pseudovitamin B_{12} 8% of the growth activity of vitamin B_{12}.

3. The amounts of these factors which combined with an 'intrinsic factor' concentrate and a sow's whey concentrate were identical with the amount of vitamin B_{12} 'bound'.

The authors wish to express their thanks to Dr N. J. Berridge for the crystalline rennin used in the preparation of the sow's whey concentrate, to Dr R. Braude and Dr K. G. Mitchell for the samples of sow's milk, to Dr E. Lester Smith for gifts of factor *B* and to Dr S. K. Kon for his interest and suggestions throughout this work.

Note. Since this paper was written, J. B. Armitage *et al.* (*J. chem. Soc.* in the Press) have identified factor *B* as vitamin B_{12} less the dimethylbenzaminazole nucleotide portion.

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The Pigments in Colour Phases of the Larvae of *Plusia gamma* L. (the Silver-Y Moth)

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Uvarov first proposed his theory of phases in 1921 (see Uvarov, 1928), one of the characteristics of insects in the 'gregarious' phase being that they are much darker in colour than those in the 'solitary phase'. The biochemical changes accompanying the colour variations in gregarious and solitary phases of *Locusta migratoria* and *Schistocerca gregaria* (order Orthoptera) have recently been investigated (see Goodwin, 1952*a*). Phases in the larvae of Lepidoptera were first observed by Faure in South Africa (1943*a*, *b*), namely, *Laphygma exigua* (the lesser army worm) and *L. exempta* (the army worm); they also probably exist in *Spodoptera abyssinia*.

Colour variations have recently been noted in this country between crowded and solitary larvae of the lepidopteran *Plusia gamma* both reared from the same batch of eggs. The solitary larvae remained pale green with thin white longitudinal stripes, whilst the crowded larvae showed forms ranging from a similar colour to a much darker green with yellow stripes. This variation was also observed in the field where unusually dark larvae were associated with mass outbreaks (Williams & Long, 1950).

This paper describes an investigation into the pigments responsible for the colour changes in *Plusia gamma*.

EXPERIMENTAL

Rearing of larvae. The larvae were reared at Rothamsted Experimental Station on sprigs of agricultural mustard stood in water in 1000 ml. short beakers fitted with fine muslin covers. A pad of filter paper in the bottom of each beaker prevented excessive dampness and provided a suitable surface for the larvae.

A few hours after hatching the young larvae from each batch of eggs were distributed at random over the breeding jars to provide both crowded cultures of eighty larvae and solitary cultures. Under these conditions the solitary larvae become a light green, whilst the vast majority of those in crowded cultures become a dark olive green.

Throughout the period of development a surplus of food was maintained in all the breeding cultures. Larvae in the middle of their last instar were then selected from the two conditions of culture and sent alive to Liverpool for examination.

Examination of haemolymph. The larvae were killed with ether and the cuticle was opened at the posterior end. The haemolymph which was squeezed out was bright green. It was collected either directly into a small test tube or, if required for spectroscopic analysis, into a dilute solution (0·1%, w/v) of KCN; this inhibited tyrosinase activity. The collected haemolymph was examined for carotenoproteins, after direct extraction by light petroleum (light petroleum, b.p. 40–60° was used throughout this investigation) had failed to reveal the presence of free carotenoids (Goodwin & Srisukh, 1949). An equal volume of ethanol was added to the lymph and the pigments thus liberated by the denaturation of the proteins were extracted from the aqueous phase with light petroleum. The extinction of the extract was measured and used to determine the amount of carotenoids present, calculated as lutein and using $E^{1\%}_{1\,cm.} = 2500$ for pure lutein (Zscheile, White, Beadle & Roach, 1942).

The aqueous residue was tested for bile pigments by extracting with ethyl acetate after acidification with HCl. The amount of bile pigment present was determined by measuring the extinction of the extract at 670 mμ. and assuming $E^{1\%}_{1\,cm.}$ for pure mesobiliverdin to be 527 (Lemberg & Legge, 1949).

Examination of the integument. The integument, freed from all internal organs, was examined for carotenoids in a number of ways. (*a*) The tissues were ground with anhydrous Na_2SO_4 and acid-washed silver sand until a fine powder was obtained; this powder was then extracted with successive portions of diethyl ether (freshly distilled from reduced iron) until all the pigment was removed. The ether was removed *in vacuo* and the residue made up to a known volume with light petroleum. This was then used for chromatographic examination after making an extinction reading at 445 mμ. in order to determine the total amount of carotenoids present. (*b*) The integument was ground with 0·067 M potassium phosphate buffer (pH 7·0) and silver sand and centrifuged; the supernatant, which would contain any carotenoproteins present, was treated in the same way as haemolymph. The residue which contained the remaining carotenoids was examined as described under (*a*). (*c*) The integument was ground with 0·2 N-NaOH and silver sand; after centrifugation, the supernatant, which contained much more material than the pH 7 extract, was treated with glacial acetic acid until just acid. A copious precipitate was produced which, when collected by centrifugation, was examined for carotenoids as described under (*a*).

In each method the material remaining after removal of carotenoids was extracted with either ethyl acetate acidified with HCl to extract any bile pigment present or with methanol containing 5% (v/v) 10 N aqueous HCl, which would remove any insectorubin (Goodwin & Srisukh, 1950).

Examination of carotenoids present. Chromatographic separation and purification of the carotenoid pigments were carried out on columns either of alumina (P. Spence, Ltd., Widnes, Grade 'O') weakened according to the method of Goodwin & Srisukh (1949) or $CaCO_3$ of analytical grade. Ordinary laboratory $CaCO_3$ is not suitable as it may contain considerable amounts of $Ca(OH)_2$ (Lederer, 1952).

Lutein (3:3′-dihydroxy-α-carotene) which was required as a reference carotenoid, was obtained from two sources, grass and leaves of tomato plants. This pigment was also purified by chromatography using the above-mentioned adsorbents.

Examination of bile pigment present. The tests for mesobiliverdin described by Goodwin & Srisukh (1951) were carried out.

RESULTS

Haemolymph

Specimens of haemolymph from both solitary and crowded *P. gamma* larvae were always bright green and did not differ significantly in their absorption spectra; a typical curve obtained by diluting haemolymph with 0·1% (w/v) potassium cyanide is given in Fig. 1.

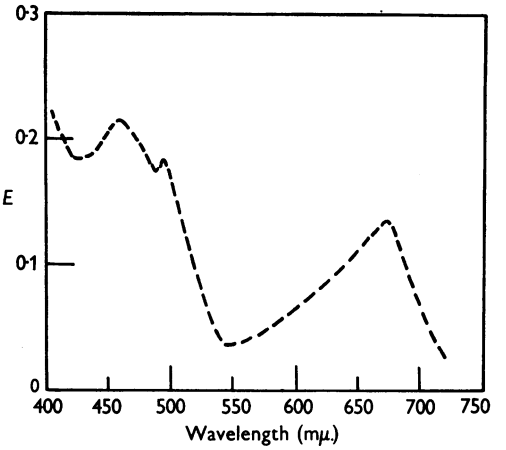

Fig. 1. The absorption spectrum of haemolymph from gregarious *P. gamma* larvae. The haemolymph was diluted with 0·1% (w/v) KCN; the exact dilution was not known but was of the order 1:10.

Carotenoids. As little or no carotenoid material was directly extractable from haemolymph with light petroleum, it appeared that, as in the case of the haemolymph of other insect species so far examined, the pigments were conjugated with protein. The haemolymph proteins were therefore denatured with ethanol, after which the carotenoids were easily extractable with light petroleum. The light-petroleum fraction was chromatographed on weakened alumina. No β-carotene was observed, thus confirming preliminary partition experiments which suggested that the pigments were entirely free xanthophylls; on elution with light petroleum containing 50% (v/v) diethyl ether, a major yellowish brown zone (A) moved slowly down the column, leaving behind a lemon-yellow band (B) which travelled down more slowly.

After purification of fraction A by chromatography on calcium carbonate, the spectrum (λ_{max} 420, 445 and 470 mμ. in light petroleum) indicated that it was lutein. This was confirmed (*a*) by com-

parison with the spectrum of an authentic specimen of lutein measured in three solvents, namely, ethanol, benzene and light petroleum and (*b*) by demonstrating that on chromatography on alumina and on calcium carbonate a mixture of the haemolymph pigment and authentic lutein did not separate. The absorption spectrum of pigment A and of lutein is shown in Fig. 2.

Fraction B only occurred in small amounts (about 10 %, at most, of the total carotenoids) and was not identified; its spectrum ($\lambda_{max.}$ 419, 441, 469 mμ. in light petroleum), and its adsorptive properties suggest that it might possibly be lutein-5:6-epoxide.

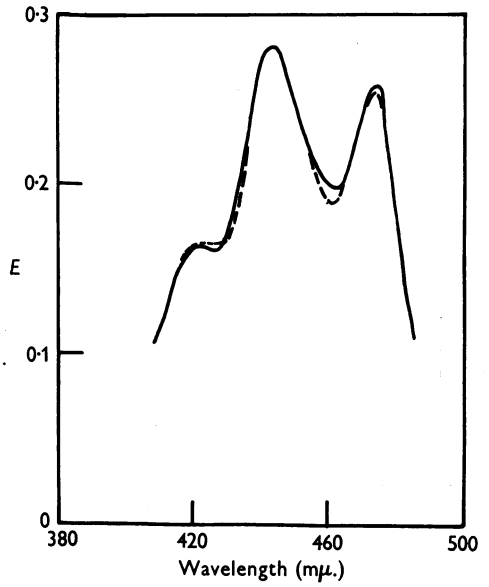

Fig. 2. The absorption spectra in light petroleum (b.p. 40–60°) of lutein and the major carotenoid from the haemolymph of *P. gamma* larvae. The *E* values of the two pigments were adjusted so as to coincide at the main peak. – – –, authentic lutein; ——, haemolymph pigment. The dotted line is shown only when it does not coincide with that of the haemolymph pigment.

Quantitative experiments showed that the concentration of lutein in the haemolymph was high and that there was no difference between solitary and crowded insects reared under otherwise identical conditions (Table 1).

Bile pigments. After removing the carotenoids, the aqueous residue was acidified with hydrochloric acid and extracted three times with ethyl acetate until all colour was removed. Except in the case of the first ethyl acetate extract of one specimen of haemolymph, which was blue, all the extracts were bluish green. It was concluded that this pigment was probably mesobiliverdin for the following reasons: (*a*) it exhibited an absorption maximum 665 mμ. (670 mμ.) in acidified solvents (methanol); (*b*) it formed a green zinc complex, $\lambda_{max.}$ 675 mμ. (685 mμ.), with no fluorescence in ultraviolet light and which on addition of iodine shifted its absorption maximum to 629 mμ. (630 mμ.) and fluoresced pink. (The absorption maxima in parentheses are those recorded for authentic mesobiliverdin by Lemberg & Legge, 1949.) Mesobiliverdin is already known to exist in the haemolymph of other insect species (Junge, 1941; Goodwin & Srisukh, 1951; Hackman, 1952).

The blue extract encountered on one occasion had its absorption maximum at 636 mμ. and not 665 mμ. The blue pigment which Goodwin & Srisukh (1951) obtained from locust haemolymph exhibited all the properties of mesobiliverdin except that the absorption-spectrum maximum in acid methanol was shifted from 670 to 647 mμ. The reason for the single appearance of this type of spectrum in one *P. gamma* extract (the remaining ethyl acetate extracts of the same haemolymph were bluish green) is not known. The amounts of mesobiliverdin in the haemolymph of solitary and crowded *P. gamma* are recorded in Table 1; there are obviously no marked differences between the mesobiliverdin content of the two colour phases.

Integument

The carotenoid extract was chromatographed on weakened alumina. On developing with light petroleum containing 50 % (w/v) diethyl ether the

Table 1. *Pigment distribution in solitary and gregarious larvae of* Plusia gamma

(The figures in brackets indicate the numbers of skins examined in each experiment.)

	Integument (mg./skin)		Haemolymph (mg./100 ml.)	
	Solitary	Gregarious	Solitary	Gregarious
Total carotenoids (measured as lutein)	1·56 (11) 1·20 (13)	2·09 (45) 1·60 (64)	2·04 1·61 1·81	— 1·63 —
Mesobiliverdin	—	0·0012* (64)	3·9 2·3 4·0	2·3 — —
Melanin	Absent	Present	Absent	Absent

* May be due to contamination of integument with haemolymph.

chromatogram described in Table 2 was obtained. Previously it had been noted that, at the beginning of the experiment, development with light petroleum containing 1% (v/v) diethyl ether removed a very small trace of pigment from the column; it could not be completely identified but was in all probability β-carotene. Fraction A, containing the bulk of the pigment (at least 90%) was eluted with ethanol, purified by chromatography on calcium carbonate and proved to be lutein by the criteria described for the haemolymph pigment. Fraction B, which occurred only in traces, had a spectrum very similar to lutein and was perhaps a *cis*-isomer of lutein. The greenish yellow band C was not completely identified, but from its absorption spectrum and its position on the column appears to be identical with the haemolymph pigment B. If the integument residue remaining after the removal of carotenoids is extracted with acidified ethyl acetate then traces of a pigment are obtained exhibiting an absorption maximum at 665 mμ. It must be concluded that the pigment is probably mesobiliverdin, although it is not possible to say whether it exists as such in the skin or was extracted from small amounts of haemolymph remaining adsorbed on the skin. This latter possibility is quite feasible, for one quantitative determination indicated the presence of about 1 μg. of mesobiliverdin in one skin; this could easily have come from slight contamination with haemolymph containing about 20 μg./ml.

Table 2. *The separation of the carotenoids of the integument of the larvae of* Plusia gamma

(Adsorbent, weakened Al_2O_3; developer, light petroleum containing 50% (v/v) diethyl ether. The zones are listed in order of increasing adsorptive power.)

Zone	Description	Absorption spectrum maxima (mμ.) (light petroleum)
A	Yellow-orange: major zone	446, 471
B	Brownish orange: small	444, 470
C	Lemon-yellow	441, 469

An aqueous extraction at pH 7 of the finely ground integument yielded only traces of combined lutein, whilst an extract made with 0·2N sodium hydroxide contained somewhat more. This shows that the major portion of the skin lutein is not in the form of a carotenoprotein. The amounts of lutein present in the skins of crowded and solitary *P. gamma* are given in Table 1.

Extraction of the skin residue with methanol containing 5% (v/v) 10N aqueous hydrochloric acid after removal of the carotenoids yielded a colourless solution, thus indicating that no insectorubin was present.

After extraction with the above solvents the remaining skin residue from crowded insects contained black patches; these were assumed to be due to the presence of melanin. Similar residues of skins of solitary larvae indicated the absence of melanin, for no black patches were observed. These findings are in accordance with those of Long (1953) who made both histological and microscopical examinations of larval integuments. Although melanin is absent from the integument of solitary specimens the melanin-producing enzyme tyrosinase is not. Aqueous extracts of the skin quickly go black on standing, as do the extracts from crowded larvae; this blackening is inhibited by potassium cyanide for both crowded and solitary larvae. Tyrosinase activity of haemolymph from both forms of larvae is also indicated by blackening on standing, which can be inhibited by cyanide. Although no precise measurements were made, it was obvious that haemolymph from solitary larvae took much longer (about 30 min.) to darken than did that from crowded larvae (which was black within 2 min. of removal from the insects).

DISCUSSION

The green pigment of the haemolymph of *P. gamma* is another example of the widely distributed composite pigment 'insectoverdin'. The major component of the yellow fraction is lutein as in *Sphinx ligustri*, *Tettigonia viridissima* and *Meconema* (Junge, 1941), *Pieris rapae* and *Cacoecia australania* (Hackman, 1952), while the blue component is mesobiliverdin as in all other insects so far found to produce insectoverdin. Although the bile pigment does not appear to alter from species to species, the carotenoid component can vary, e.g. in *Dixippus morusus* (Junge, 1941), *Locusta migratoria migratorioides* and *Schistocerca gregaria* (Goodwin & Srisukh, 1951) it is β-carotene and not lutein.

The lutein content of the haemolymph of *P. gamma* adds further evidence to support the generalization that the carotenoid levels in insect blood are very much higher than in the blood of most other animals which contain carotenoids (Palmer & Knight, 1924; Goodwin & Srisukh, 1951; Hackman, 1952), being rivalled only by that of certain breeds of cows grazing on very lush pasture (Goodwin, 1952*b*).

The reason for the preferential accumulation of either lutein or β-carotene (occasionally both can occur together but one always predominates (Hackman, 1952)) by different species of insects is not exactly known but two main possibilities exist: (*a*) one pigment is absorbed from the gut, whilst the other is for the most part excreted, or (*b*) both are absorbed but one is oxidatively destroyed to a much greater extent than the other.

The coloration of P. gamma

The overall light-green coloration of solitary specimens is almost entirely due to the bright green of the haemolymph; if this is squeezed out of an insect then the skin becomes more or less transparent, the white longitudinal stripe remaining translucent. The carotene present in the skin (and the small amounts of mesobiliverdin also possibly present) does not appear to play any major part in the coloration of the larvae. This is different from the case of solitary locusts, the green colour of which is due to the presence of an insectoverdin in the integument (Goodwin, 1952*b*).

Long (1953) found a number of biological similarities to exist between the phases of locusts and the colour phases of *P. gamma* and concluded that the biological principles involved in the production of these phases were the same. It is interesting in this respect to compare the present observations with previous investigations on locusts (Goodwin, 1952*b*).

The darker colour of crowded larvae of *P. gamma* is due to the presence of melanin in the cuticle. This, superimposed on the green of the haemolymph, produces an insect with a much darker-green aspect than the solitary type. As in locusts, the laying down of melanin in the integument of *P. gamma* is a characteristic of the crowded phase. In locusts, however, the production of insectoverdin is inhibited in crowded insects; this is not so in *P. gamma*. A further similarity between locusts and *P. gamma* is that, although melanin is not produced under solitary conditions, tyrosinase activity is not absent. An investigation into the mechanism by which melanin inhibition in the solitary phase is controlled would obviously be of prime importance in the study of the fundamentals of the phase transformations.

The narrow longitudinal line, which is white in solitary *P. gamma*, is yellow in the crowded insects. When pieces of integument containing this region are placed in acetone, the colour is rapidly removed, indicating that it is probably due to a carotenoid. The extracted tissues always show a very slight pink tint at the edge of the stripe; it is possible that this is due to the presence of minute traces of insectorubin. This would originally be present as a brown chromoprotein as in locusts (Goodwin, 1952*b*) and be denatured by the acetone. If this is so, the amounts of insectorubin present are so small as not to be detected by our normal extraction procedures.

SUMMARY

1. The green pigment in the haemolymph of the larvae of *Plusia gamma* is a typical insectoverdin, the yellow component being lutein and the blue component mesobiliverdin.

2. This pigment complex exists in the haemolymph of both the pale-green, solitary, and the dark, crowded forms of *P. gamma*.

3. The integument of both colour phases contains lutein, mostly unattached to protein. Traces of mesobiliverdin may also be present, but insectorubin appears to be absent.

4. The integument of dark, crowded larvae contains melanin, whilst that of the solitary form does not. Tyrosinase activity, however, is present in the haemolymph and integument of both crowded and solitary forms.

5. The quantitative distribution of these pigments in solitary and crowded larvae is recorded.

I am extremely grateful to Dr D. B. Long (Department of Entomology, Rothamsted Experimental Station) for bringing this problem to my notice, supplying the larvae, and allowing me access before publication (Long, 1953) to the results of his investigations on the effects of population density on the morphology, physiology and behaviour of *P. gamma* larvae. Thanks are also due to the Medical Research Council for a grant towards laboratory expenses, and to Prof. R. A. Morton, F.R.S., for his interest in this work.

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