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Biochemical Ecology of the Attine Ants

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Ecology is the study of the relationships between organisms and their environment. It is of necessity a highly eclectic field which has utilized the observations and methodologies of areas as different as bird-watching, systems analysis, and physiology.

Many aspects of a species' status within a community depend upon its characteristics as a chemical processing agent. It must utilize nutrients, avoid toxic substances, deal with the metabolic waste products of other species, and adjust to all manner of modifications in the chemical nature of its environment. Obviously the findings of biochemistry and natural product chemistry are highly significant to ecology.

This Account reviews research of a straightforward biochemical nature that has clarified the mechanistic basis for a biological phenomenon first recorded nearly a century ago in the classical literature of descriptive natural history.[1] It illustrates how an analysis of the biochemical interactions and interdependencies of organisms can contribute to the clarification of the principles which underlie the incredible complexity of natural biological communities.

The Fungus-Growing Ants

Natural History. The attine ants, commonly known as the fungus-growing ants, are native to the New World Tropics. There is an extensive literature describing the habits of this group.[2-4] All of the species which comprise the tribe Attini actively culture a fungus in their nests and exploit this fungus as their primary and probably sole food source. The simpler, more primitive genera grow their fungi on solid insect feces and insect carcasses which are collected from the forest floor by foraging workers, whereas the more specialized genera culture their fungi exclusively on fragments of leaves and flowers cut from live plants. Species of the genera *Atta* and *Acromyrmex* are commonly known as leaf-cutting ants, and are among the most abundant insects which occur in the Neotropics. Several species are serious economic pests as a consequence of their defoliating activities.

The fungus gardens of the attine ants are fragile, spongelike structures consisting of many small pieces of substrate held together by a dense mycelial growth. The fungus cultures appear to consist of a single dominant filamentous fungus along with admixtures of yeasts and bacteria.[2,4,5] Since the fungi cultivated by the attines have never been isolated from any source other than attine nests, it is generally believed that they occur only in association with their myrmecine hosts. With one exception, the fungi appear to be Basidiomycetes.

These fungi are readily isolated in pure form by standard mycological plating procedures. When grown on standard culture media, they are seen to be rather slow-growing fungi which are readily overwhelmed or replaced by other faster growing species. However, Weber[6] has demonstrated that if the ants have access to an agar culture of their fungus they can maintain it indefinitely even when it is adjacent to large areas of contamination. When the ants are denied access to the fungus garden, however, a rapid deterioration of the culture ensues, and contaminants replace the ants' fungus.

Clearly, the growth of the ants' fungus in their nests is not fortuitous, but rather depends in a critical way upon the activities of the ants. The care and maintenance of the gardens by the ants have been carefully and thoroughly observed and reported,[2-4] and several features of the ants' behavior strongly implicate a chemical basis for their success in culturing their food fungus in the face of possible competition from faster growing microorganisms present in the surrounding soil or brought into the nest on substrate or on the ants themselves.

When substrate is brought into the nest by a worker it is treated in a very characteristic way. All attine species, regardless of the preferred substrate on which they culture their fungus, deposit liquid fecal material on the substrate prior to incorporating it into their gardens. It is likely also that some species apply their saliva to the substrate while it is

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being prepared. Weber has repeatedly emphasized a likely role for fecal and salivary material in creating environmental conditions favorable to the growth of the food fungus.[4,6-8] It has been the object of our research over the past few years to establish in biochemical terms precisely how the application of fecal material contributes to the enhanced viability of the fungus.

An Ecological Perspective. The fungus-culturing activities of the ants influence the outcome of interspecific competitive interactions. Outside the ants' nest, the ants' fungus is apparently a very poor competitor and is excluded from otherwise suitable substrates by better competitors. By contrast, within the ants' nest it is such a successful competitor that it emerges as the dominant species. Interspecific microbial competition is a complex process, and its outcome is determined by a multitude of factors,[9-11] many of which are related directly or indirectly to the relative growth rates of the competing organisms. Thus, any attempt to provide a mechanistic explanation for the ability of the attine ants to maintain their fungus cultures must logically begin by considering the ways in which the ants might alter the growth rate of their fungus relative to that of potential alien competitors. One plausible hypothesis, first enunciated by Weber,[7] is that factors present in the fecal material or saliva inhibit the growth of alien fungi and enhance the growth of the ants' fungus.

Symbiosis as a Biochemical Alliance

The main thrust of our research has been aimed at clarifying the complementarity of the metabolic capabilities of the ants and their fungi. Our efforts have been rewarded by the identification in biochemical terms of contributions made by each partner in the symbiosis to the other.

Contribution of the Fungus to the Ants. Hodgson has commented that the habit of feeding on a cultured fungus has achieved for *Atta* a preeminent position among the rain forest fauna by tapping the virtually inexhaustible food supply in the plant material of the environment.[12] Implicit in this interpretation is the assumption that there is some substance present in the plant material which the ants are unable to metabolize, but which is made available to them for exploitation as a nutrient through the metabolic intervention of the fungus. Cellulose, which is probably the most abundant organic material in nature, is the logical candidate for this substance. Very few higher organisms are able to digest cellulose. Consequently they must depend upon symbiotic microorganisms to provide the enzymes active in the degradation of this polysaccharide. The attine ants are no exception to this generalization.

The digestive fluid of *Atta colombica tonsipes* is only weakly active in the degradation of carboxymethylcellulose,[13] whereas a culture of the fungus readily depolymerizes this derivatives of cellulose.[14] We have also shown that the fungus is a rich source of trehalose and mannitol, both of which are readily metabolized sugars.[15] Thus, when the ants imbibe the fluid contained within the fungal mycelia, they are obtaining nutrients constructed in part of carbon atoms which had been in the cellulose of the original substrate. Whereas most organisms which subsist on high cellulose diets harbor anaerobic microorganisms in some specialized region of the alimentary canal, the attine ants have domesticated an aerobic fungus which they cultivate in their nests.

Contribution of the Ants to the Fungus. While working out methods to grow the ants' fungus in sterile culture, we discovered an important metabolic limitation of the fungus. We established that the growth of the fungus normally cultivated by *Atta colombica tonsipes* is sensitive to the degree of hydrolysis of the polypeptide provided as the nitrogen source in a synthetic culture medium (Table I).[16] The fungus grows very poorly in a medium containing casein or a tryptic digest of casein in which hydrolysis has proceeded only to a limited extent. On the other hand, the fungus grows significantly better if provided with an enzymatic hydrolysate of casein in which extensive degradation of the protein has occurred, and which contains relatively high levels of free amino acids. The results are the same whether the fungus is grown in shaken liquid cultures or on solid agar plates.

These results suggest that the fungus cultured by *A. c. tonsipes* might lack the full complement of proteolytic enzymes needed to make efficient use of polypeptide nitrogen. This metabolic attribute of the fungus is of considerable ecological significance, since it would place the fungus at a serious competitive disadvantage in the colonization and exploitation of a substrate in which the nitrogen is present predominantly as polypeptide.

In the ants' nest the fungus grows on fresh leaf material, a material which would seem to be an unlikely substrate, since most of the amino nitrogen in leaves is present in polypeptide form. Thus the ants must compensate in some way for this critical metabolic limitation in order to grow their fungus on a substrate from which it would otherwise be excluded. We have demonstrated that the growth of the fungus in a liquid culture medium containing polypeptides as the nitrogen source is significantly accelerated by the addition of the fecal fluid of the ants.[17] The growth of the fungus in such cultures is also significantly accelerated by the addition of commercial preparations of proteolytic enzymes, such as the proteinase derived from the fungus *Streptomyces griseus* (Sigma type VI fungal protease).[17]

The findings described in the previous paragraph led us to search for proteinases in the salivary and fecal fluid of *A. c. tonsipes*.[16] No protease activity could be detected in homogenates of the thoracic salivary glands, mandibular glands, maxillary glands, or postpharyngeal glands, all of which might be re-

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Table I
Growth of the Fungus Cultured by *A. c. tonsipes* in Defined Median[a]

Nitrogen source	Concn, mg/ml	Mean dry wt of fungus, mg
A. Shaken Liquid Cultures (25°, 250 rpm, 7-day growth period)		
Casein hydrolysate[b]	1.0	47
Peptone T[c]	1.0	8
Casein hydrolysate	2.0	91
Peptone T	2.0	12
Casein hydrolysate	4.0	110
Peptone T	4.0	19
B. Solid Agar Cultures (25°, 21-day growth period)		
		Mean area of fungus, mm²
Casein hydrolysate	1.0	44
Peptone T	1.0	17
Casein hydrolysate	2.0	65
Peptone T	2.0	14

[a] Containing either an enzymatic casein hydrolysate, rich in free amino acids, or a tryptic digest of casein, poor in free amino acids.[16] [b] For Casein Hydrolyzate-Enzymatic (Nutritional Biochemicals Corp.) the following typical analysis is reported: total nitrogen, 12.7%, amino nitrogen, 4.9%. [c] Peptone T (Nutritional Biochemicals Corp.) is a tryptic digest of casein, low in free amino acids.

garded as reasonable sources of secretions applied from the mouthparts to the leaf particles during their preparation for incorporation into the garden. However, significant proteinase activity was detected in the contents of the rectum and the midgut. We also showed that the ants actually do excrete active proteolytic enzymes onto their cultures.[16]

In captivity, *A. c. tonsipes* will readily utilize cornflakes as a substrate, treating them by all appearance exactly like leaves. Untreated cornflakes have no protease activity. Pieces of cornflakes, recovered from a fungus garden just after the ants have completed the entire preparation procedure, have significant proteinase activity. Thus, by defecating on the substrate the ants add enzymes to the culture medium which contribute to the ultimate exploitation by the fungus of the proteins present in the plant material. Recently we have shown that a purified preparation of the fecal enzymes of *A. c. tonsipes*[18] significantly accelerates the growth of the fungus in a culture medium containing polypeptides as the nitrogen source.[17]

It is tempting to assume that the proteolytic activity of the fecal fluid of *A. c. tonsipes* is due to the presence of digestive enzymes produced in the midgut and concentrated in the rectum. Two other conceivable origins for the fecal enzymes have been ruled out. We have ascertained that the hindgut and rectum of *A. c. tonsipes* are lined with cuticle.[13] The presence of a cuticular lining precludes secretion of enzymes in these posterior regions of the gut. We have also detected no sign of bacterial endosymbionts either in sections prepared from the ants or in smears prepared from the rectal fluid.[13] These observations argue against the production of the fecal proteinases by a permanent gut flora. An unequivocal identification of the origin of the rectal enzymes remains an important objective of our current efforts.

We have further established that the excretion of proteolytic enzymes is a characteristic of all attine species. Protease activity has been detected in the midgut and rectal fluids of a total of 17 species of attine ants from seven genera, from the most primitive, *Cyphomyrmex,* to the most specialized, *Atta* (Table II).[19,20]

Table II
Relative Proteolytic Enzyme Activities of the Rectal and Midgut Fluids of 17 Species of Attine Ants[18,19]

Species	Ratio of rectal to midgut proteinase activity[a]
Atta colombica tonsipes	2.0:1.0 to 10.0:1.0
A. cephalotes	4.5:1.0
A. sexdens	3.5:1.0
Acryomyrmex octospinosus	4.5:1.0
A. lobicornis	13.5:1.0
A. versicolor	7.5:1.0
Sericomyrmex urichi	4.0:1.0
S. amabalis	1.5:1.0 to 15.0:1.0
Trachymyrmex septentrionalis	4.0:1.0 to 30.0:1.0
T. cornetzi	2.0:1.0 to 25.0:1.0
T. bugnioni	3.5:1.0
Myrmicocrypta ednaella	0.5:1.0
Apterostigma dentigerum	0.5:1.0 to 1.0:1.0
A. mayri	1.0:1.0
A. sp.	4.5:1.0
Cyphomyrmex costatus	2.0:1.0 to 2.5:1.0
C. rimosus trinitatus	6.0:1.0

[a] Activity was determined using Azocoll (Calbiochem) as substrate.

While there are differences in absolute magnitudes of both rectal and midgut protease levels between the various species, and even considerable variation between different colonies of the same species, significant rectal activity was detected in every species. In 14 of the 17 species examined, there was significantly higher activity in the rectum than in the midgut. In the two *Apterostigma* species, in which total rectal activity is comparable to or somewhat less than midgut activity, the midguts are distinctly larger than the rectums.[20] Thus, there is actually a higher concentration of proteolytic enzymes in the rectum than in the midgut even in these species. Only in *Myrmicocrypta ednaella* is midgut activity greater than rectal activity on both a "total activity per ant" and a concentration basis.

At the beginning of this study it was not known whether the excretion of digestive enzymes was a rare or a common phenomenon in insects. Thus, we did not know whether we had discovered a *bona fide,* evolved adaptation of the attine ants or had simply stumbled onto a widespread trait of insects which the fungus-growing ants had succeeded in turning to their advantage. We, therefore, undertook a survey of the relative levels of proteinase activity in the rectal and midgut fluids of a number of nonattine species. In a total of 35 nonattine species from 22 genera representing five subfamilies, not a single species was found to concentrate proteinases in its rectal fluid in the manner characteristic of the attines (Table III).[19,20] *Myrmica brevinodis,* in which the rectal activity was 36% of midgut activity, exhibited the highest rectal activity of any nonattine species examined. In 26 of the species it was not possible to detect any rectal activity, while in eight species there was a trace of activity present in the

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Table III
Relative Proteolytic Enzyme Activities of the Rectal and Midgut Fluids of 35 Species of Nonattine Ants[18,19]

Species	Ratio of rectal to midgut proteinase activity[a]
A. Subfamily Ponerinae	
Paraponera clavata	≤1:700
Leptogenys elongata	≤1:120
Odontomachus haematoda	≤1:290
B. Subfamily Dorylinae	
Eciton burchelli	1:34
C. Subfamily Myrmicinae	
Myrmica monticola	≤1:18
M. emeryana	≤1:60
M. brevinodis	1:3
Crematogaster cerasi	≤1:40
C. lineolata	1:7
Aphaenogaster treatae	1:45
A. rudis	1:30
Pogonomyrmex pima	≤1:16
P. rugosus	1:215
P. maricopa	1:17
P. badius	1:8
Novomessor albisetosus	≤1:360
N. cockerelli	≤1:660
Veromessor pergandei	≤1:245
Pheidole xerophila tucsonica	≤1:20
Solenopsis xyloni	≤1:20
S. saevissima richteri	≤1:17
D. Subfamily Dolichoderinae	
Dolichoderus mariae	≤1:43
Dorymyrmex pyramicus flavopectus	≤1:10
Tapinoma sessile	≤1:4
E. Subfamily Formicinae	
Paratrechina longicornis	≤1:9
Camponotus abdominalis floridanus	≤1:39
Acanthomyops claviger	≤1:40
Lasius alienus	≤1:20
L. pallitarsis	≤1:29
Polyergus breviceps	≤1:39
Myrmecocystus depilis	≤1:24
Formica montana	≤1:20
F. ulkei	≤1:28
F. obscuripes	≤1:47
F. pergandei	≤1:6

[a] Activity was determined using Azocoll (Calbiochem) as substrate.

rectal fluid. Thus, the capacity to accumulate and concentrate proteolytic enzymes in the rectum appears to be an evolved adaptation of the attines.

After establishing the presence of proteinase activity in the rectal fluid of *A. c. tonsipes,* it seemed quite reasonable to anticipate that other enzymes might be present as well. Our expectations have been confirmed. The rectal fluid of *A. c. tonsipes* is active in the enzymatic degradation of starch,[21] chitin,[21] xylan,[13] and pectin.[13] In addition there is activity characteristic of a general esterase,[13] pectin methyl esterase,[13] and an α-1,4-glycosidase.[13]

Some of these enzymes are likely to play an important role in fungus culturing. Xylan and pectin are cell wall constituents in plants. Pectin also occurs in intercellular layers and functions as an intercellular cement. The degradation of these two substances would greatly facilitate the penetration of the plant tissue and ultimately the plant cells by fungal hyphae. Thus, their application to substrate and to the gardens would certainly contribute to the exploitation of the substrate.

The chitinolytic properties of the fecal material may also play a significant role in the maintenance of the fungus gardens, particularly in the more primitive forms. Species from the primitive genera *Cyphomyrmex, Mycocepurus,* and *Myrmicocrypta* incorporate into their gardens insect carcasses, the cuticles of which contain chitin. The fungus grows on the surface of the cuticle, and judging from the mechanical fragility of such substrate, it is evident that degradation of the cuticle has occurred. Clearly, the chitinolytic action of the ants' fecal material would facilitate the utilization by the fungus of chitin present in the substrate. The chitinolytic enzymes in the fecal material might also serve to restrict the growth in the garden of some potential fungal competitors. The cell walls of most fungi are chitinous, and many are lysed by chitinolytic systems. Thus the application of the ants' fecal material to the gardens would reduce the number of fungi which could survive there, and would provide a competitive advantage to any fungus which was not susceptible to the lytic properties of chitinase.

This argument presumes that the ants' fungus, which belongs to a group which characteristically has chitin in its cell walls, is resistant to the chitinolytic action of the fecal enzymes. Although at present we have no data to confirm this presumption, it is well established that the susceptibility of chitinous cell walls to the action of lytic enzymes depends upon physical and chemical properties of the wall, and that not all chitinous fungi are lysed by chitinase.[22-26] It is worth noting that the chitinolytic enzymes present in the gut of the fungus-growing ants are not serving a digestive function, since the ants ingest only the liquid contents of the fungal mycelium and do not consume the solid mycelial walls.

In addition to surveying the enzymes present in the fecal material of the fungus-growing ants, we have also characterized some of the low molecular weight nitrogenous substances present (Table IV).[16] All of these substances are common nitrogenous excretory products in insects. Their addition to the culture would have a very beneficial effect upon the competitive status of the fungus, since their presence would permit rapid, initial growth even before the degradation of the macromolecules present in the substrate had proceeded very far. Initial growth rates on newly colonized substrate can be a significant determinant of the outcome of microbial competition.[9-11]

The Role of Growth Inhibitors

Our discussion so far has emphasized the presence in the fecal material of substances which facilitate the growth of the ants' food fungus, thereby enhancing its competitive ability. Clearly the ants might also influence the outcome of competition by adding materials to their fungus cultures which retard the

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Table IV
Nitrogenous Components of the Rectal Fluid of *A. c. tonsipes*[16]

Substance	Range, μg/ant	Average, μg/ant
Allantoic acid	1.4–2.5	1.9
Allantoin	0.7–2.0	1.3
Amino acids		0.46[a]
Ammonia	0.07–0.14	0.10

[a] The 20 protein amino acids were all present, along with small amounts of ornithine. The six amino acids glutamic acid, histidine, arginine, proline, lysine, and leucine make up over 82% (by weight) of the total.

growth of potential competitors. We have already noted that chitinolytic enzymes might serve such a function.

Recently, Schildknecht and his coworkers have detected phenylacetic acid, D-3-hydroxydecanoic acid and indolylacetic acid in the thoracic metapleural glands of the attine ant *Atta sexdens*.[27-29] These workers have suggested that these substances play an important role in fungus culturing: phenylacetic acid through its bactericidal properties, D-3-hydroxydecanoic acid through its capacity to inhibit the germination of the spores of alien fungi, and indolylacetic acid as a consequence of its properties as a plant hormone. This interesting suggestion awaits confirmation through a demonstration that the components of the metapleural glands exhibit their bacteriostatic and fungistatic properties at the still undetermined levels at which they are present in the fungus gardens.

In an earlier study using an entirely different assay system we had failed to detect any significant antibiotic activity in extracts of whole workers of *A. c. tonsipes*.[30] We did observe weak inhibition of the growth of three strains of *Staphylococcus*, two species of *Mycobacterium*, two strains of *Proteus*, and three strains of *Pseudomonas* by extracts of whole fungus gardens. We did not characterize the substances responsible for this mild bacteriostatic action, nor did we attribute any functional significance to it.

An Ecological Perspective Revisited

The obligate symbiosis between the attine ants and the fungi which grow in their nests is a spectacular example of a mutually advantageous association of two very different types of organisms. The stability and efficiency of this symbiosis are predicated upon a complex of co-evolved morphological, behavioral, physiological, and biochemical adaptations of each of the two partners. In our studies we have endeavored to unravel the biochemical interdependencies of the two organisms and to incorporate our findings into an ecological framework.

Our findings are readily placed in an ecological context by noting the effects of the ants' activities on the factors which influence the outcome of interspecific microbial competition. Garrett has identified five factors which contribute to a high degree of competitive ability in a fungus.[11] These are (i) a high inoculum potential, permitting the fungus to overcome substrate resistance readily; (ii) rapid hyphal growth, favoring rapid and extensive colonization and coverage of substratum; (iii) good enzyme secretion, favoring rapid utilization of nutrients present in the substrate; (iv) antibiotic production, reducing competition; and (v) tolerance to antibiotics produced by other organisms. The significance of many aspects of the ants' fungus-culturing activities are readily apparent when evaluated in terms of these five factors. Degradation of pectin and xylan by the enzymes present in the fecal material facilitiates penetration of the tissues and cells of the substrate, and thus effectively enhances inoculum potential. The amino acids and other low molecular weight nitrogenous components of the fecal material permit initial rapid growth of the newly planted fungal hyphae on the still sparsely colonized substrate. Fecal enzymes, such as the proteinases, chitinases, and carbohydrases, supplement those secreted by the fungus and contribute to the degradation of substrate macromolecules, facilitating exploitation of these substances by the fungus. Fecal chitinase, and possibly also the constituents of the metapleural glands, retard or prevent the growth of certain other microorganisms, thus reducing the number of potential competitors with which the ants' fungus must contend. The net effect of all of these factors is to enhance the competitive ability of the fungus to such a degree that in the narrow niche encompassed by the ants' fungus chamber it emerges as the dominant species.

Future Directions

We have recently initiated a study of the chemical and kinetic properties of the rectal proteinases of *A. c. tonsipes* and *A. texana*. We are hopeful that these studies will permit us to deduce the biochemical or physiological basis for the capacity of this tribe of ants to excrete large quantities of enzymes, a trait which so far has been detected in no other group of arthropods. We also hope to initiate a comparative study of proteinases derived from selected nonattine species and attine species representing different stages in the evolution of this fascinating group of insects. Such studies may identify the biochemical changes which accompanied the establishment of a symbiotic association between a fungus and the ancestral attines, and perhaps clarify the biochemical factors which were important in the subsequent evolution of the group. If this endeavor is successful, we believe that our investigations of the biochemical ecology of the attine ants will stand as an excellent example of the applicability of the efficient reductionist methodology of chemistry to the study of organismal biology.

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