IN VITRO CULTIVATION OF ADULT STAGES OF SOME TRICHOSTRONGYLID NEMATODES OF UNGULATES. I. AMINO ACIDS AND WATER-SOLUBLE VITAMINS

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ABSTRACT

The **in vitro** cultivation of parasitic stages of some trichostrongylid nematodes of ungulates in single amino acids indicated that L-leucine, L-alanine, glycine, L-methionine, L-threonine, L-lysine, L-glutamic acid, L-isoleucine, L-proline and L-histidine prolonged the survival times of **Ostertagia ostertagi, Trichostrongylus** sp. and **Nematodirus** sp..

It appears that the concentration of amino acids is as important as the types of amino acids utilized in the culture solutions. A detrimental effect on the worms was noted as the concentration of amino acids was increased beyond six mg per 10 ml of Ringer Locke's solution.

Further observations indicated that egg-laying was more sensitive to stress factors than were the fundamental vital processes of the worms.

Several vitamins appeared to cause an increase in egg production of the female nematodes, but an analysis of variance showed no significant difference between the vitamins used in this respect.

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The control and eradication of helminthic diseases are among the prime concerns of human and veterinary medicine. The present approach to this problem is almost exclusively empirical. It involves wholesale screening of thousands of compounds in order to come up with an active drug. Although this technique is time consuming it has been shown to be effective to some degree with parasitic nematodes. However, this approach does not recognize many aspects of the biology of these organisms. A new approach, based on physiological research, has become popular in recent years and is concerned with the cultivation or prolonged maintenance of helminth parasites *in vitro*. Difficulties have been encountered in culturing many animal parasites due to their complex biological existence, and only recently has advancement been made.

There have been very few experiments concerned with the utilization of amino acids for the production of carbohydrate reserves in parasitic nematodes. Also, very little is known as to which of the amino acids are essential to the survival of these nematodes. Weinstein and Jones (1956a) found that *in vitro* cultivation of *Nippos-*

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trongylus muris required sodium caseinate, serum and liver concentrates as well as the protein of the embryo extract. This suggests that this parasite requires amino acids in its diet.

Cavier and Savel (1954) indicated that the production of protein in the perienteric fluid of *Ascaris lumbricoides* was increased through the addition of glycine, alanine, glutamic acid and tyrosine to the culture medium in the *in vitro* culture of this nematode.

Vitamins are essential in maintaining the metabolic processes at a normal level in practically all living systems. Most animal species require the major vitamins found in the diet simply because they are unable to synthesize them from other food constituents. Certain precursors may suffice in some instances because the animal can convert these to the vitamins. Plants, various microorganisms, and certain species of animals synthesize vitamins, other animals eat the plants and store certain vitamins, and some animal tissues also serve as vitamin sources for other species.

The investigations on the vitamin requirements of parasitic nematodes have been very slender. Weinstein and Jones (1956b) found that the culture media containing several B vitamins and vitamin C were beneficial in the culture of *Nippostrongylus muris.*

The life cycles of the species of worms employed in this study are essentially the same. The eggs develop outside the host into long-tailed, sheathed larvae which are free-living through several molts. The infective third stage larvae gain access to the host by ingestion with vegetation and grow directly to maturity in the abomasum or intestinal tract. All of these worms create a condition known as "verminous gastroenteritis" when present in large numbers.

MATERIAL AND METHODS

Adult worms utilized in this study consisted primarily of *Ostertagia ostertagi, O. circumcincta* and *Cooperia onchophora.* These worms belong to the family *Trichostrongylidae* and are recognizable by the finely drawn-out head with a small buccal capsule together with a well-developed bursa in the male.

Cattle and sheep were the principal animals used as sources of worms throughout this study. There were instances when other species of nematodes were used. Some of these were: *Nematodirus sp.* and *Trichostrongylus sp.*

The incubation temperature was maintained between 36-38°C. as this approximates the body temperature of the animals from which the nematodes were obtained. The various culture solutions were adjusted to within the pH range of 4-5 with a 10% solution of hydrochloric acid.

The culture solutions included single amino acid solutions, several amino acids in solution, and single vitamin solutions.

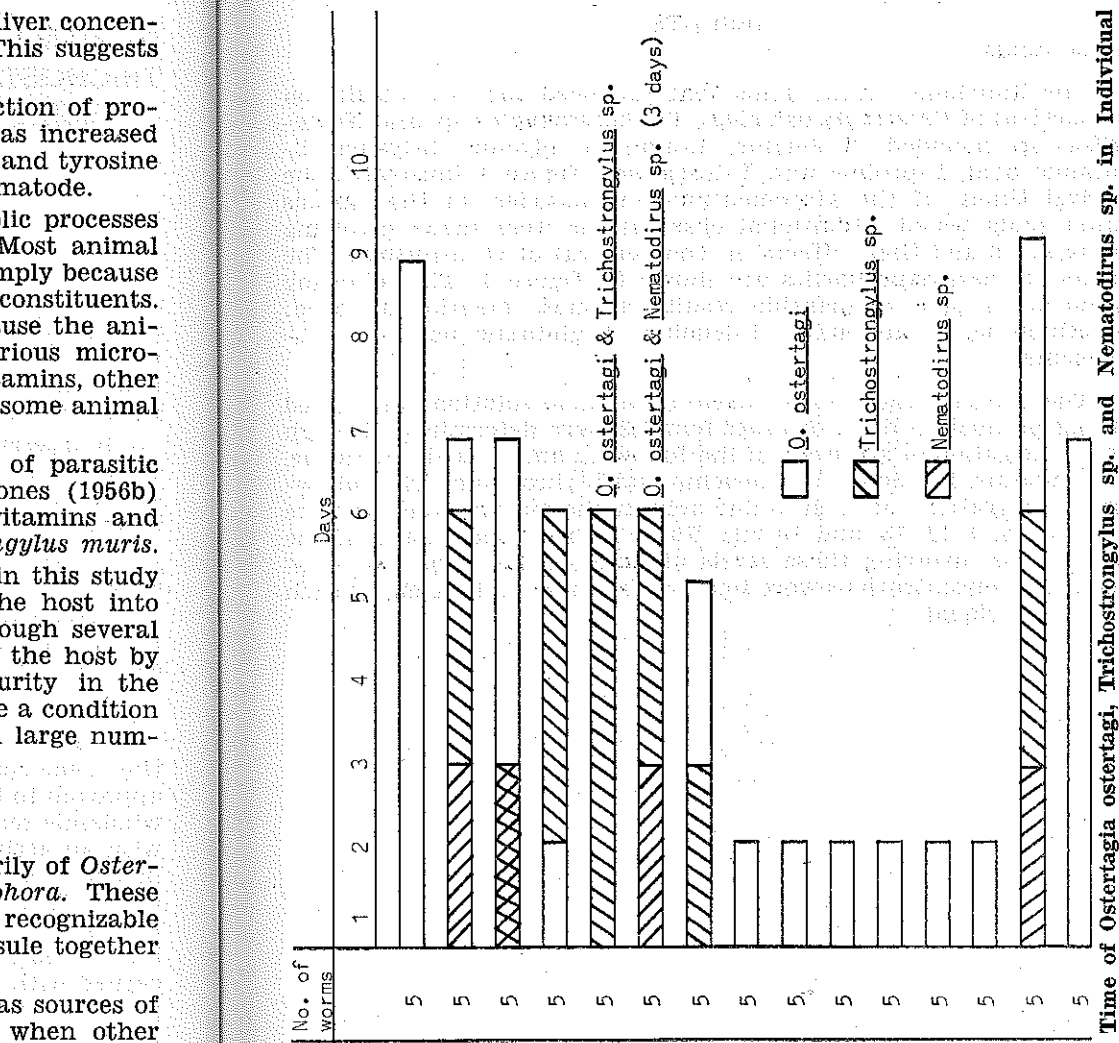

Figure 1. Survival Time of Ostertagia ostertagi, Trichostrongylus sp. and Nematodirus sp. in Individual Amino Acids and Glucose.

RESULTS

Amino Acids.

The individual amino acids that produced the best results on the survival of *Ostertagia ostertagi, Trichostrongylus* sp. and *Nematodirus* sp. included: L-leucine, L-alanine, glycine, L-lysine, L-glutamic acid, L-proline and L-histidine. Figure 1 illustrates the survival times of the aforementioned nematodes in the various amino acids tested. Additional observations were made involving amino acids and their effects on the survival of *O. ostertagi.* The results of these experiments are shown in Figure 2. The following amino acids gave comparable results to those mentioned above: L-methionine, L-threonine, L-leucine, L-glutamic acid, and L-isoleucine.

The concentrations of a mixed amino acid solution best suited for egg production of *O. osteragi* females were determined through the investigation of six levels of the following amino acids: L-valine, L-methionine, L-leucine, L-isoleucine and L-threonine. The following concentrations of each amino acid in the culture solution were tested: 3, 6, 9, 12, 15, and 18 mg. Table I shows the results of the experiments involving these levels of amino acids. The two most successful concentrations were found to be three and six mg of each in 10 ml of liquid.

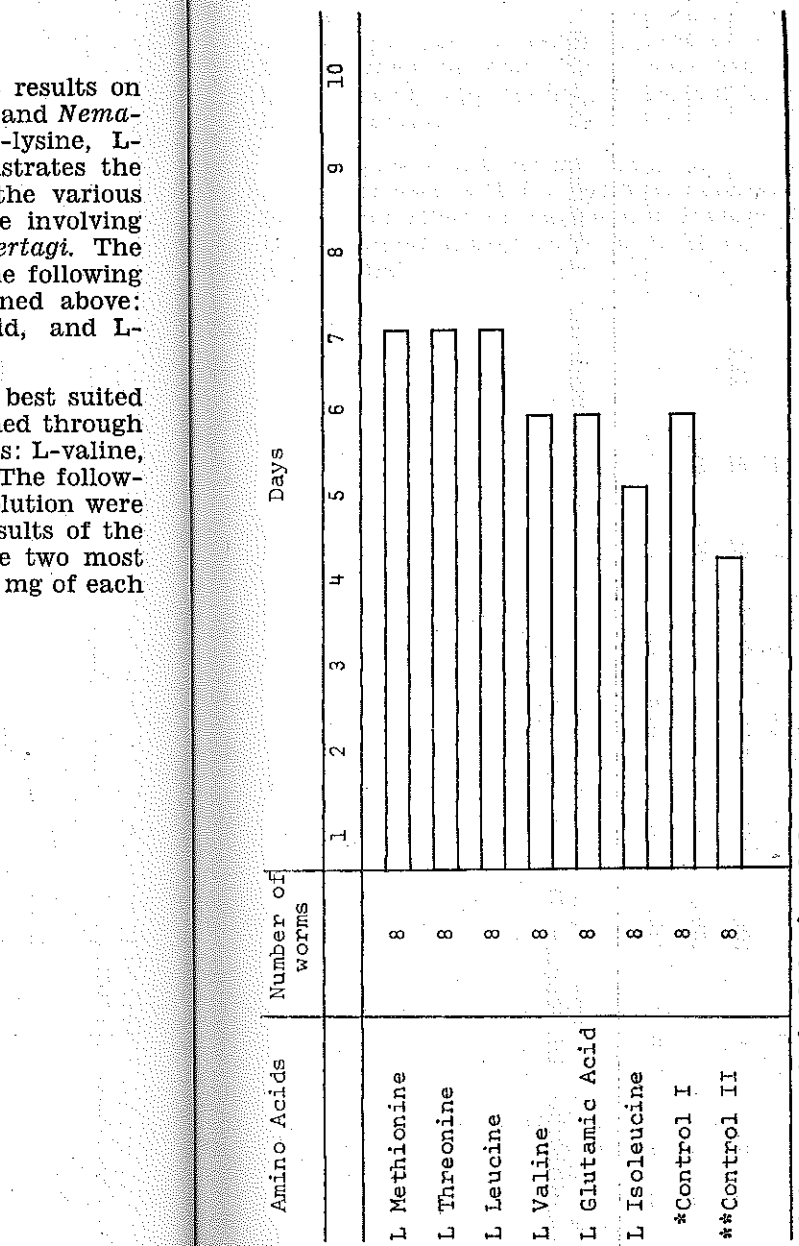

Figure 2. Survival Time of Ostertagia ostertagi in Individual Amino Acids.

TABLE I

PER CENT SURVIVAL OF *OSTERTAGIA OSTERTAGI* ADULTS IN VARIOUS CONCENTRATIONS OF SEVERAL AMINO ACIDS.

mg of Amino acid*	No. of worms	Days 1	2	3	4	5	6	7	8	9	10	11
3	8	75.0	62.5	62.5	50.0	50.0	50.0	50.0	25.0	25.0	25.0	25.0
6	8	100.0	100.0	100.0	100.0	87.5	75.0	62.5	37.5	37.5	25.0	12.5
9	8	100.0	100.0	100.0	87.5	62.5	25.0	12.5	......	......	......	......
12	8	100.0	100.0	100.0	87.5	50.0	25.0	12.5	......	......	......	......
15	8	100.0	100.0	100.0	75.0	25.0	25.0	......	......	......	......	......
18	8	75.0	75.0	75.0	75.0	25.0	12.5	......	......	......	......	......

* Represents mg of each of the following amino acids in each test medium: L-cystine, DL-valine, DL-methionine, DL-leucine, DL-isoleucine and DL-threonine.

It appears that the concentration of amino acids is as important as the type and quantity used in the culture solutions. A detrimental effect on the worms was noted as the concentration of amino acids was increased beyond six mg per 10 ml of Ringer Locke's solution.

The three and six mg amino acid concentrations mentioned above were used with *Cooperia onchophora* females in order to determine their effect on egg-laying. Figures 3-4 demonstrate that egg-laying almost ceased some 35 to 40 hours before one half of the worms died.

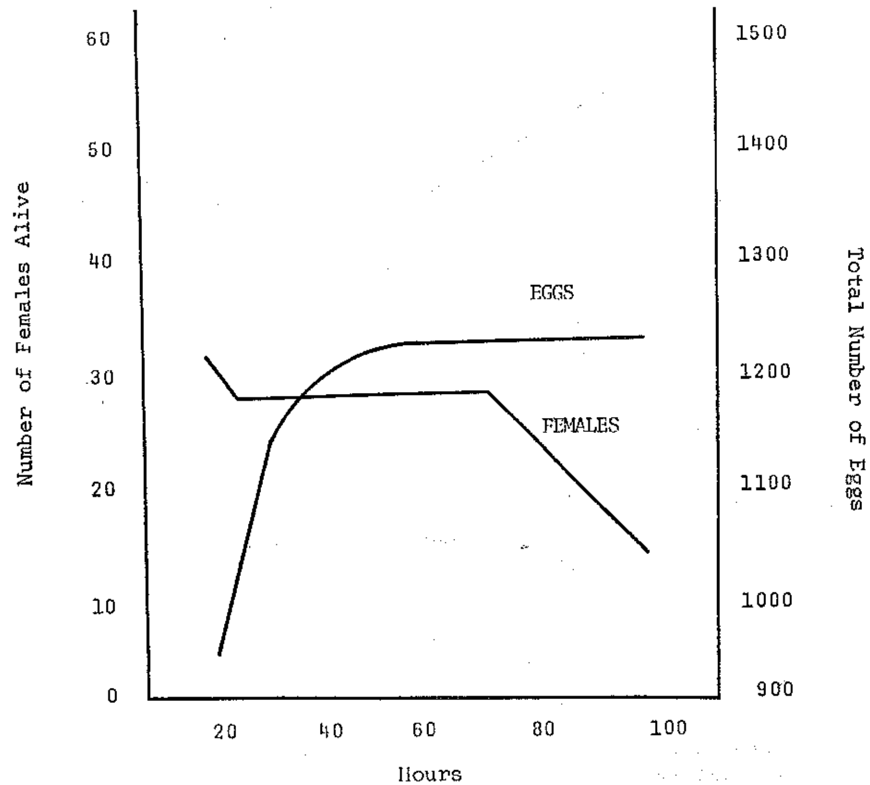

Figure 3. Comparison of Egg-Laying Abilities and Survival Time of Adult Cooperia onchophora Females in a 3 mg. Concentration of Combined Amino Acids.

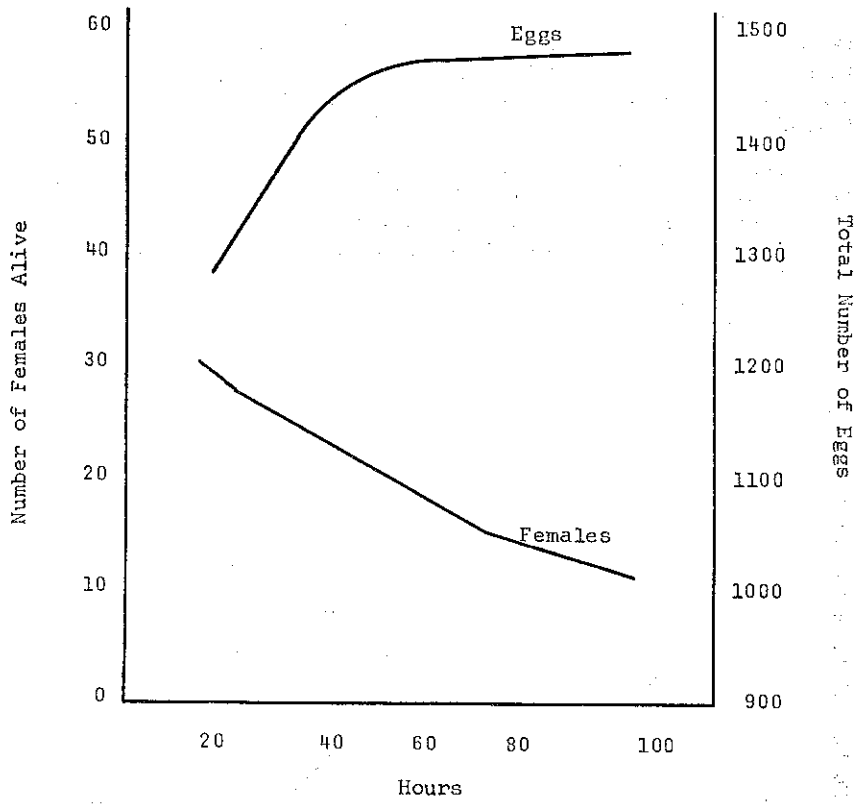

Figure 4. Comparison of Egg-aying Abilities and Survival Time of Adult Cooperia onchophora Females in a 6 mg. Concentration of Combined Amino Acids.

Water-Soluble Vitamins.

The results of the experiments concerned with the egg-laying capabilities of *O. ostertagi* and *O. circumcincta* females in the various water soluble vitamins are shown in Table 2. Although there were indications that several vitamins caused a larger egg production, an analysis of variance showed no significant difference between these vitamins in this respect.

The females that were cultured in nicotinamide produced up to 2,250 eggs while those cultured in riboflavin, thiamine and choline produced 1,305, 1,230 and 1,137 eggs respectively. The worms in all the other vitamins produced considerably fewer eggs. The rates of decrease in egg production in the various vitamin solutions were

approximately the same although the worms cultured in nicotinamide, choline and folic acid did show a less rapid rate of decrease in egg production through 72 hours of observation.

TABLE II

THE EFFECT OF WATER-SOLUBLE VITAMINS ON EGG-LAYING OF *OSTERTAGIA OSTERTAGI* AND *O. CIRCUMCINCTA* FEMALES.

Vitamins	No. of Worms	Egg Production			Total Eggs
		24 Hours	48 Hours	72 Hours	
Nicotinamide	20	2082	122	48	2250
* Control I	20	1381	102	9	1492
Riboflavin	20	1176	115	14	1305
** Control II	20	1234	114	17	1365
Thiamine	20	1109	108	13	1230
Control II	20	1234	114	17	1365
Choline	20	1009	91	37	1137
Control II	20	685	151	15	851
Folic Acid	20	751	205	36	992
Control II	20	685	151	15	851
Pantothenate	20	876	89	7	972
*** Control III	20	804	87	19	920
Biotin	20	361	79	2	942
Control III	20	899	41	2	942
Inositol	20	803	93	13	909
Control II	20	805	117	7	929
Pyridoxal	20	571	92	7	670
Control III	20	804	97	19	920

* Control I—Amino Acids and Ringer-Locke's Solution.

** Control II—Nicotinamide, Amino Acids and Ringer-Locke's Solution.

*** Control III—Nicotinamide, Amino Acids, Folic Acid, Choline and Ringer-Locke's Solution.

DISCUSSION

In this study several amino acids appeared to increase survival of the nematodes investigated. The amino acids listed by Cavier and Savel for *Ascaris lumbricoides* were included in the list of amino acids which increased survival time except L-tyrosine which did not seem to have a beneficial effect on the nematodes. Some amino acids prolonged survival, but there were many instances in which the differences in survival times produced by the various amino acids were very slight. Also, the control, which was a mixture of all the amino acids tested, approximated the best results of individual amino acids. Although the results indicated that some radical differences existed, they did not give a clear indication as to which amino acids were essential.

The information gained from the experiments concerned with the effect of a mixture of several amino acids indicated that egg-laying was more sensitive to stress factors than the fundamental vital processes of the worms. The increased egg production of the females in the various culture media would make it appear that a greater number of viable eggs could be obtained from a female than could be expected from dissection of the uterus. About five fully developed eggs are all that can be dissected from a single female, but through the use of a combination of amino acids this number may be increased from eight to ten times. Since these nematodes produce large numbers of eggs it is evident that they possess remarkable powers of protein synthesis, being able to convert absorbed protein food material into eggs. Yet little is known regarding the protein requirements and metabolism.

It appears that the concentration of amino acids is as important as the type and quantity used in the culture solutions. A detrimental effect on the worms was noted as the concentration of amino acids was increased beyond six mg per trial. Other modifications of the amino acid culture solutions such as the addition of glycerine was observed as being detrimental to both the vital processes and egg-laying capabilities of the nematodes. This would suggest that parasitic nematodes are very sensitive to even slight changes in their environment *in vitro*.

The statistical analysis of the results of the experiments with water-soluble vitamins in this study indicates that either these vitamins are not required by the nematodes in artificial media or that they are able to synthesize the vitamins that are essential to them.

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