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# SYMPOSIUM: CURRENT APPROACHES TO THE PROBLEMS OF SYSTEMATIC NEMATOLOGY

## Biochemical Approach to Systematic Studies with Axenic Nematodes[1]

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The application of biochemical systematics to nematology can provide a much needed tool to resolve problems of taxonomy and phylogeny. These problems are most apparent when the morphological characteristics merge between species, when characteristics occur in the rare form, *e.g.* male in a hermaphroditic species, or when strains show no morphological differences.

Biochemical systematics depends on elucidation of the subtle molecular differences which underlie taxonomic variation. The challenge for the nematologist is to recognize the biochemical processes that can yield information for this differentiation and then to prepare sufficient material for investigation. The challenge for the biochemist is to recognize the importance of a unique phylum, widespread yet inconspicuous, and then to modify the standard techniques to measure the small amounts of material that are available. The few attempts that have been made so far to include nematodes in an overall comparison of animal species show poor results with nematode material (32). Even with the best synergetic association of nematologist and biochemist, the problem of introducing nematode material into the broad concepts of biochemical phylogeny (16) will be dependent upon availability of material for analysis.

Since few species are sufficiently large, the problem of supply depends on either isolation of nematodes from their natural environment, a laborious task yielding non-uniform material, or upon development of pure cultures. For parasitic forms there are obvious problems, and even for free living forms pure cultures have proved to be difficult to establish and maintain. Such nematodes grow easily with a microbial flora whose presence and effect on the nematode then tends to be overlooked. Monoxenic cultures provide a greater degree of control with possibilities of providing reproducible material. They have been explored successfully in culture of plant parasites (34) using callus tissue or mycelia as the substrate. Bacteria-free animals are still a rarity not yet explored for nematode parasitism. From monoxenic cultures the supply of materials involves procedures of separation and collection. With pure "axenic" cultures, the possibilities are immediately greatly expanded. The conventional criteria of standardized material from

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TABLE 1. Growth response of microbivorous nematodes to carbohydrates.[a]

| Nematode | Number of progeny/individual at 14 days | | | | |
|---|---|---|---|---|---|
| | No CHO | Glucose | Sucrose | Ribose | Trehalose |
| Growth factor 25 μg/ml | | | | | |
| *C. briggsae* | 2 | 31 | 6 | 6 | 37 |
| *C. elegans* | 33 | 70 | 28 | 19 | 84 |
| *R. anomala* | 12 | 40 | 15 | 10 | 34 |
| Growth factor 100 μg/ml | | | | | |
| *C. briggsae* | 12 | 101 | 8 | 0 | 80 |
| *P. redivivus* | 32 | 44 | 36 | 52 | 40 |

[a] *C. briggsae* basal medium was prepared without glucose, inositol and choline. Carbohydrates were added at 6.5 mg/ml. Media were supplemented with liver growth factor precipitated at 53 C for 4 minutes (11). Three larvae of each species (seven for the dioecious nematode *P. redivivus*) were inoculated into duplicate aliquots of 0.25 ml of the test medium in 10 × 75 mm stoppered test tubes and incubated at 20 C.

TABLE 2. Growth response[a] of five nematode species under different gas mixtures.[b]

| Nematode | Progeny or stage at 7 days | | | | |
|---|---|---|---|---|---|
| | Control | $O_2$ 20% $CO_2$ 0% | $O_2$ 0.3% $CO_2$ 0% | $O_2$ 0.5% $CO_2$ 5% | $O_2$ 5% $CO_2$ 5% |
| *C. briggsae* | 500 | 500 | 500 | 500 | 500 |
| *C. elegans* | 37 | 60 | 7 | 1 | 11 |
| *R. anomala* | 17 | 10 | $L_4$ | $L_4$ | Adult |
| *P. redivivus* | 100 | 95 | 35 | 43 | 380 |
| *N. carpocapsae* | Adult | Adult | $L_4$ | $L_3$ | $L_3$ |

[a] *C. briggsae* basal medium was supplemented with 400 μg/ml (600 μg/ml yeast extract for *N. carpocapsae*) of partially purified baker's yeast extract (3). Five larvae of each species were inoculated into duplicate aliquots of 0.5 ml of medium contained in capped Leighton tubes and incubated under various gas mixtures at 23 C. Five larvae of each species inoculated into 0.25 ml aliquots of medium contained in 10 × 75 mm stoppered test tubes and incubated standing at 23 C were used as controls. At seven days the total progeny per tube were counted or the stage of maturation was recorded.

[b] The gas system of Berntzen (1) was used at a gas flow of 150 ml/min through the holding chamber.

pure cultures can be applied, and the cultures provide easily recognized differences in physiology which can then be analyzed in terms of the differing underlying biochemical process. As with the Protista, procedures can be scaled up to provide the required amounts of material.

Axenic cultures (5) have been established in continuous form (10) for very few species; *viz.*, the free-living species *Caenorhabditis briggsae, Caenorhabditis elegans, Rhabditis anomala, Turbatrix aceti, Panagrellus redivivus, Acrobeloides bütschlii* (21), *Pelodera strongyloides* (36), and the insect parasites, *Neoaplectana glaseri* and *Neoaplectana carpocapsae* (8) (12). The plant parasite *Aphelenchoides* sp. has been supported through limited reproduction (19) and the animal parasites have been maintained over certain developmental stages (31). In those cases in which chemically defined basal media are used, the cultures are relatively standardized, and so are appropriate for further biochemical study (1, 11). With the recent work on *Deontostoma californicum* (33) a start has been made on axenic culture of the phylogenetically important marine nematodes.

Information already reported from established axenic cultures can now be reviewed for its implications for biochemical taxonomy. For example, the two strains of *C. elegans* were initially shown to have different nutritional requirements at different temperatures (9). The heat sensitivity of the Bergerac strain was shown subsequently to be polygenic and sex linked (6). In cultures of *P. redivivus* the presence of a new strain was detected by a lower nutritional requirement and greater ammonia production (17).

A broader comparison of response to cultural conditions was made by comparing the response of four species to changed carbohydrates. The results are shown in Table 1. Glucose and trehalose were most readily utilized. Among these closely related species only *P. redivivus* was markedly less affected by lack of carbohydrate. The high reproduction of all the species upon addition of trehalose is of interest since this is the storage carbohydrate of animal parasitic forms. Its

application to cultures of parasitic forms has not yet been made.

Response to a changed gaseous environment might also serve to differentiate species. The response of five species is shown in Table 2. *P. redivivus* alone showed a marked response to increased carbon dioxide. Reproduction of all species except *C. briggsae* was inhibited by low oxygen. These responses to carbohydrates and gas phase exemplify the studies that are possible to show biochemical differences that can be explored to differentiate species.

Biosynthetic pathways are of great phylogenetic significance. For example the ability to synthesize certain polyunsaturated fatty acids has been lost by higher animals. It was found to be present in axenic cultures of *T. aceti* (26). Biosynthesis of sterols is lacking in most insects. Studies with nematodes so far suggest a lack of cholesterol biosynthesis but are not yet definitive because the cultures either contained cholesterol in the medium or else bacteria were present (4, 25).

The new and sensitive techniques of lipid analysis have great promise for species identification. However, since the lipid content and composition is greatly affected by environmental conditions, cultures must be standardized. Under standardized conditions we were able to show genetically-based lipid differences in a strain of *P. redivivus* selected for tolerance of an increased temperature, 32 C. The lipid composition was very different from that of the xenically cultured species (18).

Secretory and excretory products may also be important in species differentiation. The considerable differences in the excretion of nitrogenous materials has been reviewed (24). In axenic cultures of *Neoaplectana* a mucous material accumulates. The species differences in electrophoretic characteristics of this material are now being investigated as a possible aid to taxonomy of the genus.

The specific procedures for biochemical systematics that have already been applied to nematology are: comparisons by isoenzymes, comparison of proteins by immunology and electrophoresis, and DNA hybridization.

Isoenzyme studies have been made on preparations of helminths isolated from their natural environment. These studies permit comparison of enzymes of host and parasite and of related parasitic species. The heterogeneity of isoenzymes may be of particular interest to nematologists since it seems to be associated with regulatory control which must be of great importance in the adaptive mechanisms of parasitic forms. With nematodes cultured axenically, the isoenzyme patterns for esterases and phosphatases were used to distinguish two species of *Neoaplectana* (28). More information can be gained after an enzyme has been isolated. Its homology can then be examined by kinetics, thermal stability and analysis of peptide fragments and amino acid sequence. Such technics require a considerable amount of starting material.

The precipitin reaction is a powerful tool for species comparison. It has been used in identification of strains of plant parasites (35). In axenically cultured nematodes it has been applied to distinguish two populations of *N. carpocapsae,* DD 136 and Czechoslovakian (14, 23). The precipitation of proteins and cells from immune blood and sera (13, 30) on to the nematode surface possibly could be extended to differentiation and identification of a newly isolated nematode not yet in culture.

Precipitin reactions may be observed after direct diffusion or after a preliminary separation of proteins by electrophoresis. With establishment of axenic cultures, metabolic products are also available for antigenic comparison. Since with animal parasites these metabolic antigens are important in

protective immunity (29) it can be anticipated that they will also be important in species recognition. They may permit greater discrimination than is possible with the complex precipitation patterns of somatic antigens developed by immunization with whole nematode extracts. The cross reaction of somatic antigens is considerable, extending even to interaction between parasitic *Haemonchus contortus* and antiserum to free-living *Caenorhabditis* (15).

The ultimate basis of taxonomic relationship is the nature of DNA. In theory it is possible to develop mathematical indices using DNA base sequences to summarize the entire evolutionary history of a population. DNA from nematodes will be useful in examining the proposition that DNA content is proportional to histological complexity. Also of interest is the fact that nematode DNA is from a eutelic organism.

The homology of DNA is studied by hybridization, GC-content, and base sequences. A start on DNA hybridization studies of nematodes has been made (27) using *in vitro* labeling. One hypothesis under consideration is whether there has been a loss of DNA from parasitic nematodes as would be consistent with the proposition that a parasite has diminished biochemical capability. We have prepared DNA by mild extraction (20) from *N. glaseri* and *T. aceti*. Such preparations can be used for hybridization studies following *in vivo* labeling (22). Among other taxa the analysis of species divergence by the extent of DNA hybridization is the subject of considerable current literature (2). A similar analysis of *Strongyloides* would be of great interest as an aid both to identification of species and to elucidating the phylogenetic implications of the free-living rhabditoid cycle.

With the application of these biochemical techniques it is well to keep in mind certain problems peculiar to nematodes. The population from axenic culture is composed of a series of developmental stages. Comparison then has to take into account the possibility that the characteristics might be age dependent (7). The uniformity of the population can be improved by migration through selected screens, or by synchronizing development by incubation at alternate high and low temperatures. Dauer larvae, or a special infective stage, have the advantage of providing large populations of a single stage of development. Our observation of the appearance of a *de novo* strain emphasizes the caution that genetic variation can lead to differences developing between populations isolated in culture.

In summary, physiological and biochemical methods not only tend to corroborate the findings of taxonomists but they also make it possible to identify less obvious but no less important differences and similarities. They permit phylogenetic explanation on a functional and frequently on a molecular level. With development of standardized cultures to provide quantities of nematode material, biochemists will be in a position to include information from this unique phylum into fundamental phylogenetic concepts.

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