Pelodera strongyloides Schneider 1866: A Potential Research Tool

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Abstract: Axenic *Pelodera strongyloides* matured in a completely defined culture medium were homogenized and the homogenate separated into "nuclear," "mitochondrial," and supernatant fractions. Medium, homogenate and fractionates were analyzed using standard biochemical techniques. Ammonia was the only purine catabolite detected in the medium, but the electrolytes and enzymes of the major metabolic pathways in warm-blooded animals were found in the homogenate and fractionates. Nine months of artificial selection of an X-ray-induced mutant *P. strongyloides* yielded a strain with a 16-fold greater incidence of *endotokia matricida* (death of the mother due to internal birth of the young). Crossing with recently isolated wild-type individuals (low *endotokia*) reduced the frequency of *endotokia* in the succeeding generations to the level observed in the wild populations. The authors conclude *P. strongyloides* will be a suitable nematode for metabolic and genetic investigations when improved fully-defined media are developed. *Key Words: Pelodera strongyloides,* Metabolic pathways, *Endotokia matricida,* Genetic marker, Fully-defined artificial medium.

Although fundamental biochemical studies of viruses and bacteria have yielded spectacular results, the authors believe the use of relatively simple multicellular organisms would permit approaches to biological problems that cannot be solved using free-living single cells. To be appropriate, the test organisms must (i) be small to permit the use of microbiological techniques, (ii) have a short life cycle so data from thousands of individuals can be statistically analyzed, (iii) be adapted to completely defined media to assure reliable nutritional and biochemical determinations, and (iv) be dioecious to permit genetic studies.

The authors, somewhat influenced by Dougherty's culture of *Caenorhabditis briggsae* on partially defined media (5), selected the closely related *Pelodera strongyloides* Schneider for evaluation as a test organism. This paper is a report of those experiments.

METHODS AND MATERIALS

MONOXENIC CULTURE: *Pelodera strongyloides* were cultured on *Escherichia coli* growing on DIFCO Bacto-Blood Agar Base, Heart Infusion Agar, supplemented with human blood. To facilitate nematode counting, the bottom of each blood agar plate was ruled into 1-cm squares and the worms immobilized by chilling to 12 C. Counting and observation for genetic markers was performed with a dissecting microscope initially at 150 × and then more detailed observations made *in situ* at 600 ×.

When the populations of 14 petri dish cultures were estimated to total 50,000, the nematodes were Baermann-extracted using a single layer of tightly woven, cloth filter paper supported beneath gauze in the funnel. Only first and second stage larvae were able to penetrate the very minute pores. These were transferred to sterile water at 30 C, washed twice and axenized for 3–4 hr in a 1:1 v/v mixture of solutions containing 5,000 units of penicillin G and 50 mg of streptomycin per ml. Sterility was tested by plating out both washed worms and 0.5 ml of worm-antibiotic suspension on Difco Dehydrated Nutrient Agar plates.

AXENIC CULTURE: Three-tenths ml of the nematode-antibiotic suspension containing an average of 600 larvae was added to each of 50 sterile test tubes containing 1.3 ml of the modified medium of Sayre, Hansen

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TABLE 1. Modified axenic medium of Sayre, Hansen and Yarwood used for culture of *P. strongyloides*.

AMINO ACIDS	
"ESSENTIAL"	mg/L
L-ARGININE	1,300
L-HISTIDINE	377
L-ISOLEUCINE	1,148
L-LEUCINE	1,198
L-LYSINE	1,372
L-METHIONINE	518
L-PHENYLALANINE	830
L-THREONINE	956
L-TRYPTOPHANE	245
L-VALINE	1,360
TOTAL	9,304
"NON-ESSENTIAL"	mg/L
L-ALANINE	1,860
L-ASPARTIC	2,160
L-CYSTEINE	26
L-GLUTAMIC (Na)	733
L-GLUTAMINE	1,950
L-GLYCINE	962
L-PROLINE	870
L-SERINE	1,051
L-TYROSINE	363
α-ε DIAMINOPIMELIC ACID	137
TOTAL	10,112

II. NUCLEIC ACIDS	mg/L
ADENYLIC ACID	347
CYTIDYLIC ACID	323
GUANYLIC ACID	363
URIDYLIC ACID	324
THYMINE	126
TOTAL	1,483

III. VITAMINS AND GROWTH FACTORS	mg/L
N-ACETYLGLUCOSAMINE	15.00
P-AMINOBENZOIC ACID	7.50
BIOTIN	3.75
CYANOCOBALAMINE	3.75
FOLINATE (Ca)	3.75
NIACIN	7.50
NIACINAMIDE	7.50
PANTETHEINE	3.75
PANTOTHENATE (Ca)	7.50
PTEROYLGLUTAMIC ACID	7.50
PYRIDOXAL, HCl	3.75
PYRIDOXAMINE·2HCl	3.75
PYRIDOXINE·HCl	7.50
RIBOFLAVIN·$5PO_4$	7.50
THIAMINE HCl	7.50
DL-THIOCTIC ACID	3.75
GLUTATHIONE, REDUCED	408.00
TOTAL	509.25

IV. ENERGY COMPOUNDS	mg/L
D-GLUCOSE	1,315
CHOLINE CHLORIDE	885
I-INOSITOL·$2H_2O$	648
TOTAL	2,848

V. MINERALS	mg/L
NaCl	1,225.0
$(NH_4)_3PO_4$	400.0
KOH	400.0
$MgHC_6H_5O_7 \cdot 5H_2O$	500.0
$CaCl_2 \cdot 2H_2O$	50.0
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	58.8
$MnCl_2 \cdot 4H_2O$	22.2
$ZnCl_2$	10.2
$CuCl_2 \cdot 2H_2O$	6.5
KI	5.0
TOTAL	2,677.7

and Yarwood (14). Four-tenths ml of sterile, demineralized water was added to bring the total volume per tube to 2.0 ml. These test tube cultures were incubated for six days at room temperature inclined at an angle of 45 degrees. This unsupplemented medium remains clear if not contaminated.

The modification of the Sayre, Hansen and Yarwood medium is shown in Table 1. The L-leucine has been reduced to be more in balance with the L-arginine, L-isoleucine, L-lysine and L-valine. The mineral moiety has also been modified by adding NaCl, drastically reducing the K^+ and Ca^{++} ions, and including 5 mg KI/liter.

PREPARATION OF NEMATODE HOMOGENATE AND FRACTIONATES: After six days of incubation the nematodes were filtered from each tube of liquid medium. The collected filtrate was mixed and frozen for later determination of purine degradation products. A control sample of complete medium not exposed to nematodes was also frozen.

The total mass of nematodes, after being washed twice and allowed to drain, weighed 1.5 gm wet. The nematodes were macerated in a glass homogenizer immersed in ice water, and the homogenate adjusted to 10% w/v with 0.25 M sucrose. Five-tenths ml of the 10% homogenate was diluted with 7.0 ml of

0.03 M phosphate buffer at *p*H 7.4 and frozen for later analysis.

Five ml of the 10% homogenate was centrifuged 10 min at 700 *g* and the pellet washed five times and resuspended in 5 ml of 0.25 M sucrose. Five-tenths ml of this suspension was diluted to 5.0 ml with the 0.03 M phosphate buffer to constitute the "nuclear fraction" which was immediately frozen for later analysis.

The pooled supernatants from the undiluted nuclear fraction were centrifuged at 5000 *g* for 10 minutes, the pellet rehomogenized and centrifuged at 9400 *g* for 16 minutes. The final pellet was again homogenized and diluted with 0.25 M sucrose to 10 ml. Five-tenths ml of this suspension made up to 7.0 ml with buffer was designated the "mitochondrial fraction" and frozen.

Five-tenths ml of the pooled washings from the undiluted mitochondrial fraction were diluted to 5.0 ml with phosphate buffer to make up the "supernatant fraction" which was also frozen.

Although the terms nuclear, mitochondrial and supernatant were given to the various nematode fractionates, this method of fractionating was not capable of producing pellets containing only cell nuclei or cell mitochondria. In fact, microscopic examination showed some unrecognizable debris in both the nuclear and mitochondrial fractionates.

BIOCHEMICAL ANALYSIS: The tests used in this series of experiments were selected to show metabolites in the axenic growth media, electrolyte balance in the homogenate and the presence of certain metabolic pathways in the fractionates. The methods are well known and standardized, so they are listed with the results in Table 2. However, the method used for the detection of xanthine oxidase and uricase was developed from a technique originally used by Kalckar (10) for the detection of xanthine oxidase in human blood serum. The principles and our modifications of the techniques are detailed below:

Simultaneous Determination of Xanthine Oxidase and Uricase.—The principles of this determination are (i) Xanthine oxidase oxidizes xanthine to uric acid (Xanthine + $H_2O_2 + O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}}$ Uric acid + H_2O_2) and (ii) Uric acid is irreversibly oxidized to allantoin by uricase (Uric acid + $2H_2O + O_2 \xrightarrow{\text{uricase}}$ Allantoin + H_2O_2 + CO_2). The reagents used in this determination were (i) Phosphate buffer, *p*H 7.4, commercial product, The Coleman Co., Maywood, Illinois; (ii) Xanthine, crystalline powder. Nutritional Biochem. Corp., Cleveland, Ohio; (iii) Uric acid, crystalline powder. Nutritional Biochem. Corp., Cleveland, Ohio; (iv) Xanthine oxidase, eluate with activity of 10.9 U/ml. General Biochemicals, Chagrin Falls, Ohio; (v) Uricase, crystalline powder. Specific activity not furnished. International Chemical and Nuclear Corp., City of Industry, California. These solutions were prepared as follows: (i) Phosphate buffer as purchased; concentration 0.2 M (ii) Standard xanthine solution; 20.0 mg xanthine dissolved in 100 ml phosphate buffer (iii) Standard uric acid solution; 20.0 mg uric acid dissolved in 100 ml buffer (iv) Xanthine oxidase; 0.10 ml of eluate diluted to 10.0 ml with buffer (v) Uricase; 1.0 mg uricase weighed into 10 ml buffer. The optical absorption of reaction mixtures was measured using a Beckman model DB spectrophotometer equipped with a Beckman continuous recorder. Samples were scanned at slow speed with a slit aperture of 0.5 mm. The procedures in detail were as follows: (i) Dilute standard xanthine solution until 3 ml of solution yields an optimal absorption curve in the ultraviolet spectrum between 259 mμ

and 347 mμ with a definite peak at 273 mμ. In this case, the solution contained approximately 11.1 μg of xanthine per ml. This is the xanthine substrate; (ii) Dilute the uric acid solution with buffer to establish the optimal curve in the same range. A sharp peak should be seen at 293 mμ. This determines the uric acid curve for experimental determinations and establishes the purity of the compound; (iii) Dilute the xanthine oxidase and uricase solutions with buffer until a 1:1 v/v mixture of the two enzymes just shows absorbance; (iv) Mix 2 ml of the xanthine substrate with 1 ml of the enzyme mixture in the Beckman cuvettes and run the absorption curve immediately. If interference with the absorption curve of xanthine occurs, dilute the enzyme mix further; (v) Bubble oxygen into the cuvette through a plastic capillary tubing and determine absorption at 15-min intervals to establish the rate of conversion of xanthine to uric acid and the disappearance of the uric acid. At our enzyme dilution of 1:5000, one hour was required for development of the uric acid curve and another hour for the disappearance of the uric acid; (vi) Empirically determine the dilutions of the nematode fractionates required to reduce absorption interference to a minimum; (vii) Mix 2 ml of xanthine substrate with 1 ml of the predetermined dilutions of nematode fractionates and follow exactly the same procedure as in step (v); (viii) Although it was not necessary for these experiments, the absorption curves may be quantitated by standardizing known concentrations of enzymes of known specific activity, and comparing curves of the samples.

GENETIC EXPERIMENTS: Two experiments were designed to test *P. strongyloides* as a tool for genetic studies. These experiments utilized a strain of *P. strongyloides* originally exposed to X-radiation by Thomas and Quastler (19) and a recently isolated "wild strain" of the same organism. The Thomas strain consistently showed a frequency of *endotokia matricida* between 10 and 15 per thousand females compared to a frequency of one to three per thousand females in the wild strain. *Endotokia matricida* is a phenomenon in which hatching of eggs in the uterus of an oviparous nematode results in rupture of the uterus and death of the mother. We suspected this phenomenon to be a radiation-induced mutation. The first experiment was designed to see if *endotokia* could be increased by artificial selection. Back-crossing was not possible due to the certain death of the female.

Live adult females with *endotokia* were transferred to agar plates heavily seeded with *E. coli*. One endotokial female of each succeeding generation was transferred in the same way for a period of 9 months. Succeeding matings occurred among offspring of the same endotokial mother. Controls consisted of routine non-endotokial cultures of both the Thomas and the wild strain with two to four gravid females selected randomly for transfer. After 4 days, all cultures were examined daily for 4 consecutive days, and the number of living endotokial females counted. Each point on the curves in Fig. 4 represents the cumulative count of live endotokial females over 4 days.

In the second experiment, the two strains were crossed in both directions when the frequency of *endotokia matricida* in the Thomas strain had increased to approximately 100 per thousand females. Two to six third-stage and early fourth-stage F_1 females of proven endotokial origin were transferred to agar plates of *E. coli* and an equal number of immature males of wild type origin were placed in close proximity. The reverse of this cross was set up at the same time using the same techniques. Frequency of *endotokia* was determined in succeeding

generations for a period of 5 months using the same counting procedure and controls as in the previous experiments.

RESULTS AND DISCUSSION

MONOXENIC CULTURE: Three hundred adult female *P. strongyloides* averaged 1.4 mm by 0.16 mm, although those containing embryonated eggs averaged 3.5 mm by 0.42 mm. No females in these cultures measured less than 1.1 mm by 0.13 mm, although in other cultures with exhausted substrate many unfertilized females averaged 0.9 mm by 0.12 mm. Size variation is related to two factors: the tenfold increase in volume of fertilized ova in 8 hr and stunting of females from lack of *E. coli* substrate.

The same number of males selected from actively growing cultures averaged 1.0 mm by 0.08 mm ± 0.1 mm and 0.01 mm, respectively. Lack of nutritional substrate also caused slight stunting of the males, but the difference was only about 0.1 mm × 0.01 mm.

It would seem that size is a good taxonomic criterion only in males or in unfertilized females raised on adequate bacterial substrate.

With *E. coli* substrate, a complete nematode life cycle required 4 days at 25 C. The cycle was increased to 6 days at 20 C and reduced to 3 days at 28 C. Populations of individual cultures reached a maximum in 6 to 8 days at room temperature and the average population per petri dish was slightly in excess of 3,500 with blood agar substrate for the *E. coli*.

In these monoxenic culture experiments, the nematodes were counted, measured, filtered, centrifuged, and otherwise handled with slight modifications of ordinary bacteriological techniques. Although only 3500 nematodes were produced per plate in 6 to 8 days, many cultures could be established at one time for the study of mutations.

AXENIC CULTURE: *Pelodera strongyloides* were unaffected by 4-hr exposure to the concentrated antibiotic solutions used for axenization. After 6 days of axenic development in the completely defined, liquid medium, 98% of the larvae had progressed to the adult stage, and 2% died. This rate of maturation was very slow, the adults were stunted, and there was no reproduction. Females contained unfertilized oocytes and numerous unsuccessful attempts at copulation were observed. It was believed failure to copulate was mechanical and due to the mating habits of the nematode. On semisolid media copulation was easily managed but it was impossible in deep liquid culture. Since this research was completed, Yarwood and Hansen (21) proved this observation to be correct. They placed fine glass fibers in their liquid media and have had *P. strongyloides* in continuous liquid culture for almost a year. Their medium, however, still requires an undefined supplement of heated liver extract or growth factor.

Prior to successful culture of *P. strongyloides* by Yarwood and Hansen (21), no dioecious nematode, with the exception of *Turbatrix aceti* (Nicholas, 12), had been continuously cultured axenically until Cryan *et al.* (3) succeeded with *Panagrellus redivivus*. It is believed that their success with this nematode was due to a difference in mating habits. The male of *P. redivivus* coils around the body of the female during copulation so is not prevented from mating by lack of physical support from the medium.

It should be emphasized that the media used by Dougherty, *et al.* (5), Sayre, Hansen and Yarwood (14), Cryan, *et al.* (3), and Yarwood and Hansen (21) all required varying amounts of uncharacterized proteinacious supplement. Ours is believed to be the first report of nematode maturation in an unsupplemented, completely defined,

TABLE 2. Results of biochemical determinations.

DETERMINATION	REPORTING UNIT	METHOD	CONTROL MEDIUM	EXPERIMENTAL MEDIUM	NEMATODE HOMOGENATE	NUCLEAR FRACTION	MITOCHONDRIAL FRACTION	SUPERNATANT FRACTION
SODIUM	mEq/L	TECHNICON			25.0			
POTASSIUM	mEq/L	TECHNICON			2.0			
CARBON DIOXIDE	mEq/L	TECHNICON			29.0			
CHLORIDES	mEq/L	TECHNICON			19.0			
CALCIUM	mg/100ML	ELLIOTT, 1952			0.6	0.1	0.2	0.2
PHOSPHORUS	mg/100ML	FISK & SUBBAROW 1925			2.0	0.8	0.8	0.3
URIC ACID	mg/100ML	BROWN, 1957	0.0	0.0	2.0	0.6	0.3	0.9
UREA	mg/100ML	SKEGGS, 1957	0.0	0.0	11.1	0.0	0.0	11.0
AMMONIA	mg/100ML	VANSLYKE-CULLEN, 1916	5.0	60.0				
AMMONIA, SERUM	µ/100ML	SELIGSON & SELIGSON, 1961			20.0	0.0	0.0	15.0
PROTEIN BOUND I_2	µ/100ML	LEFFLER & McDOUGALD, 1964			3.0	0.0	0.0	2.0
AMYLASE	RUSSELL UNITS	HUGGINS & RUSSELL, 1948			20.0	0.0	0.0	19.0
GLUT-OXALACETIC TRANSAMINASE	SIGMA UNITS	SIGMA			5.0	0.0	3.0	3.0
GLUT-PYRUVIC TRANSAMINASE	SIGMA UNITS	SIGMA			6.0	0.0	4.6	0.0
ACID PHOSPHATASE	KING-ARMSTRONG UNITS	SIGMA			0.7	0.0	0.0	0.6
ALKALINE PHOSPHATASE	KING-ARMSTRONG UNITS	SIGMA			1.5	0.5	0.4	0.3
LACTIC DEHYDROGENASE	B-B UNITS	SIGMA			30.0	0.0	20.5	10.0
LIPASE	UNITS	COMFORT & OSTERBERG, 1947			3.0	0.0	3.0	0.0
XANTHINE OXIDASE	+ or -	MODIFIED FROM KALCKAR, 1947			+	+	-	-
URICASE	+ or -	MODIFIED FROM KALCKAR, 1947			+	+	-	-

axenic medium. The results suggest a proper mineral balance may be to some degree as important in nematode nutrition as a correct balance of amino acids and growth factors.

BIOCHEMICAL ANALYSIS: Columns 4 and 5 in Table 2 show neither uric acid nor urea is present in either the control media or the experimental media in which the nematodes had been grown. Ammonia is present at the 5.0 mg/100 ml level in the control medium, but this is undoubtedly residue from the ammonia used for adjusting the *p*H. The experimental medium shows 60 mg NH_3/100 ml. Obviously the nematode's major excretory product of purine degradation is ammonia and the major intermediate compounds, uric acid and urea, are not present in the medium.

Uric acid was found in all of the fractionates, but urea was present only in the supernatant fraction. The significance of the distribution of these compounds is uncertain,

but their presence in the nematode body indicates that a large segment of the purine degradation pathway is present in *P. strongyloides*.

The significance of 15 μg/100 ml of serum ammonia in the supernatant fraction cannot be properly evaluated at this time because not enough is known about the excretory system of *P. strongyloides*.

The amounts of sodium, potassium, carbon dioxide and chlorides (Table 2, column 6) indicate the electrolyte balance of *P. strongyloides* resembles that of warm-blooded animals. The data show that the nematode has a sodium requirement, and the relatively high carbon dioxide level suggests a carbonate-bicarbonate buffering action of the hemolymph. However, a high concentration of carbon dioxide could occur in the tissues and hemolymph if a poor gas exchange mechanism exists in this nematode.

Calcium and phosphorus were present in very small amounts and uniformly distributed throughout the fractionates. The calcium-phosphate ratio is different in *Pelodera* than in warm-blooded animals, and the small amount of uptake suggests that the large amounts of these elements in the medium of Sayre, Hansen and Yarwood (15) are unnecessary and may even be harmful.

Protein-bound iodine was detected, but no evidence was obtained indicating it is required for maturation through the larval stages. The concentration of protein-bound iodine in the supernatant fraction suggests that iodine may be in the hemolymph. No hormones have been reported in nematodes.

The comparatively large amount of amylase in this strictly bacteriophagous nematode was entirely unexpected and cannot be explained. There does not seem to be any need for this enzyme, as *E. coli* cell walls contain no starch. There are many hydrolases and several phosphorylases capable of breaking down glycogen. The Huggins and Russell Test (9) is believed to be specific for α- and β-amylase.

The two transaminases, oxalacetic and pyruvic, indicate the presence of well-known pathways involved in the synthesis of amino acids. These major pathways are also utilized by mammals.

Acid and alkaline phosphatases hydrolyze esters of phosphoric acid and are particularly effective in the recycling of phosphorus.

Lactic dehydrogenase indicates the presence of the Krebs cycle in *P. strongyloides* and suggests that these normally aerobic nematodes can build up an oxygen debt under temporary anaerobiosis.

Fairbairn (7) believed that in *Ascaris*, lipid metabolism is only concerned with egg development, but we have observed lipid globules in the intestinal cells of *P. strongyloides* during our studies of the ultrastructure of this nematode. At any rate, the positive identification of lipase indicates that this nematode has an active lipid metabolism and that the activity may be associated with mitochondria.

SIMULTANEOUS DETERMINATION OF XANTHINE OXIDASE AND URICASE: Fig. 1, 2 and 3 show the results of the xanthine oxidase-uricase determinations. Curves marked "A" in each figure show that the purified xanthine eluate absorbs characteristically at the beginning of the experiment without interference from either the highly diluted enzymes, the homogenate suspension, or the equally diluted nuclear fractionate.

The "B" curves show that after one hour, the xanthine substrate has been converted to uric acid, and the peak of highest activity is in the nuclear fractionate. These curves are positive evidence that xanthine oxidase is present in both the homogenate and nuclear fractionates and that the level of activity is approximately equal to that of the control

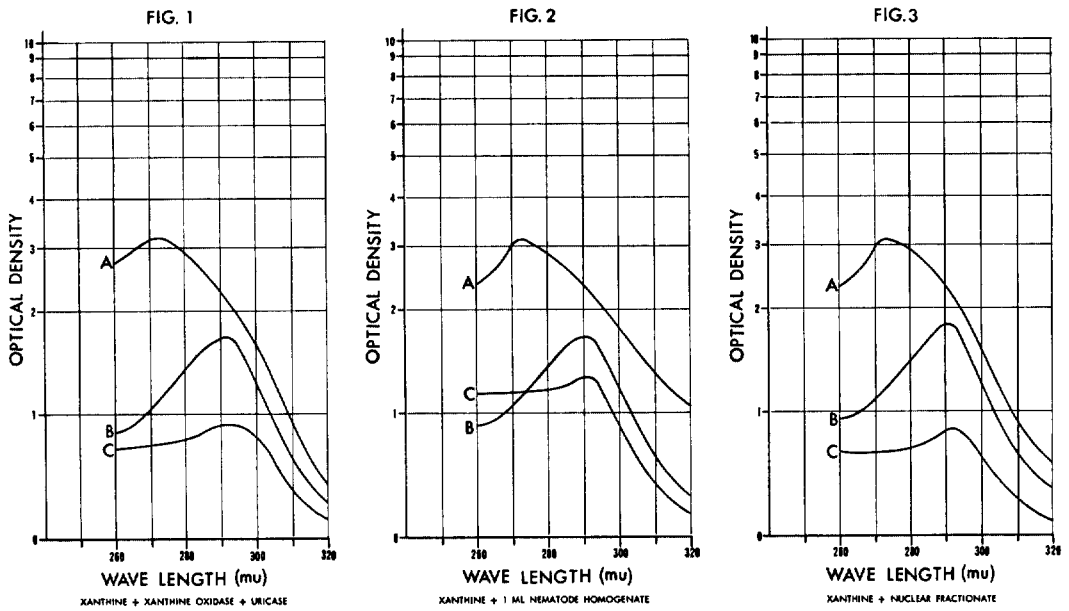

FIG. 1. Control for Xanthine-Uric Acid determinations. **A.** Characteristic peak of xanthine substrate at 263 mμ; **B.** After 1 hr. Curve is characteristic of uric acid. Note peak at 293 mμ; **C.** After 2 hr uric acid has almost disappeared.

FIG. 2. Xanthine plus dilute homogenate. Curves almost identical to Fig. 1. Curve C indicates higher nonspecific absorption at 260 mμ.

FIG. 3. Xanthine plus dilute nuclear fractionate. Curves similar to Fig. 1.

(Fig. 1). There is apparently no blocking of xanthine oxidase that would cause a "pile-up" of xanthine in the nematode as reported by Clark (1).

The "C" curves in Fig. 2 and 3 show considerable disappearance of the uric acid after 2 hr compared to the same curve in Fig. 1. The increased height of this curve in Fig. 2, particularly at 260 mμ, is believed to be due to higher background absorption by the homogenate. These data indicate the presence of active uricase in the nematode and suggest that much of the activity is limited to the "nuclear" fraction. The "mitochondrial" and supernatant fractions showed no activity.

The presence of comparatively large amounts of metabolic ammonia in the medium combined with the absence of uric acid and urea intermediates, indicates that ammonia is the only purine degradation product excreted by *P. strongyloides*. The finding of uric acid, urea, xanthine oxidase and uricase in the nematode fractionates, along with small amounts of ammonia, is strong evidence that the complete purine degradation pathway is present. Under these conditions, it does not seem possible for xanthine to ac-

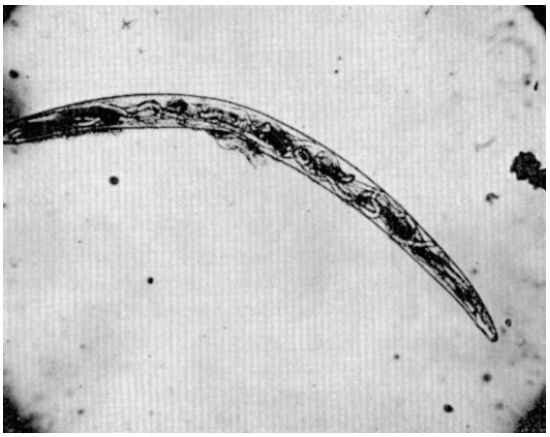

FIG. 4. *Endotokia matricida* in *P. strongyloides*. Note live larvae within cuticular sac of dead female. Vaginal plug is still intact indicating that this was a very young female, gravid for the first time. Photographed *in situ* with Eastman High Contrast Copying Film. Magnification 100 ×.

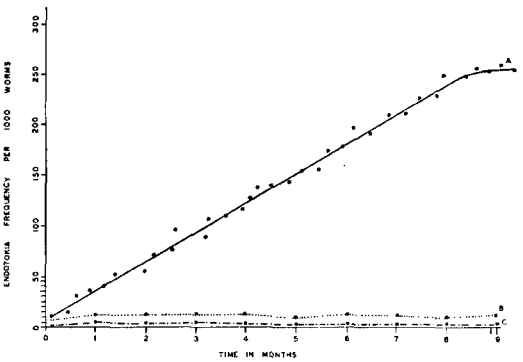

FIG. 5. Frequency of *endotokia* in *P. strongyloides*. **A**. Thomas strain. Each culture started from a single endotokial female. Each point on curve is 5-day total count of live endotokial females per 1000 females counted. Serial cultures; **B**. Randomly-mated Thomas strain used as a control. Method of counting identical, but counts were made at the beginning of each month; **C**. Wild strain control. Method and frequency of counting same as B.

cumulate in the intestinal cells as described by Clark (1).

GENETIC EXPERIMENTS: During the preliminary studies, we observed *endotokia matricida* occurred only in very young females fertilized for the first time. *Pelodera strongyloides* and several other of the free-living nematodes that we have examined have a membranous uterus and a cuticularized vagina devoid of musculature. The fertilized eggs rapidly increase in size as the embryos develop until the largest ones nearest the vagina are expelled by the elevated internal pressure. This initial elevated pressure is built up by the formation of a vaginal plug which must be lysed before the first embryonated egg can be expelled. When sufficient pressure is attained and the vaginal plug lysed, the mechanism of expulsion becomes continuous. Successive copulations do not result in the formation of vaginal plugs. Lysis of the plug must occur within narrow time limits because the expelled eggs hatch within 3–4 hours. The photomicrograph in Fig. 4 shows live larvae within the cuticle of a female that died of *endotokia matricida*. The plainly visible vaginal plug is completely intact.

We postulated that the enzyme mechanism involving lysing of the plug might have been genetically altered during irradiation of the ancestral cultures by Thomas and Quastler (19). Fig. 5 shows that continuous artificial selection over a period of 8½ months increased the frequency of *endotokia* from an already high 10–15 per 1000 to a plateau of 250 per 1000.

When artificial selection had increased the frequency of *endotokia* to 10%, males and females from these cultures were crossed in both directions with the wild strain. The frequency of *endotokia* in the progeny of these crosses dropped to 1–3% in the F_1 generation and remained constant for 5 months.

Very little information could be found in the literature relating to the genetics of *P. strongyloides*. Nigon (13) reported the num-

ber of chromosomes in *P. strongyloides* to be 22 in the female and 21 in the male. This observation has not been confirmed, but if true, sex determination must be via the *xo* mechanism, which is also found in the short-horned grasshopper and a few bugs. If the mechanism is correct, the genetic alteration must have occurred on the X chromosome and both chromosomes would be necessary for phenotypic expression in the female.

Summary and Conclusions

The results of these experiments are encouraging and suggest that it might be worthwhile to study *P. strongyloides* or one of the other free-living nematodes in greater detail for use as a metazoan research tool. The following conclusions are offered as evidence:

(i) Sufficient information was obtained to show that *P. strongyloides* metabolism and genetics can be studied with many of the techniques commonly used by microbiologists.

(ii) The 4-day life cycle is short enough to permit the study of thousands of individuals per week.

(iii) It has been shown that this nematode will grow and mature in a completely-defined liquid axenic medium, thus permitting studies of the effects of drugs, enzymes, and hormones. The medium must be further improved to permit continuous axenic culture under completely controlled conditions.

(iv) Axenically cultured nematodes have been analyzed by well-known biochemical methods and show enzymes of biochemical pathways characteristic of mammals.

(v) One phenotypic marker was found and studied by selection and crossing.

Literature Cited

1. Clark, D. T. 1955. Identification of intestinal cell inclusions in certain nematodes. Thesis. University of Illinois, Urbana.
2. Comfort, J. C., and K. Osterberg. 1934. A colorimetric method for the determination of lipase. J. Lab. Clin. Med. 20:271–280.
3. Cryan, W. S., E. Hansen, M. Martin, F. W. Sayre, and E. A. Yarwood. 1963. Axenic cultivation of the dioecious nematode *Panagrellus redivivus*. Nematologica 9:313–319.
4. Dougherty, E. C., and E. L. Hansen. 1956. Axenic cultivation of *Caenorhabditis briggsae* (Nematoda: Rhabditidae). Maturation on synthetic media. Proc. Soc. Exp. Biol. Med. 93:223–227.
5. Dougherty, E. C., E. L. Hansen, W. L. Nicholas, J. A. Mollett, and E. A. Yarwood. 1959. Axenic culture of *Caenorhabditis briggsae* with unsupplemented and supplemented chemically defined media. Ann. N. Y. Acad. Sci. 77:176–217.
6. Elliott, W. E. 1952. Volumetric determination of calcium in blood serum. J. Biol. Chem. 197:641–644.
7. Fairbairn, D. 1957. The biochemistry of *Ascaris*. Exp. Parasitol. 6:481–554.
8. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375–400.
9. Huggins, C., and P. S. Russell. 1948. Colorimetric determination of amylase. Ann. Surg. 128:668–678.
10. Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. J. Biol. Chem. 167:429–475.
11. Leffler, H. H., and C. N. McDougald. 1964. Estimate of protein bound iodine by improved technique. Clin. Chem. 10: 799–822.
12. Nicholas, W. L. 1956. Axenic culture of *Turbatrix aceti* (The vinegar eelworm). Nematologica 1:337–340.
13. Nigon, V. 1949. Modalités de la reproduction et le déterminisme du sexe chez quelques nématodes libres. Ann. Sci. Nat. Zool. Biol. Anim. 11:1–132.
14. Sayre, F. W., E. L. Hansen, and E. A. Yarwood. 1963. Biochemical aspects of the nutrition of *Caenorhabditis briggsae*. Exp. Parasitol. 13:98–107.
15. Seligson, D., and F. Seligson. 1961. A new method for the determination of serum ammonia. Clin. Chem. 7:67–69.
16. Sigma Chemical Co. 1965. Sigma Analyti-

cal Methods. Tech. Bulletins No. 500 and No. 505. The Sigma Chemical Company, St. Louis.

17. SKEGGS, L. T. 1957. An automatic method for colorimetric analysis. Am. J. Clin. Pathol. 28:311–330.
18. TECHNICON CO. 1965. Technicon Auto-Analyzer Methods. Technicon Instruction Manual, The Technicon Company, New York.
19. THOMAS, L. J., and H. QUASTLER. 1950. Preliminary reports of X-ray effects on the nematode *Rhabditis strongyloides*. Science 112:356–357.
20. VAN SLYKE, D. D., and G. E. CULLEN. 1916. The determination of urea by the urease method. J. Biol. Chem. 24:117–122.
21. YARWOOD, E. A., and E. L. HANSEN. 1968. Axenic culture of *Pelodera strongyloides* Schneider. J. Parasitol. 54:133–136.