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IRON-CONTAINING CELLS IN THE HONEY-BEE (*APIS MELLIFERA*)

I. ADULT MORPHOLOGY AND PHYSIOLOGY

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SUMMARY

Particulate iron was found within the trophocytes of the fat body of the adult honey-bee. These iron granules differed in their structure and composition from iron granules found in other biological systems. The granules had an average diameter of $0{\cdot}32 \pm 0{\cdot}07\,\mu m$ and were composed of iron, calcium and phosphorus in a non-crystalline arrangement. The granules were apparently randomly distributed within the cytoplasm of the cells, and were not associated with any particular cellular organelle.

Electron microscopy revealed the presence of cell junctions between the trophocytes. In tissues treated with colloidal lanthanum, 20-nm gaps were seen between the outer leaflets of the cells forming the cell junction. Physiological studies showed that these cells are electrically coupled, but the coupling ratio is low, as a result of extensive coupling to many cells.

INTRODUCTION

Many organisms (e.g. bacteria, homing pigeons, skates, honey-bees) have been reported to have the ability to detect small fluctuations in the Earth's magnetic field (Blakemore, 1975; Keeton, 1971; Akoev, Ilyinsky & Zadan, 1976; Martin & Lindauer, 1977). In the adult worker honey-bee, behavioural studies have revealed four reproducible effects of magnetic fields on orientation. (1) There are small *Missweissungs*, or misdirections, in the waggle dance which can be changed by altering external magnetic fields around the comb (Lindauer & Martin, 1968; Martin & Lindauer, 1977). (2) Honey-bees will, in the absence of other external cues, build a new comb in the same magnetic direction as the parent hive (Martin & Lindauer, 1977; DeJong, 1982). (3) When placed on a horizontal comb, honey-bees will gradually orient to the cardinal compass points (Martin & Lindauer, 1977).

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(4) Honey-bees can set their circadian rhythms to geomagnetic fluctuations (Gould, 1980). More recently, Walker & Bitterman (1985) have reported that honey-bees can be trained to discriminate between magnetic fields of different intensities. Although this behavioural evidence suggests that honey-bees can detect weak earth-strength magnetic fields, the sensory system involved in this behaviour is unknown.

The ability of magnetotactic bacteria to detect magnetic fields is due to the presence of a small organelle, the magnetosome (Frankel, Blakemore & Wolfe, 1979), composed of a membrane-encapsulated chain of single crystals of magnetite (Fe_3O_4). Thus, it has been suggested that other organisms may synthesize magnetite and perhaps use it as a component of the magnetic field sensory receptor (Kirschvink & Gould, 1981). Studies on magnetotactic bacteria (Mann, Frankel & Blakemore, 1984) and on chitons (Lowenstam, 1962) have shown that magnetite can be synthesized biologically. Concentrations of magnetite have been reported in a number of species, for example, chitons, dolphins, eels and honey-bees (Lowenstam, 1962; Zoeger, Dunn & Fuller, 1981; Hanson, Karlsson & Westerberg, 1984; Gould, Kirschvink & Deffeyes, 1978). In bees, the techniques used did not localize the iron to any tissue, or particular cell type. Therefore, we decided to use histological techniques to localize the iron and to determine its cellular distribution. In this way it might be possible to identify the putative magnetic field receptor.

We have described the location and identity of a specific cell type (the trophocytes), within the honey-bee abdomen, that contains a large number of iron-rich granules (Kuterbach, Walcott, Reeder & Frankel, 1982). Though the iron is predominantly in the form of a hydrous iron oxide rather than crystalline magnetite, it is a form which is a direct precursor of magnetite. Thus, the trophocytes are possible candidates for a magnetic field sensory receptor. These iron granules may also have other functions, such as iron storage or as ballast during flight. In the present study we combine anatomical, analytical and physiological techniques to investigate further these iron-containing cells.

MATERIALS AND METHODS

Adult workers of the honey-bee, *Apis mellifera*, were used for all experiments. Foraging adults were collected from local hives throughout the year and maintained in the laboratory on a diet of 50% sucrose in doubly distilled water. To minimize iron contamination, stainless-steel instruments were used for all dissections.

Bees were chilled to 4°C, placed in dissecting dishes and covered with honey-bee saline (in mmol l^{-1}: NaCl, 156·4; KCl, 2·7; $CaCl_2$, 1·8; glucose, 22·2; pH adjusted to 7·3 with $NaHCO_3$). Abdomens were dissected, by cutting the cuticle either along the dorsal midline or between the dorsal and ventral segments, and spread open. The intestinal tract, tracheae and respiratory muscle layer were removed, and the abdomen was rinsed with fresh saline. Fat body tissue could then be removed in an intact sheet.

Morphology

Tissue was prepared for electron microscopy by fixing with 2·5 % glutaraldehyde in 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·3, for 1·5 h at room temperature. For conventional transmission electron microscopy, tissue was then post-fixed for 30 min using either 1 % OsO_4 alone or with 1·5 % $K_3Fe(CN)_6$. The latter was used when preservation of lipids and membranous structures was desired. To detect the presence of gap junctions, colloidal lanthanum, at a concentration of 1 %, was added to the buffer washes. Following post-fixation, the tissue was washed in buffer, and embedded in EPOX 812 epoxy resin (Polysciences). Thin sections were cut on a diamond knife and picked up on naked copper grids. The sections were stained with 2 % uranyl acetate followed by Reynolds' lead citrate (Reynolds, 1963), and examined with a JEOL 100B transmission electron microscope.

Analytical electron microscopy

Tissue for energy dispersive X-ray microanalysis was fixed and embedded as for transmission electron microscopy except that the OsO_4 post-fixation was eliminated and the material was embedded in EPON 812 (EF Fullam, Inc.). Sections, 150–200 nm in thickness, were cut on diamond or glass knives, and picked up on 300-mesh naked copper grids. The sections were carbon-coated using an Edwards vacuum evaporator, and examined with a JEOL 200CX scanning transmission electron microscope (STEM) operating at 200 kV or a JEOL 1200EX STEM operating at 80 kV. Both instruments were equipped with Tracor Northern Si(Li) energy dispersive detectors. Specimens were held on graphite grid holders at 35–40° tilt, and spectra were acquired in the STEM mode using a 200 nm spot for 200 s.

Physiology

Tissue for physiological examination was pinned onto Sylgard in 7 mm Petri dishes and perfused with fresh honey-bee saline which flowed by gravity. The tissue was viewed with a Zeiss inverted microscope, and individual cells could be identified. The whole assembly was located inside a Faraday cage on a Micro-g flotation table (Technical Manufacturing Corporation).

Dye coupling

The tips of glass microelectrodes (5–10 MΩ) were filled with a solution of 5 % Lucifer Yellow-CH (Sigma) in 1 mol l^{-1} LiCl (after Stewart, 1978), while the shanks were back-filled with 1 mol l^{-1} LiCl. Visually identified trophocytes and other fat body cells (oenocytes) were impaled, and the dye was ejected using hyperpolarizing current pulses (10 ms duration, 2×10^{-7} A, 300 Hz) for periods ranging from 15 min to 1 h. The dye was allowed to spread for 30 min after the removal of the electrode, and the preparation lightly fixed in 4 % neutral buffered formalin before mounting in Kaiser's glycerine jelly (Humanson, 1972). Preparations were viewed and photographed using a 450 nm excitation filter on a Zeiss photomicroscope with an epifluorescence system.

Electrophysiology

Glass microelectrodes (10–15 MΩ) were filled with 3·0 mol l^{-1} KCl, and were used to impale two visually identified trophocytes, either directly adjacent to each other or several cells apart. Hyperpolarizing or depolarizing current pulses were passed into one cell through a balanced bridge circuit and the response of the second cell was recorded. The connections to the electronic apparatus were then reversed, so that the initial input electrode became the recording electrode and *vice versa*. Electrical recordings could be made in this manner for periods exceeding 1 h. The recordings were stored on a Textronix 5103 storage oscilloscope, and were photographed. All measurements were made from the photographs.

RESULTS

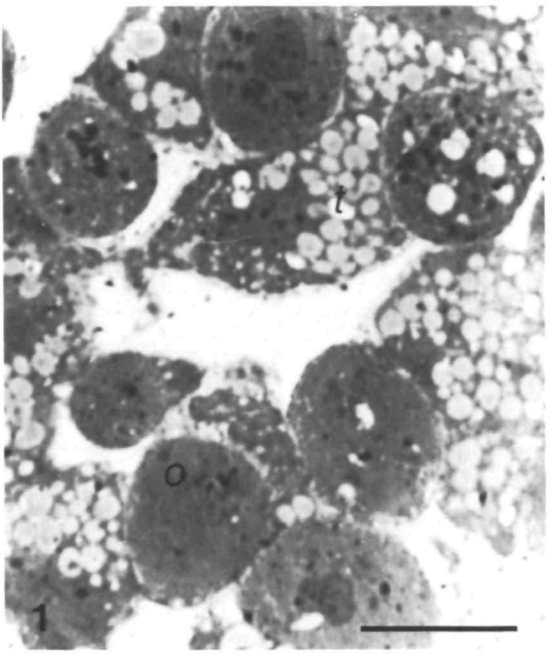
The fat body of the adult honey-bee is composed primarily of two cell types, trophocytes and oenocytes (Fig. 1). The trophocytes comprise the major cell type. These large, oval cells measure 50×75 μm, and are yellowish, suggesting a high lipid and/or iron content. Transmission electron microscopy revealed that the trophocytes possessed an extremely complex ultrastructure (Fig. 2). The surface membrane was folded in irregular processes forming a tortuous network of channels at the periphery of the cells. The nuclei were irregularly shaped and contained predominantly euchromatin with 2–4 clusters of heterochromatin. The cytoplasm contained an abundant endoplasmic reticulum, both smooth and rough, along with a large Golgi apparatus, and many vesicles. Two major classes of vesicles were present. One type was variable in size, electron-lucent, and not membrane bound, while the second type was more homogeneous in size, delimited by a unit membrane and contained an amorphous material.

The cytoplasm also contained numerous electron-dense granules, which often shattered upon sectioning. In samples post-fixed with $K_3Fe(CN)_6$, unit membranes were seen around these granules (Fig. 3), and examination at high magnification revealed no substructure within the granules. The distribution of the granules within the cell appeared to be random, and they were never seen in consistent association with any cellular organelle such as mitochondria or endoplasmic reticulum. These granules ranged in size from 0·1 μm to 0·5 μm in diameter with an average diameter of 0·32 ± 0·07 μm ($N = 900$).

Fig. 1. Light micrograph of the fat body of an adult worker honey-bee showing the two major cell types: trophocyte (t), oenocyte (*o*). Scale bar, 50 μm.

Fig. 2. Low-power transmission electron micrograph of a honey-bee trophocyte. Two vesicle types are seen; large vesicles with no apparent delimiting membrane which contain lipid (*l*) and small membrane-delimited vesicles which contain an electron-dense substance, presumably protein (*p*). Also present in the cell is a large Golgi apparatus (*g*), numerous mitochondria (*m*) and abundant endoplasmic reticulum (*er*). Scattered throughout the cytoplasm are small (0·1–0·5 μm) electron-dense granules distinguished by their tendency to shatter when sectioned (arrowheads). Scale bar, 10 μm.

Fig. 3. High magnification of an electron-dense granule from a preparation post-fixed with $K_3Fe(CN)_6$. Unit membranes can be seen around the granules (arrowheads). No internal structure can be seen within the granules. Scale bar, 1·0 μm.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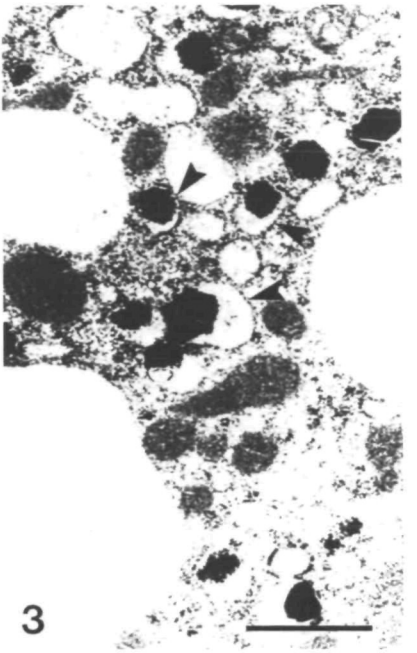
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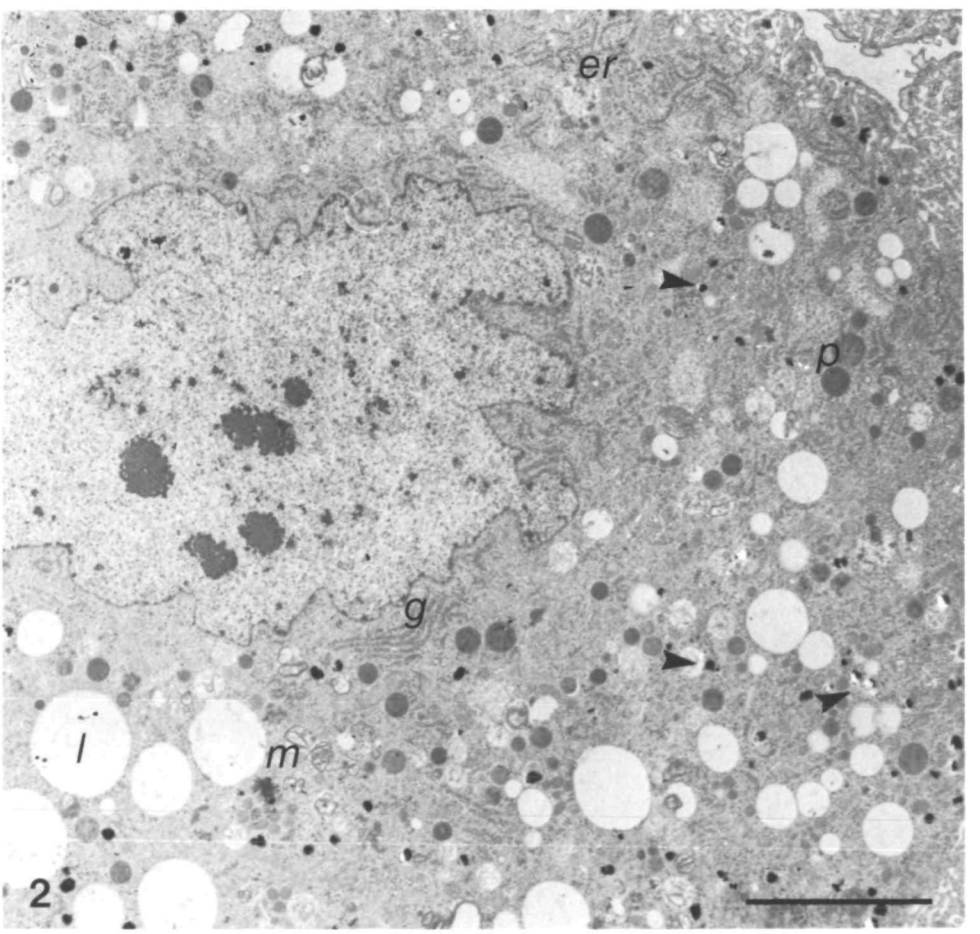
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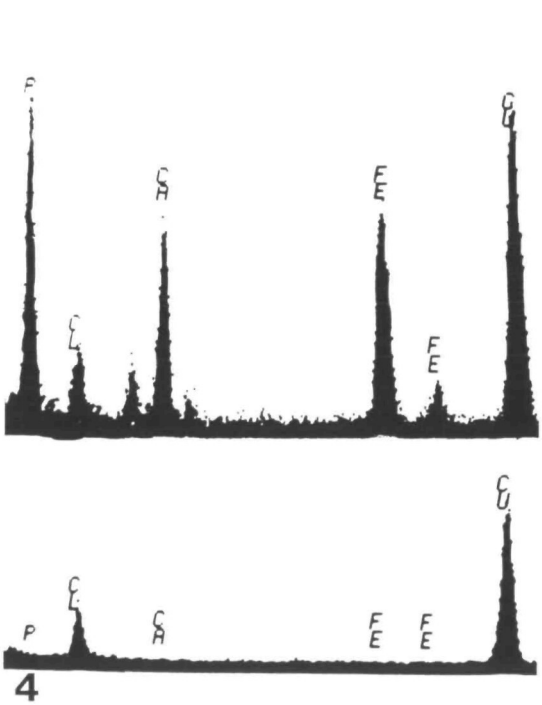
Energy dispersive X-ray microanalysis

The granules were identified in unstained, thick sections by their electron opacity, and measurements revealed the same size ranges as were found in the stained sections. The size of the electron beam was adjusted so that only the granule would be irradiated. Spectral analysis revealed the presence of iron, calcium and phosphorus within the granules, with relative concentrations of iron > phosphorus > calcium (Fig. 4). Control spectra, obtained by analysing areas of cytoplasm directly adjacent to the electron-dense granules, showed these elements to be absent from these areas. No diffraction patterns were seen when the granules were examined by electron diffraction, indicating that the material in the granule was organized into an apparently amorphous structure.

Gap junctions between trophocytes

In some regions, close apposition of the external membrane leaflets of two adjacent trophocytes was observed by conventional TEM. The use of colloidal lanthanum



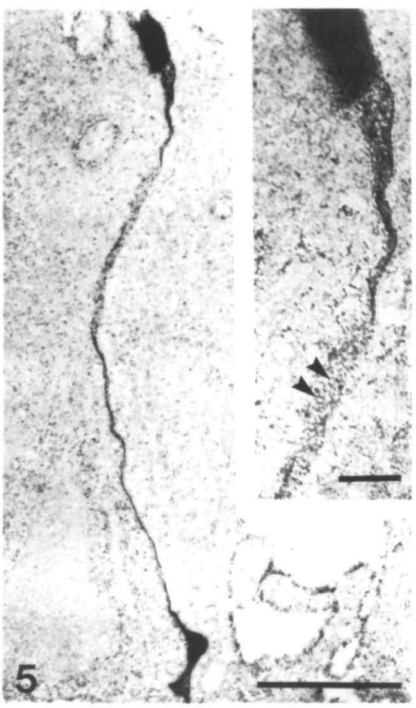


Fig. 4. The X-ray spectrum from an electron-dense granule (top) and an adjacent control area (bottom). The spectrum was obtained from a visually identified granule in a thick section of tissue not treated with osmium. Note the presence of iron (FE), calcium (CA) and phosphorus (P) in the granule and not in the control area. Copper peaks (CU) are due to the support grid.

Fig. 5. Transmission electron micrograph of a lanthanum-filled gap junction between two adjacent trophocytes showing the presence of a 20-nm gap between the outer membranes of the two cells. Scale bar, 0·5 μm. Inset: enlarged view of a tangentially sectioned area showing 5- to 11-nm diameter particles (arrowheads). Scale bar, 0·1 μm.

during fixation revealed the presence of 20 nm lanthanum-filled gaps between these external membranes of the adjacent trophocytes (Fig. 5). The extensive folding and interdigitation of the surface membranes made it difficult to determine the total area of cell contact in these sections. At high magnifications, particles were seen within tangential sections of the junctions (Fig. 5, inset). These particles were round, measured 5–11 nm in diameter, and had a small electron-lucent spot in their centre. The particles resembled the 'connexons' that have been described in other gap junctions (Gilula, 1974).

The outer membranes of the trophocytes and the oenocytes were also closely apposed, suggesting the presence of tight or gap junctions between these cells. However, when these areas were examined using the lanthanum preparations, gap junctions were not observed.

Dye coupling

The anatomical observation of gap junctions between the trophocytes suggested that these cells could be dye and/or electrically coupled. Injection of Lucifer Yellow into a trophocyte was followed by rapid, radial diffusion of the fluorescent dye to other trophocytes (Fig. 6). Each trophocyte made contact with 5–8 other trophocytes, and the dye spread equally to all cells contacted, suggesting that the junctions between the trophocytes were equivalent. This phenomenon was observed for trophocytes in all regions of the fat body within a single abdominal segment. Intersegmental dye coupling was never observed when the dye was injected into

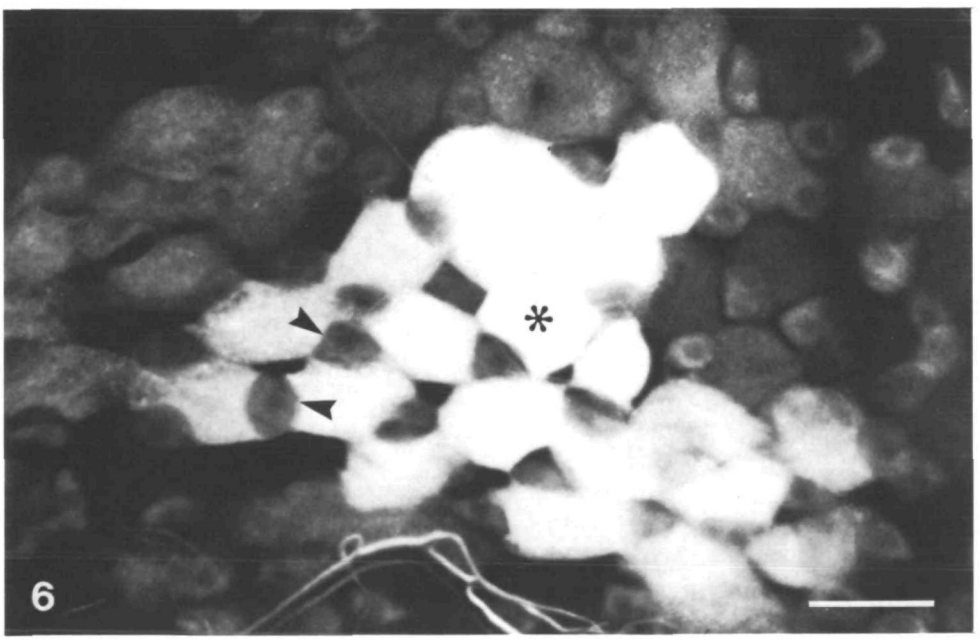


Fig. 6. Lucifer Yellow spread after a 30-min dye injection. The dye spreads from the injected cell (*) to an area encompassing roughly 15 cells. Exclusion of the dye from the oenocytes (arrowheads) shows that the trophocytes are not dye-coupled to the oenocytes. Scale bar, 75 μm.

trophocytes adjacent to the segmental border. The trophocytes were not dye coupled to the oenocytes and dye injected into an oenocyte remained within that cell.

Electrical coupling

The trophocytes routinely exhibited membrane potentials of −5 to −10 mV when impaled with KCl electrodes but never produced action potentials when depolarized. Upon application of either hyper- or depolarizing current, coupling was observed equally in both directions between adjacent cells (Fig. 7A). The time constant of the coupled cells was short, between 0·6 and 0·8 ms, indicating little capacitance. The

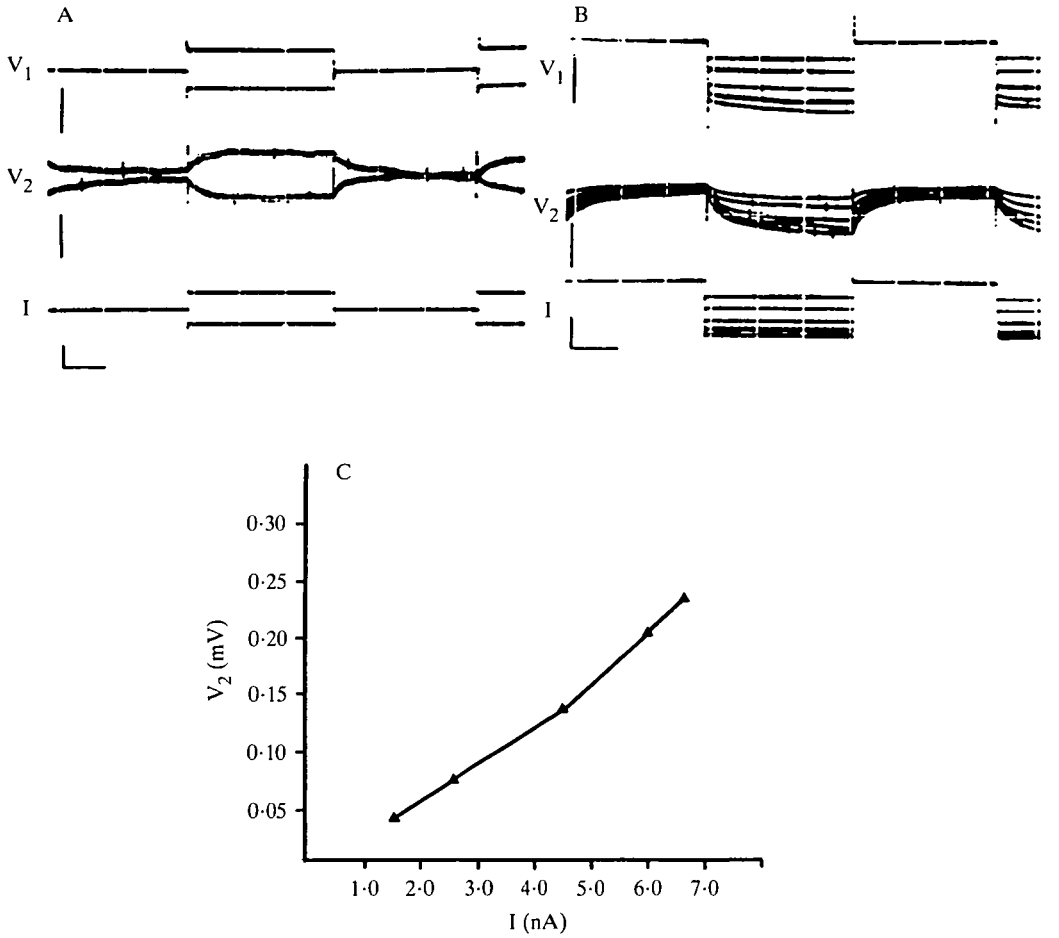


Fig. 7. Electrotonic coupling between honey-bee trophocytes. Current was injected into V_1, and responses recorded simultaneously from V_1 and V_2. (A) Coupling with depolarizing pulses. Calibration: V_1, 20 mV; V_2, 2 mV; I, 1 nA; horizontal, 5 ms. (B) Coupling with hyperpolarizing current. The response to increasing current is linear in both cells, and the time constants of the cells' responses do not change. Calibration: V_1, 20 mV; V_2, 2 mV; I = 1·5, 2·5, 4·25, 6·0 and 6·25 nA; horizontal, 5 ms. (C) Current/voltage curve of the response of V_2 from the experiment in B, showing the linearity of the response.

Table 1. *Comparison of biological iron compounds*

Granule type	Diameter (nm)	Prussion Blue reaction	Diffraction pattern	Calcium	Phosphorus	Iron
Apis trophocyte	500	yes	no	+	+++	+++
Molpadia dermal[a]	90	yes	no*	+	++	++
Haemosiderin[b]	0·8–7·2	yes	yes	−−	++	+++
Ferritin[c]	5·5	no	yes	−−	++	+++
Magnetite[d]	100	yes	yes	−−	−−	++++

* Gives a diffraction pattern upon heating.
References: [a]Lowenstam & Rossman, 1975; [b]Richter, 1980; [c]Granick, 1951; [d]Frankel, Blakemore & Wolfe, 1979.

recorded currents showed a linear current/voltage relationship and there was no increase in the time constant with step increases of applied currents of either polarity (Fig. 7B,C), suggesting the presence of a non-rectifying junction. Coupling ratios between adjacent pairs of trophocytes were calculated to be 0·02–0·04 ($N = 10$). No electrical coupling was observed when trophocyte/oenocyte pairs were examined.

DISCUSSION

The fat body of the worker honey-bee is a continuous sheet of tissue lining the internal surface of the abdomen. It is composed predominantly of two cell types, the trophocytes and the oenocytes, although occasionally other cells such as urate cells and haemocytes are present (Snodgrass, 1956). The fat body of adult insects is presumed to function in a similar manner to the vertebrate liver (Wigglesworth, 1984), and studies on the fat body of larval insects have shown it to be responsible for the elaboration, storage and secretion of hormones (King, 1972), proteins and nucleic acids (Price, 1973), carbohydrates and glycogen (Wiens & Gilbert, 1967; Jungreis & Wyatt, 1972) and lipids (Cook & Eddington, 1967) during development.

The present study confirms our earlier observations that only the trophocytes in the fat body of the honey-bee contain particulate iron (Kuterbach *et al.* 1982). This ability of the trophocytes to accumulate and sequester iron was shown as early as 1900 when Koschevnikov demonstrated Prussian Blue reactivity of the fat body of bees raised on honey or sucrose to which ferric chloride was added. The fat bodies of other insects (*Lucilia cuprina, Calpodes ethilus*) also show this ability to accumulate iron when they are raised on diets rich in iron (Lennox, 1940; Waterhouse, 1940). All of these earlier studies were performed on insects that had been fed diets artificially high in iron. This report is the first description of iron particles in insects in Nature.

The iron granules in the honey-bee are unique when compared to other forms of iron granules found in biological systems (Table 1). They do not have the characteristics of magnetite, ferritin or haemosiderin, although some similarities to the iron subunits of the dermal granules of the sea cucumber, *Molpadia intermedia*, exist. First, the honey-bee granules are larger than other forms of iron granules. This larger size may be due to enzymatic regulation of granule formation as in the formation

magnetite (Towe & Lowenstam, 1969). Second, the honey-bee granules have an apparently amorphous electron structure. This is seen as a lack of an electron diffraction pattern, which must be due to a low crystalline order within the iron complex caused by hydration. This result is consistent with our earlier Mossbauer analysis (Kuterbach *et al.* 1982), which showed that the granules were a hydrous iron oxide, similar to the iron subunits of the dermal granules of *Molpadia intermedia* (Lowenstam & Rossman, 1975). In these animals, no diffraction lines were evident until the isolated granules were heated to 500°C. This treatment drives out the water in the iron complex, revealing the presence of haemosiderin. Third, X-ray microanalysis indicates the presence of calcium and phosphorus within the iron granules. The only other granule which has significant amounts of both calcium and phosphorus is the iron sub-unit of *Molpadia intermedia* (Lowenstam & Rossman, 1975), although phosphate groups are common among other biological iron granules (e.g. ferritin, haemosiderin).

These studies, however, cannot rule out the possibility that magnetite may be present in these granules. Calculations from our previous study (Kuterbach *et al.* 1982) showed that, to obtain the magnetic remanence that Gould *et al.* (1978) measured, only 0·33% of the iron in these granules needed to be in the form of magnetite. This quantity of magnetite is well below the limit of resolution of our techniques.

Anatomical studies reveal the presence of gap junctions between the trophocytes. Gap junctions have been described in many multicellular organisms (Gilula, 1974), where they form low-resistance pathways between cells. This low-resistance pathway allows for rapid transmission of electrical and chemical signals between coupled cells. Anatomically, the gap junctions between the trophocytes are similar to those described in other systems (Gilula, 1974). The results of dye coupling studies show that these junctions between trophocytes form low-resistance pathways for the transfer of dye molecules. Further, the trophocytes in all areas of a single abdominal segment appear to be equivalent, as the dye will spread to the same extent irrespective of the location of the injected cell. There are segmental boundaries within the fat body which is shown by the failure of the dye to pass to trophocytes located in adjacent segments. Similar segmental boundaries are seen in insect epidermal cells (Warner & Lawrence, 1982), and serve to compartmentalize each segment. Thus, these segmental boundaries suggest that the fat body acts independently in each segment.

The trophocytes are electrically non-excitable cells as shown by the failure to produce an action potential and the linearity of the voltage/current relationship of the cell membrane. Their membrane potentials are typical for epidermal and fat cells (Sheridan, 1971). Electrical coupling is observed between pairs of adjacent trophocytes. The junctions are not gated by a voltage-dependent mechanism, as was reported in amphibian blastomeres (Spray, Harris & Bennett, 1979), and are ohmic in their response to current. The measured coupling ratios (V_1/V_2) are low (0·02–0·04) when compared to those seen in other non-excitable cells [e.g. liver, 0·9 (Penn, 1966); *Chironomous* salivary gland, 0·8–0·9 (Oliviera-Castro & Loewenstein,

1971)]. However, the coupling ratio is influenced by a number of factors, including cell size, cell shape, cell volume, the number of cells coupled and the area of non-junctional membrane (Sheridan, 1973, 1974). Assuming that the sizes of the coupled cells are equal and that junctional and membrane resistivity do not change, the effect of increasing the number of coupled cells would be to shunt more of the injected current away from the recording site, thus effectively decreasing the observed electrical coupling (Sheridan, 1973). Each honey-bee trophocyte is coupled to 5–8 cells based on the dye coupling results. Thus, based on geometry alone, the coupling ratio would be expected to be approximately 0·18 (i.e. 0·9/5, assuming that the junctions between trophocytes have the same resistivity as hepatocytes). The apparent coupling between cells can be decreased by cell size and shape. Sheridan (1973) has calculated that liver cells would have a junctional area/cell volume ratio of about 1/169 (estimating total junctional area to be roughly 1·5 % of the total surface area of a 15-μm cuboidal cell). Assuming that the honey-bee trophocyte is a cylinder with dimensions of 75 μm (length) $\times$ 50 μm (diameter) and that the junctional area is the same percentage of the surface area as in the liver, the ratio of junctional area/cell volume is found to be 1/833, approximately five times less than in hepatocytes. This factor would then decrease the observed coupling ratio from 0·18 to 0·036, a value close to the experimentally observed coupling ratios between honey-bee trophocytes. Thus, the observed low coupling ratio between honey-bee trophocytes is probably due to a combination of low input impedance and the three-dimensional geometry of the cells, rather than to a high junctional resistivity. The electrical characteristics of the junctions combined with the low electrical coupling of the trophocytes suggest that these junctions act mainly as intercellular corridors, as in the vertebrate liver, and mediate the transfer of metabolites.

There are several possibilities why iron would be accumulated by the fat body of the honey-bee. One is that iron may be accumulated in response to high dietary iron levels and/or for maintenance of iron homeostasis. This hypothesis is the subject of a companion paper (Kuterbach & Walcott, 1986). A second possibility is that this iron might be acting as a magnetic field sensory receptor. The results of this study show that while there is a large quantity of iron present in the honey-bee abdomen, it is neither in the form of magnetite, nor in a structure that seems likely to function as a sensory receptor [both prerequisites for the ferromagnetic hypothesis of magnetic field detection (Kirschvink & Gould, 1981)]. Additionally, neuroanatomical studies reveal that, although many nerves are seen passing through the fat body, the trophocytes are not innervated. However, these studies do not rule out the possibility that magnetic fields could have an indirect effect. For example, changes in the declination of the earth's magnetic field or in magnetic field strength can cause a decrease in enzymatic activity in some systems (Haberditzl, 1967; Welker *et al.* 1983). The best known example is in the guinea-pig pineal gland where melatonin synthesis is inhibited by artificial magnetic fields (Welker *et al.* 1983), which in turn can affect the function of the hippocampal cells (Ziese & Semm, 1985). Since the honey-bee granules may be paramagnetic, they could respond to external magnetic field fluctuations and affect local intracellular enzymatic pathways.

Thus, the presence of iron granules within honey-bee trophocytes continues to be an intriguing question.

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