tetraacetate (EDTA) have no effect on either stage. Electron microscopy and electron diffraction showed that $CH_3C(OH)(PO_3HNa)_2$ (Fig. 2), $H_2C(PO_3HNa)_2$, and pyrophosphate inhibit the crystal growth of hydroxyapatite but that the monophosphonate and EDTA do not. The inhibitory effect of the diphosphonates on the crystal growth of hydroxyapatite is shown by the pattern obtained by electron diffraction and the gel-like appearance of the calcium phosphate in the electron microscope—both features typical of the amorphous form (*7, 8*).

Pyrophosphate increases the minimum product (Ca × P) required to induce precipitation of calcium phosphate from solution under defined conditions (*1*). The two diphosphonates have similar, striking effects at concentrations as low as $10^{-7}M$; the monophosphonate has only a small effect.

Aortic calcification was induced in rats by treating them by mouth daily with 75,000 I.U. of vitamin D_3 per kilogram of body weight for 5 days (*4, 5*). The phosphonates—$CH_3C(OH)(PO_3HNa)_2$, $H_2C(PO_3HNa)_2$, or $CH_3(CH_2)_4PO_3HNa$ (10 mg of P per kilogram of body weight)—were given subcutaneously or by mouth daily from 2 days before vitamin D_3 was administered and continued until 7 days after administration of the vitamin D; the animals were then killed. Calcification of the aortas was assessed by measuring calcium content of aortas ashed at 600°C. When given orally or subcutaneously, the two diphosphonates completely prevent calcification (Table 1); the monophosphonate does not prevent calcification by either route.

Our data show that diphosphonates have effects similar to those of pyrophosphate and condensed phosphates on the inhibition of crystal growth of hydroxyapatite in vitro (*1, 9, 10*) and on the prevention of aortic calcification in rats given large doses of vitamin D_3 (*5*). In addition, they possess properties not shared with the condensed phosphates. Thus, not only do they prevent aortic calcification when given by mouth rather than subcutaneously, but they also prevent kidney calcification (*10*) when given by either route.

Studies with other phosphonate compounds have shown that there is a good correlation between the ability of any single compound to prevent hydroxyapatite crystal growth in vitro and its ability to prevent calcification in vivo (*10*). This strengthens the view that the biological activity of these compounds and of pyrophosphate is a consequence of their observed action on crystal growth. The most likely mechanism of action of the diphosphonates both in vivo and in vitro is strong chemisorption on hydroxyapatite, as demonstrated with ^{14}C-ethanehydroxydiphosphonate on synthetic apatite (*8*). The compounds that have so far proved most active have all contained the P–C–P bond; $CH_3(CH_2)_4PO_3HNa$, which contains a single C–P bond, is ineffective. The fact that EDTA has no effect shows that the inhibition of crystal growth is not determined solely by the chelation properties of the P–C–P materials and of EDTA itself. Since the molecular configuration of the P–C–P bond is close to that of the P–O–P bond, the diphosphonates, pyrophosphate, and polyphosphates may all act on crystal growth by a similar chemisorption mechanism. The observation that the diphosphonates, unlike the polyphosphates, are effective when given orally and are able to inhibit kidney calcification may be attributed to their resistance to either chemical or enzymatic hydrolysis.

The diphosphonates might provide a convenient model for investigating the action of pyrophosphate in calcium homeostasis. Since the diphosphonates are of low toxicity, they might also be used against diseases in which calcium salts deposit in soft tissues. The administration of $CH_3C(OH)(PO_3HNa)_2$ to two patients in the acute phase of myositis ossificans (*11*) has been associated with an arrest in the progress of the disease.

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Ant Alarm Pheromone Activity: Correlation with Molecular Shape by Scanning Computer

Abstract. *The ant* Iridomyrmex pruinosus *utilizes 2-heptanone as an alarm pheromone. The activities of 49 ketones and 35 nonketones as alarm pheromones for this species were determined. The molecular shapes of these compounds were assessed by submitting silhouette photographs of their molecular models to a pattern recognition machine. A highly significant correlation exists between molecular shape and alarm activity.*

The alarm pheromones employed by certain species of ants provide extraordinarily favorable tools with which to examine the relationships between chemical constitution and biological activity (*1*). One of us (M.S.B.) has collected a large amount of data on biological activity, using chemical relatives of 2-heptanone, the alarm pheromone of *Iridomyrmex pruinosus* (*2*). The results showed clearly that the behavioral response, while far from exclusive to 2-heptanone, is nevertheless specific to a restricted range of compounds. Among current theories of olfaction, the stereochemical theory seemed to hold considerable promise of being able to accommodate the experimental data (*3*). Accordingly, the compounds employed in the survey were subjected to a stereochemical analysis of their molecular shapes by another author (J.E.A.). These assessments of chemical constitution were rendered impartial and quantitative by submitting molecular model silhouette photographs to the other collaborators (G.P. and E.W.) for scanning by the PAPA pattern recognition machine (*4*).

Highly significant correlations were

observed between molecular shape and alarm activity. This suggests that the stereochemical principle plays a major and perhaps a dominant role in governing the activity of these alarm pheromones.

The ant species studied were the dolichoderine *Iridomyrmex pruinosus*, which secretes 2-heptanone from its anal glands when disturbed, and the myrmicine *Pogonomyrmex badius*, which employs a mandibular gland product, 4-methyl-3-heptanone, as an alarm releaser (*5*). For *I. pruinosus*, the compounds were evaluated on both laboratory and mature field colonies with large foraging populations. In the laboratory tests, the compound was introduced into an aspirated air stream drawn through the nest. The activity of each compound was indicated by the number of ants which emerged from the nest exhibiting alarm behavior (*6*). In the field tests, a 1-cm^2 paper treated with 10 μl of trial compound was placed upwind approximately 10 cm from a nest opening. Compounds were rated according to the numbers of ants which were attracted to the treated papers, while displaying alarm reactions, during a 2-minute period. The rating scale for *I. pruinosus* was as follows: Fewer than 25 ants alarmed, step 1 (inactive); 26 to 74 ants, step 2; 75 to 124, step 3; 125 to 174, step 4; more than 175 ants alarmed, step 5 (highest activity).

In the case of *P. badius*, the ketones were tested on laboratory colonies, each containing about 2000 workers. Ratings were assigned based on the numbers of ants which were drawn to the ketonic source, while displaying typical alarm reactions, during 3 minutes of observation. For both ant species, all compounds were tested successively on six laboratory colonies. Each compound was tested a minimum of six times, and many compounds were bioassayed at least nine times. Controls, which consisted of untreated papers, were run before each compound was evaluated. The compounds were presented in irregular order to avoid conditioned responses, and each colony was tested no more than twice daily with a 4-hour interval between tests.

The data obtained for both species were submitted to statistical analysis to establish confidence in the activity rating assigned to each compound. For example, in field tests, *I. pruinosus* was tested six times with 2-heptanone. The control papers caused responses by only 0 to 3 ants. The mean number of ants alarmed by the treated papers, however, was 229, with a standard deviation of $\pm$ 28 ants; the standard error of the mean ($N = 6$) was $\pm$ 11 ants. Hence the standard error of the bioassay is substantially less than one-half step in the five-point rating scale. For *I. pruinosus*, excellent agreement was obtained between laboratory and field bioassays for the 84 compounds which were evaluated in a total of 614 tests. Laboratory bioassays with *P. badius* were also very reproducible. These considerations demonstrate that the test procedures employed with both species of ant were entirely adequate for comparing the effectiveness of various compounds as releasers of alarm behavior.

Some of the alarm ratings have been published (*7*). (The remainder, together with the molecular similarities, will be submitted for publication elsewhere.) By prior agreement, the alarm ratings were withheld from J.E.A., G.P., and E.W. until the stereochemical assessments and computer scanning had been completed.

A scale molecular model was built for each compound and arranged in its most likely conformation. As a general rule, the fully extended form of the saturated aliphatic chain is the conformation with minimum energy (*8*). The model was placed in a conventional orientation, with the functional group facing toward the left front, and photographed in silhouette (*3*). The position of the functional group was marked on the photograph by a pinprick. For the ketones, the center of the oxygen atom was chosen as the molecular reference point, and corresponding assignments were made for the functional groups of the nonketones. Most of these compounds have articulated molecules, with many possible conformations differing by small energies. Nevertheless, a single preferred conformation could usually be selected by applying fixed stereochemical rules (*3*). Furthermore, this conformation exhibited substantial correlation with the observed biological activity. It may reasonably be anticipated that a better knowledge of the true conformation of the molecule (and eventually of the insect's olfactory receptor site) would lead to still better correlations.

The principal results of this investigation are intuitively apparent from inspecting the structural formulas, and especially the molecular silhouettes, of the more active compounds (Fig. 1). A series of isomeric and homologous ketones was examined. The natural alarm pheromone, 2-heptanone, contains a flat, zigzag chain of carbon atoms, with the keto oxygen projecting from the second position. The structural formula is shown here superimposed on a silhouette of the molecular model. As indicated by the alarm activities, a certain amount of variation in molecular architecture is tolerated. Thus, the carbon chain can be somewhat lengthened or shortened, the keto group can be moved a little, or an ethylenic linkage may be inserted, without destroying activity.

Nevertheless, any more radical departures in molecular size or shape led to compounds with virtually zero activity. Thus the straight-chain 2-alkanones with less than five or more than ten carbon atoms were all inactive. None of the cyclic alkanones, not even cycloheptanone, had any activity.

In contrast, provided that the general molecular shape of 2-heptanone is preserved, a quite remarkable amount of chemical variation is permissible, while still retaining alarm activity (Fig. 2). Using this stereochemical principle as a guide, M.S.B. was enabled to find a number of nonketones which possessed high alarm-releasing activities. Thus, the replacement of the methylene group in position 3 by an oxygen linkage results in the ester *n*-butyl acetate, which is fully as effective as the natural ketonic pheromone in releasing alarm. Another methylene replacement in position 6 farther along the chain yields an ether-ester which also was very effective.

So far, all the compounds discussed emulate the pheromone in having a carbonyl oxygen at or near the second position in the chain. However, even this key oxygen atom projection can be substituted by alternative polar arrangements, as the last three examples show. Methyl *sec-n*-octyl ether has a methyl (or methoxy) side-group, which seems to be a fairly adequate surrogate for the keto group. On the other hand, the straight-chain *n*-octyl isomer is virtually inactive. Considerable activity is still retained even if the keto group is replaced by a hydroxyl. Perhaps the most surprising iso-steric replacement is that by the bromine atom in 2-bromooctane. This totally unnatural product still is moderately active as an alarm releaser for *I. pruinosus*.

The ten compounds depicted in Figs. 1 and 2 were admittedly chosen for their intrinsic interest, or to illustrate a particular stereochemical principle. This study, however, included 150 compounds, many of which were evaluated on more than one species of ant. Every compound admitted to bioassay was later submitted to stereochemical assessment and included in the statistical analysis.

The success of the overall correlation lends confidence to our belief that this relationship, between alarm pheromone activity and molecular shape, is indeed a valid general principle.

The stereochemical similarity among the active compounds is self-evident to the unaided eye from Figs. 1 and 2. It was confirmed and quantified by an instrumental method as follows. The molecular silhouette photographs were scanned by reproducible random lines in the PAPA machine, which is basically a television camera linked directly to a computer (*4*, *9*). The PAPA machine was modified so that it could scan the silhouette photograph positive prints directly, without the necessity for producing negative transparencies. Somewhat improved results were obtained by using as the molecular reference point, not the center of gravity of the molecular silhouette (*9*), but rather the center of its functional group of atoms. By focusing on the functional group, the computer is made aware of the most polar location on the molecule. The PAPA machine was initially adjusted by using a series of 14 molecular silhouette photographs of lower fatty acids, which exhibit in varying degrees the sweat-like primary odor of isovaleric acid, for the human sense of smell (*9*). These same machine settings were then retained throughout the insect pheromone series. In this program, the machine assesses the similarity in molecular shape between each test silhouette and the silhouette of the standard molecule, the pheromone itself.

Table 1. For three species of ants, the intensity of alarm behavior released by various test compounds is correlated with their degree of similarity to the molecular shape of the natural pheromone. *r*, Correlation coefficient; *P*, approximate probability that the correlation is fortuitous.

Test compounds	Correlations	
	r	*P*
Iridomyrmex pruinosus		
49 Ketones	0.57	10^{-5}
35 Nonketones	0.81	10^{-10}
Pogonomyrmex badius		
100 Ketones	0.47	10^{-7}
Atta texana		
34 Ketones	0.32	0.05

Each molecular model was photographed and scanned from three aspects mutually at right angles. The three similarity values printed by the computer were summed to give the molecular similarity, on a dimensionless logarithmic scale ranging from about 900 for extreme dissimilarity to 1210 for identity of molecular shape. The PAPA computer print-outs are almost perfectly reproducible. The alarm activity for each compound toward a given species of ant had previously been rated on an integral scale from 1 for inactive to 5 for maximum activity (equal to the natural pheromone).

The final stage of the joint investigation was to calculate the correlation coefficient (*r*) between the alarm ratings for a series of compounds and their molecular similarities to the pheromone. The quantitative results are shown in Table 1. For *I. pruinosus* the correlations observed with the ketones, and particularly with the nonketones, are very satisfactory. The results have extremely high statistical significance. The nonketones included alcohols, aldehydes, ethers, esters, amines, and halogenated hydrocarbons, belonging to the aliphatic, alicyclic, and aromatic series. The value for the coefficient of determination (r^2) indicates that a simple linear regression of alarm rating on molecular similarity explains as much as 65 percent of the total variability in alarm rating. Hence a very substantial proportion of the variation in alarm activity among this collection of compounds can be accounted for by the stereochemical measurements alone.

With *P. badius* the correlation was not quite so high, but owing to the larger number of ketones tested, the conclusion is just as significant. We have also applied the stereochemical assessments to some alarm pheromone data collected by an independent investigator (*10*). Dr. J. C. Moser tested 34 ketones on *Atta texana*, which also employs 4-methyl-3-heptanone as alarm pheromone. The correlation coefficient is lower, but still significant.

These results give rise to optimism that the stereochemical theory could also prove successful in interpreting data on the alarm pheromones of additional species of ants, and possibly other social insects. However, any hope that the stereochemical principle could be applied to other kinds of pheromones

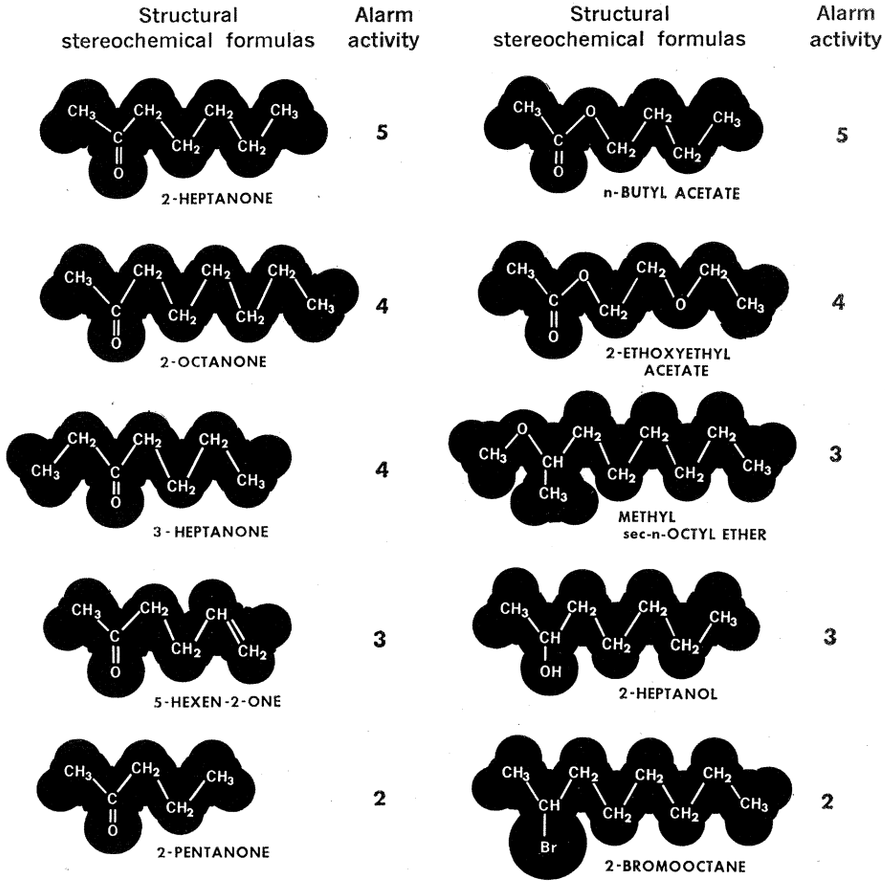

Fig. 1 (left). Ketones. Many ketones related to 2-heptanone were tested for alarm-releasing activity on *Iridomyrmex pruinosus*. Nevertheless, only a rather limited amount of variation in molecular size and shape proved compatible with alarm activity. Fig. 2 (right). Nonketones. Provided that they bear a close resemblance to the molecular shape and polar location of 2-heptanone, a remarkable variety of unrelated chemicals were found to mimic its pheromone action.

should be tempered with caution. For instance, in the sex attractant pheromones, a far higher degree of stereochemical specificity appears to prevail, and competitive inhibition may occur between close homologs or geometric isomers (*11*). Nevertheless, comprehensive conformational analysis by a fully computerized method is now becoming available (*12*). It is to be expected that further progress will be made into what we have proved to be essentially a stereochemical problem—the specificity of alarm pheromones.

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Unit Activity: Motivation-Dependent Responses from Midbrain Neurons

Abstract. *Single neurons in the midbrain tegmentum of rats showed clear discriminatory responses to three tones announcing either food or water or no reinforcement. Subjects were required to press a single lever and remain motionless for 2 seconds during which time unit activity from several brain sites was recorded. One of the tones, randomly designated, was sounded halfway through this period. Manipulation of drive states revealed that the highest activity was associated with tones announcing the reinforcement for which the animal was most strongly motivated. Thus, from a hungry animal sustained or increased firing rates were elicited by a "food" tone and differentially lowered rates by the other tones.*

The study reported here was directed at understanding neuronal activity changes in the midbrain occurring during states of expectancy. In previous studies of unit responses during anticipatory behavior (*1*), it was found that units recorded from certain areas of the midbrain often exhibited continuous accelerations of firing just prior to the accustomed time of reinforcement, particularly to food rewards. Two main questions were raised: First, were the observed unitary activity changes correlated with the expectant states or with undetected movements and different poses assumed by the subject during waiting for food and water? Second, if expectancies were involved, was it the different reinforcing value between food and water rewards or more specific factors which caused the differences in neuronal firing?

In the present experiment operant and classical conditioning paradigms were combined to decide between these possible interpretations. The aim was to have one standard form of behavior during different anticipatory states. A single, predetermined and highly stabilized motionless operant response was used, and, superimposed upon this response, auditory stimuli were conditioned to induce one of three different expectancies without allowing any change in behavior. In such an experiment there should be no differences in neuronal firing rate based primarily on behavior, but on the organism's anticipatory state.

For this purpose, rats were implanted with four to seven permanently placed microelectrodes made from insulated Nichrome wires (62.5 μ in diameter) bared at the tip. The method of implantation and recording of units by wave-form detectors has been described previously (*1*). Two weeks after surgery rats were selected for preliminary conditioning if there were clearly differentiated unitary spikes observable in recordings from at least three of the four to seven probes. During conditioning the animals were kept in circular plexiglass cages 12 inches in diameter and 11 inches high (30½ by 28 cm). The rats were first trained to press one lever for food and a different lever for water and then to remain relatively motionless for 2 seconds, at the end of which time they received the appropriate reward. During the 2-second waiting periods, movements triggered a movement detector which canceled the trial if the movements were beyond a predetermined level. Animals were kept in this training procedure for 24 hours per day and when they were able to maintain a normal diet, the unitary firing rates during each 2-second waiting period were recorded automatically. On the basis of preliminary unit counts, 12 animals were selected for the test procedure, the criterion of selection being that units recorded from the midbrain probes had to show significantly different rates of firing during responses on the two different levers.

In the test experiment there was only one lever available so that there could be only one behavioral response. Whenever the animal required food or water it had to press the lever and remain motionless for 2 seconds before reinforcement, just as during the initial training. During this 2-second interval, 1 second after its commencement, one of three tones was sounded for 1 second, and followed by reinforcement. The tones were 15, 8, and 1 khertz, respectively. Each tone was correlated with one of the three possible outcomes: food, water, or no reward. The three tones were presented on a random basis but with a predesignated probability ratio to one another. Consequently, the rat could not know at the time of lever-pressing whether the reward would be food, water, or nothing; and the rat could not make any posture correlated with the outcome of the lever response. When the tone was presented the animal's uncertainty was resolved in the sense that the type of reward it would receive was then spec-