PIGMANT EXTRACTION FROM INSECT HEADS AND HEMOLYMPH

D.K. Hayes, M.S. Schechter, and H.R. Moffitt
Beltsville Agricultural Research Center, Beltsville, Md. 20705
and Yakima Agricultural Research Laboratory, Yakima, Wash.
Agr. Res. Serv., USD A

ABSTRACT

Techniques which can be used for isolation of the carotenoids, pterins, ommochromes, and heme pigments from larvae of the European com borer, *Ostrinia nubilalis* H., the codling moth, *Laspeyresia pomonella* (L.), and the solitary locust, *Schistocerca gregaria* F., are reported. These pigments have absorption spectra consistent with action spectra for prevention or termination of diapause. Slight light-dark reversibility was observed in a pigment-protein extracted into hexadecyltrimethylammonium bromide and believed to be an ommochrome-protein complex.

We reported that light between 410-520 nm was effective in terminating diapause in the codling moth, *Laspeyresia pomonella* (L.), and the so-called oak silkworm, *Antheraea pernyi* Guerin-Meuneville (Norris *et al,* 1969; Schechter *et al,* 1971) and have observed that these wavelengths are also effective in preventing diapause in the European corn borer, *Ostrinia nubilalis* (Hubner) and the pink bollworm,Pecri«o- *phora gossyiella* (Saunders). Although Brady (1971) stated that photoreceptors for rhythms in the adult cockroach were in the eyes, the work of Lees (1964) and Williams and Adkisson (1964) indicated that the photoperiodic effects of photoreception occur in the brain in the insect species they studied. In an attempt to characterize materials which absorb light in the 410-520 nm region of the spectrum and may, therefore, be involved in the diapause response, we also determined that the dissected, intact brains of the oak silkworm and the codling moth contained materials with absorption maxima at wavelengths of light consis-

tent with the action spectra (Norris *et al*, 1969; Schechter *et al*, 1971). We, therefore, developed methods for extraction of whole insects heads, brains and hemolymph. This report summarizes 2 techniques for extraction of some of the classes of compounds in insects which have absorption maxima between 400-550 nm and indicates results of irradiation with visible light of extracts prepared using a detergent, cetyltrimethylammonium bromide.

FRACTIONATION OF WHOLE INSECTS OR INSECT PARTS

Wash in 0.25M sucrose with 0.1 g/l dithiothreitol and 0.2 g/l $CaCl_2$ (Deaerated by passing nitrogen through solution)

Residue
Homogenize in acetone
in cold

Supernatant contains
some hemolymph and
possible sol. protein

Residue
Wash with $CHCl_3$–CH_3OH (w:1)
until free of fluorescence

Supernatant contains
carotenoids and pterins

Residue
Reflux with 0.4% trichloroacetic
acid in methanol 8 hr

Supernatant contains
pterins and may contain
carotenoids

Evaporate to dryness

Take up in CH_3OH and methylate
(acidic groups of tetrapyrroles)
with BF_3 in CH_3OH

Separate products by paper or
thin layer chromatography

Fig. 1. Extraction scheme for preparation of classes of insect pigments with organic solvents.

The codling moth larvae used in this study were obtained from the Yakima Agricultural Research Laboratory, Yakima, Washington, ARS, USDA, as first and second instar larvae in thinning apples. The European corn borer larvae were obtained from the European Corn Borer Research Unit, Ankeny, Iowa, ARS, USDA, on the medium of Lewis and Lynch (1969) and were held under a short-day photoperiod, LD 12:12, until extracted. Freeze-dried locust hemolymph was obtained from Alissa Tietz, Israel Institute for Biological Research, Ness Ziona, Israel.

Two types of extraction were developed, the first being with organic solvents, and the second an extraction with a surface-active agent described by Ajami and Riddiford (1971, 1971b). The solvent extraction process is outlined in Fig. 1. Materials with a typical yellow carotenoid absorption spectrum (absorption maxima at 420, 450 and 480 nm) were found in the hexane fraction from locust hemolymph, and heads of codling moth and European corn borer larvae. In aqueous extracts of the adult of the imported cabbageworm, ***Pieris rapae*** (L.), we obtained bile pigments with the maxima of the absorption spectra in the red portion of the spectrum similar to spectrum of mesobiliverdin. Such materials were also reported from other species of insects by Hackman (1952). These materials were obtained as described by Wieland and Tarter (1940), but we did not report further here because the absorption spectra were not consistent with the action spectra we have reported. Yellow carotenoids and blue bile pigments may account for the green color observed in lepidopteran larvae and in solitary locusts (Goodwin, 1954; Hackman, 1952).

In all the preparations made with organic solvents, fluorescent substances were found in the acetone, chloroform/methanol and methanol fractions, and in the aqueous residue remaining after evaporation of the acetone followed by extraction with hexane. Absorption and fluorescent spectra and the behavior on paper chromatograms were consistent with data on pteridines reported by Hadorn and Mitchell (1951). These materials do not have absorption maxima above 400 nm but do exhibit considerable absorption between 400-450 nm.

Exhaustive extraction with methanol under nitrogen in a Sohxlet apparatus was required to remove the pteridines from the residues to be extracted for hemes and bile pigments. When care was taken to conduct the methanol extraction and the extraction with 0.4% TCA in methanol under nitrogen, no color could be observed in the TCA-methanol extract. However, when such precautions were not taken, the TCA-methanol

extract was pink and exhibited a broad absorption from 400 to 520 nm, similar to the ommochrome found by Ajami and Riddiford in heads of saturniid moths (Ajami and Riddiford, 1971; 1971b). We, therefore, believe that in the extracts we studied the ommochrome pigment was produced in the presence of oxygen after the homogenate had been prepared. Thus, in these insects a precursor to the ommochrome is present, but the ommochrome itself is found only in low concentrations *in vivo*.

When the TCA-methanol extract was examined for fluorescence that might be characteristic of tetraphyrroles, a substance was found with excitation and emission maxima which correspond to those obtained with protoporphyin IX (excitation 345 or 440 nm; emission at 580, 620 nm) which is found as a prosthetic group of many heme enzymes.

The second method of preparation involved the use of the surface-active agent, hexadecyltrimethylammonium bromide (CTMB). Heads were prepared as for the solvent extraction, with care taken to exclude atmospheric oxygen. After homogenization and centrifugation at 12,000 rpm, the precipitate was washed with the homogenizing solution until washings were free of fluorescence when viewed under long-wave UV light (about 360 nm). The procedure for extraction described by Ajami and Riddiford (1971; 1971b) was then followed. Pink material was obtained which was similar to or identical with the ommin and/or ommin-protein complexes reported by Ajami and Riddiford (1971; 1971b). Effects of light were determined using a Beckman DK-2 spectrophotometer and quartz cuvettes with ground glass stoppers. To obtain an anaerobic sample, oxygen was removed by passing UHP nitrogen through the solution, the cuvettes were filled to overflowing and ground glass stoppers were inserted.

Light induced modifications in the pink extract; Fig. 2 shows that after irradiation with 11,000 lux of daylight fluorescent light, the peak at 408 nm is depressed; the peak appeared to be shifted and sharpened after freezing in dry ice and thawing anaerobically. Subsequent attempts to dialyze the preparation were not successful in increasing resolution of the peaks; the increase in absorption from 360-600 nm was due to oxidation by contaminating o-phenoloxidases.

When a second extract was exposed to 11,000 lux of daylight fluorescence for 155 minutes, absorption was enhanced slightly (Fig. 3). When the same extract was thawed anaerobically and the absorption spectrum was measured immediately the absorption decreased and did not quite return to the original intensity.

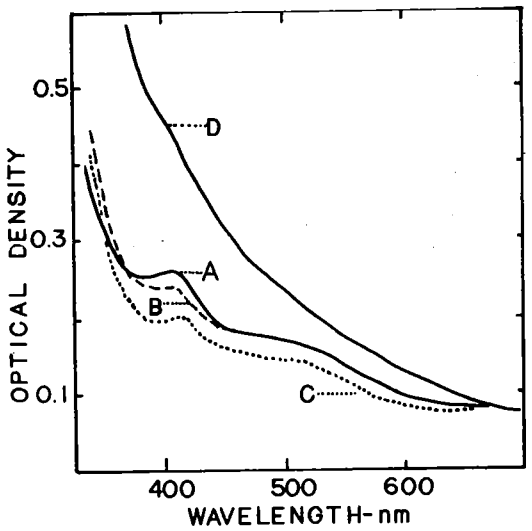

Fig. 2. Effect of irradiation under anaerobic conditions and of aerobic dialysis on absorption of CTMB extract from European corn borer heads. Daylight fluorescent lamp, 15 w, 11,000 lux. A. Prior to irradiation, thawed in light at room temperature; B. After 1,5 hr irradiation, 11,000 lux. C. After holding in dry ice, darkness 18 hr, thawing in dark anaerobically. D. After dialysis overnight at 4°C in dark and presence of O_2.

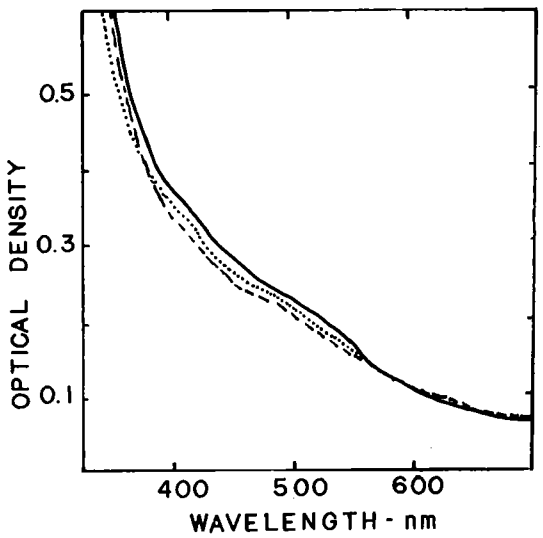

Fig. 3. Changes occurring during one light to dark exposure of CTMB extract of heads of the larvae of the European corn borer. ———Extract thawed anaerobically under room light; ----extract exposed 155 minutes anaerobically to 11,000 lux from daylight fluorescent tub; .... extract thawed anaerobically and in dark after being held in dry ice, darkness 18 hr.

The changes we observed were less dramatic than those reported by Hendricks and Borthwick (1955) for the phytochrome system but visible light did change the characteristics of the absorption spectra. In our extracts, the subcellular architecture has been destroyed; perhaps similar changes could occur with greater facility and with lower energy requirements *in vivo*. In the intact cell inhibitors may not be in contact with material interacting with light and an excess of molecular oxygen would be excluded; thus the phenomenon illustrated in Fig. 4, curve D. would be prevented.

We concluded from this series of tests that the following classes or compound can be identified in extracts from insect tissue: carotenoids, pteridines, protoporphyrin containing materials, and ommins.

REFERENCES

Ajami, A. M. and Riddiford, L. M. 1971. Identification of an ommochrome in the eyes and nervous systems of saturniid moths. *Biochemistry* **10**:1451-1455.

Ajami, A. M. and Riddiford, L. M. 1971. Purification and characterization of an ommochrome-protein from the eyes of saturniid moths. *Ibid.* 1455-1460.

Brady, J. 1971. The search for an insect clock. *In* "Biochronometry". National Academy of Sciences, Wahsington, D.C. 517-526. (M. Menaker, Ed.).

Goodwin, T. W. 1954. Carotenoids, their comparative biochemistry. Chemical Publishing Co., Inc., 212 Fifth Ave., New York. 217.

Hackman, R. H. 1952. Green pigments of the hemolymph of insects. *Arch. Biochem. Biophys.* **41**:166-174.

Hadorn, E. and Mitchell, H. K. 1951. Properties of mutants of *Drosophila melanogaster* and changes during development as revealed by paper chromotography. *Proc. Nat. Acad. Sci.* U.S. **37**:650-665.

Hendricks, S. B. and Borthwick, H. A. 1955. *In* "Aspects of Synthesis and Order in Growth". Princeton Univ. Press, Princeton, N.J. 149-169. (D. Rudnick, Ed.).

Lees, A.D. 1964. The location of the photoreceptors in the aphid, *Megoura viciae* Buckton. *J.Exp. Biol.* 41: 119-133.

Lewis, L. C. and Lynch, R. E. 1969. Rearing the European corn borer, *Ostrinia nubilalis* (Hubner), on diets containing corn leaf and wheat germ. *Iowa State J. Sci.* **44**:9-14.

Norris, K. H., Howell, F., Hayes, D. K., Adler, V. E., Sullivan, W. N., and Schechter, M. S. 1969. The action spectrum for breading diapause in the codling moth, *Laspeyresia pomonella* (L.) and the oak silkworm, *Antherea pernyi* Guer. *Proc. Nat. Acad. Sci. U.S.* **63**:1120-1127.

Schechter, M. S. , Hayes, D. K., and Sullivan, W. N. 1971. Manipulation of the photoperiod to control insects. *Israel J. Entomol.* **6**:143-166.

Wieland, H. and Tarter, A. 1940. Uber die Flügelpigmente der Schmetterlinge. VIII. Pterobilin, der blaue Farbstoffe der Pieridenflugel. *Ann. Chem. (Liebigs).* **545**:197-208.

Williams, C. M. and Adkisson, P. L. 1964. Physiology of Insect Diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm, *Antheraea pernyi. Biol. Bull.* **127**:511-525.

With, T. K. 1968. Bile Pigments: Chemical, biological, and physical aspects. Academic Press, N.Y. 654-655.