standing overnight haemolymph from both phases darkens, that from *solitaria* being slightly bluer than that from *gregaria*. This darkening is almost certainly due to the action of tyrosinase for such an action has previously been noted by Dennell (1947) in isolated haemolymph of a number of insect species. The slight blue appearance assumed by aged *solitaria* haemolymph may be due to the presence of small amounts of blue pigment, although most appears to be destroyed. In both phases the haemolymph contains considerable amounts of a carotenoid attached to a protein, but no free carotenoids. The carotenoid was identified as β -carotene in both cases; no astaxanthin was detected. Most of the β -carotene occurred in the yellow coagulum which was always formed when lymph was being collected.

The *gregaria* haemolymph proteins yielded no insectorubin or blue pigment, whilst the solitary

haemolymph contained a blue pigment but not insectorubin. This was obtained only in minute amounts, but appeared to be identical with the blue pigment extracted from the integument.

Fig. 1. The absorption spectrum of locust haemolymph. —, haemolymph from solitary locusts—fresh; ---, haemolymph from solitary locusts—after standing 24 hr.; - · - · -, haemolymph from gregarious locusts.

The green pigment complex of the integument of solitary locusts. This pigment, which is water-soluble and shows an absorption spectrum ($\lambda_{\max}$ 673 m μ .) very similar to that of the green *solitaria* haemolymph (see Fig. 1), can be separated into two major fractions: (1) yellow, soluble in ether; and (2) blue, soluble in ethyl acetate; a third and minor yellow fraction soluble in water also exists.

The ether-soluble fraction contained a mixture of two carotenoids, attached to protein. After chromatographic separation of these pigments they were identified as β -carotene and free (unesterified) astaxanthin by their chromatographic, spectrographic and partition behaviour.

After chromatographic purification, the blue ethyl acetate-soluble pigment exhibited, in different solvents, the spectra recorded in Fig. 2. When dissolved in methanol and treated with methanolic zinc acetate the solution became green and exhibited a spectrum with maxima at 625 and 685 m μ . (Fig. 3); addition of iodine alters the spectrum considerably and only one band (at 632 m μ .) is observed (Fig. 3). These and other properties recorded in Table 1 make it extremely likely that the blue pigment is mesobiliverdin. The properties of authentic mesobiliverdin are also shown in Table 1 for comparison.

The water-soluble material occurs in amounts much too small for full study. It is heat-stable, does

not occur as a protein complex, it fluoresces bluish-purple in ultraviolet light, retains its fluorescence in 0.1 N-sodium hydroxide and glacial acetic acid and on the addition of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), and

Fig. 2. The absorption spectrum of the bile pigment (probably mesobiliverdin) present in the integument of green solitary locusts as the prosthetic group of a blue chromoprotein. —, in dioxan; - · - · -, in methanol containing 5% (v/v) of conc. HCl.

Fig. 3. The absorption spectrum of the zinc acetate bile pigment complex. - · - · -, before the addition of I_2 ; ---, after the addition of I_2 .

exhibits an absorption spectrum with a band at 282 m μ . It appears to be very similar to the pterin-like pigment present in the eyes of locusts (Goodwin & Srisukh, 1951).

Table 1. *A comparison of the properties of the blue pigment obtained from green locusts and of mesobiliverdin*

Property	Locust pigment	Mesobiliverdin*
Absorption maxima in methanol containing 5% (v/v) conc. HCl	367, 647 m μ .	363, 670 m μ .
In neutral solvents (ether, dioxan)	636 m μ .	635 m μ .
Stability in conc. H ₂ SO ₄ at 100°	Stable for at least 15 min. then gradually changes to green	Stable
Gmelin reaction	Positive	Positive
Diazo reaction	Negative	Negative
Complex with zinc acetate		
Colour	Green	Green
Absorption maxima	625, 685 m μ .	685 m μ .
Fluorescence	Nil	Nil
Addition of I ₂ to complex		
Fluorescence	Pink	Red
Absorption maximum	632 m μ .	630 m μ .

* Lemberg & Legge (1949).

DISCUSSION

The green pigments of the haemolymph and integument of solitary locusts are undoubtedly chromoprotein complexes of the insectoverdin type (Junge, 1941). They differ from each other in one major respect, for although they both have the same blue component (mesobiliverdin-protein complex), the yellow component of the haemolymph yields on denaturation only β -carotene whilst that of the integument yields both β -carotene and free astaxanthin. This indicates either that the integument contains two yellow chromoproteins or that β -carotene and astaxanthin are attached to the same protein. Although no evidence is available to settle this point, the first possibility is, on general grounds, more likely. The small amounts of yellow pterin-like pigment which are also present in the extracts, can make little contribution to the final colour of either the haemolymph or the integument. Chauvin's (1941) claim that the green pigment of the integument is a flavone cannot, on the basis of the present work, be accepted.

The absence of astaxanthin from the haemolymph, but its presence in the integument, seems to offer further proof of the conversion of β -carotene, and not xanthophylls, into astaxanthin in locusts, a fact previously noted in developing locust eggs (Goodwin, 1949). It also appears that the site of conversion in the insect is the integument itself.

The data recorded in Table 1 make it reasonably certain that the bile pigment in green locusts is that noted by Junge (1941) in a number of other insects, namely mesobiliverdin. The only differences in properties between our pigment and Junge's are (a) that his appeared to be less stable in concentrated

sulphuric acid at 100°, and (b) that his could not apparently be extracted from ether with *N*-hydrochloric acid; in spite of these minor differences, it appears that our pigment is the same as that investigated by Junge.

The only point at which we diverge from the recorded data for mesobiliverdin is the position of the absorption maximum of the long-wave band when the solvent is acid methanol. We find $\lambda_{\max}$ to be at 647 m μ ., whereas Pruckner & Stern (1937) record a peak at 670 m μ .

As carotenoproteins are present in both gregarious and solitary insects a characteristic difference between the two phases is thus the production of mesobiliverdin in solitaries. This cannot be accepted as a hard and fast differentiation because very occasionally green haemolymph (but not green integuments) are encountered in gregarious insects and solitaries are not always green. The production of the green pigment is always accompanied by a failure to produce integumental melanin, another characteristic of solitary hoppers (Goodwin, 1950), and it is interesting to note in this connexion that the change in isolated haemolymph of insects to a grey-black colour occurs in both solitary and gregarious haemolymphs. This suggests that solitary hoppers are still potentially capable of producing melanin and that their failure to do so is due rather to an inhibition of tyrosinase than to a complete disappearance of the enzyme.

Insectorubin could never be detected in the haemolymph and this strongly suggests that it is not synthesized in the gut but in the integument from a precursor present in the haemolymph. This observation also provides further proof against Chauvin's (1941) idea that this pigment is absorbed by the insect from its food.

SUMMARY

1. The haemolymph and the green parts of the integument of solitary locusts contain typical insectoverdins which can be resolved into yellow and blue components which are chromoproteins.

2. The prosthetic group of the blue chromoprotein is in each case probably mesobiliverdin. The yellow component of the haemolymph is a β -carotene-protein complex whilst that of the integument contains both β -carotene and free astaxanthin; this probably means the co-existence of two yellow chromoproteins in the integument, although the

possibility of both carotenoids being attached to the same protein has not been ruled out.

3. A small amount of a yellow water-soluble pterin-like material also occurs in the haemolymph and integument; its contribution to the green coloration can only be slight.

4. Neither astaxanthin nor insectorubin occurs in haemolymph. Both are therefore probably synthesized in the integument, the former from β -carotene and the latter from unknown precursors.

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Derivatives of Adrenaline and Noradrenaline in an Extract of an Adrenal Medullary Tumour

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Although pharmacological evidence of the occurrence of noradrenaline in the body has been accumulating since Barger & Dale's (1910) classical work on the sympathomimetic amines (for references, see Bergström, Euler & Hamberg, 1950), the first unequivocal demonstration of its presence in the animal body was made by Holton (1949). Using a paper chromatographic technique devised by James (1948), Holton found that the main pressor component of extracts of adrenal medullary tumours was noradrenaline, adrenaline also being present but in much smaller quantity. This finding has been confirmed by Goldenberg, Faber, Alston & Chargaff (1949), who further showed the presence of noradrenaline in U.S.P. adrenaline prepared from cattle suprarenals. Euler & Hamberg (1949) have also demonstrated chemically the occurrence of nor-

adrenaline, in addition to adrenaline, in extracts of cattle suprarenals. Moreover Bergström *et al.* (1950) and Tullar (1949) have isolated it from this source.

Through the kindness of Prof. G. W. Pickering, of St Mary's Hospital, London, the author had the opportunity of examining an extract of a human adrenal medullary tumour by paper chromatography. Adrenaline, noradrenaline, and a number of related compounds may be detected on the developed chromatogram, by spraying the dried paper with a solution of potassium ferricyanide in phosphate buffer pH 7.8 (James, 1948). With this reagent the compounds yield red or purplish-red derivatives. A chromatogram of the tumour extract, on spraying with the ferricyanide reagent, showed the presence of four substances yielding red-coloured derivatives. Of these, two occupied positions which corresponded