SYMPOSIUM ON BIOCHEMICAL BASES OF MORPHOGENESIS IN FUNGI[1]

II. Nemin and the Nematode-Trapping Fungi[2]

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Introduction

There is a biological balance in nature controlled by a complex system of checks and counterchecks. The equilibrium is dynamic and the struggle for survival may be described by the verb *to eat*, conjugated in both the passive and active voice. Where there is prey, there will be a predator. However, these roles are interchangeable, and the successful predator of today is frequently the susceptible prey of tomorrow. Microbiologists join the laity and marvel at the ability of carnivorous plants to capture and consume insects, yet they neglect the carnivores of the microbial world, which in many ways are more amazing than the sundew, Venus' flytrap, or pitcher plant.

Included in the abundant and diverse microbial population of soil are fungi which are taxonomically distinct but ecologically a natural group, united by their adaptation to the predacious habit (7, 8). These remarkable microorganisms are able to capture, kill, and consume microscopic animals (11, 12). Their prey include amoebae, rotifers, nematodes, and springtails. We have been concerned with those organisms that are known collectively as nematode-trapping fungi. Some are phycomycetes and at least one is a basidiomycete (9), but many are Fungi Imperfecti of the order *Moniliales* (14). *Arthrobotrys*, *Dactylaria*, *Dactylella*, and *Trichothecium* are the genera most commonly represented. The cell walls of these fungi are chitinous (28), and the structures they produce for the capture of prey are remarkable. They vary greatly in form, but trap nematodes by either adhesion or occlusion.

Organelles of Capture

Adhesive networks are produced by hyphal branching. In some species, networks are simple and consist mainly of short branches which fuse occasionally. In others, there is considerable anastomosis to form a three-dimensional system. The mycelium is covered with a secretion, and nematodes contacting the network are captured by adhesion and entanglement. Certain species form one-celled sticky processes (Fig. 1), and others produce spherical knobs on short aerial hyphae. These knobs, which have been referred to as *lethal lollipops*, are also coated, and capture nematodes by adhesion. There are a number of species in which the hyphal branches curl, fold, and fuse to produce clusters of rings or loops (Fig. 2). The surfaces of these specialized hyphae are in all cases coated with an adhesive, and the

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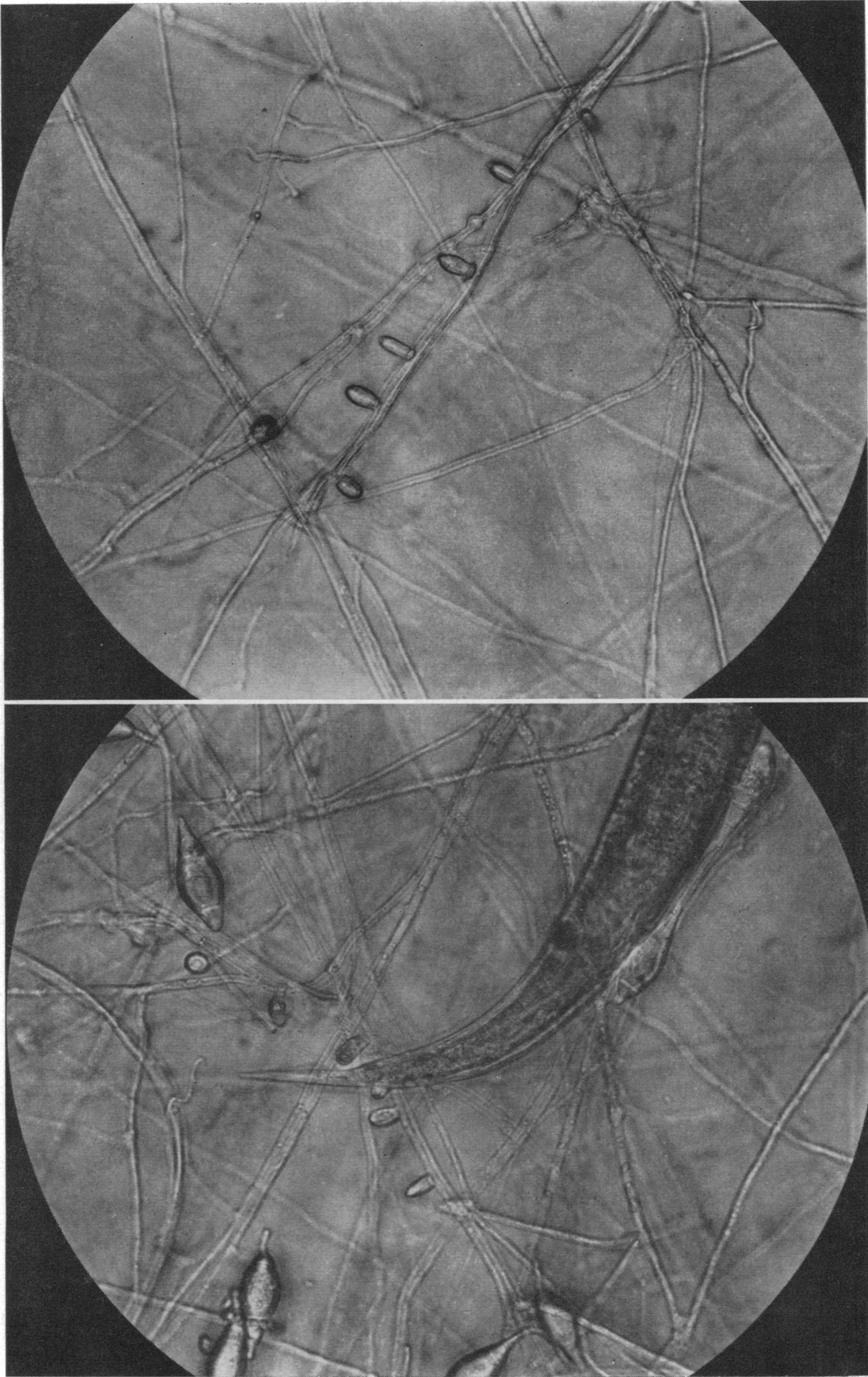

FIG. 1. *Dactylella cionopaga forms one-celled processes (top) which capture nematodes by adhesion (bottom). Young cultures have unicellular traps, but as the fungus ages the organelles of capture elongate and become multicellular. Magnified 520 ×.*

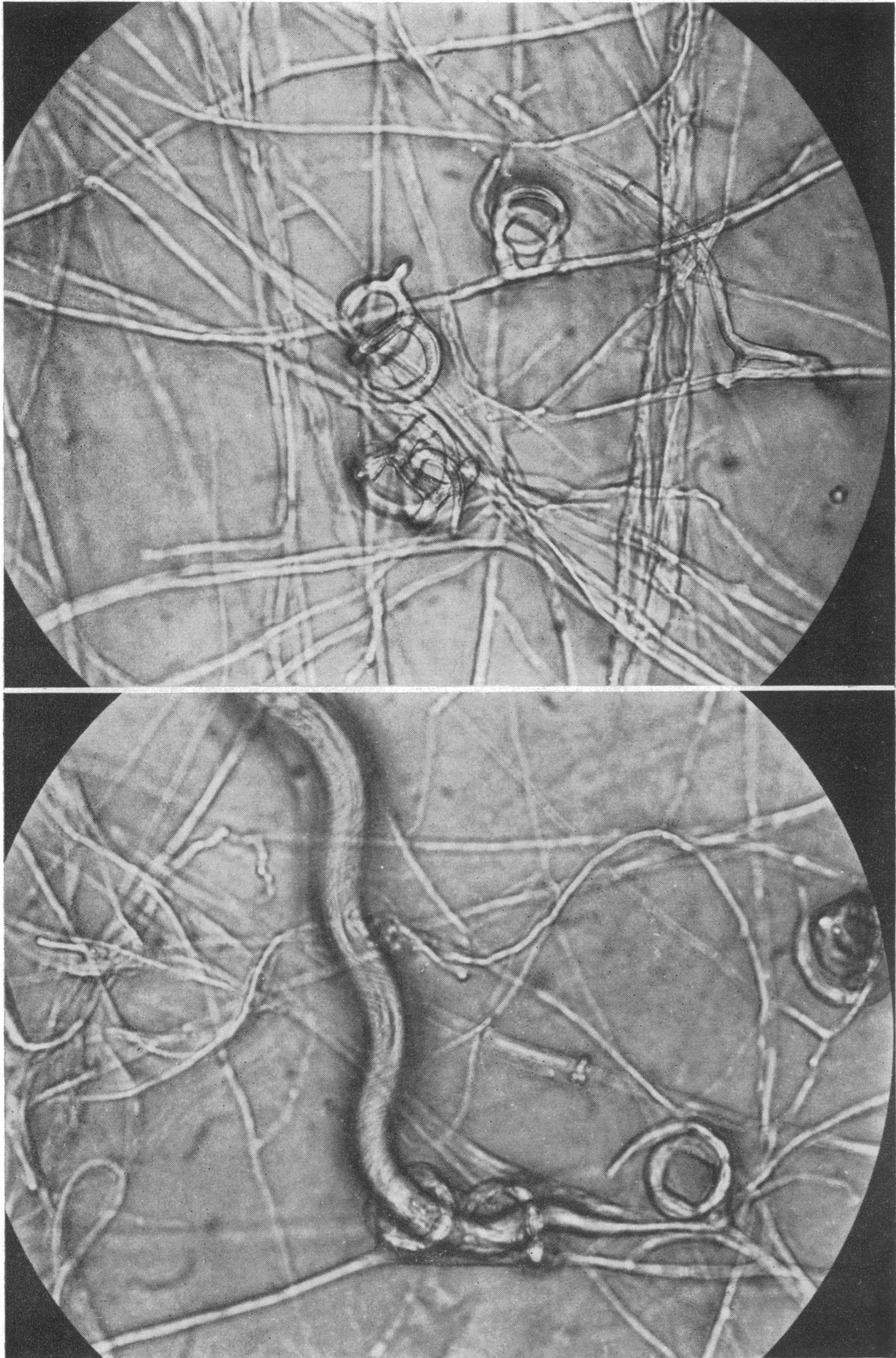

FIG. 2. *Adhesive networks of hyphal loops formed by Arthrobotrys conoides (top). Nematodes are caught and held on contact (bottom). Magnified 520 ×.*

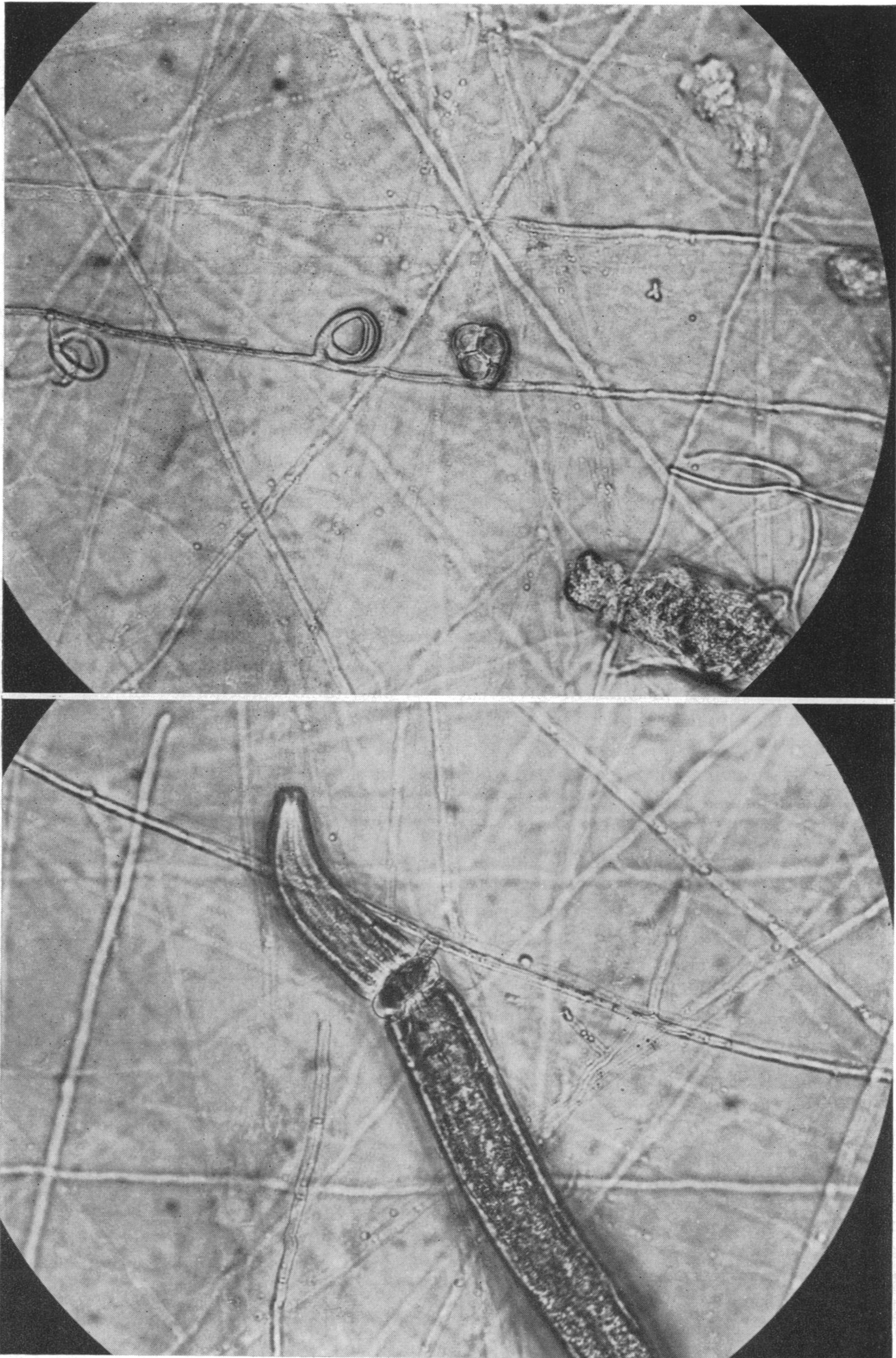

FIG. 3. *Constricting rings, as formed by Arthrobotrys dactyloides, appear to be the most highly developed organelle of capture. Each is composed of three cells and is attached to the mycelium by a stalk (top). When a nematode enters the ring, the cells swell rapidly, obliterating the opening, and gripping the worm so that it cannot escape (bottom). Magnified 520 ×.*

fate of a nematode, which in the course of its wanderings makes contact, is similar to that of a fly that encounters flypaper.

Mechanical traps are composed of three cells which join to form a closed ring at the end of a short mycelial branch. They are not adhesive. Entrapment can be passive, in that a nematode that enters a ring and attempts to force its way through, becomes firmly wedged and unable to escape. However, some species of nematode-trapping fungi produce constricting rings. These appear to be the most highly developed and in some aspects the most remarkable organelle of capture. Constricting rings are active rather than passive. When a nematode enters, the cells swell to approximately three times their normal volume, obliterating the opening and constricting the nematode so that it cannot escape. Ring closure does not require more than 0.1 sec and appears to be a thigmotropic response (3, 21), triggered when the nematode contacts the inner surface of any cell of the ring (Fig. 3).

Whatever the species of nematode-trapping fungus or the trapping mechanism involved, the fate of the nematode is similar. It struggles for a time and then appears dead or moribund. The surface of the nematode is penetrated (24), and the fungus hyphae ramify throughout the carcass and absorb its content. Under favorable conditions, nematodes may be captured in large numbers, especially by those fungi that form adhesive networks of hyphal loops. The actual cause of death of a trapped nematode is uncertain. It may be due to mechanical damage and exhaustion during its struggle for liberty, but this is unlikely. The production by the fungus of a toxin (25) is a possibility that would better account for the observation that nematodes are frequently reported as dead before their surface is penetrated.

ISOLATION AND CULTURAL CHARACTERISTICS OF *ARTHROBOTRYS CONOIDES*

In spite of their remarkable morphological adaptation, nematode-trapping fungi are not obligate predators. They will grow on various complex media (4, 5, 27) and can be isolated with ease (13). For this purpose, one need only place a small quantity of soil, root tissue, or partially decomposed organic matter on the surface of a dilute medium such as cornmeal extract agar and observe microscopically the sequence of organisms that develops (2, 15). There will be some growth of bacteria and many common fungi will be recognized, but their numbers will be limited by the low nutrient level of the medium. Nematodes present in the inoculum will multiply, and eventually fungi which prey on nematodes will be observed. They are isolated most readily by micromanipulation of spores.

By these procedures, we have obtained the nematode-trapping fungus *Arthrobotrys conoides* (7) in pure culture. The organism develops saprophytically on a variety of laboratory media employed commonly for the maintenance of fungus cultures. Of particular interest is the fact that in pure culture *A. conoides* rarely produces traps. However, if nematodes are added, the fungus mycelium differentiates, and within 24 hr a network of adhesive hyphal loops is produced. The phenomenon is unique in that the presence of prey *somehow* induces the predator to undergo morphological change essential to the predatory relationship. Therefore, involvement in the biochemistry of morphogenesis was an inevitable consequence of our interest in nematode-trapping fungi.

All scientific endeavor is directed by a series of simple questions. The investigator asks *what?* and *how?*, and it is within this rather colloquial framework of inquiry that our aims and progress are presented. A reply to *what?* does not necessitate an explanation. It requires observation only and is the essence of descriptive biology. The foregoing discussion indicates in *what* we are interested: the nematode-trapping fungi, a remarkable group of microorganisms. More specifically and relevant to the subject of this symposium, we were intrigued by the fact that nematodes cause a change in fungus form, and it was not possible to refrain from asking *how?* This is the question of molecular biology. To reply is to provide an explanation.

What follows is a somewhat detailed presentation of our inquiry into the biochemical basis of morphogenesis in nematode-trapping fungi. *A. conoides* was employed as the test organism for investigations that have spanned approximately 4 years. The task has been arduous but gratifying. There were many difficulties to overcome and the work is not complete. The challenge of intricately organized systems must be met. Answers are obtainable and we look forward with optimism to the time when, in reply to *how?*,

it will be possible to provide a complete and meaningful explanation of trap formation by *A. conoides* specifically, and nematode-trapping fungi in general.

NEMIN

The fact that some species of fungi do not form traps when grown in pure culture, but do so in the presence of nematodes, suggested that some morphogenic substance is produced by the worms. Evidence in support of this possibility existed at the time that our interest became active. It had been demonstrated by Comandon and de Fonbrune (1), Couch (3), and by Lawton (20) that water in which nematodes had been suspended was able to induce trap formation. Moreover, French investigators reported (6, 23) that various animal sera and tissue extracts were also adequate stimulants. Plant extracts were described as inactive (6), and it appeared that some substance, or group of related substances, capable of causing morphogenesis in the nematode-trapping fungi occurs commonly throughout the animal kingdom but is absent from higher plants.

Unequivocal evidence that a metabolic product of nematodes acts as a chemical signal for trap formation was obtained from studies performed in conjunction with N. R. Stoll of the Rockefeller Institute in New York. Dr. Stoll has developed media and procedures for the axenic cultivation of the nematode *Neoaplectana glaseri* (26). Broths, which were inoculated with approximately 100 worms and supported a population of 110,000 nematodes after 6 weeks of incubation, caused *A. conoides* to form traps, whereas uninoculated broth was inactive. *Nemin* was proposed as the name for the morphogenic substance causing trap formation by predacious fungi (22).

INQUIRY INTO THE NATURE OF NEMIN

To determine the identity of nemin, three different approaches were made simultaneously: (i) the nutrition of *A. conoides* was investigated to determine whether differentiation was subject to nutritional control; (ii) nemin was purified from an active extract of worms; and, (iii) when the *boarding house* practice of testing everything within reach appeared justified, it, too, was included in our efforts. The rate of progress toward our goal has been uneven, and rapid advances along one or another avenue of approach have been determined by technological development and individual enthusiasm.

Nutritional Studies

Despite its ability to capture, kill, and subsist on nematodes, *A. conoides* did not demonstrate a unique nutritional pattern. Yeast extract which was required for growth in a glucose-inorganic salts medium could be replaced by biotin, thiamine, and zinc (2, 17). Studies showed that biotin biosynthesis by the fungus is blocked at the desthiobiotin-to-biotin conversion. *A. conoides* is unable to synthesize either of the two moieties of thiamine but, when supplied with pyrimidine and thiazole, it completes biosynthesis of the vitamin via a coupling reaction. This experimental nutrition enabled us to describe a chemically defined medium that supports rapid and abundant growth of *A. conoides*, but it did not identify nemin as any of the amino acids, vitamins, purines, or pyrimidines tested.

Extraction from Ascarids

Bioassay. Before it was possible to proceed with isolation and identification of nemin, it was necessary to develop an assay procedure and select a suitable starting material. The assay method employed is based on a series of studies in which the influence of various environmental factors on growth and trap formation by *A. conoides* was determined (29). Cornmeal extract agar is used as the assay medium. It is prepared in 0.022 M phosphate buffer at pH 5.8 and supplemented before autoclaving with 10 ppm of novobiocin and 20 ppm of bromcresol purple. The antibiotic prevents bacterial contamination of assay plates. The buffer maintains the reaction of the agar at pH 5.5 to 5.8, the region in which spontaneous trap formation (3, 16, 20) is minimal. Pure cultures of *A. conoides* produce some traps at times, but these are few in number and no difficulty is encountered in differentiating between control plates, on which traps are produced without provocation, and treated plates, on which trap formation is induced by nemin. Bromcresol purple provides visual evidence of any change in reaction caused by the preparation being assayed or by growth of the fungus. The antibiotic, buffer, and pH indicator at the concentrations employed have no influence on growth and trap formation by *A.*

conoides. The assay agar is added in 10-ml quantities to sterile petri plates and permitted to solidify. Each plate is inoculated at the center of the medium surface with an agar plug on which growth of *A. conoides* is 1 to 2 days old. Inoculated plates are incubated for 5 days at 28 C.

Center-punched absorbent paper discs are then placed equally distant from each other around the circumference of the agar surface. Each plate receives five discs, located such that the mycelium exposed through the center holes is 1 to 2 days old. Solutions for assay are sterilized by filtration through membranes or sintered glass and diluted in sterile water blanks. Quantities (0.1 ml) of the test solutions are added directly to the fungus mycelium visible through the holes in the center of the paper discs. All solutions are tested in triplicate. The plates are returned to the incubator for approximately 36 hr, and the treated areas are then examined microscopically for traps. Observation is neither tedious nor prolonged, since the amount of growth made by the assay organism is seldom abundant and traps are distinguished easily.

The total solids content of preparations assayed is determined by drying an appropriate quantity at 105 C for approximately 18 hr. Nemin activity is measured in terms of dilution units/mg. The nemin unit is the reciprocal of the highest dilution of the preparation that causes the fungus to form traps. If a preparation has a total solids content of 10 mg and is active at the dilution of 1:1,000, it contains 1,000 dilution units/10 mg, or 100 units/mg.

This assay procedure was used to determine the nemin activity of various animal sera, aqueous extracts of nematodes, and culture broths of *N. glaseri*. It is apparent from the results summarized in Table 1 that water extracts of the free-living nematode *Panagrellus redivivus* had the greatest nemin activity. Sera derived from the cow, horse, pig, and sheep had similar activity, and dried bovine serum did not differ greatly from normal serum. The activity of water extracts of ascarids obtained from the swine intestine at the time of slaughter was relatively high. These nematode worms are numerous, individually large, and can be collected in quantity. Therefore, they were selected as starting material for chemical fractionation and the isolation of nemin.

TABLE 1. *Nemin activity of various blood sera and nematode extracts*

Preparation assayed	Highest active dilution	Dry weight	Nemin
		mg/ml	*units/mg*
Sterile sera			
Bovine	1:1,000	86	11.6
Horse	1:2,000	93	21.5
Porcine	1:2,000	87	23.0
Sheep	1:1,000	82	12.2
Bovine serum			
Liquid	1:500	58	8.6
Dried*	1:1,000	85	11.8
Ultrafiltrate	1:100	15	6.7
Dialyzed	1:100	72	1.4
Water extracts of nematodes			
Panagrellus redivivus	1:1,000	5	200.0
Swine ascarids	1:1,000	27	37.0
Culture broth of *Neoaplectana glaseri*†	1:100–1:1,000	37	2.7–27.0

* Reconstituted in sterile distilled water saturated with CO_2.

† Obtained from N. R. Stoll of the Rockefeller Institute, New York, N.Y.

Purification procedures. Since we were concerned with a complete unknown, numerous preliminary tests of a qualitative nature were required. However, it soon became apparent that we were dealing with a complex substance of high molecular weight rather than with a simple or small molecule. The activity of aqueous extracts of ascarids failed to partition to any significant extent into organic solvents. Moreover, it was not dialyzable and was precipitated from aqueous solution by ethanol, acetone, and ammonium sulfate at concentrations in excess of 50%.

A considerably purified nemin preparation was first obtained by the following procedure (19). A quantity of thoroughly washed ascarids was homogenized in cold 50% aqueous ethanol, by use of a Waring Blendor. The homogenate was centrifuged and the sediment re-extracted with additional aqueous ethanol. The combined extracts were concentrated approximately 27-fold in vacuo. Insoluble material was removed

by centrifugation and discarded, since the bioassay showed activity to be localized in the soluble portion of the concentrate. This was diluted to 30% by volume with cold acetone, and the precipitate that formed was harvested, washed, and dried. Bioassays indicated that nemin activity was concentrated in the acetone-dried powder, and gel filtration of a water solution of this powder was performed in a column containing Sephadex G-50. The effluent was collected in 60 separate 2.7-ml fractions. The absorbancy at 280 mμ, intensity of the ninhydrin reaction, and biological activity of each fraction were determined. The results are summarized in Fig. 4.

Nemin activity was concentrated primarily in tubes 19 to 23, and associated with the maximum at 280 mμ, which is characteristic of proteins. When the contents of tubes 19 to 23 were pooled and subjected to electrophoresis on cellulose acetate film using Veronal buffer at pH 8.6, five components stained with Ponceau S, but assays indicated that biological activity was associated with the fastest moving acidic fraction only. Isolation of this material in quantity was accomplished by preparative zone electrophoresis using Sephadex G-75 as the supporting medium. The buffer was 0.1 M triethylamine carbonate at pH 8.3. Bands were located by placing cellulose acetate film in contact with the gel surface. The film was subsequently

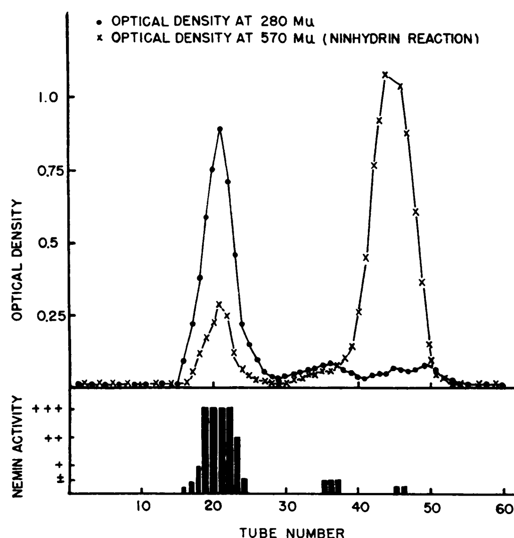

FIG. 4. *Gel filtration on Sephadex G-50 of an acetone-dried powder from swine ascarids.*

dried and stained with Ponceau S. That portion of the gel which contained the active fraction was collected with a spatula, eluted with water, and the eluate was lyophilized. A yield of 122 mg of biologically active material from 100 g of ascarids was obtained. Approximately 40 μg of this preparation were required for a definite response in the bioassay.

The preparation showed maximal absorption in the ultraviolet region at 278 mμ ($E_{1\%}$ = 10.2 at pH 7.3), and the ratio $A_{278\,m\mu}/A_{260\,m\mu}$ was 1.45. It appeared homogeneous when examined by moving boundary (NaCl-phosphate buffer at pH 7.3, I = 0.1), cellulose acetate film (pH 4.0, 5.4, 7.3, 8.6), disc, and polyacrilamide gel electrophoresis. The sedimentation coefficient ($S_{20,w}$) for a 1.0% solution in 0.1 M phosphate buffer (pH 7.3) was 1.92. Although the preparation sedimented as a single boundary, the ultracentrifugation pattern was polydisperse.

A second product, with which further studies have been made relating to biological activity, was obtained by homogenizing and thoroughly extracting ascarids with water. The water extract was dialyzed and then lyophilized to yield a beige-colored powder capable of *inducing allergy in the chemist* as well as morphogenesis in nematode-trapping fungi. A quantity of this material was dissolved in 0.005 M phosphate buffer (pH 7.2) and fractionated by anion exchange chromatography on diethylaminoethyl (DEAE) cellulose. Development was by stepwise gradient elution, and the following sequence was employed: I, 0.005; II, 0.01; III, 0.02; IV, 0.05 M phosphate buffer; V, 0.1 M NaCl in 0.05 M phosphate buffer; VI, 0.5 M NaCl in 0.1 M phosphate buffer, all at pH 7.2; and VII, 0.5 M NaCl in 0.1 M NaH_2PO_4. The eluate was obtained in 25-ml quantities with an automatic fraction collector, and absorbancy measurements were made at 280 mμ with a Beckman DU spectrophotometer.

These procedures revealed nine distinct peaks. The fractions that comprised each component were pooled, dialyzed, and lyophilized. Each product was tested for total nitrogen (micro-Kjeldahl), protein (Folin-Ciocalteu reagent), carbohydrate (anthrone reagent), and for nemin activity. It is apparent from the results summarized in Fig. 5 that ability to induce trap formation by *A. conoides* was not limited to any one fraction. Eight of the nine preparations were

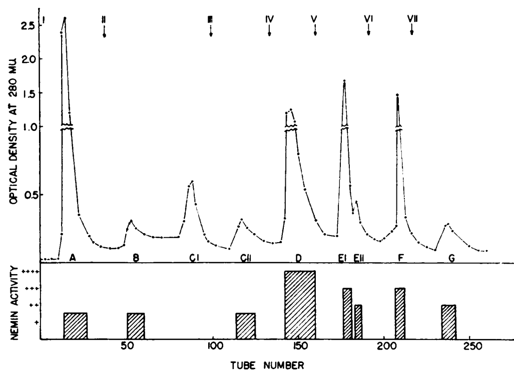

FIG. 5. *Anion-exchange chromatography on diethylaminoethyl cellulose of the nondialyzable portion of a water extract of swine ascarids. Development was by stepwise gradient elution as described in the text.*

morphogenic and, to prevent this divergence of activity from dissipating our effort, it was necessary to select a single preparation for further study. The most reasonable choice was fraction D. Analyses indicated that this material was free from carbohydrate and had a nitrogen content approximating that of purified protein. Moreover, it demonstrated the greatest biological activity per unit dry weight and appeared similar, if not identical, to the preparation obtained by zone electrophoresis on Sephadex G-75 and described previously.

The ability of fraction D to induce trap formation by *A. conoides* was not decreased significantly by treatment with 9 M urea or 8 M guanidine HCl. A temperature of 100 C for 30 min did not inactivate preparations dissolved in 0.1 M phosphate buffer at pH 7, but acid (0.1 N HCl) and alkaline (0.1 N NaOH) hydrolysis at 100 C for 30 min resulted in a marked and total loss of titer, respectively. Performic acid oxidation caused complete inactivation but reduction by thioglycolic acid did not modify morphogenic potency.

We have employed fraction D as starting material in a number of attempts to further purify the active principle. Included in the techniques used were anion-exchange chromatography on DEAE cellulose with gradients and eluents that differed in composition, concentration, and pH; preparative electrophoresis using agar, starch, and polyacrylamide as supporting media; and electrophoresis on vertical columns packed with ethanol-treated cellulose. Some progress has been made, particularly by electrophoresis in agar, but at this time insufficient material is available for bioassay and it is apparent that our present preparations are not as yet homogeneous. Unfortunately, these studies are incomplete and it is possible to state only that fraction D contains at least three proteins which remain to be isolated and characterized. Since a lack of definitive results is frequently license for speculation, we feel at liberty to discuss briefly our present ideas concerning the nature of nemin.

Discussion and Concluding Remarks

Throughout these investigations, the association of biological activity with the water-soluble protein fraction of ascarids was conspicuous. Jackson (18) has observed precipitation at the excretory pore of nematodes immersed in specific antisera, and perhaps proteins excreted by nematodes are morphogenic as well as antigenic. However, we wish to suggest that nemin is a peptide of relatively low molecular weight or possibly a single amino acid, of rather common occurrence in the animal kingdom, at least. It is difficult to visualize how an exogenous protein can induce morphogenesis. Specific action at the fungus surface is plausible, but proteins supplied to microorganisms are usually hydrolyzed enzymatically. Degradation due to fungus proteases may result in liberation of the same biologically active entity from different substrates. Therefore, any protein that embodies our hypothetical amino acid or sequence of amino acids would be potentially capable of inducing morphogenesis in nematode-trapping fungi. This possibility is offered as a working hypothesis only, but it is consistent with our observation (Fig. 5) that at least eight different ascarid proteins are biologically active, as well as with the literature (6, 20, 23, 29) that describes a great variety of animal sera, tissue extracts, and fecal extracts as able to cause predacious fungi to form traps. Moreover, there is no lack of agreement between published data (6, 22) which indicate that the active principle excreted by, rather than extracted from, worms has some solubility in organic solvents, and the suggestion that nemin is a peptide of low molecular weight or possibly an amino acid.

Recent studies in our laboratory have provided evidence in support of this thesis. It was

observed that the biological activity of ascarid proteins treated with pepsin remained unchanged. However, if enzymatic hydrolysis was followed by dialysis, the digest was then incapable of inducing morphogenesis. Some, but not all, of the original activity was recovered from the dialyzed portion, indicating that the active principle (nemin) is a substance liberated by enzymatic hydrolysis of protein and capable of passage through cellophane film. Additional support for our proposal is derived from assay results which demonstrate that the nemin activity of an ultrafiltrate of bovine serum is not significantly different from that of whole serum (Table 1). This is conceivable only if the size of the biologically active molecule does not restrict movement through cellophane. If our supposition is to be proven incorrect, then time must reveal the true nature of nemin. In so doing it would provide us with our objective, and echo the statement of Duclaux (10) that, "One sometimes reaches the truth by error, and sometimes the error by truth."

The possibility that nemin is a low molecular weight peptide or an amino acid occurred to us soon after it was apparent that biological activity was associated with the water-soluble protein fraction of ascarids. To expedite our work and identify nemin in the shortest possible time, it appeared reasonable to obtain and test commercially available proteins, peptides, and amino acids. This has been done and, in all, 13 proteins, 49 peptides, and 27 amino acids were examined empirically. Included in these tests were various blood fractions, animal hormones, and peptide antibiotics, but all were devoid of activity.

For the moment there is little more that can be presented with any degree of confidence. We are persisting in our efforts but it is not anticipated that the work will be easy. Moreover, we are fully aware that if and when nemin is identified it will not be the end, but rather the time for a fresh start to our inquiry into the biochemical basis of morphogenesis in nematode-trapping fungi. We conclude with a rhyme composed by Robert Frost, for it describes succinctly our present circumstance.

We dance round in a ring and suppose,
But the secret sits in the middle and knows.

ACKNOWLEDGMENTS

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