forms the largest, most aggressive colonies of any potential competing native ant species. It also employs a swift, precise trail system which is initiated by scouts when they discover food or new nest sites (*6*). It is of advantage to the *Pheidole* colony to strike hard and fast when a fire ant scout is discovered near the nest. The danger is sufficient to commit major workers to destroy the intruder and to search the surrounding area for the presence of additional scouts. Other ant species are less threatening, and evidently the *Pheidole* minor workers are able to subdue scouts and small parties of these insects without help. It will be of interest to learn whether alarm-recruitment systems occur in other ant species in addition to pure recruitment systems, as is the case in *P. dentata*, and whether they are specifically directed at principal enemies.

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1. For a review of general recruitment in ants and alarm-recruitment in termites, see E. O. Wilson, *The Insect Societies* (Harvard Univ. Press, Cambridge, Mass., 1971). M. C. Cammaerts-Tricot has independently found alarm-recruitment in the ant *Myrmica rubra* but does not report on enemy specification; see *Anim. Behav.* **23**, 124 (1975).
2. W. S. Creighton, *Bull. Mus. Comp. Zool. Harv. Coll.* **104**, 1 (1950).
3. Colonies collected in the field are placed in containers 28 by 45 cm and 16 cm deep, the sides of which are coated by Fluon (ICI America, Inc., Stamford, Conn.) to prevent escape. The ants are permitted to move into test tubes 148 mm long with 23-mm inner diameter, kept moist by tight cotton plugs that trap water at the bottoms of the tubes. The colonies are fed with synthetic diet [A. Bhatkar and W. H. Whitcomb, *Fla. Entomol.* **53**, 229 (1970)] and freshly killed insects.
4. A fuller account of the pheromone assay and other aspects of the alarm-recruitment response is being prepared for publication elsewhere.
5. A positive response is defined as the recruitment of major workers by minor workers following the introduction of alien ants 25 cm from the *Pheidole* nests. The number recruited is defined as the maximum number of majors seen in a 20 by 20 cm sector around the invaders for 30 minutes following the introduction minus the maximum number of majors seen in the same area during the 15 minutes immediately preceding the introduction.
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24 March 1975

Orientation of Water in Striated Frog Muscle

Abstract. *Proton and deuterium nuclear magnetic resonance spectra of striated frog gastrocnemius muscle exhibit angular dependence, indicating partial orientation of water in the muscle. Nonzero static dipolar and quadrupolar interactions resulting from the anisotropic motion of the water molecules modulate the spin echo decays, contributing to their nonexponential behavior.*

Partial orientation of water molecules in several biological systems has been observed using the technique of nuclear magnetic resonance (NMR) (*1–4*). The orientation produces a nonzero static dipolar interaction (*5*) for the protons in H_2O and a nonzero static quadrupolar interaction (*5*) for each deuterium in D_2O. These interactions may cause splittings in the NMR spectra (*6*). The splittings are determined by the average orientation of the water molecules. The orientation factor is related to the angle θ between the axis of the ordering matrix and the magnetic field by the relation $3\cos^2\theta - 1$ (*7*). Such splittings have been demonstrated in hydrated collagen (*1, 2*), in oriented DNA (*3*), and in model membrane systems, such as lecithin multilayers (*4*). The orientation of water molecules on nerve fibers has been a subject of dispute (*8, 9*). The nonzero interactions can also be detected by pulsed NMR experiments, because they would modulate the exponential decay of the spin echoes (*10, 11*).

We have studied both the continuous wave (CW) and pulsed NMR of ^1H and ^2H in striated frog gastrocnemius muscle at various orientations in the magnetic field. The results show that the water molecules in muscle have partial orientation; they also enable us to gain new insight into the interpretation of spin echo data for biological systems.

Pulsed NMR experiments were performed using a home-built NMR spectrometer with a 12-inch high-resolution Bruker magnet. Spin echo trains were obtained by the Carr-Purcell method (*10*) with the Meiboom-Gill modification (*12*). The spin-lattice relaxation time, T_1, was measured by the standard 180°-τ-90° technique (*10*) with full magnetization plots. Continuous wave and Fourier transform spectra were obtained with a Varian XL-100 spectrometer equipped with pulse and Fourier transform accessories manufactured by Nicolet Technology Corporation.

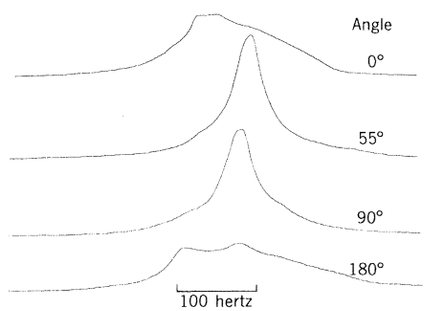

Fig. 1. Proton NMR spectra at 100 Mhz and 32°C for frog gastrocnemius muscle oriented in a magnetic field.

Frogs weighing about 20 g were used for the measurements. To introduce D_2O into the muscle, the frogs were kept in a container with 10 percent D_2O in H_2O for 3 days, then in 30 percent D_2O for 3 days, and finally in 50 percent D_2O for 1 to 3 weeks. Immediately after the frogs were killed, the gastrocnemius muscle was excised from the leg, and a striated piece was cut and carefully placed on a special Teflon plug. The plug was then fitted into the lower end of a sample tube which was cut open. Thus, the sample could be rotated about an axis perpendicular to the fiber axis so that the muscle fibers could form specific angles with respect to the magnetic field. Although small variations were observed for different frogs, the essential features of all results were reproducible for different samples.

The CW proton spectra for a normal frog muscle are shown in Fig. 1. The chemical shift of the signal is practically the same as that for liquid water. Since normal muscle contains over 70 percent water by weight, and the rest is mostly nonmobile macromolecules, one can safely assume that the CW proton signal observed is essentially due to H_2O in the muscle. When the muscle fibers were oriented parallel to the magnetic field ($\theta = 0°$ or 180°), the water signal was broad and split; when the sample was oriented at other angles, the line width was reduced, and it roughly obeyed the relation $3\cos^2\theta - 1$. The ^2H spectra for partly deuterated frog muscles (Fig. 2) did not show obvious splittings, but the line width had a similar angular dependence.

The features in the proton and deuteron NMR spectra of frog muscles indicate that the water molecules may be partly oriented in the muscle fibers. In addition to the change in line width, both the ^1H and the ^2H signals also show changes in chemical shift with the angle of orientation. Since water in different parts of a cell that are separated by physical boundaries may have different spin-lattice relaxation times (*13*), it is possible that the different fractions of water may have slightly different chemical shifts. The NMR peak may then be a composite signal of water molecules in separate parts of the cell, plus that due to extracellular water. Each part may change with the angle of orientation differently, resulting in an overall angular dependence for both the line width and the resonance position. Another possible cause of the change in chemical shift is the angular dependence of the magnetic susceptibility of the heterogeneous system.

There are two aspects in the spectra that deserve discussion. First, at physiological

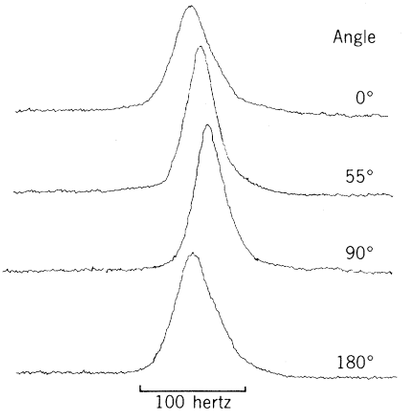

Fig. 2. Deuterium NMR spectra at 15.4 Mhz and 32°C for partially deuterated frog gastrocnemius muscle oriented in a magnetic field (each spectrum was an accumulation of 32 pulses with Fourier transform).

*p*H and ambient temperature, the mean time between proton jumps for H_2O is about 2×10^{-3} second (*14*). Thus, dipolar splittings of less than 150 hertz would be averaged by the proton exchange to give a single broadened peak. It is very likely, however, that the observed water signal is a time-averaged signal of two fractions, one hydrated to the macromolecules and highly ordered and the rest isotropic (*15*). If the residence time for the water protons of the hydrated fraction is longer than that for ordinary water, dipolar splittings of smaller magnitude may be observed without being averaged to a single peak. Second, in systems where water orientation is more definite (*1–4*), the deuterium quadrupole splitting is usually larger in magnitude than the proton dipolar splitting. In the present case and for water on nerve (*8, 9*), splittings were not observed in the deuterium NMR spectra. Nevertheless, this does not preclude the possibility of preferred water orientation, because for a particular set of ordering parameters it may be impossible to observe deuterium quadrupole splitting at any angle (*9*).

It was suggested (*9*) that the ambiguity mentioned above may be circumvented by using pulsed NMR techniques, which distinguish susceptibility shifts from dipolar splittings (*10*). The pulsed NMR results are plotted in Figs. 3 and 4, which clearly show that the spin echo decays are dependent on the orientation of the muscle fibers and, in the case of deuterium, on the pulse spacing (*16*). On the other hand, T_1 for both ^1H and ^2H did not show any angular dependence. The results therefore indicate that there must be a certain favored molecular orientation for water in the muscle fiber. However, it must be emphasized that this does not imply that the bulk of the cell water is necessarily in an "ordered" state, because the nonzero dipolar and quadrupolar interactions can be the time-averaged result of a fraction of water molecules undergoing anisotropic motion while the other water molecules move isotropically. In fact, detailed studies of T_1 for both ^1H and ^2H over wide ranges of frequency and temperature (*15*) indicated that over 80 percent of the water in muscle undergoes relaxation similar to ordinary liquid water.

The results in Figs. 1 and 3 show that nonzero static dipolar interaction must be an important factor in modulating the proton spin echo decay for water in muscle. The nonexponential behavior observed by previous investigators was explained by the superposition of three exponential decays presumably due to three fractions of water undergoing slow exchange (*17, 18*). Values of the spin-spin relaxation time T_2 of about 0.2, 0.04, and less than 0.01 second were derived for these three fractions. If this approach were used, the data in Fig. 3 would have to be interpreted by a change in the relative amounts of water for the different fractions and a corresponding change in their T_2's with the change of the angle of orientation, which is difficult to understand.

It was indicated above that the observed water signal in a biological tissue is a composite signal due to water in different parts of the tissue. It is most likely that the rates of exchange between water molecules separated by physical boundaries (for example, intracellular and extracellular water) are slow on the NMR time scale. Depending on the relaxation times and relative amounts of the different fractions, their values of T_1, $T_1\rho$ (the spin-lattice relaxation time in the rotating frame), and T_2 may or may not be resolved from the composite signal. In most cases, only a single T_1 is observed. For spin-spin relaxation measurements, preferential orientation of the water molecules would be an important factor contributing to the nonexponential decay of the spin echo train and the shortening of the observed T_2 (*19*), and one must be careful in attempting a quantitative analysis of the experimental data. Even though the present experiments were carried out with oriented striated muscle fibers, the effect of preferential orientation of water molecules should be considered in the interpretation of all T_2 data for water in biological systems, because microscopic nonzero static dipolar and quadrupolar interactions would be important even when samples are randomly oriented in the magnetic field.

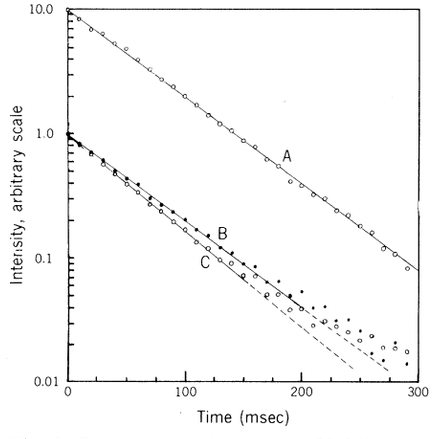

Fig. 3. Proton spin echo data at 30 Mhz and 25°C for (A) H_2O with $5 \times 10^{-4}M$ $Mn(ClO_4)_2$, (B) frog gastrocnemius muscle oriented at 90° with respect to the magnetic field, and (C) frog gastrocnemius muscle oriented parallel to the magnetic field. The data were recorded with a Nicolet 1080 signal averager with eight scans per spectrum (*16*).

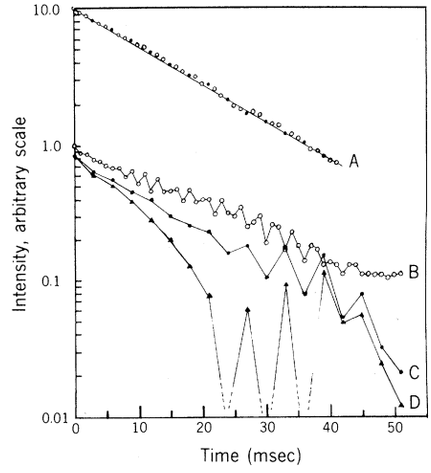

Fig. 4. Deuterium spin echo data at 9.8 Mhz and 25°C for (A) D_2O with $5 \times 10^{-3}M$ $Mn(ClO_4)_2$; (B and C) frog gastrocnemius muscle oriented at 55° with respect to the magnetic field; and (D) frog gastrocnemius muscle oriented parallel to the field. The open symbols represent data for 1-msec pulse spacing, and the closed symbols represent data for 3-msec pulse spacing. The data were recorded with a Nicolet 1080 signal averager with 16 scans per spectrum (*16*).

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16. The data for samples of doped H_2O and D_2O in Figs. 3 and 4 serve as a check of the accuracy of the experiments. The amount of water in each sample was adjusted so that its signal intensity was comparable to those of the muscle samples. The data were plotted on a scale ten times larger than the scale for the muscle data for the sake of clarity. The angular dependence for both the ^1H and ^2H echoes and the fluctuation for the ^2H echoes of the muscle samples were observed for several different frogs and were well within experimental uncertainty. The fact that the deuterium spin echoes for doped D_2O (curve A, Fig. 4) did not depend on pulse spacing but those for muscle did (curves B and C, Fig. 4) further indicates that the spin echo train of D_2O in muscle is modulated by nonzero static quadrupolar interaction.
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Direction Finding by Hornets Under Gravitational and Centrifugal Forces

Abstract. *The effect of centrifugal and gravitational forces whose resultant ranged between 26° and 45° on comb construction by hornet workers was assessed experimentally. Comb construction by hornets exposed to centrifugation at 1 to 2 days of age differed from that of hornets similarly exposed at 3 to 7 days of age. Juvenile hornets built their cells in the direction of the resultant force, whereas adults resisted the centrifugal force and tried to build in the direction of the gravitational force. Juveniles started their comb from the side walls, whereas adults started from the roof, as did nonspinning, control hornets. The findings suggest that hornets rapidly learn the gravitational force during the first days of life, and that they are aided by geometric cues of the breeding box to build in the direction of the force to which they had become habituated.*

Ordinarily the first comb in the nest of Vespinae is built by the queen in the spring, following the hibernation period. The queen initially constructs a stem or pedicle which is attached to the roof of the nest and from which a comb made up of 15 to 30 cells is suspended. The queen oviposits one egg within each cell. The eggs are glued to an inner wall of the cell to prevent their falling out, and the queen attends the brood until eclosion of the first worker hornets from their pupal cases (*1*). Thereafter, the worker hornets assume the construction duties while the queen engages primarily in oviposition. Ishay (*2*) observed that young workers of *Vespa orientalis* which are kept in groups of 5 to 40 individuals without a queen also build a comb similar to the queen's vernal one. In previous studies (*3, 4*) it was suggested that hornet comb construction is influenced by gravitational cues.

The aim of the study reported here was to observe how hornets build the comb and cells under the influence of gravitational and centrifugal forces. For this purpose a four-armed centrifuge was designed to accommodate regular artificial breeding boxes (ABB's) (*5*). Five such boxes were attached firmly to each arm at different distances from the center of rotation, with the top and bottom walls of the ABB vertical to the direction of the earth's gravity. When the centrifuge rotated at 25 rev/min, a centrifugal force of $0.325g$ acted on the first box, placed 75 cm from the center. The direction of the resultant force acting on this box was 26° from the normal; the angle is given by

$$\tan \phi = (2\pi f)^2 r/g = 4\pi^2(25/60)^2 r/980$$

where $2\pi f$ is angular frequency, r is the radial distance from the center, and g is the constant of gravity. The corresponding angles for the second to fifth boxes were 32°, 35°, 41°, and 45° (the fifth box was attached 144 cm from the center).

Concomitantly two controls were used: (i) a rotating control consisting of two ABB's placed in opposition at a distance of only 5 cm from the center of the centrifuge and (ii) a stationary control consisting of two ABB's placed immediately adjacent to but not in contact with the centrifuge.

Twenty hornet workers were introduced into each ABB. These were either juvenile (1 to 2 days old) or adult (3 to 7 days old) and were provided with a clump of clay soil as building material and with a 200-cm^3 cotton-plugged bottle of 30 percent sucrose solution, fastened to one side of the ABB, which served as a liquid food source. Once every 2 days, the centrifuge was stopped for 5 to 10 minutes to introduce solid protein food (honey bees or hornet pupae) into each ABB and to service the machine. To prevent possible hornet adaption to the rhythm of activation or interruption of the centrifuge (*6*), these stops were made at different hours of the day or night. Acceleration of the centrifuge to constant speed required 7 seconds, and deceleration 6 seconds. Each experiment continued for 3 weeks, a period sufficient for most vesparium-reared hornets to build a comb and rear brood up to the pupal stage. In the vesparium, the hornets ordinarily start building on the third to fourth day of life and continue intensive construction for 2 to 3 weeks, and then the building activities diminish (*7*).

In two experiments, the centrifuge was exposed to direct sunlight and the temperature in the attached ABB's ranged from 22°C at night to 30°C in the daytime. In another two experiments, the centrifuge was activated within a room with a dome-shaped ceiling, under fluorescent illumination and at a constant temperature of 27°C. All the hornets used in these experiments were laboratory-eclosed specimens derived equally from queenright colonies (colonies in which a living queen is present) and queenless colonies (orphan colonies whose queen was lost or died), and were fully supervised from the moment of eclosion from the egg. Juvenile hornets were maintained for several days in ABB's at 27°C before being transferred to the centrifuge on the third to seventh day of life. In all instances, the experimental hornets were placed in ABB's in which there was no prior comb construction.

The direction of the cells with respect to the normal was measured with a goniometer within ½° (although such precision was not necessary). It was found that in an undisturbed environment—that is, when building in nature or in the ABB's of a vesparium—hornets build their cells toward the center of the earth, but there is a scatter of ± 3° in the hornets' determination of this direction. Such a scatter was also observed for the direction of the comb stalk. We found that the stalk was, in general, orientated in the direction of the central cell. Around the central cell, an initial ring of three to six cells is usually built, whose direction is 6° to 8° away from the central cell, but the average orientation is the same as that of the central cell. A second circle of cells is then built, comprising 8 to 12 cells orientated 12° to 14° away from the normal, but again the average orientation is the same as for cells of the first ring. Only in the subsequent outermost ring are cells again orientated in the normal direction.

Guided by these observations, we adopted the following procedure for measuring the direction in which the hornets build within the centrifuge. Three different measurements were made: (i) the direction of the stalk with respect to the normal, (ii)