Magnetite-Based Biological Effects in Animals
Biophysical, Contamination, and Sensory Aspects
TR-111901

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EPRI Project Manager
C. N. Rafferty
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This report was prepared by

The California Institute of Technology
Division of Geological & Planetary Sciences
Pasadena, California 91125

Principal Investigators
J. L. Kirschvink
J. Brassart

Oregon State University
Department of Biochemistry
Corvallis, Oregon 97331-6503

Principal Investigator
M. H. Nesson

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REPORT SUMMARY

As part of its mission to understand potential effects of electric and magnetic fields (EMF) on human health, EPRI conducts research on biophysical mechanisms of interaction. This report provides evidence supporting the existence of a magnetic field sensory system in animals based on the magnetic iron compound, magnetite. The investigators also identify iron particle contamination as a potentially important uncontrolled factor in current in vitro EMF biological experiments.

Background
A fundamental problem in assessing the human risk of exposure to EMF is the lack of an accepted mechanism of interaction at the molecular and cellular levels. One physically plausible action of magnetic fields on biological systems involves possible coupling through a magnetic compound called magnetite. Magnetite is found in all animals including man. It is a biologically synthesized crystalline mineral (Fe₃O₄) that when organized in long chains readily orients in the earth’s magnetic field. Furthermore, weak, extremely low frequency magnetic fields can perturb the orientation of these chains and if structurally coupled to nerve cells could constitute the biological basis of a magnetic field sense. Other biological effects of EMF involving magnetite or other iron compounds are also possible but would be unnatural in the sense that these effects are not the result of biological evolution and do not have adaptive value. Such effects might include microwave absorption by endogenous magnetite or cellular effects reported in in vitro studies in which the presence of contaminating iron particles is probable.

Objective
To seek evidence for the existence of a magnetite-based magnetic field receptor in animals and to evaluate other possible EMF biological effects due to interaction with magnetite or other magnetic particles.

Approach
Biophysical models and calculations were made to describe a simple directional magnetoreceptor at the membrane level and to evaluate the possibility of biological effects due to absorption of microwaves by magnetite. Laboratory experiments included tests to quantify the presence of magnetic particles in cell cultures and to evaluate their origin; magnetometry experiments performed on salamanders; and evaluation of mouse brains for the presence and localization of magnetite using a variety of magnetometric and histological methods. Researchers also considered an hypothesis in which animals use their magnetic sense to anticipate earthquakes.
Results
Based on the results of this project as well as other research, the investigators state, “Tiny crystals of single-domain magnetite are now known unequivocally to be the biophysical transducer between the geomagnetic field and the nervous system of higher animals . . . .” This conclusion is supported by several findings of this project:

- Biophysical modeling fully accounts for the sensitivity and small number and occupied volume of magnetite-based sensory receptors.
- Single domain magnetite was detected by magnetometry in animals that show clear behavior responses to magnetic fields.
- The mean magnetite concentration in the mouse brain is $4.8 \pm 4.0$ ng/g tissue. At least some of the magnetite in the mouse brain occurs as intracellular aggregates.

The investigators also conclude that biological systems might also respond to microwaves due to absorption by magnetite. Finally, their research indicates that exogenous magnetite or other magnetic iron compounds are likely to contaminate most in vitro systems used in EMF effects research. This contamination is expected to be variable and uncontrolled and may provide a biophysical basis for some reported EMF effects as well as explain the poor reproducibility of many published studies.

EPRI Perspective
In making fundamental advances in understanding the remarkable ability of some animals to detect and respond to weak magnetic fields, this project represents progress towards understanding possible biophysical mechanisms of interaction, of vital importance in EMF research today. Also of major significance—and warranting immediate investigation—is the proposal that contamination by magnetic particles might affect the outcome of EMF in vitro studies.

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ABSTRACT

The question of whether weak magnetic fields are able to influence living organisms has been a controversial subject for as long as humans have known about magnetism. However, the presence of biogenic magnetite (Fe₃O₄) in animal tissues, and the widespread presence of magnetic contaminants in our environment, both offer simple and easily-understood mechanisms through which weak, extremely low frequency (ELF) magnetic fields, and perhaps microwave radiation, can affect living organisms or cells growing in tissue culture. Tiny crystals of single-domain magnetite are now known unequivocally to be the biophysical transducer between the geomagnetic field and the nervous system of higher animals, and the major components of the sensory system in Vertebrates are gradually coming into focus. Other magnetic particles exist in the environment, and can compromise in vitro experiments aimed at understanding whether, how, and why magnetic fields influence biology. Support from EPRI during the past 5 years has contributed greatly to this understanding, ranging from biophysical models of the dynamics of magnetite interacting with cellular structures like ion channels, to the interaction of microwave radiation with intracellular magnetosomes.
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INTRODUCTION AND OVERVIEW: ON THE EVOLUTION OF MAGNETORECEPTION

Pigeons, turtles, cetaceans, and numerous other animals have an uncanny ability to find their way home when released in an unfamiliar area. How they manage this is one of the largest mysteries in the behavioral sciences. Suggestions that the geomagnetic field might play a role in these long-range navigation and homing abilities were at first met with intense skepticism, for good reasons. First and foremost is the fact that most humans are not consciously aware of the surrounding geomagnetic field. Biomagnetism also received a bad rap early on from the legacy of Franz Anton Mesmer and members of the French mesmerite movement (~1775 - 1784), who claimed that they could cure disease by exposing patients to magnetized objects. After this was thoroughly discredited by a commission appointed by King Louis XVI (which included Benjamin Franklin), animal magnetism fell into nearly two centuries of disrepute, serving mostly as a romping ground for Charlatans. This may still be the case in some biomedical areas.

In the intervening years, numerous sensory systems that humans apparently do not possess have popped up in interesting places within members of our own phylum. Some are straightforward extensions of known senses – like the echolocation in bats and cetaceans, infra- and ultrasound detection in birds, and the sex pheromone receptors (the vomeronasal system) in the nose of most higher vertebrates. Others, such as the electroreceptive organs of sharks and rays and the infrared detectors of snakes, are based on newly discovered types of highly-specialized receptor cells. These discoveries generated little scientific controversy.

Magnetoreception has not had such an easy ride. During the 1940s, experiments suggesting that pigeons might use geomagnetic cues during homing generated great interest, but were difficult to reproduce. Early conditioning experiments designed to elicit magnetoreception in laboratory settings also failed. However, the most stinging criticism came from biophysicists concerned with how a living organism could actually detect the weak geomagnetic field. All known sensory systems have specialized receptor cells designed to respond to the external stimulus, and these are always coupled to appropriate neurons to bring this information to the brain. The problem lay in the initial conversion of the geomagnetic stimulus into a signal that an individual cell could detect; back-of-the-envelope calculations, done numerous times in many flavors, ruled out most of the obvious methods (paramagnetism, electrical induction, the Hall
effect, nuclear magnetic resonance, for example). The simplest strategy – that of having a small permanent magnet – was dismissed out of hand on the simple assertion that there were no physiological ferromagnetic materials (Griffin 1944). But the behavioral experiments did not agree with these biophysical analyses, and apparent geomagnetic effects on animal behavior kept being discovered (reviewed recently by Wiltschko and Wiltschko, 1995). It took nearly two decades to realize that the geomagnetic compass used by adult birds was programmed to be ignored if other orientation cues (the sun or star compass, polarized skylight, infrasound, ultrasound, etc.) were present. Follow-up studies have shown that these orientation cues are a complex but consistent web of interacting responses, present not only in birds but in all major vertebrate groups and many invertebrates (Wiltschko and Wiltschko, 1995).

The first clue toward solving the biophysical problem came in an obscure place – the teeth of a small mollusk. A paleontologist, the late Heinz A. Lowenstam of the California Institute of Technology, discovered that massive amounts of the mineral magnetite (Fe₃O₄) were capping the major lateral teeth of *Polyplacophoran* mollusks (the chitons) (Lowenstam 1962), who use it as a hardening agent (Figure 1-1). Discovery of much smaller amounts of magnetite in other animals had to wait for the development of ultrasensitive superconducting moment magnetometers; their use led to the recognition that ppb levels of biogenic magnetite are naturally present in animals as diverse as insects, birds, fish, and even humans (Kirschvink et al. 1985; Kirschvink et al. 1992). The final crack in the biophysical arguments against magnetoreception came with the discovery of the magnetotactic bacteria and protists (Figure 1-2), which possess linear chains of either single-domain magnetite (Frankel et al. 1979) or greigite (Fe₃S₄) (Heywood et al. 1990). They provide clear and easily reproducible examples of the geomagnetic field influencing biological activity.
Figure 1-1
Magnetite-bearing teeth of the Polyplacophoran mollusk, Cryptochiton stelleri. Each tooth is about 1 mm in size, and capped with a layer of black magnetite (Photo credit: H.A. Lowenstam)
At present, magnetite and greigite are the only known ferro- or ferrimagnetic minerals formed by living organisms under intracellular biological control (Lowenstam and Weiner 1989). Magnetotactic bacteria form these minerals within a proper lipid-bilayer membrane vacuole termed a ‘magnetosome’ (Gorby et al. 1988) which allows the bacterium to control precisely the size, shape, and crystallographic orientation of the particles. In every species of magnetic bacteria examined to date, the size and shapes of the individual crystallites fall within the known constraints needed to produce single magnetic domains which are uniformly magnetized crystals, or perfect little bar magnets (Diaz-Ricci and Kirschvink 1992; Kirschvink and Lowenstam 1979); these range from about 0.03 to 0.1 µm in length, and are from equant to needle-like in shape. For magnetosomes made of magnetite, many of the crystal morphologies are starkly different than the typical octahedral shape most often found in volcanic and other inorganic natural sources. Biological morphologies range from cubo-octahedral sets of intersecting {100} and {111} and equivalent faces in the Magnetospirilla (Mann et al. 1984) to pseudohexagonal prisms in magnetococci and peculiar tear-drop, arrowhead, and bullet-shaped forms in some other species (Mann 1985; Vali and Kirschvink 1991). It is clear that a matrix-mediated biomineralization process controls the deposition of these minerals within the magnetosome membrane, as some of the crystallites express chemically equivalent faces with radically different morphologies. Within a single bacterium or bacterial strain, the crystals also tend to have very similar sizes and shapes. Furthermore, they are usually organized in long chains, with the magnetically easy {111} crystal axis of magnetite aligned parallel to the chain length, thereby
maximizing the magnetic moment of each cell for a given quantity of iron. A similar pattern of crystal morphology and alignment is also present in higher organisms, reflecting the natural functions of magnetotaxis in microorganisms and magnetoreception in higher animals (Mann et al. 1988). More recently, scanning laser confocal reflection microscopy coupled with neural staining techniques has been used to localize probable magnetite-containing receptor cells in the vertebrate trigeminal system (Kirschvink 1997; Walker et al. 1997), culminating a 20-year search for the receptors which were first detected with magnetometric techniques (Gould et al. 1978; Walcott et al. 1979).

As illustrated by the magnetotactic microorganisms, magnetoreception is an obvious role for magnetite. It is biophysically simple. Although only a few micrometers in size, the typical magnetotactic bacterium contains enough magnetite to make its rotational energy in the geomagnetic field exceed the thermal background energy by factors of 20 or more. The cells are very good, passive compasses, and will align with the earth’s magnetic field even when dead. For higher animals, the equivalent of only one magnetotactic bacterium, connected to a single sensory neuron, could give an ant, a honey bee, a trout, or even a whale an extraordinarily good magnetic compass sense. If this were all of the magnetite used for magnetoreception, finding and characterizing the receptor would be a fruitless, needle-in-the-haystack operation. However, the behavioral literature indicates that there are at least two types of response to the geomagnetic field – a simple compass, and another which responds to small fluctuations in total intensity of the background field. This latter sense has been implicated as a component of the navigational ‘map’ used by whales, turtles, and birds (Wiltschko and Wiltschko 1995). Extensions of the biophysical analyses like that presented here in Chapter 2 indicate that an array of a few thousand to a million magnetite-containing cells could yield responses to total intensity fluctuations of better than 0.1%, as is observed behaviorally (Wiltschko and Wiltschko 1995), and that this entire receptor system could fit within a 1 mm cube and yet have the magnetite present at no more than 1 ppm (Kirschvink et al., 1985, and Chapter 2 here). And there is still no requirement for the receptors to be concentrated into such a small volume. Although it would still be a needle in the haystack, the odds of finding them are much better than previously thought. Simple experiments using short but strong magnetic pulses (which exceed the coercive force of the magnetite) have shown that both the magnetic compass and intensity sensory systems involve the use of permanently magnetic materials like magnetite (Beason and Semm 1996; Kirschvink et al. 1997). Linear chains of single-domain biological magnetite crystals suitable for magnetoreception are easy to extract from animals and image (Mann et al. 1988) (Figure 1-3), and electrophysiological studies in birds have consistently identified fibers in the ophthalmic branch of the trigeminal nerve as the one carrying magnetic field information (Beason and Semm 1996; Walker et al., 1997).
Introduction and Overview: On the Evolution of Magnetoreception

At present, very little is known about the genetics of magnetite biomineralization. One protein (dubbed ‘Mag-A’) has been identified by phage mutagenesis in a *Magnetospirillum* strain (Matsunaga et al. 1997). Mag-A’s function appears to relate to the transport of Fe both into the cell and into the magnetosome membranes (Nakamura et al. 1995). Two other proteins with strong resemblance to ferritin have also been sequenced, but they have two weird properties (Bertani et al. 1997). Not only do their open reading frames on the DNA actually overlap by one base pair, implying that they cannot be transcribed in sequence by the same ribosome, but their amino acid residues near the active iron nucleation site, which are usually highly conserved, differ markedly from those of other bacterioferritins. This argues that the mineral product being nucleated (if, indeed, that is the case) is not the mineral ferrihydrite. As no ferritin cores have ever been detected in the cytoplasm of magnetotactic bacteria (Vali and Kirschvink 1991), and as a magnetosome-bearing organism would have no use for an iron storage protein like ferritin anyway, Bertani et al. (1997) suggest that these modified ferritin proteins might be involved in magnetite formation.

Despite this lack of knowledge concerning the biochemistry and genetics of magnetosome formation, the very fact that the crystals are intracellular precipitates with biologically distinctive morphologies indicates that they can be recognized as fossils, should they happen to be preserved properly in sediments. Shortly after the discovery of the magnetotactic bacteria (Blakemore 1975) and the confirmation that they contained magnetite (Frankel et al. 1979), Kirschvink & Lowenstam (1979) predicted that bacterial production of magnetite could explain the mysterious presence...
of fine-grained magnetite in deep-sea sediments. This provided a clear explanation of how many deep-sea sediments preserve a stable record of geomagnetic reversals and other features of the Earth’s ancient magnetic field (Kirschvink 1982) and led to the eventual discovery and naming of ‘magnetofossils’ composed of both magnetite (Kirschvink and Chang 1984) and greigite (Demitrack 1985). Improvements in extraction and TEM imaging techniques eventually led to the recovery of intact magnetosome chains (Petersen et al. 1986) with all of the same distinctive crystal morphology, size, and shape constraints observed by magnetosomes in extant magnetotactic bacteria (Vali et al. 1987). Amusingly, greigite magnetofossils were discovered 5 years before the greigite-precipitating magnetotactic bacteria (Heywood et al. 1990). Subsequent work has shown that the relative proportions of cuboidal to hexagonal magnetosome forms is even a useful indicator of paleoclimatic conditions in deep sea sediments (Hesse 1994), including organic carbon flux (Yamazaki and Kawahata 1998). Of course, the most important magnetofossil-related debate at present is their putative presence in the carbonate blebs of the Martian meteorite ALH84001 (McKay et al. 1996). Magnetosome chains composed of single-domain magnetite crystals with any of the biologically-unique features of Earthly magnetotactic bacteria would be unambiguous proof of the existence of early life on Mars.

On Earth, the oldest known magnetofossils date back about 2 billion years (Chang and Kirschvink 1989), approximately the time that the eukaryote cells evolved (Han and Runnegar 1992). The widespread presence of magnetosome chains in eukaryotic algae (Torres de Araujo et al. 1985), Chordates (Mann et al. 1988), and numerous other animal groups (Kirschvink et al. 1985) argues that the ancestral eukaryotes inherited the ability to form magnetosomes through the process of serial endosymbiosis, in much the same manner as they gained mitochondria and chloroplasts. This single observation is exceedingly important, as many other biomineral systems in higher organisms, including vertebrate bone and molluscan shells, are made using vacuole-based systems very similar to that used by the magnetotactic bacteria. Evolution usually does not re-invent the wheel; when a need arises for a new system, natural selection tends to copy and modify an existing system rather than producing it from scratch (Gould and Vrba 1982). Hence, the sudden appearance at the Precambrian/Cambrian boundary of many diverse animal phyla with the ability to make mineral hard parts should not be a mystery – they merely needed to modify their existing magnetosome system and substitute their other ion transportation systems for calcium, phosphate, or carbonate to yield a new mineral product. This is actually a testable hypothesis, as it predicts an underlying biochemical similarity between mineral formation systems in the magnetic bacteria and many separate phyla. The recent observation that pure molluscan nacre does not stimulate an immune response when injected in living human tissues (Westbroek and Marin 1998), but in fact stimulates bone repair, is certainly consistent with this hypothesis.

From an evolutionary perspective, it now seems clear that magnetite-based magnetoreception is a sensory modality which dates back at least to the last common ancestor of Chordates and Arthropods (~ 600 to 900 Myr ago), and the existence of
magnetotactic protists argues that this genetic ability may go back even further to the evolution of the first eukaryotes nearly two billion years ago. Despite the presence of magnetic field sensitivity in fish, amphibians, reptiles, birds, and mammals (Wiltschko and Wiltschko 1995), humans seem to be an exception [but see the great ‘debate’ over this issue: (Baker 1980), and section V in the Kirschvink et al., (1985) volume]. If humans are really an exception, it would imply that this rather useful ability was lost rather recently in our evolutionary history. We certainly have a trigeminal nerve, with an ophthalmic branch, and we also have the ability to make biogenic magnetite (Kirschvink et al. 1992). At least one other vertebrate sensory system thought to have been lost in the final stages of human evolution – the vomeronasal organ with its sex pheromone receptors – has recently been found to be both present and functional (Berliner et al. 1996); human vomeronasalins now form a booming perfume industry. Non-sensory magnetic effects on human have recently been discovered, such as the ability of a 1 mT static field to elicit epileptiform activity in patients preparing for brain surgery (Fuller et al. 1995), so the final word on the existence of human magnetoreception has certainly not been written. And some humans, particularly Polynesian navigators, seem able to judge direction correctly in situations where all obvious cues (the sun, moon, stars, waves, etc.) are absent (Finny 1995).
2

BIOPHYSICAL CONSTRAINTS ON MAGNETITE-BASED MAGNETORECEPTION

Rationale

This chapter extends previous theoretical and experimental analyses on the role that biogenic magnetite plays in the transduction of electromagnetic fields to both sensory and non-sensory processes. The first section is a back-of-the envelope calculation for the role of a simple directional compass system, to set constraints on the size and number of magnetite-based receptors which would be required to produce observed behavioral responses in honey bees, newts, and turtles. The receptor system can fit within a remarkably small volume. Second, a long-standing and controversial biomedical application of magnetic fields has been in the area of analgesia, dating back to the time of Anton Mesmer and the French Mesmerite movement. Although widely regarded as a romping ground for Charlatans, it is worth exploring the limits of possible magnetite-based involvement in one study which might be plausibly related to the motion of biogenic magnetite. And finally, effects of microwave absorption on biogenic magnetite is a completely uninvestigated area, and we present here some initial data on the frequency specific absorption properties of biogenic magnetites in the cellular-telephone range.

Biophysics of a Simple Directional Magnetoreceptor

The widespread presence of biogenic magnetite in a variety of animals (Kirschvink et al. 1985) leads to the question of how sensitive a magnetite-based receptor system might be, and what volume of tissue an array might be needed to house it. The actual constraints on this system are illustrated best by the honey bee, for which numerous magnetic effects are known (reviewed by Kirschvink, 1992). In this animal the receptors have been localized to a small volume in the anteriordorsal abdomen both by magnetic and behavioral techniques (Walker and Bitterman 1989a). In particular, Walker and Bitterman (1989b)’s two-choice force-alternative experiments on honeybees indicate that they can detect a magnetic anomaly of only 25 nT against the background static field, or a fluctuation of only 0.6%. In the past, the interpretation of this (and similar behavioral effects in other animals) has relied on the assumption that the animals were detecting the intensity fluctuation in the magnetic field, rather than the directional shift
(e.g., Kirschvink and Gould 1981; Kirschvink and Walker 1985; Yorke 1981). This interpretation was justified on the basis that to detect such a small directional shift would require having the ability to maintain track of an animal's spatial orientation to within an orientation at least as precise as that being used to discriminate the small angular shift in the magnetic field direction. More recently, however, serious suggestions have been made that sea turtles (Lohmann and Lohmann 1994) and migrating newts (Phillips and Deutschlander 1997) might rely on minute fluctuations of the geomagnetic inclination component as part of a multi-coordinate magnetic map system. This, of course, requires that the animals would have the ability to detect the vertical axis (e.g., gravity) to a high degree of precision, and use this as a reference for pulling out subtle directional changes in the magnetic field orientation.

We present here a very simple model of a magnetite-based system which is aimed at answering the question of how large an array of magnetite-based receptor cells might be need to be to resolve this fine-scale fluctuation, using as our model the honey bee data of Walker and Bitterman (1989b). Figure 2-1 shows the configuration. A crystal (or chain of crystals) of magnetite is linked via a cytoskeletal filament to a mechanically-activated trans-membrane ion channel. In the model shown here, we assume the crystals are similar to those in typical magnetotactic bacteria, e.g., rectangular magnetite prisms ~ 50nm x 50 nm x 100 nm in dimension, with magnetic moments of \( 1.2 \times 10^{16} \text{ Am}^2 \), and magnetic/thermal energy ratios in the geomagnetic field of ~ 1.5. As the average magnetic/thermal energy ratio of the honeybee compass receptor has been measured at ~ 6 (Kirschvink 1981), each receptor cell would need a chain of four such crystals. Howard and Hudspeth (1988) have shown that typical mechanically-activated ion channels are activated at roughly the kT threshold (where k is the Boltzmann constant and T the absolute temperature). Channel activation requires a force of approximately 1 piconewton, moving the channel gate through a distance of ~ 4 nanometers (1 piconewton x 4 nm ~ kT). The maximum angular deflection in a 50 \( \mu \text{T} \) background field produced by a 25 nT anomaly is simply \( \tan^{-1}(25 \text{ nT} / 50 \mu \text{T}) \), or an angle \( \theta \) of 0.03 °. If one edge of this crystal is linked to the gate of the ion channel, this deflection will move it though a distance of 50 nm x \( \tan(\theta) \), or 25 picometers.

Energy balance can now be used to estimate the number of such receptors needed to average out background noise. Each organelle like those diagrammed on Figure 2-1, when moved through an angle \( \theta \), will contribute a small energy to the receptor array; this energy (\( \Delta E \)) is given simply by the force x distance, or 1 piconewton x 25 picometers, or \( 2.5 \times 10^{-23} \text{ Joule} \), or 0.006 kT. The question now is simply how many, \( N \), of these identical receptor cells would be needed to bring the signal/noise ratio of the array up to unity. If each receptor structure acts independently of all of the others, the signal to noise ratio should improve as the square-root of the number of discrete receptors present. Hence, \( \Delta E / kT \sim 1 / \sqrt{N} \), so \( N = (kT / \Delta E)^2 \). For the value of \( \Delta E \) estimated above, on the order of 25,000 ion channels/magnetosome chain structures would be needed to bring the signal/noise ratio up to unity.
The next question concerns the volume that this structure could be located in. The magnetosome crystals should be spaced far enough apart such that the magnetic field of one grain on its neighbors would be less than that of the signal needed to detect. A simple calculation shows that a spacing of 20 µm will reduce the peak magnetic field from one of these 4-crystal structures to less than 18 nT, just less than the ∼ 25 nT detected by the best bee in the Walker and Bitterman (1989b) experiment. Assuming that each receptor cell is a 20 µm cube, and that they are packed like blocks into a volume of tissue, 25,000 cells would occupy a block with dimension of
about 30 x 30 x 28 unit cells; hence, this entire structure would fit within a tissue cube of about 600 µm on an edge. The total quantity of magnetite in this array would only be about 31 picograms, with a volume density of 32 ppb.

Detection of tiny quantities of magnetite has been one of the major factors in the construction and improvements of the magnetically-shielded, dust and particle free clean laboratory at the California Institute of Technology. The total of 31 picograms of single magnetite estimated here will produce a saturation magnetic remanence of \( \sim 1.5 \text{ picoAm}^2 \) \((1.5 \times 10^{-9} \text{ emu})\), which is now within the levels measured easily with DC-SQuID moment magnetometer systems, which have SQuID noise levels in the 0.1 picoAm\(^2\) range. Recent improvements in the laboratory techniques to handle and measure such weakly magnetized materials without introducing contamination (discussed in section 3) have actually allowed us to approach this noise limit in real dissection experiments.

**Magnetic Analgesia**

In a provocative paper, McLean *et al.* (1995) report a series of intracellular recording experiments with static magnetic fields on isolated neural cells, recording their firing rates when stimulated with a sub-threshold potential from a second microelectrode. The static field conditions were those produced by quadrupole array of NdFeB disk magnets, arranged in a planar array of alternating polarity; this configuration was one that appeared to produce an analgesic effect on human fingers and skin. Similar results were produced using a smaller array of NdFeB magnets. The most dramatic decrease in cellular firing rates occurred when the magnets were positioned such that the cells were exposed to high field gradients, with little to no effect when positioned in a region of strong static field and minimal magnetic gradient. Peak effects required several minutes to reach their maximum levels.

Although not mentioned by McLean *et al.* (1995), a biological response which depends only on the static spatial gradient of the geomagnetic field, without a strong dependence on the total intensity of the field, is quite plausibly produced by single-domain ferromagnetic materials. Electrical induction requires time varying fields, and a paramagnetic response would depend upon the product of both the static field strength and the spatial gradient of the field (Adair 1991). Free-radical (Schulten 1982) and magnetochemical (McLauchlan 1989) mechanisms should depend only on the strength of the field at the site of action. Although the rotational torque of a single-domain ferromagnetic material will depend on the total field strength, the translational force \( F \) on it is given simply by \( F = \mu \partial B / \partial x \), where \( \mu \) is again the magnetic moment of the particle. As of this writing, we know of no other magnetic field interaction mechanism which has this simple dependence on field gradient.
Back-of-the-envelope calculations can be of use here to provide some insights on the quantity and configuration of biogenic magnetite which could be involved in this analgesic effect. At a fundamental level, motion of the magnetite could pull on intracellular structures such as the cytoskeleton, or cause organelles to wander from their most effective site of operation. Following the lead taken above, we will focus our analysis around the known properties of mechanically-activated trans-membrane ion channels, using the data of Howard and Hudspeth (1988) as a guide (e.g., a gating force of ~ one piconewton, and a gating distance of ~ 4 nanometers, with a net energy use of ~ kT). In this model, the magnetic particles are physically dragged by the large field gradients, and the force on the cytoskeletal filaments is responsible for opening the channels. On Figure 2-1, this is equivalent to the particle opening the ion channel by being dragged to the right, rather than doing it by rotation. Generalizing from this, it is possible to trade off the force vs. the distance through which the magnetic structure moves, keeping the overall drop in energy at the kT level. Even though this would abandon the ion channel model per se, it can provide a simple guideline on the amount of magnetite which might be needed to yield any effect via this type of mechanism. Also, the time required for the magnetite arrays to move through the larger distances could be the cause of the time delay between application of the gradient and the analgesic effect.

Following the estimate outlined above, we will again model this situation with standard 50 x 50 x 100 nm crystals of single-domain magnetite, each with a magnetic moment of 1.2 x 10^{-16} Am^2. If there are N of the identical crystals in a magnetite-bearing intracellular structure, the total force on them from a gradient of ∂B/∂x will be F = Nµ ∂B/∂x. As the energy drop (ΔE) is simply the force times the distance (FD), the number of magnetosomes needed to produce a 1 kT drop in energy is calculated easily as shown in Table 2-1, for the 4 values of field gradient in the experiments of McLean et al. (1995).

Table 2-1
Estimated numbers of the 50x50x100 nm prismatic magnetosome crystals needed to yield an energy drop of 1 kT when they are displaced a given distance in the gradient magnetic fields produced in the McLean et al. (1995) experiment. The ion channel model, in which the gate of an ion channel moves 4 nm, would require 2652 of the standard magnetosomes in the strongest gradient value used (3.3 T/m) to produce the gating force of 1 piconewton. The numbers of magnetosomes needed is calculated as N = kT/(µD ∂B/∂x), where µ is the moment of the individual magnetosome (1.2 x 10^{-16} Am^2), D the distance moved in meters, and ∂B/∂x the values of the field gradient used by McLean et al. (1995).

<table>
<thead>
<tr>
<th>∂B/∂x, in tesla/m</th>
<th>.004</th>
<th>2652</th>
<th>19444</th>
<th>109375</th>
<th>486111</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance moved, in micrometers</td>
<td>.04</td>
<td>265</td>
<td>1944</td>
<td>10938</td>
<td>48611</td>
</tr>
<tr>
<td></td>
<td>.4</td>
<td>27</td>
<td>194</td>
<td>1094</td>
<td>486</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>19</td>
<td>109</td>
<td>486</td>
</tr>
</tbody>
</table>
These numbers illustrate some interesting constraints on the system. To activate a single ion channel with the strongest field gradients used (3.3 T/m) would require 2652 of the standard magnetite crystals to be linked to the same ion channel. The size scales involved suggest that this is unlikely to be the actual physical mechanism involved. On the other hand, if the magnetite-bearing structure were to move through a distance of 4 micrometers to yield the effect, only 3 magnetosomes would be needed to reach kT. McLean et al. (1995) report that the gradient levels of 3.3 and 0.45 T/m were effective at producing the analgesic effect, whereas the 0.08 and 0.018 T/m experiments did not. Following Table 2-1, a plausible candidate structure would be something with between about 20 and 2000 magnetite crystals, moving between 40 nm and 4 µm within the cells. For the range of smaller structures involved in this model, the time for them to migrate through this distance might account for the delay between application of the field and the reduction of firing rate.

This is obviously a testable hypothesis. Numerous dissection studies performed at the Caltech biomagnetic clean lab over the past two decades have identified the presence of magnetic materials in skin samples, although the problem of contamination to any surface exposed to magnetic ‘dirt’ has inhibited the intensive efforts of the sort which led to the discovery of biogenic magnetite in the human brain (Kirschvink et al. 1992). However, as the individual cells used in by McLean et al. (1995) were grown in culture, it should be possible to test for the presence of ferromagnetic particles within them using the laser confocal reflection microscopy techniques as described by Walker et al. (1997).

**Magnetite and Microwave Radiation: Background and Adsorption measurements**

The presence of trace amounts of biogenic magnetite (Fe₃O₄) in animal and human tissues, and the observation that ferromagnetic particles are ubiquitous in laboratory materials (including tissue culture media), provide a physical mechanism through which microwave radiation might produce, or appear to produce, biological effects. Magnetite is an excellent absorber of microwave radiation at frequencies between 0.5 and 10 GHz through the process of ferromagnetic resonance, where the magnetic vector of the incident field causes precession of Bohr magnetons around the internal demagnetizing field of the crystal; it also has a variety of absorption characteristics which result from electron hopping between adjacent iron centers. For these reasons, Kirschvink (1996) suggested that biogenic magnetite might be a reasonable candidate for the biophysical transduction mechanism for microwave effects, should any such things be found (for an introduction to this literature, see Guy 1984, Barnes 1989, Michaelson 1991, Bernhardt 1992, Stuchly et al., 1988; Tenforde & Liburdy 1988; Liburdy & Tenforde 1986; Liburdy et al., 1988; Cleary et al., 1992; Liburdy, 1992). It is the goal of this section to review briefly from a theoretical perspective the possibility outlined by Kirschvink (1996) that local absorption of microwave radiation by small crystals of
Biophysically precipitated magnetite (Fe₃O₄) might be a mechanism capable of producing biological effects; the biophysical review which follows is adapted from that paper. In the final section of this chapter, we present new experimental data on the relative efficiency of microwave absorption of biogenic magnetite vs. other naturally-occurring ferro- and ferri-magnetic minerals in the frequency band from 1-40 GHz.

**Microwave Biophysics.** In tissues which do not contain ferromagnetic materials, only a small fraction of the incident energy passing through a cell is absorbed, primarily through dielectric interactions of polar and charged molecules with the E vector of the microwave field. This type of interaction results in penetration depths generally in the cm to decimeter range (depending upon frequency). It is easy to show that the absorption on the cellular level is small. For example, in a study of the ~ 835 MHz radiation produced from cellular telephones, Anderson & Joyner (1995) measured a 10-fold decrease (90% loss) in power over a distance of approximately 5 cm in phantom models of the human head. If we assume the typical cell is ~ 10 µm thick, this attenuation would happen over ~ 5,000 cells. If X is the fraction of energy passing by each cell to the next one in line, then [1-X] is the fraction absorbed by each cell. For an incident field with an initial power level of 1, after passage through 5000 cells the transmitted power will be $X^{5000}$. Thus, $X^{5000} = 0.1$ for a 90% reduction in power, implying that the fraction of energy absorbed by each cell is $\{1-10^{-5000}\}$, or 0.046%. Hence, normal cells are generally transparent to the microwave radiation going through them.

This relative microwave transparency does not hold for tissues or cells containing ferromagnetic materials such as magnetite. Due to the process of ferromagnetic resonance (Kittel 1948; Bloembergen 1956), these materials can absorb microwave radiation strongly. At ferromagnetic resonance, the imaginary term of the susceptibility (which determines the energy dissipation within a ferromagnetic crystal) becomes infinitely large. This holds particularly for single-domain particles, where other damping processes are minimal (for a review, see Smit & Wijn 1959, section 23). Thus, the important parameter to determine is the fraction of the cross-sectional area of a cell which might contain magnetite. Typical magnetotactic bacterial cells usually contain up to a percent magnetite by volume, although some exceptional organisms, like the 8 µm long *Magnetobacterium bavaricum* (Spring et al., 1993; Vali & Kirschvink 1990) are in the 5-10% range. For a simple model with 1% by volume of magnetite, consider a cubic cell 10 µm on edge containing $10^4$ magnetosomes, each of which is a 0.1 µm cube. If arranged as a continuous square sheet in the cell, these particles would form a 100 x 100 layer, 10 x 10 µm in dimension, of thickness 0.1 µm. If oriented perpendicular to the incident radiation, this sheet would be capable of screening 100% of the area of the cell. If this plane were arranged normal to the incident radiation, a minimum of only 1% of the cell’s area would be intercepted. More realistically, a random crystal arrangement would probably shield something more like 10 to 30% of the cell volume, which is similar to that present in published TEM images of these organisms. With perfect ferromagnetic resonance, therefore, we should expect absorption efficiency of ~ 10 to 30% for this cell, in contrast with the 0.046% estimated above for tissues dominated by water absorption. In practice, however, a uniform internal
demagnetization field (see next page) would only be present in single-domain ellipsoidal particles; fan-like dispersion near the edges of other crystal shapes would act to detune parts of the crystal volume, reducing slightly the volume of the crystal which is perfectly on resonance. Nevertheless, one should expect roughly a factor of 1000 larger energy dissipation from a magnetocyte of this sort.

It is instructive to express this absorption of microwave energy by a single 0.1 μm crystal of biogenic magnetite in terms of the background thermal energy, kT (where k is the Boltzmann constant and T the absolute temperature; kT = 4 x 10^{-21} Joule at 300 K). A microwave field of 10 mW/cm^2, which is near the upper limit generated by commercially-available cellular telephones, corresponds to an energy flux of 2.5 x 10^{18} kT/cm^2·sec. At the peak resonance for a magnetite crystal (see next page), this implies that on the order of 10^{18} kT/sec is dissipated into the cellular environment around the crystal. As the carbon-carbon bond energy is ~ 140 kT, and typical hydrogen bond energies are ~ 10kT, there is at least the conceptual possibility that local effects of the absorbed energy could exceed thermal noise. This stands in sharp contrast to the related biophysical problem of extremely low-frequency (ELF) radiation influencing biological systems, where the major problem is simply to reach the kT level with any interaction (Adair 1991). Magnetite also has metallic resistivity (~ 5 x 10^{-5} Ω·m), which makes it roughly 6,000 times more conductive than any other biological material, and broadens its interaction with microwave fields through electrical effects.

Ferromagnetic Resonance. Magnetite is a member of a broad class of materials called ferrites, which over the past 40 years have become intimately involved in the control and tuning of microwave devices. The book by Smit & Wijn (1959, hereafter referred to as S&W) provides a thorough review of the basic physical theory, as well as in-depth discussions of the many forms of ferromagnetic resonance effects which were discovered in a flurry of activity in the decade after the initial prediction by Kittel (1948). Using this work, it is possible to make a first-order estimate of the resonance frequencies expected for a magnetite crystal of known shape, size, and crystallographic orientation.
Figure 2-2
Schematic representation of the precession of a magnetization vector \( \mu \) in the demagnetization field, \( H \). In a uniformly magnetized solid, the alignment of the Bohr magnetons at each atomic locus leads to an effective magnetic charge separation. The field external to the crystal (the magnetic induction) is precisely what would exist if it were generated by an array of magnetic charges fixed on the surface of a particle. Hence, these effective charges also generate a real magnetic field inside of the crystal (\( H \)), which is oriented in the opposite direction from that of the magnetization, hence the name ‘demagnetizing field’. (Dotted lines on the left diagram indicate field direction, not magnetization direction.) This demagnetization field is felt by each of the unpaired Bohr magnetons within the crystal lattice, as indicated by the right-hand diagram. As such, there is a torque on the magneton, \( \mu \times H \), which always acts at right angles to the \( \mu \) and \( H \) vectors. Thus, the tip of the \( m \) vector precesses over the surface of circular cone with a resonance frequency given by equations (2-1) and (2-2).

Figure 2-2 illustrates the basic principle of ferromagnetic spin resonance, which is most applicable to the single-domain magnetites produced biologically. Inside a ferromagnetic crystal, strong magnetic fields (\( H \)) are generated by three types of anisotropy: (1) that which is present in the crystallographic structure, \( H_a \), (2) that which is from the shape of the particle, \( H_s \), and (3) that which is produced by stress from defects in the crystal lattice. Thus, the magnetic moment of each unpaired electron (\( \mu_B \), or Bohr magneton) of the iron atoms in the crystal will experience a torque, \( \mu_B \times H \), which acts perpendicular to both \( \mu_B \) and \( H \) (\( H \) being the vector sum of \( H_a \), \( H_s \), and \( H_d \)). As each electron also has quantized angular momentum (\( J \)) in addition to its magnetic moment, it will precess around the direction of the \( H \) vector just like a spinning top in a gravitational field. Thus, the angular precession frequency, \( \omega \), is given by:

\[
\omega = \gamma H, \tag{eq. 2-1}
\]
(S&W, eq. 18.4, p. 78) where \( \gamma \) is the gyromagnetic ratio (\( \mu_b/J \)) for an electron. Converting this to frequency, \( f \), and including the value for \( \gamma \), S&W (eq. 18.5, p. 78) provide the useful relationship

\[
f = 35.2 \ H \ \text{kHz} \quad \text{(eq. 2-2)}
\]

when \( H \) is measured in \( \text{A/m} \). [This has been converted from the Gaussian CGS units used by S&W to SI units; 1 Oe = (1/4\( \pi \)) x 10\(^3\) A/m. Also note that in a vacuum \( B = \mu_o H \), where \( \mu_o (= 4\pi \times 10^{-7} \text{ henries/m}) \) is the permeability of free space.] Thus, if the total magnetic field resulting from the anisotropy and any external fields are known, the peak resonance frequency can be found.

For several reasons, biogenic magnetite presents a simple system for the application of this theory. First, virtually all crystals of biogenic magnetite which have been studied with transmission electron microscopy (TEM) have sizes and shapes which fall within the single-domain stability field (Diaz-Ricci & Kirschvink, 1992), implying that they are uniformly and stably magnetized. This also implies that only the spin resonance need to be considered, as other effects like domain wall resonance (reviewed by S&W, section VI) should not happen. Second, high-resolution TEM (HRTEM) studies of the crystal structure of most biogenic magnetites reveals that the crystals are usually slightly elongate, with the \{111\} axis parallel to the long axis. This has been observed in numerous magnetotactic bacteria (Towe & Moench 1981; Mann 1985; Vali & Kirschvink 1990), Salmon (Mann et al., 1988), and in many of the magnetite crystals from the human brain (Kirschvink et al., 1992ab). This coincidence of the “easy” direction of the magnetocrystalline and shape anisotropies implies that the internal magnetic fields they produce add together linearly, making computations easy. Finally, all of the HRTEM studies indicate that the biogenic crystals are usually free of crystal lattice defects, which allows effects from stress anisotropy to be ignored.

For magnetite at physiological temperatures, the internal field produced by the magnetocrystalline anisotropy in the \{111\} orientation, \( H_a \), is given by:

\[
H_a = \frac{1}{M_s} \left[ \frac{4}{3} K_1 + \frac{4}{9} K_2 \right] \quad \text{(eq. 2-3)}
\]

(S&W, Table 11.I, p. 46), where \( M_s \) is the saturation magnetization, and \( K_1 \) and \( K_2 \) are the magnetocrystalline anisotropy constants. Banerjee & Moskowitz (1985) give best values for these parameters of 480 emu/cm\(^3\), -1.35 x 10\(^5\) and -0.44 x 10\(^5\) erg/cm\(^3\), respectively (note that 1 emu = 10\(^3\) Am\(^2\), and 1erg = 10\(^7\) joule). In the absence of shape anisotropy, equation (2-2) predicts a 1164 MHz resonance frequency.
For shape anisotropy, $H_s$, the internal field depends upon the relative value of three orthogonal demagnetization factors, $N_a$, $N_b$, and $N_c$, where $N_a + N_b + N_c = 4\pi$. If the particle is elongate and equant, $N_b = N_c$, and the internal field produced by the shape anisotropy, $H_s$, will be given by:

$$H_s = (N_b - N_a) M_s$$  \hspace{1cm} \text{(eq. 2-4)}$$

(S&W, p. 80). Calculation of exact values for these demagnetization factors depends on the detailed shape of the particles, and closed-form expressions have been worked out for both equant rectangular prisms and ellipsoids (see Diaz-Ricci & Kirschvink, 1992, for a thorough summary of the earlier literature). For elongate needles, however, $N_a = 0$ and $N_b = N_c = 2\pi$, yielding a maximum for $H_s$ of $2\pi M_s$ equivalent to a resonance at 8445 MHz. As $H_s = 0$ for either a perfect cube or sphere (where $N_a = N_b = N_c$), the shape contribution to the ferromagnetic resonance will vary from zero to 8445 MHz.

**Figure 2-3**

Theoretical peak ferromagnetic resonance frequencies for rectangular single-domain crystals of magnetite. As described in the text, the magnetocrystalline anisotropy alone leads to a constant resonance frequency of 1165 MHz. The shape anisotropy (neglecting magnetocrystalline anisotropy) leads to frequencies between 0 and 8445 MHz. If the $\{111\}$ axis of magnetite is aligned with the particle elongation (as is the case in many biogenic magnetites), the two anisotropy fields will sum, yielding peak resonance frequencies up to 9609 MHz. Note that these are only the peak of the resonance curves, which, as described in the text, ought to be rather broad.
Figure 2-3 shows results of a detailed calculation of the peak resonance frequency for equant, rectangular parallelepipeds as a function of particle shape. For crystals in which the \{111\} axis is the elongate direction, the anisotropy fields add linearly (S&W, p. 81), yielding a theoretical peak resonance between 1,164 and 9,609 MHz. Elongation of particles in other crystallographic directions would result in vector combinations of the anisotropy fields, resulting in slightly lower frequencies.

Calculating the width or sharpness of the ferromagnetic resonance is not a simple matter, as it depends in a complex fashion on the particle shape, volume, Fe\(^{2+}\) content, and packing arrangement of adjacent crystals. Although it appears that no ferromagnetic resonance parameters have yet been measured for any biogenic magnetites, there are at least two reasons to suspect that the resonance absorption will be broad. First, the intrinsic width of the absorption peak increases with the Fe\(^{2+}\) content in ferrites, due to electron hopping between adjacent Fe\(^{2+}\) and Fe\(^{3+}\) centers and the increased dielectric constant (S&W, p. 292). Second, all known biogenic magnetites have been found either in linear chains, as in magnetotactic bacteria, algae, and salmon, or in clusters like those in chiton teeth (Kirschvink & Lowenstam 1979; Nesson & Lowenstam 1985), although additional arrangements may also exist (Ghosh \textit{et al.}, 1993; Kobayashi \textit{et al.}, 1993, 1994). Although the average concentration of biogenic magnetite in human tissues is small (5-100 ppb), magnetic interaction data (Kirschvink \textit{et al.}, 1992) imply that the crystals are in interacting clumps of some sort, as would be expected if they were localized in specialized cells as noted earlier.

As the magnetic field of a neighboring grain will add vectorially to the internal field controlling the precession frequency of each Bohr magneton in the crystal, a random assortment of magnetically interacting particles should act to broaden the resonance. Note that the initial state of an interacting assemblage of semi-mobile magnetosomes should be such that the external magnetization should be near zero. Exposure to a strong external field, such as that from an MRI device, will leave a net remanence on an interacting cluster, potentially changing the resonance characteristics. This could be tested experimentally.

\textit{Experimental Measurements of Microwave Absorption Spectra from Magnetite.} Kirschvink (1996) was unable to locate any detailed information concerning the spectral absorption of magnetite in the microwave range, although a few point observations at the ‘oven’ frequency of 2.45 GHz were known. These include reports of Chen \textit{et al.} (1984) who noted that magnetite was relatively opaque to the transmission of 2.45 GHz microwave radiation. Walkiewicz \textit{et al.} (1988) conducted microwave heating studies on a variety of materials in a 1 kW, 2.45 GHz oven. A 25 g sample of magnetite powder reached 1258 °C in only 2.75 minutes, making it one of the best microwave absorbers of the 150 reagent-grade elements, compounds, and natural minerals tested. This work has led subsequently to the development of microwave sintering of iron ore, wherein the rapid thermal expansion of magnetite cracks the surrounding rock matrix, reducing the energy required in the ore grinding process (Walkiewics \textit{et al.}, 1991).
A more desirable goal is to know the absorption characteristics of magnetite and other materials across the entire spectrum of possible human exposure frequencies, and to compare these absorption properties with those of other materials. These data would form a basis for biophysical inference concerning the possible role of microwave absorption within specialized cells containing magnetite. Towards this goal we have initiated a collaboration with Prof. David Rutledge, director of the millimeter wave laboratory at Caltech. Through much trial and error, we finally developed a simple method for measuring the absorption spectra of small quantities of mineral powders placed in the core of an air-gap connector (basically, a short coaxial connector with a gap between the central lead and the shield). Using a Hewlett-Packard Network Analyzer, we were able to measure the intensity and phase relationships of a low-intensity microwave beam as it interacted with the material in the air gap, including both reflected and transmitted waves. From these data the power absorbed at frequencies between 1 and 40 GHz can be determined, as shown here on Figure 2-4.

In agreement with the above analysis, we found that fresh biogenic magnetite precipitated by the magnetotactic bacteria is by far the strongest broad-band absorber of microwave radiation of all the mineral powders we tested, at all frequencies up to 40 GHz. However, comparison of the absorption spectra with that of water reveals that it is only in the 0-5 GHz region where magnetite dominates over it; at higher frequencies water stops penetration of the microwave energy better than magnetite does. Hence, at the cellular telephone frequencies of about 0.9 and 2 GHz, the mechanisms considered by Kirschvink (1996) remain a potential concern.
Figure 2-4
Microwave absorption spectra in the 1-40 GHz frequency range for important magnetic minerals. Mineral powders were packed into the air-gap of an HP SMA, 50-Ω male-male gender changer, forming a powder cylindrical shell (located between the 3 mm diameter coaxial outer shield and the 1.6 mm i.d. central conductor, approximately 10 mm long). The gender changer had been previously calibrated (zeroed) with an HP Network Analyzer for four frequency ranges (1-10, 9-20, 19-30, and 29-40 GHz), each with 200 intermediate frequencies. For each frequency, data on the microwave transmission from input to output of the circuit \((S_{12})\) and reflection \((S_{11})\) were recorded, allowing the adsorbed power to be calculated as \(1-S_{12}^2-S_{11}^2\) (e.g., energy that is neither reflected by, nor transmitted through, the sample is adsorbed). This absorption data was normalized for the mass of the magnetic mineral in the exposure device. For the maghemite, hematite, pyrrhotite, and goethite samples, the mass of material was measured by difference before and after loading the powder. Samples of bacterial magnetite were obtained by freeze-drying bacterial pellets grown in pure culture, and then packing a measured volume of the powder into the gender changer. Magnetite-bearing teeth from the Polyplacophoran mollusk, Cryptochiton stelleri, were dissected and pooled from the radulae of 6 individuals (about 400 teeth). Particle size for the single-domain magnetite crystals is approximately 0.12 μm for the chitons (Kirschvink & Lowenstam, 1979), and is approximately 40 nm for both bacterial strains. The mass of magnetite for the biological samples was measured from the saturation isothermal remanent magnetization.
FERROMAGNETIC CONTAMINATION AND EMF BIOEFFECTS

Rationale

During the past decade, there has accumulated a substantial literature dealing with the effects of extremely low frequency (ELF) electromagnetic fields (EMF) on in vitro systems. The cellular responses studied have been many and varied. They include such phenomena as calcium-ion release from chick brain tissue (Blackman et al., 1982; 1991), increased calcium-ion uptake by lymphocytes (Lyle et al., 1991), induction of fibroblast differentiation to a non-mitotic state (Rodemann et al., 1989), inhibition of bone cell response to hormonal stimulation (Luben et al., 1982; Cain et al., 1987), and alteration of protein synthesis in several systems (Goodman & Henderson, 1988; 1991; Blank & Goodman, 1989; Blank et al., 1993). Goodman and Henderson and their coworkers were pioneers in examining the effects of extremely low frequency electromagnetic fields (ELF EMFs) on cell transcription, initially with dipteran polytenic salivary gland cells (Goodman & Henderson, 1986; Goodman & Shirley-Henderson, 1991; Goodman et al., 1992c; Weisbrot et al., 1993), and then in a series of studies (Goodman et al., 1989; 1992a; 1992b; Goodman & Shirley-Henderson, 1991; Wei et al., 1990) with HL-60 cells, a human lymphocytic cell line established from a patient with acute promyelocytic leukemia (Collins et al., 1977). They reported the elevation of transcript levels for several genes, including b-actin, histone H2B, c-myc, c-src, and b-tubulin, upon exposure of cell cultures to EMF. However, there have been significant problems in the ability of investigators to replicate the Goodman & Henderson (referred to from here on as GH) group results. Some attempts (Greene et al., 1991; Krause et al. 1991) have reported stimulation of RNA synthesis, not of the specific mRNAs detected by the GH group, but rather of 45S precursor ribosomal RNA. Other investigations have failed to detect any response to EMF, and have suggested fundamental methodological flaws in the original experiments (Saffer and Thurston, 1995a,b).

In 1995, our attention was drawn to a different possibility: the entire literature of in vitro EMF effects had been oblivious to, or at least silent on, the subject of a possible role for ferromagnetic contamination in the production of cellular responses to EMF. As we pointed out in a short letter to Nature, (Kobayashi et al., 1995), there had been no attempts to control, measure, or reduce the levels of ferromagnetic particulates in the samples which undergo EMF exposure and are then analyzed for response. This criticism applies not only to the HL-60 cell experiments, but to all of the in vitro systems...
that have been the subjects of EMF exposure studies. In the remaining portions of this chapter, we will review this problem of ferromagnetic contamination and the response of the community to it. As of this writing, we know of no EMF in vitro experiments which have even attempted to control for this problem.

Biophysics of Cellular responses to magnetic fields. It is difficult to rule out a priori the possibility that ELF magnetic fields might be having a direct effect on cell biology. These laboratory studies have triggered much speculation on the fundamental question of how weak, extremely low-frequency magnetic fields might be capable of producing biological effects of any sort. Numerous authors have proposed transduction mechanisms invoking effects like calcium ionic resonance (Liboff 1985; Liboff & Mcleod 1988), quantum-mechanical interaction with bound states of ions ‘trapped’ in macromolecular cages (e.g., Lednov, 1990), as well as organized assemblages of cells in electrical contact with each other (e.g., Weaver & Astumian 1990, 1992). However, many prominent physicists have had trouble on fundamental principles with virtually all of the proposed mechanisms (e.g., Adair 1991abcd,1992, 1993, etc.), principally with the difficulty of producing an effect which can stand out above thermal noise. Although there are certainly quantum-mechanical processes which can influence chemical reactions through hyperfine interactions at reasonably low field strengths (e.g., Mclauchlan 1989), the difficulty with replicating many of the critical experiments does not provide confidence that the correct mechanism(s) has yet been found. It is interesting to note, however, that we have not found any reported effects of EMF fields on cells which are incompatible with the hypothesis of ferromagnetic interactions as described next. These effects are possible both through biogenic magnetite (Fe₃O₄), produced intracellularly through biochemical processes, or from ferromagnetic contaminants present in cell culture media.

Biophysics of ferromagnetic materials: A much simpler, and overlooked hypothesis. Most materials found in organisms are generally thought of as being non-magnetic, i.e., either diamagnetic (repelled weakly from a magnetic field, like water and most fatty substances), or paramagnetic (weakly attracted to a magnetic field, like the deoxyhemoglobin in blood cells). For materials of these types, the direct physical influence of the Earth’s magnetic field is extraordinarily weak, with the energy of magnetic interaction being many orders of magnitude below that of the background thermal energy, kT (where k is the Boltzmann constant and T the absolute temperature). However, another category of materials, termed ferromagnetic, interact very strongly with the earth’s magnetic field. Unlike diamagnetic and paramagnetic substances, quantum-mechanical interactions acting on unpaired electrons within ferromagnetic materials force the electron magnetic moments (Bohr magnetons) to form long-range alignments. The magnetic moments from each Bohr magneton within such a crystal are added vectorially, and in many materials a crystal of only a few tens of nanometers in size will have magnetic interaction energies with the geomagnetic field greater than the background thermal energy (kT). Motion of this material in response to external magnetic fields can in principle account for a variety of magnetic effects at the cellular level, such as opening mechanically sensitive trans-membrane ion channels (Kirschvink, 1992b, Kirschvink et al. 1993) as illustrated on Figure 2-1.
The conceptual model of a magnetite crystal connected to an ion channel (illustrated in Figure 2-1) forms the basis for a variety of back-of-the-envelope calculations which indicate that a typical 0.1 µm sphere of magnetite in cytoplasm could contribute 1 kT of energy to an ion channel at each 1/2 cycle of a 60 Hz ELF magnetic field, at field amplitudes of less than 0.1 mT rms (Kirschvink 1992; Kirschvink et al., 1993). If the particles were in a liquid with the viscosity of water, this number reduces even more. Effects at even lower fields are possible if several particles become attached to (or are present in) the same cell. Hence, this is a plausible biophysical mechanism which could account for many reported effects of EMFs on biology. A major question, however, concerns the nature of the ferromagnetic particles. If they are natural biogenic magnetites synthesized by the cells, then it is a reasonable inference that effects observed in tissue culture would be of concern for humans in vivo. On the other hand, if the ferromagnetic particles are contaminants inadvertently concentrated in the nutrient media used in cell growth, the observed biological effects could be purely artifactual, without any implications for human health. Hence it is fundamentally important to determine which, if either, might be responsible.

We next review briefly the evidence for magnetite biomineralization in higher organisms, as well as the common knowledge (at least in the field of rock magnetism) of ferromagnetic contaminants in virtually all laboratory plastics.

*Magnetite Biomineralization.* Magnetite, a ferrimagnetic inverse spinel, was first discovered 30 years ago as a biochemical precipitate capping the teeth of a primitive group of mollusks, the chitons (class Polyplacophora; Lowenstam, 1962). It was subsequently found to be a biochemical precipitate in 3 of the 5 Kingdoms of living organisms, including the magnetotactic bacteria (Frankel et al., 1979), protoctists (Torres de Araujo et al., 1985), and a variety of animals such as honeybees (Gould et al., 1978), pigeons (Walcott et al., 1979), oceanic fish (Walker et al., 1984; 1988; Mann et al. 1988), and many others. More recently it has also been found in the soft tissues of the human (Kirschvink et al., 1992). In comparison with other biogenic magnetites, the human brain crystals share many of the unique features of biogenic magnetite. In many organisms, magnetite crystals are individually encapsulated by lipid-bilayer membranes to form structures termed magnetosomes (Gorby et al., 1988; Nakamura et al., 1991; Vali & Kirschvink 1990). Magnetosome chains in the microorganisms and fish cause their individual dipole moments to sum vectorially, providing a net magnetic torque large enough to produce their magnetotactic or magnetoreceptive behavior. However, magnetic material is also present in some animal tissues which can have no conceivable sensory role (e.g., tumors, Kobayashi et al., 1997), so there may be non-sensory functions for this material as well.

*Laboratory instrumentation and facilities.* In higher organisms other than the chitons, the presence of ferromagnetic particles was first discovered using ultra-sensitive magnetometers based on Rf-biased Superconducting Quantum Interference Devices (SQuIDs), particularly the type originally developed for use in rock magnetism (Fuller et al., 1985). The use of these instruments for detecting magnetite in animal...
Ferromagnetic Contamination and EMF Bioeffects

tissues and for assessing contamination in culture materials has been a major focus of our clean-lab magnetic facility (Walker et al., 1985; Kirschvink et al., 1992; Kobayashi et al., 1995). Largely through EPRI support, background noise levels on the DC-SQuID magnetometer system is such that nearly a picogram of magnetite can be detected.

Ferromagnetic Contamination. The presence of ferromagnetic contaminants in many laboratory plastics and other materials has been common knowledge in the geophysical field of rock and mineral magnetism for well over 20 years, ever since the development of the SQuID moment magnetometers. It has also been a major headache for the field. Many rock samples are only weakly magnetized, and it is necessary to find materials for constructing sample holders that are less magnetic than the rock samples they support. It appears that virtually all laboratory plasticware is injection molded into metallic forms, and many solid plastics (like “pure” teflon) acquire a few ppm of sub-micron sized ferromagnetic inclusions during their manufacturing process. In practice, it is never possible to get a perfect, non-magnetic plastic sample holder. The best that can be done is to soak the plastics in concentrated HCl, and hope that the remanent moment is stable enough to subtract from the measured values. Although the absolute concentration of magnetic material may seem low, the number of particles is quite large. A concentration of 1 ppm of 0.1 µm diameter particles corresponds to 1.9 x 10^9 per ml, easily comparable (or exceeding) the typical cell density in EMF experiments. The best material that we have found for use in the clean-lab magnetic studies is an ultra-pure quartz glass, which we have made into thin (~ 50 µm thick) fibers which are then soaked in concentrated HCl.

The significance of this contamination problem became apparent to us during the course of our work with human T-cell leukemic (Jurkat) cells. It is customary to use disposable, pre-sterilized plastic labware (flasks, pipettes, centrifuge tubes, etc.) and commercially-prepared culture media because of their convenience and the assumption of a high level of quality control and cleanliness. However, we have found that none of these materials are free of ferromagnetic particulate contamination. We found that ferromagnetic contaminants are ubiquitous in the culture media, on the disposable plastic tissue-culture vials, and in the centrifuge tubes used during each time the nutrient media is replaced. Magnetic particles wash off of these materials, and are concentrated in the cell pellets during each centrifugation step. In an effort to adapt the methodology of tissue-culture to our magnetic clean-lab environment, we did a simple experiment which dramatically illustrates the magnitude of this contamination problem (Kobayashi et al., 1995). Starting with 100 ml of fresh medium, we conducted a ‘sham’ growth experiment in which the liquid was used to wash an equivalent number of flasks and tubes which would normally yield ~ 0.1 gram of Jurkat cells (~ 15 disposable 250 ml flasks and centrifuge tubes). After the final centrifugation step, a diffuse, gray cloudy clump of material was visible at the bottom of the tube. This junk could be moved around the bottom of the tube with a small hand magnet. Subsequent SQuID measurements indicated that the magnetic material was extremely fine-grained, well dispersed, and probably a combination of magnetite, maghemite, and perhaps some other ferromagnetic phases. We detected the equivalent of 160 ng of magnetite in the
rinsate, and the magnetic data indicated that the contaminants are small particles, usually in the sub-100 nm size range. 160 ng of magnetite equates to about 32 million 100 nm cubes, which can be compared to the ~ 1 million cells which would have been produced in a culture of this volume.

Matsunaga et al. (1989) have demonstrated that 100 nm particles of magnetite, whether naked or coated with bovine serum albumin, are readily taken up by human white blood cells, including non-phagocytic lymphocytes as well as phagocytes. Because the ferromagnetic particles interact strongly with magnetic fields, their presence in cell cultures, at a number density far higher than that of the cells, may provide a simple mechanism to account for links between EMF exposure and \textit{in vitro} biological effects.

A simple calculation shows that the mechanical energy present in a single 0.1 µm magnetite crystal exposed to a 60 Hz, 0.1 mT magnetic field is many times the thermal background noise (Kirschvink 1992). Such particles, if adsorbed on cell surfaces or ingested by the cells, could conceivably transfer this energy to contiguous cell structures such as mechanically-activated ion channels which operate with a gating force close to the thermal noise limit (Denk and Webb 1989), and thereby alter cytoplasmic ion concentrations sufficiently to produce the observed bioeffects.

\textit{Aftermath of the Kobayashi et al. (1995) Nature letter}

In any normal experimental science, identification of an uncontrolled and potentially fatal variable would lead to a flurry of activity aimed at testing the hypothesis that it may have compromised previously published results. Furthermore, studies conducted after identification of the problem ought to include at least minimal efforts to control or mitigate the confounding variable. Unfortunately, it would appear that the field of \textit{in vitro} EMF bioeffects is not a normal experimental science.

Table 3-1 shows the results of a Science Citation Index search on the peer-reviewed literature for articles which refer to the Kobayashi \textit{et al.} (1995) letter. As of October, 1998, this index yields thirteen papers, five of which deal with cultured cells, of which three report a positive EMF effect. Although two of these (Belayaev \textit{et al.}, 1997 and Belayaev \textit{et al.}, 1998) mention ferromagnetic contamination in a discussion of several possible mechanisms (motility and pattern formation of E. coli cells; production of stable chemical messengers; secondary radiation in cell-to-cell communication during the response to ELF, etc.), the problem of ferromagnetic contamination is never mentioned again nor considered as an explanation for their results. Only Blackman \textit{et al.} (1995), in a note added in proof, argue that the complexity of their results implies that they are “… not magnetite based and therefore is beyond the scope of objections recently raised to \textit{in vitro} magnetic field studies (Kobayashi \textit{et al.}, 1995)”. We disagree with this assessment. Blackman \textit{et al.}’s (1995) data basically show minimal effect in low intensity fields, maximum effects at intermediate combinations, and minimal effects at stronger values. This pattern could be mimicked by a modified magnetosome/ion-channel model in which no response is produced before the magnetosome energy is brought up to a threshold level for opening a channel, and in which the magnetite
becomes detached from the channels in the highest energy situation. The only method to rule out the contamination hypothesis is to actually test it, which they have not done. Dr. Blackman has also politely refused our request for his data, thereby preventing us from attempting to model them with a magnetite-based system.

But the citation search only flags those articles which cite the Kobayashi et al. (1995) letter. A related question concerns the continued publication of studies reporting positive EMF effects from cell culture experiments which simply ignore the problem. As a crude assessment of this, we have examined all issues of Bioelectromagnetics from August, 1995 (5 months after publication of Kobayashi et al. (1995) to the fall of 1998). We found 22 papers which both reported a positive EMF effect and used isolated cells of any sort. Of these, 16 involved the use of ELF EMF, and the other 6 used pulsed, RFR, EHF or DC EMFs. Twelve of these 22 papers consist only of a report of the effect without discussing any eventual mechanism. The ten others discuss several mechanisms, but none of them examine or attempt to test the contamination possibility.

Demonstration that a positive EMF bioeffect is not the result of an uncontrolled artifact in an experimental procedure is an impetus which rests squarely on the shoulders of the authors which make such claims. Our mini-review of the recent literature of in vitro EMF experimentation suggests that this onus is being ignored in the field of Bioelectromagnetics. This is not a healthy situation, and is certainly consistent with labels of ‘pathological science’ which have been applied to the field (Adair, 1991). As stated by Kobayashi et al., (1995), “…any effect of EMF exposure on cultured cells, if it is due to the presence of ferromagnetic contaminants, would have no relevance to in vivo biology. Data used to establish human exposure standards to electromagnetic fields must rely on properly controlled experiments.” As of this writing, it appears that there are no such data from cell culture experiments which meet this standard. As the primary goal of the NIEHS/DOE RAPID program was to give the U.S. Congress advice on EMF levels to set for human exposure, virtually all funds expended by this program for in vitro studies have been wasted.
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<tr>
<th>Paper</th>
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<td>1. Wilson et al. (1998)</td>
<td>Size characterization of emulsion particles; does NOT deal with the issue of EMF biological effects</td>
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<td>5. Kirschvink (1996)</td>
<td>Theoretical considerations on the microwave absorption by magnetite</td>
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<td>7. Saffer and Thurston (1995a)</td>
<td>Comment on the lack of controls in studies claiming positive EMF effects</td>
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<tr>
<td>11. Belyaev et al. (1997)</td>
<td>Cell exposure: VH-10 fibroblasts and lymphocytes</td>
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MAGNETORECEPTION

Rationale

Of all known methods through which living organisms might perceive weak, ELF magnetic fields, magnetite-based magnetoreception is clearly the simplest and has the most experimental support. Following the initial studies using superconducting moment magnetometers which initially revealed the presence of biogenic magnetite in higher animals (Gould et al. 1978; Walcott et al. 1979), bulk extraction methods were developed which eventually allowed the extraction and identification of chain-like structures of magnetite crystals, which turned out to be very similar to those present in the magnetotactic bacteria (Kirschvink et al. 1985; Mann et al. 1988; Walker et al. 1984). Following on these discoveries were behavioral tests of this magnetite-based magnetoreception hypothesis (Kirschvink and Gould 1981) involving the use of short magnetic pulses, similar to those which can convert North-seeking magnetotactic bacteria to South-seekers (Kalmijn and Blakemore 1978). Honey bees (Kirschvink and Kobayashi-Kirschvink 1991) and a variety of birds (Beason et al. 1997; Munro et al. 1997a; Munro et al. 1997b; Wiltschko et al. 1994; Wiltschko and Wiltschko 1995) display clear pulse-related effects on their behavior, definitively flagging the role of a ferromagnetic receptor system in animals. As these data strengthened, the biophysical theory and experimental constraints also improved our understanding of how a magnetite-based sensory system could yield ELF responses in animals (Kirschvink et al. 1992; Kirschvink 1992a; Kirschvink 1992b; Kirschvink et al. 1993; Kirschvink et al. 1997; Kirschvink and Walker 1985). Electrophysiological studies also followed suit, with successful reports of magnetically-influenced activity in the ophthalmic branch of the trigeminal nerve in both birds (Beason and Semm 1996; Semm and Beason 1990) and fish (Walker et al. 1997). And finally, (Walker et al. 1997) (see also (Kirschvink 1997)) make a reasonably solid case for having isolated a magnetite-containing receptor cell connected to the trigeminal nerve in trout. Throughout the past decade, EPRI support has played a major role in the general advancement of this field.

In this chapter, we take the opportunity to present two results of EPRI support which extend the field of magnetoreception. The first is a detailed study of the magnetic properties of the spotted Newt, for which a variety of geomagnetic effects are known but for which the presence of magnetite had been unclear. The second addresses the question of pattern recognition in the geomagnetic field, with particular focus on the question of whether or not animals may have developed an ability to recognize
geomagnetic fluctuations which happen prior to Earthquakes, such as that which preceded the Loma Prieta event in central California (Fraser-Smith et al. 1990). Both of these have either been submitted for publication in peer-reviewed journals, or will be shortly.

**A magnetite-based map component of homing in the Eastern red-spotted newt?**

**Abstract**

Behavioral results obtained on the Eastern red-spotted newt (*Notophthalmus viridescens*) led to the suggestion of a hybrid homing system involving signals from both a light-dependent and a non-light-dependent mechanism (Phillips 1986b; Phillips and Borland 1994). To evaluate the possible role of a receptor based on biogenic magnetite in this animal, we performed magnetometry experiments on a set of newts previously used in behavioral assays. The natural remanent magnetization (NRM) carried by these newts was strong enough to be measured easily with a DC-SQuID moment magnetometer. Isothermal remanent magnetizations (IRM) were two orders of magnitude higher than the NRM and gave evidence for the presence of a ferromagnetic material consistent with magnetite in the newt’s body. The NRM has no preferential orientation among the animals when analyzed relative to their body axis, and the demagnetization data show that the magnetic material grains are not aligned parallel to each other within each newt. Although the precise localization of the particles was not possible, the data indicate that magnetite is not localized in a restricted “punctual” area. A quantity of single-domain magnetic material is present which would be adequate for use in either a magnetic intensity or direction receptor. Our data, when combined with behavioral results, suggests a link between the behavioral choice and the ferromagnetic material, providing some support to the idea that magnetite plays a significant role at least in the map component of homing of the Eastern red-spotted newt.

**Introduction**

The Eastern red-spotted newt is a terrestrial vertebrate known to exhibit two forms of orientation behavior based on the Earth’s magnetic field. One is a simple-compass orientation (Phillips 1986a), in which a fixed (shoreward) trained directional heading is maintained despite changes in the background field direction. The other is a magnetic effect on navigation or homing (Phillips 1987), which requires both some form of ‘map’ information to determine the correct geographic position relative to home, as well as a compass to orient in the homeward direction. In a subsequent study (Phillips et al. 1995), newts were found to use true navigation, *i.e.* to return to the origin of a displacement (‘home’) without access to familiar landmarks or goal-emanating cues, and without knowledge of the displacement route.
One could have thought that the newt’s compass component of homing is derived from the same pathway (or mechanism) as the one involved in the simple compass orientation. Nevertheless, subsequent results strengthened the hypothesis that the newts were using separate magnetoreception systems in these two forms of orientation. When a simple compass is needed, newts appear to use an axially sensitive receptor system (i.e., one which responds only to the magnetic field inclination), whereas their homing response uses a compass which can detect the polarity of the field (Phillips 1986b). Subsequent studies indicate that shoreward and homeward magnetic compass orientations are affected by the wavelength of light (short-wavelength and full visible spectrum vs. long-wavelength) in different ways (Phillips and Borland 1992; Phillips and Borland 1994).

Based on their findings, Phillips and Borland (1992, 1994) suggested that a light-dependent magnetic compass was used for the shoreward orientation response, whereas the homing response would be produced by a “hybrid mechanism”. This latter response would combine information from the previous light-dependent compass with input from a non-light-dependent system. However, the debate continues as to whether the light effect is a primary feature at the level of the biophysical magnetic compass transducer, or an influence of light on the animal’s subsequent behavior (Kirschvink et al. 1997). There is agreement that the sensitivity needed for the magnetic navigation response is incompatible with a response produced by an optically-driven radical pair recombination system (Schulten 1982), whereas it is within the theoretical range for a magnetite-based system (Kirschvink and Walker 1985).

In the 35 years since Lowenstam (1962) proposed the hypothesis that ferromagnetic material could serve as a possible magnetic transducer, biogenic magnetite particles have been found in a wide variety of organisms (e.g., (Gould et al. 1978; Kirschvink et al. 1985a; Walcott et al. 1979; Wiltschko and Wiltschko 1995a)), some of which display behavioral and neurophysiological evidence for an Earth-strength magnetic field sensitivity (e.g. (Beason and Semm 1987; Semm and Beason 1990; Walker et al. 1997)). In parallel with these discoveries, numerous biophysical models were developed of how small magnetite crystals might be transduced into neurological signals which could be used by an animal (Kirschvink et al. 1993; Kirschvink and Gould 1981; Kirschvink et al. 1992a; Kirschvink and Walker 1985; Yorke 1979). Given the properties of the newt’s homing response, Phillips and Borland (1994) assumed that the non-light-dependent part of the “hybrid mechanism” could be magnetite-based.

In order to assess this suggestion, the present experiments took advantage of the EPRI-sponsored magnetometry facility at the California Institute of Technology. A set of newts which had been tested for homing ability by the Phillips group at Indiana University was used to test for any presence of ferromagnetic material. Our data show that the red-spotted newt contains magnetic particles with properties that are consistent with magnetite, thereby supporting the hypothesis that it uses a magnetite-based receptor for navigation.
Methods

The investigations have been carried out on a set of adult male newts *Notophtalmus viridescens* previously subjected to magnetic orientation experiments (the results of which will be discussed elsewhere: Phillips *et al.*, manuscript in preparation). These animals were collected in ponds SSW and ESE from Bloomington, Indiana. They have been prepared at Indiana University and analyzed at Caltech. They were anesthetized with MS-222 at least 24 hours after testing, frozen in liquid nitrogen, and then shipped on dry ice. The experiments used ultrasensitive moment magnetometers (2G Enterprises®) employing DC-biased superconducting quantum interference devices (SQUIDs). These devices are designed to measure the total ferromagnetic moment of samples placed within a Helmholtz-coil pickup loop (Fuller *et al.* 1985). In order to assess the characteristics of any ferromagnetic material, newts were subjected individually to several rock magnetic experiments: The NRM vector was initially measured and demagnetized progressively with alternating magnetic fields (Af demagnetization). They were then given an anhysteretic remanent magnetization (ARM) in progressively stronger biasing fields, the strongest of which was then Af demagnetized. Next, they were given an isothermal remanent magnetization (IRM) in a peak pulse field of 100 mT, which was then Af demagnetized. Finally, a complete IRM acquisition spectrum was conducted in log-spaced steps up to 1 Tesla. During the measurements the newts were maintained frozen by a flow of dry, cold-filtered air within the sense region of the magnetometers. The temperature was at least lower than -40/-50 °C (223/233 K). All measurements were performed in a magnetically shielded environments, dust-free in the case of IRM and ARM experiments. The newts were suspended on a thin nylon monofilament line, moved by a stepping motor between the loading and measurement regions of the magnetometer, and eventually the magnetization and demagnetization coils. The measurements, except for the NRM, were conducted routinely. As we cannot control the temperature precisely inside the magnetometer, we tentatively performed a rough “warming experiment” on two newts. Each sample was given an IRM with a 1T pulse after being soaked in liquid nitrogen. Following this, the sample magnetization was monitored every few seconds for the following 11+ minutes as when warming in an atmosphere which temperature was between -18 and 0 °C (255 and 273 K). This procedure was then repeated once without the 1T pulse and finally once with this pulse.

Results

A natural magnetic remanence was detected in all the newts examined, as indicated in Table 4-1. The NRM ranges from 0.57 to $25.4 \times 10^{-11}$ Am$^2$, with an average of $4.40 \times 10^{-11}$ Am$^2$, whereas the background noise levels were in the range of $1.1 \times 10^{-12}$ Am$^2$. The NRM directional analysis relative to the anterior head shows no particular direction of alignment among the newts ($p > 0.30$, test of uniformity (Fisher *et al.* 1987) applied on the 18 NRM directions). Experiments utilizing the Af demagnetization of the NRM are designed to detect multiple magnetic directions.
within a sample; these gave a qualitative indication of the directions present in each newt (Figure 4-1). This distribution is also statistically random (positive test of uniformity (Fisher et al. 1987)) except for three newts. For these latter, however, though the previous statistical test gives a probability of uniform distribution lower than 5%, the scatter in the grain distribution is high. Thus, in each newt the magnetic grains are not more or less aligned along a specific direction, instead their directions are scattered rather randomly. The characteristics of the magnetometer did not allow even a rough estimate of their localization in the newt’s body. However, the magnetic signal displayed along the newt body (obtained by measuring the magnetization given for different longitudinal distances between the newt’s nose and the magnetometer sense region; data not shown) has a very consistent pattern among the newts and is definitely not compatible with the idea that the magnetic particles are clustered in a unique area (at least less than 0.5-1 mm long). The magnetization carriers seem rather to be spread out in a large volume of the newt’s body. However, the anatomy of their distribution and the volume they occupy are both unknown.

Table 4-1
Intensity and direction of the NRM.
NRM, intensity of the NRM and its standard error; D and I, are the declination and inclination of the NRM, respectively; α95 and k, 95% confidence cone and the precision parameter of the Fisher’s (1953) statistics. The NRM shows no preferred direction of orientation among the newts (n=18, p>0.30; probability of uniform distribution; Fisher et al., 1987; non significant preferred orientation= p>0.05).

<table>
<thead>
<tr>
<th>Newt</th>
<th>NRM (10⁻¹¹ A.m²)</th>
<th>D (deg)</th>
<th>I (deg)</th>
<th>α95</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.47 ± 0.05</td>
<td>347.9</td>
<td>32.0</td>
<td>1.1</td>
<td>1166.6</td>
</tr>
<tr>
<td>C</td>
<td>25.44 ± 0.10</td>
<td>136.5</td>
<td>-35.7</td>
<td>0.9</td>
<td>1946.1</td>
</tr>
<tr>
<td>E</td>
<td>5.92 ± 0.03</td>
<td>284.4</td>
<td>23.8</td>
<td>0.8</td>
<td>2763.8</td>
</tr>
<tr>
<td>F</td>
<td>1.94 ± 0.06</td>
<td>305.5</td>
<td>26.2</td>
<td>1.3</td>
<td>976.7</td>
</tr>
<tr>
<td>G</td>
<td>6.26 ± 0.08</td>
<td>146.8</td>
<td>-1.5</td>
<td>0.5</td>
<td>8438.3</td>
</tr>
<tr>
<td>H</td>
<td>7.25 ± 0.04</td>
<td>47.3</td>
<td>28.2</td>
<td>0.9</td>
<td>2309.2</td>
</tr>
<tr>
<td>J</td>
<td>2.79 ± 0.12</td>
<td>298.5</td>
<td>-18.6</td>
<td>2.2</td>
<td>982.6</td>
</tr>
<tr>
<td>L</td>
<td>1.88 ± 0.03</td>
<td>357.9</td>
<td>5.6</td>
<td>1.6</td>
<td>671.5</td>
</tr>
<tr>
<td>M</td>
<td>1.65 ± 0.02</td>
<td>92.6</td>
<td>-1.3</td>
<td>1.6</td>
<td>564.0</td>
</tr>
<tr>
<td>N</td>
<td>1.94 ± 0.01</td>
<td>128.3</td>
<td>-2.3</td>
<td>1.3</td>
<td>1296.5</td>
</tr>
<tr>
<td>O</td>
<td>0.58 ± 0.03</td>
<td>291.3</td>
<td>-11.5</td>
<td>2.2</td>
<td>244.7</td>
</tr>
<tr>
<td>P</td>
<td>1.37 ± 0.01</td>
<td>97.2</td>
<td>-11.3</td>
<td>1.7</td>
<td>410.3</td>
</tr>
<tr>
<td>Q</td>
<td>8.89 ± 0.03</td>
<td>40.6</td>
<td>39.5</td>
<td>0.7</td>
<td>3978.5</td>
</tr>
<tr>
<td>T</td>
<td>1.02 ± 0.03</td>
<td>14.9</td>
<td>-9.6</td>
<td>1.1</td>
<td>1579.5</td>
</tr>
<tr>
<td>W</td>
<td>1.05 ± 0.03</td>
<td>4.3</td>
<td>-39.1</td>
<td>1.9</td>
<td>343.1</td>
</tr>
<tr>
<td>X</td>
<td>4.76 ± 0.08</td>
<td>133.8</td>
<td>66.6</td>
<td>1.0</td>
<td>1506.0</td>
</tr>
<tr>
<td>Y</td>
<td>1.30 ± 0.03</td>
<td>247.3</td>
<td>14.6</td>
<td>2.0</td>
<td>437.3</td>
</tr>
<tr>
<td>Z</td>
<td>3.66 ± 0.06</td>
<td>105.0</td>
<td>53.3</td>
<td>2.0</td>
<td>471.1</td>
</tr>
</tbody>
</table>
Figure 4-1

Equal-area projection of mean magnetic grain directions deduced from the af demagnetization of the NRM. The procedure used here is to view the magnetization vector directions as radiating from the center of a unit sphere and to display the intersection of these vectors with the sphere. The sphere and the points of intersection of the vectors with it are then projected onto the horizontal plane. Black solid squares are for negative inclinations and grey solid circles for positive inclinations, i.e. downward and upward relative to the horizontal plane, respectively. The declination is measured around the perimeter of the projection, clockwise from 0° (0° and 180° are oriented toward the nose and the tail of the newt, respectively). The inclination is measured from 0° at the perimeter of the projection to ±90° at the center of the projection. Each point represents the mean direction for grains with coercivities within the range defined by two following step of demagnetization. This gives a qualitative idea of the distribution of the grain orientations.
There is a consistency in the characteristics of acquisition and Af demagnetization for IRM and ARM among the newts; Figure 4-2 displays the mean patterns with their standard deviation. The fact that the newts gain and lose magnetization in such experiments yields evidence for the presence of ferromagnetic materials in the newt’s body. The magnetic characteristics given by IRM acquisition and af demagnetization are displayed in Table 4-2. The saturated IRM values are two orders of magnitude higher than the NRM, with a mean of $2.08 \pm 1.13 \text{nAm}^2$, with saturation arising between applied fields of 400 and 600 mT, but mainly around 400 mT. The ARM acquisition pattern (Figure 4-2b) shows that there is a high intergrain interaction effect. All these results are consistent with interacting SD grain magnetite (Cisowski 1981) and suggest that the ferromagnetic crystals are disposed in small interacting clumps. On another hand, the decrease in magnetization exhibited in our “warming experiment” (Figure 4-3) suggests that the newts also contain superparamagnetic (SPM) grains, as do other organisms (Kirschvink and Gould 1981; Kirschvink and Woodford 1991).

**Table 4-2**  
Magnetic characteristics as deduced from the IRM acquisition and af demagnetization experiment.  
sat. field, saturation magnetic field; mdf, medium destructive field; Hrc, remanent coercive force; sIRM, saturated IRM.

<table>
<thead>
<tr>
<th>Newt</th>
<th>sat. field (mT)</th>
<th>Mdf (mT)</th>
<th>Hrc (mT)</th>
<th>sIRM ($10^9 \text{ A.m}^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>400</td>
<td>32.2</td>
<td>51.3</td>
<td>2.87</td>
</tr>
<tr>
<td>C</td>
<td>400</td>
<td>32.2</td>
<td>48.7</td>
<td>4.57</td>
</tr>
<tr>
<td>E</td>
<td>400</td>
<td>41.0</td>
<td>59.9</td>
<td>1.41</td>
</tr>
<tr>
<td>F</td>
<td>400</td>
<td>38.9</td>
<td>56.9</td>
<td>1.38</td>
</tr>
<tr>
<td>G</td>
<td>520</td>
<td>37.6</td>
<td>55.0</td>
<td>2.04</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>36.9</td>
<td>52.2</td>
<td>4.26</td>
</tr>
<tr>
<td>J</td>
<td>600</td>
<td>32.2</td>
<td>52.2</td>
<td>3.71</td>
</tr>
<tr>
<td>L</td>
<td>400</td>
<td>29.0</td>
<td>43.2</td>
<td>2.83</td>
</tr>
<tr>
<td>M</td>
<td>400</td>
<td>33.3</td>
<td>52.2</td>
<td>1.55</td>
</tr>
<tr>
<td>N</td>
<td>460</td>
<td>33.9</td>
<td>52.2</td>
<td>1.10</td>
</tr>
<tr>
<td>O</td>
<td>460</td>
<td>47.0</td>
<td>71.2</td>
<td>1.85</td>
</tr>
<tr>
<td>P</td>
<td>400</td>
<td>36.3</td>
<td>53.1</td>
<td>1.90</td>
</tr>
<tr>
<td>Q</td>
<td>400</td>
<td>30.0</td>
<td>46.2</td>
<td>0.75</td>
</tr>
<tr>
<td>T</td>
<td>400</td>
<td>39.6</td>
<td>59.0</td>
<td>0.76</td>
</tr>
<tr>
<td>W</td>
<td>400</td>
<td>38.9</td>
<td>61.0</td>
<td>1.65</td>
</tr>
<tr>
<td>X</td>
<td>400</td>
<td>37.0</td>
<td>59.9</td>
<td>1.11</td>
</tr>
<tr>
<td>Y</td>
<td>400</td>
<td>34.5</td>
<td>52.2</td>
<td>1.68</td>
</tr>
<tr>
<td>Z</td>
<td>400</td>
<td>39.6</td>
<td>58.9</td>
<td>2.04</td>
</tr>
</tbody>
</table>
Figure 4-2
Mean pattern of magnetic behavior of the newts magnetization carriers.

a) The curve labeled IRM acquisition shows the relative magnetic moment remaining after a brief exposure to a magnetic pulse of the indicated strength. The tendency of the curve to flatten at high field levels is characteristic of the magnetite-maghemite solid solution series; most other ferromagnetic minerals saturate in fields higher than 1T. The curve labeled af of sIRM shows the progressive alternating-field demagnetization of the saturation IRM. The magnetic field value at which these two curves cross is a good estimate of the remanent coercive force (Hrc). The ordinate of the intersection point for non-interacting particles occurs at the 50% value; a depression or shift in this position is an indication of particle clumping effects.

b) The acquisition of ARM. The upper control curve shows data from a sample of magnetotactic bacteria in which the magnetite crystals are aligned in linear chains and have few interparticle interactions, whereas the lower control curve is from a sample of magnetite from chiton teeth, which are SD crystals but are highly interacting. The solid squares bound by a dashed line represent the mean pattern for the newts ferromagnetic material. sIRM, saturated IRM.
Figure 4-3
Evolution of the magnetization during a warming from a low temperature. Néel (1949) has shown that the relaxation time for a magnetic domain will increase exponentially as the temperature is lowered. Thus some grains which are SPM at room temperature (= 300K) will behave as single domains capable of holding remanence at much lower temperature. Conversely, any net remanence held by these domains at low temperature will disappear as they warm in field-free space across their SPM/SD transition (their blocking temperature). For the first warming cycle, the sample was given an IRM at low temperature, and the magnetization then monitored through time (curve 1) as the sample warmed inside the field-free region of the magnetometer. Then, curve 2 was obtained after the sample has been cooled down, but not given an IRM. Curve 3 shows a subsequent warming cycle following the same procedure as for the first cycle. See text for more details. The sharp decrease in curves 1 and 3 compared to curve 2 suggests the presence of SPM ferromagnetic grains in the newt.
Discussion

This study indicates the presence of ferromagnetic material in the newt body. The consistency of the magnetic property data among the newts makes it unlikely that the observed magnetizations could be the consequence of occasional external contamination. Although it was impossible to define the spatial disposition of the magnetic particles, our results suggest they form clumps that could be situated in rather different areas of the body. This would not be unusual given that some animals, and even humans, have been shown to contain ferromagnetic particles in different parts of their body (e.g. (Grassi-Schultheiss et al. 1997); Kirschvink et al. 1992b; Kirschvink and Walker 1986; Wiltschko and Wiltschko 1995a)). Our magnetometry results, along with the fact that ferromagnetic material found in animal and human tissue was mostly identified as the iron oxide magnetite, suggest that SD/SPM magnetite particles are most likely the magnetization carrier in the Eastern red-spotted newt (Notophtalmus viridescens). This remains, however, to be confirmed by extraction and clear characterization (using e.g. transmission electron microscopy and electron diffraction).

(Phillips and Borland 1992) proposed that the simple compass orientation (used in shoreward orientation) in newts is light-dependent, explaining their results by two antagonistic spectral mechanisms with a reduction in the sensitivity of the long-wavelength one. Using the similarity of the response spectral differentiation between shoreward and homing experiments, they also proposed (Phillips and Borland 1994) that the compass component of homing uses inputs from the previous pathway, thus giving rise to the idea of a hybrid navigational system in the newt.

Despite the consistency of newts’ shoreward orientation responses with a light-dependent, radical pair-based compass model (Deutschlander et al. 1997; Phillips and Borland 1992; Phillips and Borland 1994; Phillips and Deutschlander 1997), these results are also consistent with a secondary interaction between the visual system and the behavioral response (Kirschvink et al. 1997). Proponents of these optical effects have not yet designed a biophysical experiment capable of testing their role in the magnetic transduction process, in the same fashion that the now classic Kalmijn and Blakemore (1978) pulse-remagnetization experiment has been used to demonstrate the role of a ferromagnetic receptor in the birds and bees (Beason et al. 1997; Diaz-Ricci et al. 1991; Kirschvink and Kobayashi-Kirschvink 1991; Kirschvink et al. 1985b; Munro et al. 1997; Wiltschko et al. 1994; Wiltschko and Wiltschko 1995b).

As an alternative, Edmonds (1996) described a model of an optically detected magnetic compass, formed by the incorporation of needle-shaped ferrimagnetic single-domain (SD) crystals, such as magnetite, within liquid crystalline oil droplets that could be contained in retina’s cones. This model could also take into account the axial sensitivity and light-dependent patterns of the newt’s behavioral response to magnetic field. The presence of colored oil droplets in the inner segments of cones in avian retinas has been known since the last century (Goldsmith et al. 1984). Some reptiles also possess this kind of droplet (Liebman and Granda 1975) through which light must pass before reaching the visual pigment. But whether they are liquid crystals containing magnetite has not been demonstrated.
Even if the specific results of the shoreward experiments (axial magnetic sensitivity, a 90° shift between short- and long-wavelength responses (Phillips 1986b; Phillips and Borland 1992) suggest involvement of a radical pair-based mechanism, this is far from being obvious in the homing experiments. In these latter experiments, Phillips and Borland (1994) explained the random orientation of newts under long-wavelength light and the magnetic polar sensitivity of the compass as due to the interaction between the mechanisms forming the hybrid system of navigation. But the random orientation can as well be interpreted as the consequence of a “switch effect” of light on a non-light dependent mechanism: the homing compass working normally in the presence of short-wavelengths, and conversely being inhibited in their absence. Assuming this non-light-dependent compass system involves magnetite-based receptors under appropriate conditions (Kirschvink and Gould 1981), it would explain the polar sensitivity of the newt homing compass. Nevertheless this “switch effect” would bring up a puzzling point, namely the integration of an indirect effect of light in this magnetic compass, thus complicating the response pathway to the magnetic field. If a light-dependent shoreward compass actually exists, it would imply that the newt’s magnetoreception system is unnecessarily complex with two magnetic compasses and one magnetic intensity (or inclination) detector, as already noticed by Phillips and Borland (1994). The results of the most recent behavioral experiments (Phillips et al., manuscript in preparation), which involved newts trained under long-wavelength light, seems to be contradictory to such a complex system with a “switch effect”. At present, the question still remains open and would require other kinds of biophysical, behavioral, and neurophysiological data to resolve.

Regardless of the debate on the compass mechanisms, the main implication of our study is for the navigation system of the Eastern red-spotted newt. Though our data suggests that magnetite may be found in different anatomical areas of the newt’s body and, thus, may have different physiological functions, the presence of magnetite, together with the pattern of the homing behavior, suggests that magnetite-based magnetoreceptors are very likely involved in newt’s map component of homing. Indeed, this is the most appropriate explanation to the newt’s homing behavioral characteristics.

The newts have been demonstrated to display true navigation after being deprived of magnetic, visual, olfactory and inertial cues during displacement from their home pond (Phillips et al. 1995). Given that in this case no route-based directional information is available to them, it suggests that they rely, when homing, on, at least, a bicoordinate map. Following their findings, Phillips and Borland (1994) suggested that the geomagnetic field plays a role in this map. Yet an important requirement of a system deriving map information from the geomagnetic field is a high level of sensitivity (Gould 1980; Moore 1980). The radical pair recombination mechanism that could be involved in part of the newt’s orientation behavior (Phillips and Borland 1994) does not provide this kind of sensitivity (Schulten 1982), whereas a magnetite-based system does (Kirschvink and Walker 1985). Our data, along with the fact that magnetite is almost the only ferromagnetic biomineral found in animals and humans, suggests magnetite as the most plausible magnetization carrier in the newt. Moreover, magnetite-based
magnetoreceptors are compatible with the magnetic polar discrimination the newt displays when homing is experimentally elicited (Phillips 1986b). Thus, the involvement of magnetite in the newt’s map component of homing seems, at present, to be the best fit between the available data and theoretical considerations.

An additional interesting implication lies in the current debate about possible health effects of electromagnetic fields. When homing under natural conditions, the eastern newt moves over relatively short distances (i.e. < 5 km, generally 2-3 km). In its natural habitat in the northeastern USA, the regional spatial gradient in the geomagnetic field is 3.4 nT/km in total intensity, with a maximum of up to 16 nT/km around Ithaca, New York (Lednor 1982) and generally around 0.005 °/km in inclination. Therefore, the eastern newt represents an helpful means by which we could establish the threshold of sensitivity of biological systems to variation in an earth-strength magnetic field.

**Conclusion**

Magnetic sensitivity has been shown in a large number of animals (for a review see e.g. Wiltschko and Wiltschko, 1995a). But with the possible exception of the rainbow trout (Walker et al. 1997), the search for magnetosensitive cells has been frustrating. Thus, the magnetoreception mechanisms still rely on assumptions that ought to be consistent with the available data. Our study reveals the existence of a ferromagnetic biomineral in the Eastern red-spotted newt’s body. The magnetic properties of this material are consistent with SD/SPM grain-sized magnetite. This is the first direct evidence for the presence of ferromagnetic particles, most likely belonging to the maghemite-magnetite family, in an amphibian, thus expanding the variety of animal species containing such materials. Theoretical considerations associated with our results and behavioral data, make magnetite the best candidate, at present, for the putative magnetoreceptors involved in the map component of homing of this amphibian.
Earthquake Prediction by Animals: Evolution and Sensory Perception

Joseph L. Kirschvink

Division of Geological & Planetary Sciences, California Institute of Technology
170-25, Pasadena, CA 91125, U.S.A.; kirschvink@caltech.edu; 626-395-6136

Abstract

Animals living within seismically active regions are subjected episodically to intense ground shaking, the effects of which can kill individuals through burrow collapse, egg destruction, and tsunami action. Although anecdotal and retrospective reports of animal behavior suggest the ability of many organisms to detect an impending seismic event, no evolutionary plausible scenario has been presented so far through which such behaviors might have evolved. However, the evolutionary mechanism of exaptation can do this in a two step process. The first is to evolve a vibration-triggered early warning response, acting in the short time interval between the arrival of p- and s-waves. Anecdotal evidence suggests this exists. Next, if precursory stimuli exist well prior to the earthquake itself, similar evolutionary processes can link an animal’s perception of these stimuli to its p-wave triggered response, yielding a true predictive behavior. A population-genetic model indicates that such a seismic escape response system can be maintained against random mutations as a result of episodic selection that operates with time scales comparable to that of strong seismic events. Hence, additional understanding of possible earthquake precursors which are presently outside the realm of seismology might be gleaned from the study of animal behavior, sensory physiology, and genetics. A brief review of possible seismic precursors suggests that tilt, hygroreception (humidity), electric, and magnetic sensory systems in animals could be linked into a seismic escape behavioral system. Several testable predictions of this analysis are discussed, and it is recommended that additional magnetic, electrical, tilt, and hygro-sensors be incorporated into dense monitoring networks in seismically active regions.

I. Introduction

One of the major goals of seismology is to determine whether fault zones generate any hint of impending earthquakes. Towards this end, the geological study of rocks and layered sediments has proven useful for providing a chronicle of earthquakes prior to written history (Sieh, 1996), and new geophysical techniques like GPS provide real-time information on the buildup of strain across major faults. Unfortunately, these data shed little light to the more fundamental question of whether precursory phenomena exist which might be used for an accurate forecast in the period of a few hours to days before an earthquake. Recent discussions in the literature on this topic have been mixed, with much debate about whether large earthquakes are by their very nature unpredictable (e.g., (Geller, 1991a; Geller, 1991b; Geller et al., 1997a; Geller et al., 1997b)), or whether we simply need a more complete record of geophysical and geochemical data in
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earthquake prone areas (Aceves & Park, 1997; Park, 1996). Unfortunately, gathering data of this sort has progressed slowly, due to the rarity of large seismic events, the limited spatial distribution of continuously recording instruments, and the large number of variables which could be recorded.

It is clearly impractical and prohibitively expensive to record continuously a random variety of physical and chemical parameters near all possible earthquake epicenters. Even focused work on the San Andreas Fault at Parkfield, California, which was the probable epicenter of the 1857 Fort Tejon earthquake (Sieh, 1978) and the site of semi-periodic magnitude 6 earthquakes (Roeloffs & Langbein, 1994) demonstrates the cost and elusive nature of this recording problem. Hence, virtually all inferences about possible precursory phenomena must come from the occasional lucky observation, or must be gleaned from anecdotal reports obtained after the fact. In an attempt to systematically evaluate such observations, the International Association of Seismology and Physics of the Earth’s Interior (IASPEI) organized a sub-committee on earthquake prediction with the task of evaluating these precursory events through a peer-review process, and creating a preliminary ‘List of Significant Precursors’ (Wyss, 1991; Wyss & Booth, 1997), commonly referred to as the ‘List’. Between 1989 and 1997, a total of about 40 nominations were made and evaluated by this sub-committee, of which 5 cases were placed on the preliminary List, including foreshocks, preshocks, seismic quiescence before large aftershocks, radon decreases in ground water, and ground water level increases (Wyss, 1997). Six of the nominations which were not accepted for the List in the first two rounds dealt with various electromagnetic phenomena.

Given these difficulties in directly measuring pre-seismic phenomena, it seems worth asking whether information about seismic precursors might be available from a totally different, non-geological source: animal behavior. No nominations for the IASPEI List were considered which involved data from animal behavior, although the topic has certainly generated past interest [e.g., Gans, (1976); Tributsch, (1982)]. Claims of human sensitivity prior to earthquakes have also muddled this field, perhaps irrepairably given some of the patently absurd claims that have been made (e.g., headaches in northern California predicting earthquakes in Nicaragua, as described by Clarke, (1996)).

In discussions with my geophysical colleagues, a commonly-raised objection to the concept that animals might detect earthquake precursors is the fact that the life spans of most animals are much shorter than the typical repeat time of large seismic events. Hence, they are unlikely to remember any precursory signals. However, animal behavior is particularly susceptible to genetic control; so much so that an entire scientific journal is devoted to the topic (e.g., Behavior Genetics). Through the course of random mutation and natural selection, rare events which episodically kill or reduce the fitness of even a small fraction of the breeding individuals of a species can lead to the evolution of mechanisms to avoid such mortality; this is best known in the case of disease. Organisms can also evolve behaviors which enhance survival and fitness, such as the well-known escape responses from predators and fire. Many of these involve sophisticated pattern recognition abilities. Work during the past 50 years has demonstrated that a large number of complex behavioral responses exhibited by animals are under genetic control. Behavioral genetic systems, like all genes, are ancient
fossils which preserve information that has helped survival of the lineage over the past 3.5 billion years. Behaviors as complex as the honey bee waggle dance language are instinctive, appearing spontaneously even in populations of the insect raised without the benefits of experienced, adult worker bees. Many examples are known where a sensory input (through vision, hearing, touch, smell, etc.) elicits a ‘fixed-action’ response, causing an immediate and essentially involuntary, reflex-like reaction. These range from escape responses to mating behavior, and they occur in animals as diverse as flatworms, insects, and mammals. Note that behaviors of this sort are NOT learned – they are inherited, shaped through the long, slow process of random mutation and natural selection. It is therefore not necessary for the event which triggers the response to have happened within memory of any living individuals within a given population. The proper question is therefore best expressed within the context of population genetics: Is it reasonable for a seismic escape behavioral pattern to evolve, and can the genetic system be maintained in the face of selection pressures operating on the time scales of damaging seismic events?

Previous biological analyses dating back to the mid-1970’s concluded that an innate (genetically-based) seismic escape response was unlikely to have evolved in animals, due to the impression that earthquakes were ‘… too rare to establish a selective advantage that might permit genetic fixation of such a characteristic’, and the question of ‘… whether most species could take successful evasive action even if they had advanced knowledge of an impending earthquake’ (Gans, 1976). Both of these concerns need re-evaluation. First, in 1975 we had only rudimentary knowledge of the frequency of moderate and large seismic events. In California and many other areas we now realize that great earthquakes occur with average repeat intervals of 100 years or so (Dolan et al., 1995; Sieh, 1996). Although moderate earthquakes of M ~ 6+ affect smaller geographic areas, they are more numerous and may dominate the local seismic hazards for an area. Furthermore, zones of high seismic activity have existed on Earth for at least the past 2 billion years or more, as they are a byproduct of plate tectonic processes. A small selection pressure acting over a vast interval of geological time can be as effective at gene fixation as is stronger selection acting over a shorter time interval. Second, it occurs to this author that evasive action can, in many instances, reduce mortality during an earthquake. Earthquakes can kill animals or reduce their fitness in a variety of ways, ranging from the direct physical shaking (e.g., causing the collapse of burrows, shaking eggs out of nests, breaking honey comb, etc.) or indirectly through the action of mudslides and tsunamis. Fitness could also be reduced in the interval after an earthquake, as a result of the disruption of normal behavior from aftershocks. For many organisms, behavioral action taken prior to an earthquake ought to reduce mortality, such as fish and cetaceans leaving coastal zones, rodents exiting from collapsible burrows or dwellings, bees swarming, parents delaying egg-laying, etc.

My preparation of this article stems from similarities in this list of possible pre-earthquake behavior to those which have been reported as unusual for animals in the days, hours, and minutes prior to an earthquake. The book by Tributsch, (1982) documents many of these observations in depth, from many cultures. The similarity of reported behaviors, observed from cultures as diverse as those in the Middle East,
South America, and Asia lends at least some credibility to the hypothesis that a biological earthquake warning system may exist. If so, there must be a plausible route by which such a system could have evolved.

One major process through which complex biological systems evolve is to take an existing genetic pattern which evolved for one function, and to link or adapt it for a different role. The new system is then gradually debugged and improved through the process of random mutation and natural selection. This evolutionary pattern has been termed ‘exaptation’ (Gould & Vrba, 1982). For a seismic escape response to develop in this fashion, an organism would need to combine an existing escape, panic, or ‘exit from the burrow’ behavioral pattern with one or more appropriate sensory inputs to trigger the reaction. This reduces to three fundamental questions: (a) Has anything like a ‘seismic escape response’ already been established in the behavioral genetic repertoire of animals? If so, how could it have evolved? (b) Are there occasional precursory physical or chemical events, detectable at the surface of the earth, which might signal an impending earthquake? And if so, have animals evolved the sensory abilities needed to detect them? A final question (c) concerns the ability of any seismic escape response gene system to be maintained within a population, in the face of random mutation and genetic drift between appreciable seismic events. Each of these questions is addressed below.

II. Has a seismic escape or ‘early warning’ response already evolved?

Virtually all animals possess instinctive responses to escape from predators and (for land animals) from fire; in humans these responses are known as panic, and are associated with the rapid release of adrenaline which heightens sensory awareness and temporarily blocks sensation of pain. Numerous observations exist of animals displaying panic in the few seconds prior to the onset of strong ground shaking. Tributsch, (1982) lists many such examples, including dogs barking, nervous cats jumping out of windows, birds screaming, rats running out of their holes, bees swarming, etc. Such behavior immediately prior to an earthquake is not difficult to explain, as seismic p-waves travel faster through the crust than the associated s-waves by roughly 2-4 km/sec. If organisms are sensitive enough to vibrations to detect the arrival of the p-waves, that could provide enough of a warning to trigger a death-avoiding response immediately prior to the arrival of the more damaging s-waves. Only in very close proximity to the epicenter will the shaking start without appreciable warning. As moderate to large earthquakes like Loma Prieta can cause liquefaction at distances of 50+ km (Pease & Orourke, 1997), it is reasonable to infer that burrow collapse could be triggered in loose topsoil at greater distances. Hence, animals which live tens of km from the epicenter have several seconds after detection of the p-wave to escape the effects of the energetic s-waves. Although anecdotal in nature, these observations support the hypothesis that a seismic escape response is present in the behavioral repertoire of animals, and that it can be released at least by the sensory perception of low-frequency vibration.
In the case of p-wave arrivals, the sensory ability which triggers the seismic escape response is clearly acoustical or mechanical in nature, and is even felt by humans on occasion (K. Sieh, personal communication, 1998). At least some extant rodents (e.g., California kangaroo rats) use low-frequency seismic ‘footdrumming’ as a method of communication between burrows to mark territorial boundaries, and to notify predatory snakes that their presence has been discovered (Randall, 1997; Randall & Lewis, 1997; Randall & Matocq, 1997). As both the snakes and rodents have the ability to detect and respond to these vibrations, and sensory systems are in general highly conserved, the ability to detect these low-frequency signals is probably a primitive feature of all vertebrates.

The evolution of a p-wave triggered seismic escape response is not difficult to conceive, particularly via the process of exaptation described in the previous page. A cursory survey of the field of neurophysiology demonstrates that predator-prey interactions are largely responsible for driving the ability of animals to detect environmental signals down essentially to the thermal noise limit (Block, 1992). Evidence of predatory activity in the fossil record extends back to the latest Precambrian at about 545 My ago, as indicated by predatorial borings in the shells of the first primitive mollusks (Bengtson & Zhao, 1992). Given the enormous selection pressure of predator/prey interactions, evolution should have perfected the auditory and tactile sensitivity of animals to the point where p-wave arrivals could be perceived within a geologically short interval of time. Evolutionary exaptation of these senses to yield a seismic escape response would then be a very small change, linking these two existing systems (vibration sensitivity and predator escape) to yield a new behavior that minimizes mortality from seismic activity. As a wide variety of vertebrates (fish through mammals) display behaviors consistent with the presence of this basic system (Tributsch, 1982), it probably evolved prior to mid-Ordovician time.

III. Are there other physical or chemical events at the Earth’s surface which signal impending earthquakes, and is it plausible for animals to detect them?

Once a p-wave triggered seismic escape system is in place (e.g., it reaches genetic fixation within a population), a similar process of exaptation can occur to link it to any other sensory signals that an animal might perceive prior to an earthquake. This step, if it has occurred, represents the transition from an early warning response to a true predictive response. My reasoning is as follows. Close to an earthquake epicenter, the p-waves may be energetic enough to cause liquefaction and burrow collapse on their own, prior to arrival of the s-waves (Lin, 1997). In these areas, a p-wave triggered response alone would not be of much use for avoiding or minimizing seismic effects. On the other hand, if there are indeed other signals which are within the realm of an animal’s sensory perception which herald an impending seismic event prior to the actual rupture, random genetic mutations in the behavioral genes may occasionally link detection of this signal to the p-wave triggered early warning response. This evolutionary exaptation could then be refined and improved by natural selection.
During the past billion years, this may have happened many times, in many disparate lineages, depending upon how good an organism’s sensory perception processes had become.

As noted in the previous page, the IASPEI preliminary list of significant earthquake precursors includes five geophysical nominations, three of which are seismic in nature (foreshocks, preshocks, and seismic quiescence before major aftershocks). The other two relate to a decrease in radon concentration and an increase in ground water levels (Wyss, 1997). All of these precursors probably relate to dilatancy and development of micro-cracks, particularly as elastic strain in the fault zone approaches the failure point. As noted by Wyss, (1997), this list does not preclude the existence of other geophysical or geochemical precursors; it simply represents the set of lucky observations obtained so far that meet minimal thresholds for statistical reliability. The problem at hand is to try to extend these suggestions in a fashion which may lead to testable predictions concerning animal behavior.

For this purpose, it also is useful to examine the set of ancillary cases (some of which have not yet even been nominated for the IASPEI consideration), as well as the admittedly anecdotal descriptions of animal behavior prior to earthquakes. Note that the goal of this exercise is NOT to nominate these for IASPEI consideration, but to encourage experimental tests of the relevant stimuli on suitable animal populations to see if they are capable of releasing panic-like seismic escape behavior. Viewed from this perspective, at least four possible candidates for animal sensitivity can be gleaned: (i) ground tilting, (ii) humidity changes, (iii) electrical currents, and (iv) magnetic field variations. These are considered in detail next.

i. Apparent tilt precursors prior to strong earthquakes have been reported both in Japan and China, and several were included in formal IASPEI nominations (e.g., cases #5, #6 and #16 reviewed in Wyss, (1991). However, the magnitude of these precursors appear to be in the range of a few micro-radians, acting over several hours before the earthquake. In the vertebrates, the vestibular system in the inner ear is the main organ which mediates the detection of verticality, and the sensitivity of this response has been best studied in humans. Using a joystick-controlled gymbal system, Bisdorff et al. (1996) found that normal human subjects typically needed tilts of 6 ° (~ 0.1 radian) or more from verticality to respond to this stimulus. Humans are therefore unlikely to be able to detect tilting prior to earthquakes. On the other hand, many organisms have much better vestibular systems than do humans. Anatomically, these are particularly well-developed in subterranean rodents as compared to aboveground rats (Lindenlaub et al., 1995), and the use of a high-resolution vertical sensitivity as part of a multi-component navigational system in animal homing has been suggested seriously (Phillips, 1996). Unfortunately, few behavioral measurements of tilt sensitivity have been made in non-human animals, so there are at present no published data capable of refuting the hypothesis that animals close to an epicenter could detect an impending event in this manner. On the other hand, extraordinarily accurate tilt meters are inexpensive, and easy to implement (Westphal et al., 1983).
ii. The recognition that changes in ground-water level might sometimes provide clues to an impending earthquake suggests that associated changes in local humidity might be detected by animals. If the groundwater level rises significantly due to pre-seismic dilatancy, it must displace air from the pore spaces in the process. This moist air would then escape upwards and increase the humidity of air in soil and burrows, and perhaps in the surface boundary layer on top of the soil. If water level changes are detectable in groundwater wells, it ought to be detectable via humidity measurements in the soil.

The process of humidity reception in animals is known as hygroreception. Spiders and insects possess hygrosensitive sensilla which consist of specialized receptor cells with hygroscopic hair-like structures that detect humidity and/or temperature fluctuations (Sayeed & Benzer, 1996; Tichy & Loftus, 1996). Vertebrates appear to detect humidity through their olfactory system. Controlled laboratory experiments have shown that desert rodents are able to detect seed caches buried in dry sand based on variations of only a few percent of their water contents (Vanderwall, 1993).

Animal detection of impending earthquakes through hygroreception might therefore be possible in arid environments. However, it is difficult to see how this method would work in rainy areas like Japan, which have uniformly high levels of humidity both in the soil and in air. It is also difficult to understand how the pattern of a pre-seismic humidity change would differ from that generated by an impending storm. On the other hand, Tributsch (1982) notes that some of the behaviors displayed by animals before earthquakes resemble their pre-storm behavior, so this may be a component in their pre-seismic behavior. Digital hygrometers are on many commercially-available home humidifying systems, and ought to be inexpensive to put in the field.

iii. The presence of electrical and optical precursors prior to earthquakes is at present an area of intense, if controversial, research. Specific claims have been made in Greece of successful predictions, but the seismic community is understandably skeptical (see the May 27, 1996 issue of Geophysical Research Letters, which was devoted to this ‘VAN’ technique). However, the existence of ‘earthquake lights’ associated seismic events has been noted and discussed often (Derr, 1973; Derr & Persinger, 1986; Hedervari & Noszticzius, 1985; Lockner & Byerlee, 1985; Lockner et al., 1983; Ouellet, 1990). Earthquake lights were photographed during the extensive 1966 earthquake swarm in Matsushiro, Japan (Derr, 1973; Derr, 1986) and in southern Washington state (Derr & Persinger, 1986). Derr (1973) notes that lights have been reported before, during, and after the earthquakes. Although laboratory studies of rock fracture suggest optical emission via an exoelectron excitation process (Brady & Rowell, 1986), good explanations for how the atmospheric luminescence can persist for periods of several minutes have not been provided. Presumably, some form of crack propagation coupled with ion flow and perhaps fluid movement could be responsible for both electrical and optical effects.
For terrestrial animals, electrical sensitivity is rather low compared to marine or freshwater animals, due to the high resistivity of air. High voltages are perceived through the secondary effects of shock and/or the electrostatic action on feathers or hairs. In contrast, aquatic animals such as sharks, rays, and some fish often have exquisite electrical sensitivity due to specialized organs used both for communication and prey location (Bullock, 1982). In the elasmobranch fish (sharks and rays), a specialized receptor system in the ampullae of Lorenzini has, in fact, reached the thermal noise limit with the ability to perceive nano volt changes in electrical fields (Kalmijn, 1974); these are comparable to the voltage of a flashlight battery applied across the Atlantic ocean. Thus, the extensive reports reviewed by Tributsch (1982) suggesting an electrical link to anomalous behavior in fish before earthquakes merits serious experimental consideration, and could well have been incorporated into pre-seismic behavioral triggering in these and other aquatic animals. Similarly, nocturnal animals would have no difficulty detecting earthquake lights by simple visual signals. If some of these signals happen prior to significant seismic events, exaptation could link them to a pre-existing escape response.

iv. Ultra-low frequency (ULF) magnetic field variations are perhaps the least understood class of possible earthquake precursors, representing about 6 of the 40 nominated cases in the IASPEI List (Wyss, 1997); none has yet made the short List. Many other examples and thorough discussions of possible mechanisms for producing these magnetic precursors are reviewed by Mueller & Johnston (1998), Park (1996) and Park et al., (1993). Furthermore, and as noted below, some studies have employed proton-precession magnetometers that are not capable of recording the most interesting data in the .01 - 5 Hz region (e.g., (Mueller & Johnston, 1998)). Most of these studies arose from luck — magnetic observatories just happened to be recording some features of the geomagnetic field for other reasons when the earthquake occurred, and as such they often experienced power failures and data gaps as a result. (Co-seismic data loss is one factor preventing inclusion on the formal List.) It should be noted that a major problem with recording ULF magnetic signals of this sort is the fact that the magnetic field anomalies tend to follow an inverse cube power law with distance from the source, as expected from a dipole. Hence, meaningful data can only be obtained when recording instruments with adequate sensitivity and bandwidth just happen to be present very close to the epicentral area. Two of these lucky observations include the recording of a persistent 23 nT magnetic field anomaly a few days prior to the 1978 M, 7.0 Alay earthquake (Shapiro & Abdullabekov, 1982), also discussed by Park et al. (1993), and a very fortuitous, broad-band spectral recording of the 1989 M, 7.1 Loma Prieta earthquake made by a group at Stanford University (Bernardi et al., 1991; Fraser-Smith et al., 1990).

As noted in the next page, the Alay and Loma Prieta events are particularly important for the question of earthquake prediction by animals, as the precursory signals lie within, or very close to, the measured behavioral threshold limits for magnetic field perception by animals. The Loma Prieta data particularly merit detailed discussion.
here, as the recordings provide information about the spectral characteristics and amplitudes of the geomagnetic field changes in 9 frequency bands between 0.01 and 10 Hz, at a distance of only 7 km from the epicenter. Rather than recording the data continuously (as is typical for a seismogram), magnetic field characteristics were stored as ‘geomagnetic activity indices’, which were the set of logarithms (base 2) of the 30 minute averages of the power in each frequency band. R.M.S. field amplitudes can be reconstructed using a calibrated conversion table (Bernardi et al., 1991; Fraser-Smith et al., 1990). These data show a significant elevation in magnetic activity in the .01 to 5 Hz frequency range starting about 2 weeks prior to the earthquake, with peak amplitudes in the 1-3 nT range. About 3 hours before the event, however, the largest signals exceeded the dynamic range of the instrument, with peak r.m.s. fields exceeding 6 nT as measured in the 0.01-0.02 Hz band. As this represents a time average, peak field levels could easily have been much higher. Unfortunately, about 8 hours worth of the co-seismic and subsequent data were lost due to power failure caused by the earthquake.

Sensory detection of this level of geomagnetic variation by some animals is neither an improbable or an impossible event. One of the most surprising developments in the field of sensory neurophysiology during the past 25 years has been the discovery of geomagnetic influence on behavior in a phyletically diverse assemblage of organisms [a topic which has been reviewed extensively elsewhere (Kirschvink, 1997; Kirschvink et al., 1985; Kobayashi & Kirschvink, 1995; Wiltschko & Wiltschko, 1995)]. In parallel with the growing experimental data on magnetoreceptive organisms, the biophysical basis of the response was found to be due to small crystals of the ferrimagnetic mineral, magnetite (Fe₃O₄), which are formed biochemically. Crystals of this biogenic magnetite that were used for orientation were first discovered in the magnetotactic bacteria (Blakemore, 1982; Blakemore, 1975; Frankel & Blakemore, 1984), where they are held together in linear chains so that their individual magnetic moments will sum together. The resulting magnetostatic orientation energy per cell typically exceeds thermal noise (kT) by factors between 10 to several thousand. Neurophysiological studies have also revealed similar magnetite crystals in honey bees (Gould et al., 1978), pigeons (Walcott et al., 1979), and fish (Walker et al., 1984), which, in the best preparations, are also aligned in linear chains as they are in the magnetotactic bacteria (Mann et al., 1988). Subsequent electrophysiological studies in fish and birds have identified the ophthalmic branch of the trigeminal nerve as the main conduit of magnetic field information to the brain, from magnetite-based receptors located in the frontal regions of the head (Semm & Beason, 1990; Walker et al., 1997). More recently, lipophillic dyes and scanning laser confocal reflection microscopy have been used to identify some of the magnetite-containing cells at the distal termini of the trigeminal nerve, which appear to be highly specialized for magnetoreception (Walker et al., 1997).

As is the case for many other sensory systems, behavioral data indicate that some animal groups have extended the sensitivity of their magnetoreceptor systems down essentially to the thermal noise limit. Biophysical models of this limit for magnetointensity perception, given the typical quantities of magnetite measured in
animals, are on the order of 100 pT (Kirschvink & Walker, 1985). At least two plausible evolutionary driving factors can be envisioned for producing a ultrasensitive magnetoreceptor system. The first involves selection for long-distance homing ability in migratory animals. Magnetic field inclination and total intensity of Earth’s dipole field vary in a regular fashion with latitude, and sensory perception of these components can be selected upon to improve homing and navigation abilities in an essentially continuous fashion. As the resolution of the overall system improves (presumably through the addition of additional receptor cells and better information processing), other navigational features present in the geomagnetic field can aid in their navigational abilities, spurring additional selection for increased sensitivity. Birds have long been known to be disoriented at magnetic anomalies (Walcott, 1978), just as cetacean (dolphin & whale) stranding events preferentially happen at magnetic anomalies along the coastlines (Kirschvink, 1990; Kirschvink et al., 1986; Klinowska, 1985). Most of the positional variance observed in fin whales migrating at sea is explained by their avoidance of high magnetic fields and field gradients, suggesting that they use the marine magnetic lineations as a normal part of their navigational system (Walker et al., 1992). Analyses of these data imply sensitivity to intensity fluctuations in the range of a few nT.

The second evolutionary factor which could drive magnetoreception to, or help keep it at, the thermal noise limit involves use of the diurnal variations in the geomagnetic field as a timing cue. Nocturnal animals and those which live or nest in dark cavities (like honeybees) are not always able to set their internal circadian clocks with sunlight, as is typically done by most animals. Although the geomagnetic field at most localities on the earth’s surface is fairly stable at night, solar heating of the ionosphere begins at daybreak and produces electric currents which are active during most of the daylight hours. These lead to a periodic shift in the magnetic field components at the surface on the order of 50 to 100 nT, with regular variations according to season and latitude (see Skiles, 1985) for a review). Direct evidence exists that honey bees can actually use this information as a timing cue. Lindauer (1977) presented compelling evidence that bees raised in a constant-condition flight room were able to maintain track of their internal biological clocks, despite the absence of visual, thermal, humidity, and other signals relating to day/night cycles. This time-keeping ability was disrupted on days with magnetic storms, implying that their time-keeping abilities were based on the 20-50 nT diurnal variations of the geomagnetic field. This basic effect was replicated by Gould (1980), who was able to shift the diurnal cycle artificially through the use of a 23-hour synthetic geomagnetic diurnal variation generated by a fluxgate-controlled coil system. In mammals, further evidence for this link between circadian rhythms and ULF magnetic variations comes from studies of melatonin synthesis. Melatonin is the main hormone in animals which controls the sleep/wake cycles; its production by the pineal gland is suppressed both by light and by weak, ULF shifts in the magnetic field (Reiter, 1994).
Experimental data from honeybees further demonstrate that the geomagnetic sensory system has evolved to the level where the Alay and Loma Prieta magnetic anomalies could probably be detected, both in frequency and sensitivity. The discovery that bees can be taught to discriminate artificially-generated magnetic fields in laboratory settings (Walker & Bitterman, 1985) led to measurements both of the threshold sensitivity (Walker & Bitterman, 1989), and the frequency response of the receptor systems (Kirschvink et al., 1992; Kirschvink & Kobayashi-Kirschvink, 1991; Kirschvink et al., 1997). Figure 4-4 shows a compilation of these data, which indicate that the honeybee magnetoreceptor system is tuned to respond best to frequencies below 10 Hz, with sensitivity in the low nT range. Tributsch (1982) reports observations of unusual swarming behavior of bees ~ 15 minutes prior to the onset of strong earthquakes.

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Figure 4-4
Behavioral measurements of the ability of honeybees to discriminate extremely low frequency a.c. magnetic fields. This figure shows the proportion of honeybees able to discriminate the given field stimulus. The data for the nine honeybees exposed to the d.c. field are from Walker & Bitterman (1989), and those from 11 honeybees tested at 60 Hz a.c. and 15 honeybees at 10 Hz a.c. are from Kirschvink et al. (1997). Shaded intervals at the lower end of each curve show the area of uncertainty between the performance of the best animal at that frequency and the subsequent level at which the animal did not discriminate. For comparison, the black arrow indicates the approximate magnitude of the d.c. geomagnetic field in Pasadena (45 µT). The honeybee magnetoreception system is tuned for maximum sensitivities below 10 Hz.
Evolution of this highly-sensitive magnetoreception system is an interesting problem. Primitive, early eukaryotes presumably inherited their magnetite-based magnetotactic responses from their bacterial ancestors, maintaining its use for providing a constant rudder against the randomizing influence Brownian motion (Torres de Araujo et al., 1985). The presence of nearly identical magnetosome-chain structures in higher animals, and their use in geomagnetic orientation, is most easily explained as the result of common descent (Chang & Kirschvink, 1989; Kirschvink, 1989). However, as the musculature and locomotor abilities improved in primitive animals, there would be selection for more accurate homing and orientation abilities. In most sensory systems this is accomplished by increasing the number of receptors and the complexity of the neural circuits which extract environmentally relevant information. An increased number of neurons not only results in better accuracy of the directional signals present in the magnetic field, but it also permits extraction of the total intensity of the local magnetic field (Kirschvink & Gould, 1981; Kirschvink & Walker, 1985), which is a scalar parameter and hence independent of the orientation of an animal. As the average north/south gradients in total intensity are in the range of 5 to 10 nT/km, natural selection for enhanced navigational accuracy ought to drive the system to the point where magnetic anomalies in the Earth’s crust could be used as navigational reference signals. The ability noted above of honey bees to keep track of time in dark cavities by detecting diurnal variations in the geomagnetic field is probably an example of where this high-resolution sensory system was exapted for a completely different purpose than it originally evolved for. A similar exaptation of this high-resolution magnetic sensitivity for time keeping is not unlikely in nocturnal burrowing animals, some of which are known to have a good magnetic sensory system [e.g., African mole rats (Burda et al., 1990; Wiltschko & Wiltschko, 1995)]. Once this low-frequency, high-resolution magnetic sensory system has evolved, its further evolutionary exaptation to trigger an existing seismic escape response is possible. Highly-evolved pattern recognition is also possible, as is common in the vertebrate hearing and visual systems. As species of all major sub-groups of the sub-phylum Vertebrata display this high-resolution magnetic intensity sensory system, it must have evolved prior to their divergence (Ordovician time).

IV. Can a seismic escape response system be maintained within a population?

The time scale for a genetic system to evolve and be fixed in a population depends upon the relative selection pressure (the differential survival) that the ability provides the individuals within the population, as well as the length of time over which the selection pressure operates. Hence, a behavioral trait which is nearly neutral in terms of natural selection for most of the time, but during a rare event provides a significant chance of increased survivability, can in theory produce a highly evolved system. In fact, modern refinements of the principal model of evolution (the neutral theory of Kimura) have led to what is now called the ‘nearly neutral theory’, in which most competing copies of a particular gene (alleles) in a population drift along with no net positive or negative effect over the course of many generations except when a fluctuation or event in the
environment selects heavily for their presence (Gillespie, 1984; Ohta, 1996; Ohta & Gillespie, 1996). This pattern of ‘episodic selection’ could arise as a natural consequence of mortality generated by the repeat patterns of earthquakes.

A simple Monte-Carlo simulation demonstrates how a system of this sort can lead to the fixation and maintenance of a seismic escape response gene. For the model presented here, we consider a population of burrowing animals, such as California’s Kangaroo Rats (Dipodomys sp.), which often live in and around the San Andreas Rift (Best et al., 1996). In the terminology of population genetics [e.g., Crow & Kimura, (1970)], fixation is the process through which a trait or gene initially present in only a small fraction of the population comes to dominate, or ‘fix’ itself, as being present on both copies of the proper chromosome in each breeding individual of a population. Once fixed, it is fairly stable against being displaced by new mutation. In the absence of selection pressure or mutation, the ability of any particular copy of a gene to make it to the next generation is a chance process. As most animals are diploid (two copies of each chromosome), each parent randomly contributes only one of their two copies of each gene to their progeny. Consider now a situation where one of the behavioral genes (call it ‘A’) acts to link a sensory input to an established response which will minimize mortality resulting from an earthquake. Individuals with only the other allele for this gene, denoted, ‘a’ (e.g., the ‘aa’ genotype), do not produce this response, whereas either heterozygous (‘aA’ or ‘Aa’) or homozygous (‘AA’) individuals with the trait will respond. According to the neutral theory of population genetics (Crow & Kimura, 1970), in the absence of selection and mutation the relative levels of the gene will do a random walk, with less frequent alleles usually being lost from the population. We are interested, however, in the ‘nearly neutral’ case, where only once in many generations the trait ‘A’ enhances the survivability of any individual that possesses it. Given the behavioral evidence that a p-wave triggered escape response has already evolved in animals, the most important question concerns the level of relative selection which is required to maintain the trait in the population.

Figure 4-5 shows typical results for a simple, first-order Monte-Carlo simulation in which the breeding population size is held constant at 10,000 individuals, with a 50-generation (or year) recurrence interval for a seismic event large enough to cause mortality via burrow collapse, run for 1,000 generations. At each seismic event, we assume that any individual with at least one copy of allele ‘A’ survives and is able to reproduce in the next generation. During each seismic event, 10% of all individuals without any copies of the ‘A’ allele are ‘killed’ (e.g., blocked from reproducing in the simulation). Each simulation was repeated 10 times, and the results were shown as the mean occurrence of the ‘A’ phenotype, ± one standard deviation at each generation.
Figure 4-5
Results of Monte-Carlo simulations on the stability of a seismic escape response trait under conditions of episodic selection. In these simulations, the effective breeding population is held constant from generation to generation at 10,000 diploid individuals, with equal numbers of males and females. To move from one generation to the next (breed), one female and one male are selected randomly from the ‘parental’ generation using a uniform random-number generator and each parent randomly gives one of its alleles to the progeny. (Individual parents can have many offspring). This process is repeated until the new generation has reached 10,000 individuals, whereupon it is randomly divided into equal numbers of males and females. After every 50 generations, a ‘seismic event’ is introduced in which the phenotype of each individual is examined. Individuals with at least one ‘A’ allele are allowed to reproduce as normal, but 10% of those which lack it (‘aa’) are killed randomly (e.g., marked so that they are skipped in the next breeding cycle). Each simulation was allowed to run for 1000 generations, and repeated with identical starting conditions 10 times. Results were averaged at each generation and are shown on the figure as families of three curves, the mean (bold lines) ± 1 standard deviation at each generation.

In order to test the genetic stability of a seismic escape response, and its ability to be fixed within a population using these parameters, individuals in the initial populations were all homozygous (either all ‘AA’ or ‘aa’). This was done to simulate the situation where a small group of genetically similar individuals migrated into a new area, thereby infecting the existing population with seeds of the other genotype. In this figure, the five simulations started with (‘aa’,‘AA’) ratios of (10 / 9990), (100 / 9900), (1000 / 9000) (2500 / 7500), (5000 / 5000), and (9900 / 100), respectively, and can be distinguished by their initial starting values on the ordinate. Note that the phenotype fractions shown after one generation are much higher than in the seed population, as the initial homozygosity is lost completely in
the first random breeding operation. Individuals with genotypes of ‘aA’, ‘Aa’, and ‘AA’ all have the seismic escape gene, and are protected from the random seismic death.

In the first simulation, which starts with only 10 individuals having the ‘aa’ genotype, virtually all individuals in every subsequent generation are protected from seismic death with at least one copy of the ‘A’ gene, and all of the ‘a’ alleles are gradually lost within a few hundred generations. (This curve is indistinguishable from the top line on the graph.) In the first two simulations, so few ‘aa’ phenotypes exist in the population that the net results approach those expected from simple random genetic drift. In the following simulations, however, a significant number of ‘aa’ individuals are blocked from breeding every 50 generations so that the fraction of individuals protected by the ‘A’ gene increases rapidly with time.

In these simulations, a 10% mortality for the ‘aa’ genotype once every 50 generations is a rather small factor. As an example in the (2500 / 7500) simulation near the middle of the chart, only about 230 of the ‘aa’ individuals were blocked from breeding between the 50th and 51st generation, out of a total of 500,000 random matings during the 50 generations involved; this is an average selective factor of only 0.046%.

Simulations in which the ‘a’ trait (no seismic escape response) is present in small amounts indicate that it is quickly eliminated from the population even after only a few dozen seismic events, even for only a 10% advantage of the competing ‘A’ genes expressed only once every 50 years (a net selective advantage of only ~ 0.05%). Hence, a seismic escape response system should be genetically stable once fixed within a population. Similarly, when homozygous ‘AA’ individuals are introduced at low levels into a population of initially pure ‘aa’, the ‘A’ phenotype has a very high chance of reaching fixation within a geologically short interval of time.

V. Discussion

The analysis presented above implies that if there are occasional precursors to earthquakes that animals could detect, behavioral patterns could evolve to minimize associated mortality. Several considerations exist which make this more plausible than might otherwise be thought. First, plate tectonics (the driving force for most earthquakes) has operated for at least the past 2 billion years on Earth. Hence, the surface environment has been subjected to repeated strong shaking with repetition on the 100 to 10,000 year time scales, perhaps even higher on active plate margins. Second, these effects are not limited to plate boundaries. Even many mid-continent areas experience seismic events in this time frame [e.g., New Madrid, Missouri AD 1811-1812; Lisbon, Portugal AD 1755 (Demoulin, 1996; Johnston & Schweig, 1996)]. Third, and as noted above, strong seismic events are indeed capable of inducing significant mortality in existing populations, particularly in near-shore, burrowing, and egg-laying organisms. Fourth, if taken at face value, anecdotal reports of peculiar animal behavior before earthquakes (Tributsch, 1982) are compatible with evolution of a basic seismic escape response sometime before the divergence of the animal phyla nearly
1 billion years ago (Runnegar, 1982; Wray et al., 1996). As the geological evidence of burrow formation goes back nearly 540 million years, as shown from trace fossil evidence in rocks of Early Cambrian age (Mcilroy & Heys, 1997), burrow collapse as a mechanism of seismic mortality should date back at least this far. The distant ancestors of all mammals (synapsids) who survived the Permian/Triassic mass extinction 250 million years ago were apparently burrowing animals living along the tectonically active margin of the ancient supercontinent of Gondwanaland (Smith et al., 1993). Their fossil remains are almost always found as pairs of animals in collapsed burrows (Smith et al. (1993), and personal communication, 1997). Thus, mammals in particular have had over 250 million years in which to refine their seismic escape response, and link it via exaptation to additional sensory signals. As a general rule, genes that control evolutionarily ancient processes evolve much more slowly, and are influenced far less by genetic drift, than are more recent additions to the genome. Hence, one would expect a seismic escape response system to be evolutionarily conserved.

Several lines of experimentation could help test the above ideas. First, we need to study animal behavior associated directly with the ground shaking which is associated with seismic events. Although it is obviously impractical to sit and wait for suitable earthquakes to happen, the introduction of broad-band seismometers during the last decade has provided excellent and detailed recordings of ground motion during several large earthquakes (Landers, Northridge, Kobe, etc.). Just as these records are being used to test building designs and revise earthquake safety guidelines [e.g., Heaton et al. (1995)], controlled shake-table experiments could be done on laboratory populations of burrowing animals from seismically active zones; some of California’s endemic kangaroo rats would be good candidates. These experiments would establish a baseline of animal behavior for comparison with reactions of other stimuli. Following this, a variety of field-based experiments could be done on the same species, in which candidate precursory geophysical and geochemical signals are given artificially to determine which, if any, are capable of triggering similar behavioral reactions. The exaptation model outlined above would predict some similarity in the evoked behavioral response between shaking and other stimuli linked to seismic escape activity. In particular, simple back-of-the-envelope calculations indicate that it would be relatively easy to modulate the magnetic field within a 100-m diameter coil system to the levels indicated by the Alay and Loma Prieta magnetic precursory events; this could be done with minimal disturbance around a natural kangaroo rat warren in the field. Even though the exact seismomagnetic patterns were not recorded prior to these events, enough information is available from the Loma Prieta earthquake to produce a family of complex waveforms with similar spectral characteristics to those recorded just prior to the main event. Studies like this should be done. Ultimately, knowledge of the complete genome sequence of these animals might yield clues to the triggering mechanism and sensory patterns which elicit seismically-triggered fixed-action responses, but we may need to wait 20 - 50 years before the genetic basis of innate pattern recognition is understood.
Finally, seismologists should not limit their recording and monitoring efforts solely to ground motions, which has historically been the case. For the ultimate goal of earthquake prediction, there is no substitute for detailed records of possible precursory signals associated with strong seismic events. As such, it is silly to depend upon serendipitous observations such as those from Alay and Loma Prieta. Although some focused magnetic studies exist [e.g., Mueller & Johnston (1998)], the proton precession magnetometers typically used do not operate in the interesting frequency range (0.01 - 5 Hz) flagged by the Loma Prieta data. In Southern California, the TriNet/Terrascope stations maintained by Caltech, the U.S. Geological Survey and the CDMG have several 24-bit data channels which are not presently in use. These ought to be equipped with inexpensive sensors to monitor tilt, geoelectrical potential, humidity, and magnetism, and the magnetic signals should be recorded continuously at least through 10 Hz. As an example, the per-unit cost of fluxgate magnetometers, with sensitivities in the 50-100 pT range, has dropped precipitously in recent years due their development for use in intelligent vehicle highway systems (IVHS). Although not as sensitive as the induction coil systems used to record the Loma Prieta event (Fraser-Smith et al., 1990), they are far less expensive, have adequate sensitivity to detect nT-level changes, and are unlikely to go off-scale as did the Stanford equipment. These could be deployed easily on the TriNet/Terrascope system in Southern California, and perhaps elsewhere.

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MOUSE BRAIN MAGNETITE STUDIES

Rationale

Magnetite (Fe₃O₄) is permanently magnetic at body temperature and interacts with external magnetic fields up to 10⁷ times more strongly than other physiological material. The presence of biogenic magnetite crystals in the human brain (Kirschvink et al. 1992) raises questions as to magnetite’s role in normal human biochemistry, physiology and behavior, and opens for study the possibility that environmental electric and magnetic fields might produce physiological effects by perturbation of tissue magnetite. Knowledge of the cellular distribution and ultrastructure of brain magnetite is necessary to further our understanding of its biological functions.

Because of the difficulty of obtaining fresh human brain tissues, especially under appropriately controlled magnetically-clean conditions, as well as the necessity of extensive safety precautions in handling human tissues, we have shifted to the laboratory mouse as the focus of our research into brain magnetite. Mice are readily available commercially, they are inexpensive, and their small size can be considered an advantage for histological and ultrastructural studies, in which good fixation is of major importance.

Our objective in these studies can be simply stated: we wish to locate and identify biogenic magnetite in situ within the tissues of the mouse brain.

SQuID Magnetometry

Methods

All magnetometric analysis was performed with the Superconducting Quantum Interference Device (SQuID) moment magnetometer located within a magnetically-shielded, dust- and magnetic particle-free clean lab at the California Institute of Technology. General methods for the handling and dissection of biological samples to prevent their contamination with magnetic particles has been described previously (Kirschvink 1983; Walker et al. 1984, 1985).
Adult female breeder mice (CF-1 strain) were sacrificed by cervical dislocation, their skulls were immediately opened, and each entire brain was removed, rinsed briefly with phosphate-buffered saline solution, and individually frozen. Samples were stored frozen at -15 °C. Prior to analysis, the brains were thawed just sufficiently to remove them from the storage vial, rinsed briefly with magnetically-clean water, and refrozen in the sample holders. The holders consist of a small quartz cup (approximately 1 ml volume) with an attached 6-10 cm long quartz fiber bent into a hook at the end. The holders were cleaned by treatment with concentrated HCl and rinsed with magnetically-clean water.

Before each use with a tissue sample, an empty holder was pulse magnetized and its moment determined for background subtraction. Some brain samples were pulse magnetized and used solely to determine intensity of magnetization (total magnetic moment per gram); other samples were subjected to rock-magnetic analysis, consisting of a progressive Isothermal Remanent Magnetization acquisition and alternating field (Af) demagnetization sequence (as first described in Cisowski 1981) to provide information about the composition, size range, and interactions of the magnetic materials present.

**Results**

Our initial studies, prior to the recent magnetometer upgrade, required pooling of two to four whole brains for sufficient signal to provide meaningful data. Measurements of these samples indicated that the concentration of magnetite in mouse central nervous system (CNS) tissue was in the same range as reported (Kirschvink et al. 1992) for human brain tissues (3-10 ng/g tissue). As well, the rock magnetic analyses of the pooled mouse brain samples provided evidence for the occurrence of interacting aggregations of magnetic particles, just as had been previously described for human brain (Kirschvink et al. 1992). These early studies provided some confidence in the shift from human to murine tissue.

The upgraded magnetometer/clean room facility has resulted in a substantial increase in detection sensitivity. The improvement now allows the collection of significant magnetometric data from a single entire mouse brain. We have carried out analyses of a total of 21 individual CF-1 female mouse brains. The mean magnetite concentration is 4.8 ± 4.0 ng/g tissue, with a range from 0.6 to 17.1 ng/g tissue. The mean weight of the 21 brains was 0.48 ± 0.04 g. Thus, on average, an individual mouse brain contains 2.3 ng of magnetite.
Magnetic Extraction

Methods

We have described in detail the methods that we have developed for the preparation and examination of magnetically concentrated extracts from tissues (cf. page 2-3 in Kirschvink et al. 1993). In the earlier NIH-funded program which resulted in the discovery of biogenic magnetite in the human brain, the extraction protocols (Kirschvink et al. 1992) were intentionally designed to provide a magnetic fraction totally free of any cellular debris or other organic residue. These purely mineral concentrates were desirable for high-resolution transmission electron microscope (TEM) lattice imaging studies. We carried out similar extractions on murine tissues for our initial TEM preparations. Pooled mouse brain samples (from CF-1 female breeder mice) were treated either with toluene/quaternary ammonium hydroxide (TS-1 tissue solubilizer, Research Products International Corp.) or with household bleach (5.25% NaOCl) to release the insoluble magnetic particles. Magnetically concentrated material was examined on a Philips CM12 scanning transmission electron microscope, equipped with a Kevex Level IV Delta energy dispersive x-ray spectrometer for elemental analysis.

Since magnetometry analysis indicated that the biogenic magnetite crystals in the CNS tissues of man and mouse were present in interacting aggregations, we reasoned that gentler tissue disruption methods might be helpful to address the question of the arrangement of the magnetic material within brain tissues. Pooled samples of from 3 to 10 whole mouse brains (from CF-1 breeder females) were subjected to multiple freeze-thaw cycles with vortexer mixing during each thaw cycle to disrupt the tissues. Magnetically-clean 95% ethanol was added to the crude homogenates to a final concentration of 50% v/v, to terminate any enzymatic activities and prevent microbial contamination. We immersed a glass-sleeved probe, containing a NdFeB magnet, into the liquid to concentrate and isolate the magnetic fraction of the homogenate. The magnetic concentrates were transferred directly to carbon-coated formvar films on copper specimen grids, air dried, and examined in the TEM.

Results

The initial, and not unexpected, conclusion from the mouse brain extraction studies is that magnetite, presumably biogenic, occurs in the organic material-free extracts of mouse brain tissues. We have identified magnetite both by selected area electron diffraction of clumped electron-dense particles (Figure 5-1) and by microdiffraction of individual crystals (Figure 5-2) in the magnetic concentrates from several replicate preparations. Note that these pure mineral phase extracts exhibit aggregations of particles of various shapes and sizes, generally clumped in close contact, presumably by magnetic interactions.
Figure 5-1
a. Electron micrograph of magnetite crystal assemblage from magnetically concentrated extract of mouse brain tissue, digested with household bleach (5.25% NaOCl).
b. Selected area electron diffraction pattern obtained from the material in Figure 5-1a. Camera length, 550mm; 100KeV. The d-spacings calculated from the diffraction rings match those of a magnetite standard.
Figure 5-2
a. Electron micrograph of small clump from magnetic extract of mouse brain tissue, prepared by digestion with bleach.
b. Selected area electron diffraction pattern obtained from the single large crystal indicated by the arrow in Figure 5-2a. The spot pattern indicates a single crystal of magnetite.

Of greater interest, the preparations isolated from the freeze-thaw disrupted tissues often exhibit an entirely different appearance (Figures 5-3a and 5-3b). These images show material (characterised as magnetite by electron diffraction) arranged in a loose collection of near-equant crystals, predominantly between 20 to 100 nm across, associated with what appear to be cell fragments. In Figure 5-3b, some of the electron-dense crystals appear to be present within membranous vesicles, and additional empty vesicles are also visible nearby. These observations suggest that at least some of the magnetite present in the mouse brain occurs as intracellular aggregates, similar in appearance to the membrane-bound magnetite crystals that comprise the magnetosomes of magnetotactic bacteria. These observations are consistent with the rock-magnetic magnetometry results which indicate the presence of interacting magnetite crystals within the intact frozen human and murine brain tissue. Taken together, these findings suggest the existence in the CNS of a population of one or more types of cell that contain large numbers of magnetite crystals. We refer to these hypothetical magnetite-rich cells as ‘magnetocytes’.
Figure 5-3
a. Electron micrograph of an apparent tissue fragment, containing numerous magnetite crystals, obtained by magnetic extraction from a freeze-thaw disrupted preparation of mouse brain tissue.
b. Higher magnification electron micrograph of preparation as in Figure 5-3a. Note the numerous vesicle-like membranous structures, some with magnetite crystals within, others appearing empty.
Histological studies

Iron-Staining Methods

The detection and localization of iron-containing structures and deposits within cells and tissues of diverse biological systems has long been of interest. The classic method for the detection of ferric [Fe$^{3+}$] iron is the formation of Prussian Blue (ferric ferrocyanide) by acid-ferrocyanide treatment (Perls 1867). Many techniques to increase the sensitivity of this method have been described. The value of 3,3´-diaminobenzidine (DAB) to intensify the ferric-ferrocyanide reaction product has long been known (Nguyen-Legros et al. 1980). Enhancement of the DAB reaction product by post-intensification with silver-gold treatment (Rodriguez et al. 1984) and with uranyl nitrate (Goto et al. 1992) were both originally developed for amplification of the DAB signal produced by the immunoperoxidase staining method. Recently, all of the techniques mentioned above have been combined into a sensitive method for the detection of iron in the rat CNS (Moos & Møllgård 1993).

Citrate-Bicarbonate-Dithionite (CBD) Treatment

CBD treatment was first described (Mehra & Jackson 1958) as a technique for the removal of free iron oxides from soils and clays. The strong reducing agent, sodium dithionite (Na$_2$S$_2$O$_4$), combined with the iron chelator, citrate, buffered to pH 7.3 with bicarbonate quickly solubilizes fine-grained iron oxides when reacted at 80 °C for 10-15 minutes. However, at 20-25 °C, fine-grained magnetite is stable for an extended time, while powders of hematite (α-Fe$_2$O$_3$), goethite (α-FeOOH), and lepidocrocite (γ-FeOOH) are all solubilized (Kirschvink 1981).

Histological Methods

By combining the ultra-sensitive iron-staining methods described above with the selective solubilization properties of the CBD treatment, we hoped to be able to develop a histological staining technique specific for magnetite, allowing us to detect tissue magnetites at the optical microscope level. We performed vascular perfusion fixation of anaesthetized Swiss-Webster female mice, with standard glutaraldehyde/formaldehyde fixative and a gravity-flow perfusion apparatus. The entire brains were dissected free from the skull cavity and rinsed and stored on ice. We prepared 50 or 100 µm slices (either coronal or sagittal) with a Vibratome™ (courtesy of Dr. Frank Moore, Zoology Department, OSU). Individual slices were collected and stored in the order of cutting in 24-well tissue culture plates in phosphate-buffered saline at 4 °C. Much effort was taken to determine the optimal times and conditions to maximize the sensitivity of the entire long sequence of iron-staining and intensification steps, using adjacent sections for comparisons. As well, various methods for the reduction of non-specific background staining by DAB were compared. However, when
we compared adjacent slices, one treated with CBD at room temperature and the other with 80 °C CBD treatment (to solubilize all the iron oxides), we were unable to discern any consistent indications of stainable material within localized regions of the brain slices which we would expect to result from the presence of biogenic magnetite crystals. We can not distinguish between two possible explanations for these negative results: One, we have not sufficiently optimized the dithionite dissolution procedure or the multi-step iron-staining sequence of reactions to allow the selective staining of magnetite; or two, the magnetite is present in concentrations still too low to be detected by the protocol that we have developed. The latter possibility would argue against the existence of the presumed ‘magnetocytes’, or at least suggest that they are sufficiently rare and/or scattered to escape detection by our histological procedures.

Reflection Confocal Microscopy

Magnetotactic Bacteria

Discrete particles of matter that have a diameter much smaller than a single wavelength of light exhibit a phenomenon called Rayleigh scattering when illuminated with light (cf. Cogswell 1995). The size of the particle and its chemical composition, as well as the wavelength of the light, determine what portion of the illuminating beam is scattered forward, to the side, or backward (Born & Wolf 1980). For particles having a diameter •0.1λ, approximately equal amounts of light are scattered forward and backward. Thus, particles of the dimensions of the biogenic magnetite crystals observable in magnetic extracts of brain tissue (i.e., 20-100 nm) could possibly be detectable by a sensitive reflection-mode optical microscope. Though their true size cannot be determined because they are much smaller than the ~ 200 nm resolution limit of a light microscope, they could be detected (and perhaps quantitated) by their tendency to backscatter the incident light.

We prepared samples from two parallel cultures of the magnetotactic bacterium, *Magnetospirillum magnetotacticum*, one grown under conditions which allowed the synthesis of the characteristic intracellular magnetosome chains (comprised of membrane-bound magnetite crystals about 40 nm across), and the other under iron-limited growth conditions so that the bacteria contained essentially no magnetosomes. Each culture was pelleted, resuspended in a small volume of 4% low-melting-temperature agarose, cooled and then processed by standard TEM methods of fixation, dehydration and plastic embedment into Spurr’s resin. Ultrathin sections of each block were prepared and examined in the TEM to confirm the presence and absence respectively of magnetosomal chains in the two samples. Thicker sections (1 to 3 µm) of each were then microtomed and mounted on glass microscope slides and coverslipped with Cytoseal 60™ mounting medium (Stephens Scientific Inc.). The sections were examined with the Leica TCS 4D Confocal Laser Scanning Microscope at the Center for Gene Research and Biotechnology at OSU. Using 488 nm light from a Kr-Ar laser, we were readily able to detect a robust reflection signal from the bacteria known to contain
magnetosomes (Figure 5-4), while the parallel, iron-limited sample gave no signal at the same settings of laser intensity, pinhole diameter, and photomultiplier gain and offset. Although the individual magnetite crystals that comprise a magnetosome chain can not be resolved, it is easily seen in Figure 5-4 that the reflection image of many of the individual bacterial cells is elongate and comparable in length to the length of the magnetosome chains (up to 2.5 µm), as observed in TEM images.

![Figure 5-4](image)

**Figure 5-4**

a. Reflection confocal microscope image of *Magnetospirillum magnetotacticum*. The image is an overlay of two channels: 1) a projected stack of 12 reflection confocal slices, which appears as the bright white regions and 2) a similar stack of 12 transmitted light images, which, since they are non-confocal, appears as the somewhat blurry gray to black regions. The bacteria themselves are visible as the darker structures, while the bright reflection images arise from the magnetite-containing magnetosome chains within the bacterial cells. Similar bacterial preparations, grown under iron-limited conditions, and observed in the TEM to lack essentially all magnetosomes, give no reflection signal (not shown).

b. Higher magnification image similar to Figure 5-4a. Note that the reflection signals frequently appear elongate, as would be expected for Raleigh scattering from the elongate magnetosome chains.

**Mouse brain samples**

We have examined mouse brain Vibratome slices, prepared as described in the Histology Methods section immediately above. Untreated slices were dehydrated in a graded ethanol series, transferred to Hemo-De™ clearing agent (Fisher Scientific Co.), and cover-slipped with Cytoseal 60™. In the absence of suitable positive controls, we have concluded that these brain tissue preparations do not provide any clear
information. With these preparations, we have observed many reflection images that exhibit relatively diffuse intensities, frequently detectable over large areas of many brain subregions. The complex CNS tissue appears to contain various subcellular structures or deposits that reflect or scatter light. Discrete particles with a markedly different refractive index from their surrounding milieu may provide a source of scattered light. When we did observe intense foci of reflected light, such as might be expected to arise from ‘magnetocytes’, they always proved to come from non-cellular particulate material, likely just dirt and dust particles.

The major difference between the bacterial preparations and the brain tissue slices is that the bacteria are completely infiltrated with the polymerized epoxide resin, while the brain slices are immersed in the liquid resin solution of the mounting medium. It is possible that much of the background reflection signal of the brain samples will disappear if the tissue is infiltrated with the higher refractive index solid plastic. We are in the process of preparing mouse brain tissue samples that have been prepared by the traditional TEM plastic embedment method (into Spurr’s resin), to determine whether reflection microscopy might yet be of value in our attempts to localize the biogenic magnetite in mouse brain.

‘Magnetocyte’ concentration

Cell Dissociation

If brain ‘magnetocytes’ actually exist, we might be able to utilize magnetic separation methods to concentrate them for further characterization. Our initial strategy was to follow protocols for the dissociation of live brain tissue into single cell suspensions, developed originally as a method for generating primary cell cultures of neurons from rat visual cortex (Huettner & Baughman 1986). Entire mouse brains, from CF1 breeder females sacrificed by cervical dislocation, were immediately removed and minced with acid-washed glass coverslips. The minced tissue was then incubated at 37 °C with gentle agitation in a solution containing 20 units/ml of activated papain, a proteolytic enzyme. Following incubation, the tissue fragments were gently triturated by filling and emptying the barrel of a 5 ml pipette. Although vigorous trituration of neuronal tissue results in a high yield of cells, most are spherical and devoid of processes; gentler treatment results in more undissociated tissue fragments and a lower cell yield, but many of the cells retain their proximal processes.

The dissociated cells were gently pelleted from the papain solution and immediately resuspended in an ovomucoid solution containing DNase: ovomucoid is a papain inhibitor and the Dnase digests the viscous DNA released from broken cells. The intact cells were then separated from cell membranes by centrifugation through a single-step discontinuous albumin density gradient. The pelleted intact cells were resuspended in buffered saline solution. Since our initial goal was not to prepare living cell cultures but rather to carry out the possibly lengthy process of magnetic concentration, at this point
in the procedure, we wished to kill and stabilize the dissociated cells. Hence, the cell suspension was fixed by the addition of glutaraldehyde to a final concentration of 2.5% (v/v). After overnight fixation, the cells were transferred through a stepwise ethanol concentration series to a final 50% ethanol in buffered saline.

**Magnetic Concentration**

Magnetic concentrates were by our previously developed contamination-free methods, using acid-washed glass vials with a glass-sleeved NdFeB magnet probe. The magnetically concentrated fixed cells were then suspended in liquified 4% low-melting-temperature agarose and chilled to gel the agarose. The agarose plug was sliced, and after standard dehydration and infiltration, embedded in Spurr's resin to provide polymerized plastic blocks for preparation of ultra-thin sections for TEM.

**Results**

Examination of sections prepared with heavy-metal staining (Pb/U double staining) revealed numerous profiles of myelinated neural processes and various supporting cells, presumably oligodendrocytes and astrocytes. In additional unstained sections (Figure 5-5), iron-containing electron-dense granules, mostly in the 20-40 nm size range, were seen in some of those support cells most closely adherent to myelinated processes, which were themselves devoid of such granules. The consistent presence of these granules within several adjacent serial sections (each 70-100 nm thick) of an individual cell profile provides strong evidence that they are not merely contaminants introduced at any point subsequent to the fixation step.

However, our attempts to produce electron diffraction patterns, either by selected area diffraction of contiguous grains or by microdiffraction of individual grains, were ineffectual. We are thus unable to know whether or not the electron-dense grains are magnetite: failure to observe characteristic magnetite diffraction patterns may be because the granules do not contain magnetite, but may also be a result of the small size of the granules and their presence within the epoxy resin matrix. We have only been able to detect the iron-containing electron-dense granules in unstained sections. We cannot distinguish them in stained sections, even with a knowledge of their probable location by comparative examination of an immediately adjacent unstained section. Consequently, we have been unable to determine whether the granules are associated with specific subcellular structures such as cytoskeletal elements or even whether they occur within membrane vesicles.
Figure 5-5
Electron micrograph of ultrathin section of magnetically concentrated fraction from dissociated and fixed mouse brain tissue. n: profile of myelinated neuronal process; s: supporting cell, containing electron-dense objects. Unstained.
**Magnetophoresis**

We have continued to pursue the development of magnetic separation techniques to attempt to concentrate the hypothesized ‘magnetocytes’ for further characterization. In the past, we have relied on two distinct strategies for magnetic concentration. In the first, we simply immerse a glass-sleeved NdFeB magnetic finger into a liquid suspension of a tissue preparation to fish for a magnetic fraction. After a sufficient time for accumulation to occur, usually with concomitant gentle stirring of the vessel by rotation, the probe is removed and placed into a receiving container and the magnet is removed from the glass sleeve. This results in transfer of the magnetically concentrated fraction to the new container for additional manipulations to prepare samples for transmission electron microscopy (TEM) characterization. The entire operation is performed ‘blind’; there is no easy way to carry out visual observation through a stereo microscope to follow the progress of the concentration phase. In the second strategy, the initial sample suspension is placed into a suitable acid-cleaned vial. A strong NdFeB magnet (18 x 30 x 12 mm) is placed against the exterior of the vial wall and the process of accumulation of magnetic material to the high field gradient region at the edge of the pole of the external magnet can be observed and followed through a stereo microscope. Then, ideally, the magnetically concentrated fraction can either be taken up by pipetting or by immersing a glass-sleeved magnetic probe close to the accumulated material, while, at the same time, withdrawing the external magnet. Although both of these strategies would seem to be straight-forward, in our experience, the transfer step turns out to be frustratingly unreliable; too frequently, the miniscule magnetic concentrate is either not removed from the original container or is lost entirely.

Recently we have devised a magnetic separation method that has its origin in a published report by Zborowski et al. (1995) which describes the use of a flow cell combined with a high gradient magnetic field to isolate lymphocytes heavily labeled with paramagnetic ferritin (10⁷ ferritin molecules per lymphocyte). Our flow cell is inexpensive and easy to construct. A standard 25 x 75 mm glass microscope slide is modified by drilling two small holes (about 35 mm apart) to which are attached 15 mm lengths of glass tubing (1.5 mm inner diameter) with cyanoacrylic glue. Suitable sized plastic tubing can then be attached to the tubing stubs to form the top of the flow cell. The chamber volume itself is defined by a gasket of adhesive-coated silicone rubber (Grace Biolabs, Inc., Bend, OR); the 2.5 mm thick gasket stock is cut into a slide-sized rectangle with an oval hole about 35 mm long by 18 mm wide. The gasket is attached to the slide so that the inlet and outlet tube fittings connect to the chamber. The bottom of the flow cell consists of a 22 x 50 mm glass cover slip which is approximately 0.17 mm thick. The flow cell assembly is mounted with rubber bands onto the magnet so that the interior of the flow chamber is separated from the very high gradient magnetic field by only the thickness of the cover slip. The magnet assembly consists of two trapezoidal NdFeB magnets, each 18 x 20 x 7 mm, bonded to a plate so as to leave an 8 mm air gap between the 18 mm edges of the two poles of the assembly. The flow cell is attached to
an arrangement of plastic tubing, two-and three-way valves, and glass reservoirs and
the liquid flow is produced by a controllable rate, slow-flow peristaltic pump (Rainin
Rabbit, Rainin Instrument Co., Inc.). The system is initially cleaned by running
20% HCl through it in a recirculating path.

In the first trials of the separation apparatus, we ran a pooled sample of three papain-
dissociated, glutaraldehyde-fixed mouse brains. Examination with a stereo microscope
of the flow cell illuminated at a very shallow angle with a fiber optic illuminator
allowed us to observe the slow accumulation of material directly over the edges of the
pole piece air gap, which are the regions of highest field gradient. It also became clear
that the entire assembly should be operated in a vertical position, to reduce any
interference due to gravitational sedimentation of the cell suspension. However, our
efforts to remove the magnetic concentrate by pumping after removal of the flow
chamber from the magnet assembly were not successful; redilution and retention were
the problems.

In our more recent trials, we realized that it might be possible to avoid the hazards of
the transfer steps entirely. Since our major goal in attempting to isolate magnetic
fractions of cell preparations has been to carry out examination of the concentrates in
the TEM, we reasoned that we might be able to perform the additional preparative
steps for plastic embedding of the samples directly in the flow cell itself. Dissociated
brain cell suspensions were first pumped through the flow cell, resulting in the
accumulation of material at a position overlying the air-gap edges. Then, dehydration
was carried out by successively pumping through a series of ethanol dilutions from
30% to 100% while the flow chamber remained affixed to the magnet assembly. Next,
several changes of the monomer resin formulation (Spurr’s resin) were put through the
chamber to infiltrate the concentrate with the plastic. Then, the entire flow cell/magnet
assembly was closed off by valves and was gently removed from the pump and
reservoirs and placed into a 60 °C oven for 24 hours for polymerization. After
disassembly of the flow cell, the end product was a piece of polymerized Spurr’s resin,
in the oval shape of the flow cell chamber, with two narrow lines, visible as light-
scattering zones upon oblique illumination, which comprise the magnetically
concentrated material now embedded near the coverslip side of the resin block.

The entire plastic block could be taped onto a microscope slide, with the former
coverslip side up, and covered with a drop of immersion oil and a fresh coverslip and
examined in a standard optical microscope or with a reflection confocal microscope.
The cell clumps, fragments and other debris observable by these optical methods were
not very informative. However, the concentration zones were trimmed and mounted
and ultrathin sections were cut and examined in the electron microscope. We noted the
presence of only highly fragmented cellular debris, although we were somewhat
cheered by seeing numerous fields which contained small clumps comprised of roughly
7 nm electron dense structures of iron-rich material of the correct size and spacing to be
ferritin molecule clusters. This finding suggests that the magnetic separation apparatus
has a strong enough field strength and gradient to allow it to concentrate the
paramagnetic ferrihydrite cores, which have a much lower magnetic moment than an
equivalent volume of magnetite. The severe fragmentation of the cells was most likely a result of two faulty design features of the apparatus as configured in this preparation. First, the peristaltic pump had been placed on the inlet side of the flow cell, and, hence, the cell suspension was pumped through the flow chamber, exposing the cells to the stresses and pressures of the peristaltic drive mechanism. Second, we had recirculated the cell suspension many cycles to try to maximize the yield of magnetic concentrate.

Our most recent preparations were carried out as described directly above, but with the peristaltic pump on the outlet side of the flow cell, pulling the dissociated cell suspension through the flow chamber. As well, the magnetic concentrates are the product of only a single pass of the suspension through the apparatus. Thus, the dissociated cells never pass through the pump. We are currently working our way through two such preparations, cutting ultrathin sections, examining them in the TEM and hunting for iron-rich structures as determined by energy-dispersive X-ray spectrometry and seeking identification of magnetite crystals by electron diffraction. Although the appearance of the cells is much improved, we have not yet observed any candidate ‘magnetocytes’.

Discussion

This report summarizes a long, multimodal search for in situ mouse brain magnetite crystals, a search that has been frustratingly short on significant positive results. We have never observed so much as a single identifiable magnetite particle within the central nervous system tissues of the mouse. We are not alone in this frustration; except for the case of the family of species of magnetotactic bacteria and a single species of algae, there have been no published reports of success in identifying magnetite within the tissues of any higher organism. None of the many claims of discovery of candidate magnetite-based sensory receptor cells have provided conclusive evidence of the presence of identified magnetite.

The frustration, but also the goad to continue the search, arises from the solid evidence, from magnetometry and from TEM studies of tissue extracts, that biogenic magnetites are present in brain tissue, even though their concentration is at the parts per billion level.

The most significant advance from our studies is the observation of apparent cellular fragments containing numerous magnetite grains in extracts from crude freeze-thaw homogenates of mouse brain. These TEM images strongly suggest that at least a part of the tissue biogenic magnetite is intracellular, and more weakly indicate that there may exist a population of cells, the hypothetical ‘magnetocytes’, which contain many such crystals.

The failure, up to now, to locate and identify magnetite in situ, though frustrating, is not wholly unexpected. Consider, for example, in regard to attempts to develop sensitive iron-staining techniques, that the quantity of iron contained in the hemoglobin of a single red blood cell is equivalent to the amount of iron present in 500 crystals of
magnetite of 50 nm diameter. Similarly, total moment measurements with the SQuID magnetometer are extremely sensitive: our ability to detect and characterise the magnetic material contained within a single murine brain means we are working with a sample with only 2 ng of magnetite, equivalent to less than $10^6$ 50 nm crystals. If the hypothetical ‘magnetocytes’ were each to contain 1000 crystals, only 1000 such cells would be expected within the entire mouse brain.

Several tools may yet provide the necessary micrometer scale resolution that could provide sufficient localization to allow the conclusive identification of magnetite grains by electron diffraction in the TEM. Although we have not yet been successful in our attempts with reflection confocal microscopy on brain tissue samples, our ability to detect magnetosomes in magnetotactic bacteria offer some hope of eventual progress. Several laboratories are developing scanning SQuID magnetometer microscopes, which are expected to have the necessary sensitivity and the requisite resolution to be of value in the search for tissue magnetites. As well, micrometer resolution magnetic resonance imaging microscopes are also under development.
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