Adenosine Triphosphate Quantification as Related to Cryptobiosis, Nematode Eggs, and Larvae[1]

HARVEY W. SPURR, JR.[2]

Abstract: Sonification was the most effective method used for disintegrating nematode eggs and larvae for adenosine triphosphate (ATP) determinations. Sensitivity of the assay was sufficient to measure ATP in one larva. Second-stage larvae of *Anguina tritici* averaged 1 x 10^5 femtograms (fg) ATP and *Meloidogyne incognita* eggs, 0.8 x 10^5 fg ATP. Larvae of *Panagrellus redivivus,* a saprobe, averaged 12.2 x 10^5 fg ATP, a measurement which was considerably higher than the ATP levels in plant parasites. Endophytic bacteria and fungi from wheat galls were detected as background organisms associated with *A. tritici* activated by hydration. Also, bacteria in suspensions of eggs from *M. incognita* prepared with NaClO were measured by the use of butanol extraction and ATP determination. Second-stage *A. tritici* larvae increased in ATP content within 40 min after being activated from cryptobiosis by hydration. In the cryptobiotic state, larvae had 50% less ATP than when active. ATP concentrations were similar in galls of different ages. Apparently, ATP concentrations do not change during cryptobiosis. Starvation results in a decline in ATP concentration/larva. Subjecting *A. tritici* larvae to the lethal temperature of 60 C resulted in a three-fold increase in the decay rate of ATP over that of larvae sonified, then heated at 60 C. These results suggest an association between ATP decay and the mechanism that causes death of larvae at elevated temperatures. *Key Words: Anguina tritici, Meloidogyne incognita, Panagrellus redivivus,* starvation, thermal inactivation.

Living organisms depend on adenosine triphosphate (ATP) and similar compounds to energize vital metabolic activities, and nematodes in various states of hypobiosis must preserve adequate ATP, or a mechanism for synthesizing it, to survive. The measurement of ATP in nematodes has been hampered by the lack of suitable techniques. When a sensitive instrument was developed for the quantification of bacterial ATP and populations (1, 2), I designed these experiments to: (i) extract and quantify nematode ATP, (ii) compare ATP in different larvae and eggs, and (iii) determine ATP in cryptobiotic larvae before and during hydration and activation.

Received for publication 26 August 1975.

[1] Cooperative investigations of the Southern Region, Agricultural Research Service, United States Department of Agriculture and the North Carolina State University Agricultural Experiment Station, Raleigh. Paper No. 4830 of the North Carolina Agricultural Experiment Station, Raleigh. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or North Carolina State University, and does not imply its approval to the exclusion of other products that may be suitable.

[2] Research Plant Pathologist, Southern Region, Agricultural Research Service, U. S. Department of Agriculture, Oxford Tobacco Research Laboratory, Oxford, North Carolina 27565 and Professor of Plant Pathology, North Carolina State University, Raleigh. The author appreciates the technical assistance of Eleanor T. Howard and thanks Dr. T. T. Hebert for providing wheat galls and W. M. Williams for the photographs.

MATERIALS AND METHODS

Sources and species of nematodes: To obtain active larvae of *Anguina tritici* (Steinbuch) Filip., wheat galls ("cockles") were soaked in water, opened with a teasing needle, and the second-stage larvae squeezed out with forceps into water in a watch glass. Hydration caused the larvae to take shape and become active. When galls were soaked 1 h or less before excision, larval movement began 8-24 h after excision and fewer viable larvae were obtained than when galls were soaked for longer periods before excision. When galls were soaked for 1-3 days prior to excision, high numbers of active larvae were observed after 45-60 min.

A 24-h soak, followed by excision, and a 24-h incubation were used for most experiments (Fig. 1).

Panagrellus redivivus (Linn) T. Goodey was cultured with yeast on autoclaved oatmeal (30 ml oatmeal + 60 ml water). Cultures were incubated for 3-4 weeks at 26 C. Larvae were carefully decanted from the culture flasks, and active larvae were separated from debris by Baermann funnel.

Meloidogyne incognita (Kofoid & White) Chitwood was maintained on greenhouse-grown tomato, *Lycoperscion esculentum* 'Saturn'. Eggs, harvested by the NaClO (sodium hypochlorite) procedure (5), were used for inocula and ATP studies. Roots collected 45 days after the inoculation of young plants were washed and shaken in 200 ml of 1.05% NaClO for 4 min. The NaClO solution was quickly passed through a 200-mesh (75-μm) sieve nested in a 500-mesh (26-μm) sieve on which the freed eggs were trapped. Eggs were rinsed in a stream of cold tap water to remove residual NaClO and transferred to a 2-liter stainless steel container. Approximately 1,800 ml of deionized water were added; the mixture was stirred for 30 min and then poured through a sieve. The eggs were washed into a stainless steel container, and the concentration adjusted by dilution with deionized water.

Butanol extraction of ATP: This procedure was adapted from the Instruction Manual for the DuPont 760 Luminescence Biometer (E. I. DuPont De Nemours & Co. Inc., Instrument Products Div., Wilmington, Delaware 19898). The Biometer and the DuPont vacuum filter stand with 0.45-μm pore filters and filter assemblies were used to make ATP determinations (1). The nematodes to be extracted were pipetted onto the 0.45-μm filter. Vacuum was applied and released as soon as the liquid disappeared through the filter. Nematodes on the filter were washed with 1 ml of 0.01 *M* morpholinopropane sulfonic acid (MOPS) buffer (DuPont), pH 7.4, containing 0.01 *M* $MgSO_4$ at 25 C. The drop clinging to the bottom of the filter was shaken off. A clean, 12-ml glass centrifuge tube was placed under the filter, and 0.2 ml n-butanol (Fisher Certified Grade A-400, Fisher Scientific Co., Chemicals Manufacturing Div., Fair Lawn, N. J.) was added to the filter. After 20 sec, the solution was vacuumed off, and the procedure was repeated. Then 0.5 ml MOPS was added to the filter for 10 sec, vacuumed off and the procedure repeated. Next, 8 ml of 1-octanol [(washed in deionized water) Fisher Certified Grade A-402] were added to the tube containing the butanol and MOPS filtrate. The tube was shaken vigorously for 10 sec and centrifuged at 1085 *g* for 3 min to speed separation of the layers. The top octanol-butanol layer was removed by aspiration or pipetting. The bottom, aqueous layer was used for ATP determinations.

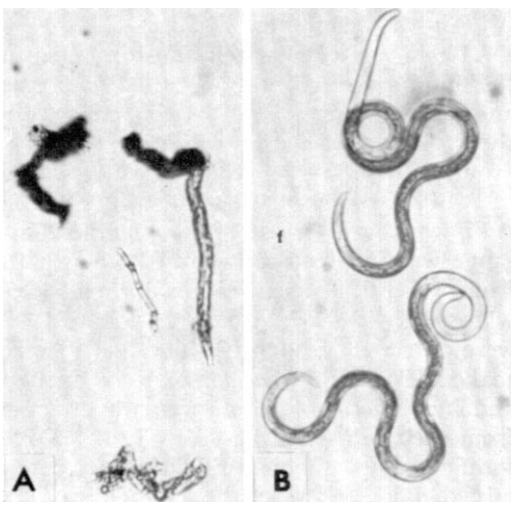

FIG. 1. Hydration of cryptobiotic *Anguina tritici*. *A)* Hydrating larvae taking shape 2 min after adding water. *B)* Fully hydrated, active larvae.

Sonification to extract ATP: The following general procedure was used to disintegrate larvae and eggs to release ATP. The desired numbers of nematodes were added to a 12-ml graduated centrifuge tube. Volume was adjusted to 5 ml with MOPS, and the tube was placed in crushed ice. The sonifier probe was inserted and the solution sonified. A Branson Sonifier (Model S125, Branson Instruments, Inc., Danbury, Conn.) provided satisfactory disintegrations: 2 min, No. 2 setting, 3 amps disintegrated *A. tritici* and *P. redivivus* larvae and *M. incognita* eggs (1,000 per ml); 5 min, No. 5 setting, 6.5 amps disintegrated *M. incognita* eggs and the larvae contained within. If larval concentrations were above 80/ml and egg concentrations above 1,000/ml, increases in time and power were required for satisfactory disintegrations. One ml of sonicate was

filtered with an 0.45-μm pore filter as described previously, and the amount of ATP in the filtrate was determined. The sonifier probe was cleaned by being rinsed 3 times with 2*N* HCl, deionized water, MOPS, and deionized water.

ATP measurement: ATP was quantified by using a sensitive photometer (DuPont 760 Luminescence Biometer) to measure the light produced in the luciferase-luciferin firefly reaction (2). Deionized water was equivalent to low response water since it did not contain measureable ATP. A tube containing 0.1 ml of reaction mixture (0.01 *M* MOPS pH 7.4, 0.01 *M* $MgSO_4$, 0.71 *m*M crystalline luciferin, 100 units DuPont purified and stabilized luciferase) at 25 C was inserted into the photometer. Ten μliters of extract containing ATP were injected into the tube and the readout in femtograms (fg) ATP/ml recorded. One femtogram equals 10^{-15} gm ATP. Three readings were made of each extract. Additional readings were made if disparities were present. The average reading in fg ATP/ml was divided by the number of individuals/ml (larvae or eggs) to obtain fg ATP/nematode.

RESULTS

Extraction of ATP: Butanol did not adequately rupture and remove ATP from larvae and was inadequate, especially if nematodes/ml were 80 or more. This solvent was used to monitor bacterial populations associated with nematode preparations.

Sonification at low temperature, 0-5 C, was efficient and practical for disintegrating nematode larvae and eggs. Sonification alone yielded ATP values equivalent to treatment with 3% perchloric acid followed by sonification, and values were higher than with heat extraction (100 C for 10 min).

ATP in nematodes: ATP was determined in larvae of *A. tritici* and *P. redivivus* and eggs of *M. incognita* (Fig. 2). The technique used for determining ATP was sufficiently sensitive to quantify the ATP in a single larva. Since each species examined had individual characteristics related to the determination of ATP, the results are presented by species.

Anguina tritici: The ATP content of larvae from galls soaked 1 h and galls soaked 3 days prior to excision were compared after 1, 2, 3, 4, and 9 days of incubation. The ATP/larva fluctuated from 1.3 x 10^5 to 2.4 x 10^5 fg. This fluctuation was not influenced by the length of the soak period. The water the larvae were incubated in contained 1.4 x 10^5 fg ATP/ml. When this water was spread on yeast extract agar medium, many bacterial colonies developed. The incubation water averaged about 2.8 x 10^5 bacteria per ml (based on the average conversion figure for growing, viable bacteria of 5 x 10^{-1} fg ATP per cell) (1, 2).

Wheat galls were tested for microflora. Galls were surface-sterilized in 1% NaClO for 1, 5, and 10 min; rinsed 3 times in sterile water; incubated in sterile water for 1 h; and asceptically cut in half. One half was incubated in water for 24 h and the larvae proved to be active. The other half was plated on potato dextrose or yeast extract agar and incubated. Fungi and bacteria grew from these galls and were similar in numbers for all NaClO treatments. When *A. tritici* were incubated in water for 11 days, the background in the incubation mixture was fairly constant at 2.6 x 10^5 fg ATP/ml (5.2 x 10^5 bacteria/ml) throughout the 11 days, if it is assumed that the background was due primarily to bacteria. Larvae ranged from 1.0 x 10^5 to 2.4 x 10^5 fg ATP/larva after the contribution from bacteria sonified with larvae was substracted. Background was considered low and relatively constant, compared to ATP in the nematodes.

There was a linear relationship between the numbers of second-stage *A. tritici* larvae and the amount of ATP extracted from them. After a correction was made for background, these larvae averaged 1.8 x 10^5 fg of ATP. A range of 0.2 x 10^5 to 2 x 10^5 fg ATP was characteristic of these larvae. This range was further substantiated when galls from several years were examined. Five wheat galls for each year from 1970 to 1972 were selected randomly. The galls were soaked 24 h and the larvae were excised and incubated for 24 h. Seventy-five larvae from each gall were placed in 5 ml MOPS, sonified, and their ATP content determined. For each year, the average was 1 x 10^5 fg ATP/larva (Table 1). Larvae were fairly similar in ATP from gall to gall and from year to year.

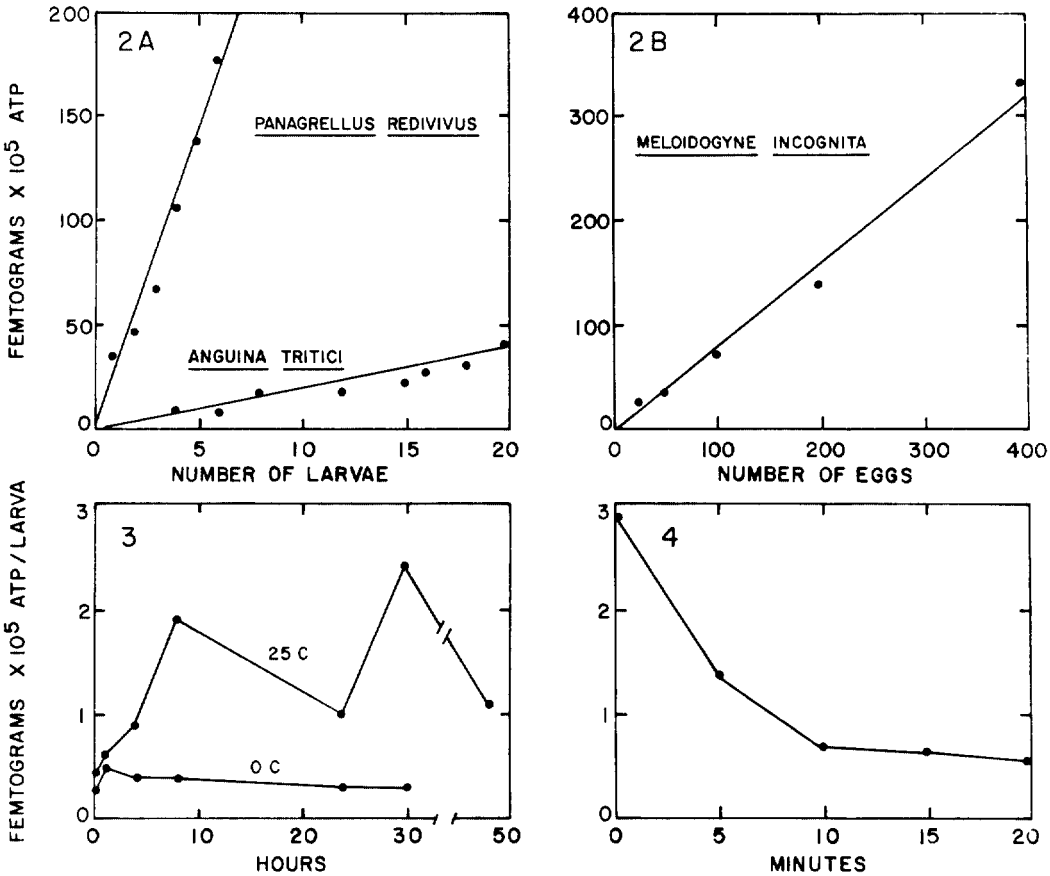

FIG. 2-4 *2)* Quantification of ATP in nematode larvae and eggs. *A)* Free-living larvae of *Panagrellus redivivus* and second-stage larvae of plant-parasitic *Anguina tritici*. *B)* Eggs of *Meloidogyne incognita*. *3)* Quantification of ATP in second-stage larvae of *A. tritici* during hydration from the cryptobiotic state at 0 and 25 C. *4)* Decrease in ATP concentration during heat treatment at 60 C of second-stage larvae of *A. tritici* before sonification.

A dry wheat gall was excised and half the amorphous larvae were dropped into 2 ml of MOPS in a tube immersed in boiling water. After 30 min, the tube was removed and cooled rapidly with ice to 25 C. The other half of the excised larvae were dropped in 2 ml of MOPS at 25 C. Seventy-five larvae from each treatment were sonified after 40 min and 18 h of incubation. The heated larvae had from 20 to 50% less ATP than larvae incubated at 25 C.

TABLE 1. Average ATP in second-stage larva of *Anguina tritici* from galls of different ages.

Gall	ATP/larva (fg x 10^5)		
	Age (years)		
	5	4	3
1	2.0	1.6	1.0
2	0.7	0.6	1.1
3	0.7	0.6	0.2
4	1.0	1.3	1.4
5	1.0	0.7	1.3
Mean	1.0	0.9	1.0

The level of ATP in the cryptobiotic larvae was quantified further in the following experiment. A large dry wheat gall was halved; one-half was added to 2 ml of sterile water in a tube and incubated at 0 C and the other half was added to 2 ml sterile water in a tube at 25 C. At intervals, 75 larvae from each treatment were placed in 5 ml of MOPS and sonified. Larvae incubated at 0 C hydrated but did not become active (Fig. 3). After 32 h at 0 C, they were warmed to 25 C and held 16 h but they did not move. Larvae at 25 C initially had the

same amount of ATP as larvae at 0 C. However, within 1 h, net synthesis of ATP was apparent, and ATP thereafter averaged 3 times higher than in larvae held at 0 C. Fluctuations in ATP in *A. tritici* larvae at 25 C are typical of the type of fluctuations which were observed in these experiments. These fluctuations seem related to physiological changes in these larvae and not to variations in experimental techniques. Thus, ATP, 0.4 x 10^5 fg, is found in larvae in the cryptobiotic state, and net synthesis occurs soon after hydration at favorable temperatures.

Free-living Panagrellus: P. redivivus averaged 27.1 x 10^5 fg ATP/larva (Fig. 2A). This average represents a mixture of larval stages. Larvae of the L2 class (3), separated on a column 3.3 cm in diam and 25 cm long filled with 0.45-mm glass beads, averaged 12.2 x 10^5 fg ATP at the time of separation from culture and 6.2 x 10^5 fg ATP after 10 days of incubation in water at 25 C.

Meloidogyne: Sonified eggs of *M. incognita* averaged 0.8 x 10^5 fg ATP/egg. Repeated washing of the eggs with water gradually removed the gelatinous matrix with ATP. The bathing water was heated to 100 C for 15 min and then placed in a flash evaporator at 27 C under partial vacuum to reduce the volume. The ATP concentration in the water was determined at intervals. The flash and decay of light from the reactions was recorded to confirm that Biometer readings were from ATP and not other chemicals. As water was evaporated, ATP concentration increased (Table 2).

When water from egg suspensions was plated, bacterial colonies grew, a fact which indicated the NaClO solution used to remove egg masses from roots did not completely sterilize egg preparations. Determinations of bacteria filtered with a 0.45-μm filter showed the number of bacteria was initially very low in the egg suspension. When eggs were incubated in sterile water, the bacterial count remained relatively constant until the ninth day and then increased slightly.

Single eggs of *M. incognita* ranged from 0.34 x 10^5 to 0.91 x 10^5 fg ATP. The two sonification intensities used did not affect ATP concentrations. Two min at 3.5 amps broke the eggs but not all of the larvae, whereas 5 min at 6.5 amps disintegrated eggs and larvae. Eggs of *M. incognita* stored for 6 months at −20 C were low in ATP (0.1 x 10^5 fg). Also, these eggs were browner than fresh eggs.

TABLE 2. ATP in water after filtering eggs of *Meloidogyne incognita*.

Solution	Volume (ml)	ATP (fg x 10^5/ml)
Egg filtrate	200	12.0
Heated filtrate[a]	200	12.0
Evaporated filtrate[b]	100	23.6
Evaporated filtrate	67	26.1
Evaporated filtrate	25	99.0
Evaporated filtrate	4	620.0

[a]Heated 100 C for 15 min.
[b]Evaporated under vacuum at 27 C.

Decay of ATP: ATP decays in solution at a rate dependent upon concentration, composition of the solution, and conditions to which it is subjected. Standard ATP solutions in the 1,000- to 100-fg/ml range, prepared with MOPS buffer and 0.01 *M* $MgSO_4$ and held at 0 C, decayed at the rate of 1 x 10^5 to 2 x 10^5 fg/min/ml during the period from 20-40 min after thawing. The same solution decayed 5 times faster at 100 C. The ATP solution obtained from sonified *A. tritici* larvae, 15 larvae/ml, decayed at a rate of 0.1 x 10^5 fg/min at 25 C. This rate was similar even in larvae tested up to 43 days after excision from galls. Thus, ATP in sonified preparations and solutions of ATP maintained in an ice bath decayed at a rate slower than did a standard solution of ATP at ambient or higher temperatures.

After these observations were made, it was surprising to note that the ATP concentrations were so low when heat was used to extract ATP from larval suspensions of *P. redivivus*. When *P. redivivus* larvae were held at 60 C for 10 min and then sonified, the ATP level decreased from 13 x 10^5 to 1 x 10^5 fg/larva. The same procedure was tested on *M. incognita* eggs and *A. tritici* larvae. The treatment at 60 C for 10 min prior to sonification reduced ATP by 90% in both. Sonification and then 60 C heat treatment reduced ATP 12% in *M. incognita* eggs but did not reduce it in *A. tritici* larvae.

Treatment at 60 C for 10 min was assumed to kill the three nematode species. This was confirmed by visual observation of the *A. tritici* larvae. The decay of ATP was quite rapid initially; it tapered off to a constant level 10 min after the initiation of the heat (Fig. 4). At this time, the larvae were dead and the decay mechanism seemed inactive.

DISCUSSION

The efficient and practical disruption of nematode larvae and eggs by sonification freed ATP which could be reproducibly measured with the highly sensitive Luminescence Biometer. Differences in size and metabolic activity may result in the lower amounts of ATP in larvae and eggs of plant-parasitic nematodes, in comparison with ATP levels in the larger, free-living *P. redivivus* larvae (15-30 times higher). Nematodes contain considerably more ATP than bacteria, which average 0.5 fg ATP/cell. The sensitivity of this method was sufficient to measure ATP in a single larva, and the number of larvae or eggs in a uniform suspension was accurately calculated from an ATP determination. This is an efficient method for assaying nematode numbers in many circumstances.

Any population of nematodes may be associated with bacteria and fungi. Both were associated with *A. tritici* larvae and appeared endophytic in wheat galls, since surface-disinfection procedures designed to remove epiphytes did not eliminate all microorganisms (7). A few microorganisms, primarily bacteria, were also present in both *A. tritici* larval suspensions and *M. incognita* egg suspensions. This ATP assay is also a reliable procedure for quantitatively measuring numbers of bacteria. By differential filtration, butanol extraction, and ATP measurement, the numbers of bacteria in the nematode suspensions were obtained. The background measurement provides a necessary check or monitoring system for measuring ATP background concentrations in many experiments with nematodes. These background data emphasize the difficulties in preparing pure or "bacteria-free" nematode suspensions but provide a means for assessing some of the effects of microbial associations.

Several factors influence the quantity of ATP in nematodes: physiological state (active vs cryptobiotic), nutritional state, and species differences. Concentrations of ATP were fairly constant and reproducible in larvae and eggs of *M. incognita,* but changed under a physiological stress such as starvation. Fluctuations in ATP, such as those noted in *A. tritici* incubated at 25 C, were repeatedly observed. The introduction of light may explain these fluctuations. When larvae were illuminated with light from the microscope, they seemed to become more active. It was necessary to use light to select larval samples for determinations of ATP during time-course studies.

It is interesting to note the presence of ATP in the water in which *M. incognita* eggs were suspended. Since successive washings of the eggs had lower ATP levels, it is likely that the ATP dissipated with the matrix. How or why ATP was present in the matrix is unknown. Probably ATP was synthesized in the eggs and then leached into the matrix.

Variation in ATP in *M. incognita* eggs was not dependent upon time after inoculation of the plant, but may reflect physiological condition and/or amount of matrix dissolution from washing during preparation. After egg suspensions were prepared, some eggs hatched or broke. In assessing egg concentrations, we could count eggs, egg cases, and larvae in a sample. An accurate determination of ATP depends upon an accurate assessment of the population in suspension.

The presence or absence of metabolism in larvae in the cryptobiotic state is often discussed in regard to survival. Recent reviews suggest that metabolic activity must be low or nondetectable (4, 8). When larvae of *A. tritici* were examined after hydration at 0 and 100 C, they assumed a recognizable form from their amorphous, cryptobiotic form. At both temperatures, 50% less ATP was present than was present in the active state at 25 C. When hydration was initiated at 25 C, the larvae took on a recognizable form within a few min and a net increase in ATP was detected in 40 min. Therefore, in the cryptobiotic state, ATP and the mechanism for synthesizing ATP are present. Since ATP concentrations were similar in galls of different ages (3, 4, and 5 years), it is likely that metabolism is nondetectable

because it is nonexistent in the cryptobiotic state.

Rate of ATP decay was examined partly to relate ATP to nematode survival. Standard solutions of ATP were fairly stable at 0 C, and the decay rate did not increase substantially when the temperature was increased to 100 C. However, when live larvae of *A. tritici* were subjected to the lethal temperature of 60 C before sonification, the ATP decay rate was 3 times faster than when similar larvae were sonified and then subjected to 60 C. These results indicate that the death of larvae at 60 C may result directly or indirectly from a rapid depletion of ATP.

As suggested previously (6), the sensitive measurement of ATP has proved useful for studies of nematode survival in active and in hypobiotic states.

LITERATURE CITED

1. ANONYMOUS. 1970. Instruction Manual, 760 Luminescence Biometer, E. I. DuPont De Nemours & Co. (Inc.), Instrument Products Div., Wilmington, Del.
2. ALLEN, P. D., III. 1972. Development of the luminescence biometer for microbial detection. Developments in Industrial Microbiology 14:67-73.
3. CHOW, H. HU, and J. PASTERNAK. 1969. Protein changes during maturation of the free-living nematode, Panagrellus silusiae. J. Exp. Zool. 170:77-84.
4. COOPER, A. F., and S. D. VAN GUNDY. 1971. Senescence, quiescence and cryptobiosis. Pages 297-318 *in* B. M. Zuckerman, W. F. Mai, and R. A. Rohde, eds. Plant Parasitic Nematodes, Vol. 2, Academic Press, London and N. Y.
5. HUSSEY, R. S., and K. R. BARKER. 1973. A comparison of methods of collecting inocula of Meloidogyne spp. including a new technique. Plant Dis. Rep. 57:1025-1028.
6. SPURR, H. W., Jr. 1973. ATP in second-stage larvae of Anguina tritici activated from cryptobiosis. Phytopathology 63:806 (Abstr.).
7. SPURR, H. W., JR., and R. E. WELTY. 1975. Characterization of endophytic fungi in healthy leaves of nicotiana spp. Phytopathology 65:417-422.
8. VAN GUNDY, S. D. 1965. Factors in survival of nematodes. Annu. Rev. Phytopathol. 3:43-68.