Evaluation of culture media for axenic growth of *Romanomermis culicivorax*

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SUMMARY

A method for surface sterilization of large numbers (> 5 000) of preparasites of *Romanomermis culicivorax* is presented. Grace's insect tissue culture medium (GITC) was supplemented with fetal bovine serum and bovine albumin and used as a basal test medium (BB) for *in vitro* culture attempts with *R. culicivorax* preparasites. Experiments were run in 1 ml of test media in sealed glass tubes for 21 days at 27 ± 1° at densities of about 146 parasites/tube. Various modifications of the test medium were evaluated. The mean growth of *R. culicivorax* was measured with the Bradford protein assay and compared to a growth equation attained *in vivo*. One of the best media tested was GITC medium with the salt concentrations altered to that reported for the hemolymph of fourth instars of *Culex pipiens*, the amino acid concentrations based on hydrolyzed female *R. culcivorax* postparasites, and reduced glutathione (200 mg/l) was added (RCg). Average growth in this medium was 944.4 ng protein/parasite which was equivalent to 4.2 days growth *in vivo*. A significant decrease in growth ($P < 0.05$) was noted when parasite densities per tube were less than 71. Deletion of isoinositol and choline chloride did not clearly affect the mean protein per parasite in the various media tested. However, parasites cultured in the absence of these compounds showed more cuticular aberrations. Growth was poorest in medium BB and a medium (CCg) with the amino acid and salt ratios based on hemolymph analysis of fourth instar *C. pipiens* plus the addition of reduced glutathione. Substitution of trehalose for sucrose or the omission of sucrose from GITC medium did not significantly affect the growth of *R. culicivorax in vitro*.

RÉSUMÉ

Évaluation de milieux destinés à l'élevage axénique de Romanomermis culicivorax

Une méthode de stérilisation superficielle convenant à de grands nombres (> 5 000) de larves préparasites de *Romanomermis culicivorax* est présentée. Le milieu de culture de Grace pour les tissus d'insectes (GITC), supplémenté par du sérum de fœtus bovin et de l'albumine bovine, est utilisé comme milieu test de base (BB) pour les essais d'élevage *in vitro* de larves préparasites de *R. culicivorax*. Les expériences ont été conduites en tubes de verre scellés contenant chacun environ 146 parasites dans 1 ml de milieu, pendant 21 jours à 27° ± 1°. Diverses modifications du milieu ont été testées. La croissance moyenne de *R. culicivorax* a été mesurée par le test protéinique de Bradford et comparée à l'équation de croissance obtenue *in vivo*. L'un des meilleurs milieux essayés (RCg) était un milieu GITC dont *i)* la concentration en sel était modifiée pour correspondre à celle de l'hémolymphe du quatrième stade larvaire de *Culex pipiens, ii)* la concentration en amino-acides était fondée sur les hydrolysats de femelles de *R. culicivorax* et *iii)* auquel était ajouté du glutathion (200 mg/l). La croissance moyenne dans ce milieu était de 9 444,4 ng protéines/parasites, ce qui équivaut à une croissance de 4,2 jours *in vivo*. Une diminution significative ($P < 0,05$) de la croissance a été notée lorsque la densité des parasites était inférieure à 71. La suppression de l'isoinositol et du chlorure de choline dans les différents milieux testés ne modifie pas de façon nette le contenu moyen en protéines du parasite. Cependant les parasites élevés en l'absence de ces composés montrent plus fréquemment certaines anomalies cuticulaires. La croissance a été la plus faible dans le milieu BB et dans un milieu (CCg) pour lequel les pourcentages d'amino-acides et de sels étaient fondés sur l'analyse de l'hémolymphe du quatrième stade larvaire de *C. pipiens* et auquel était ajouté du glutathion. La substitution du tréhalose au glucose ou la suppression de ce dernier dans le milieu GITC ne modifie pas significativement la croissance *in vitro* de *R. culicivorax*.

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The mermithid nematode, *Romanomermis culicivorax*, is an obligate endoparasite that spends about eight days of its life cycle in the hemolymph of the larva of a mosquito. *In vitro* culture of this nematode has been actively pursued in recent years for several reasons. First, successful *in vitro* culture might make inundative biological control of mosquitoes by *R. culicivorax* more feasible. Secondly, *in vitro* culture would provide the opportunity for studying mermithid-insect host relationships and for understanding the environmental clues responsible for sex determination in these nematodes. One obstacle to a successful *in vitro* culture media screening program has been the lack of an effective technique for the surface sterilization of large numbers of *R. culicivorax* preparasites. In all previous *in vitro* culture attempts surface sterilization was limited to transfer of individual preparasites through solutions of antiseptics or antibiotics or the collection and surface sterilization of eggs (Roberts & Van Leuken, 1973; Sanders, Stokstad & Malatesta, 1973; Finney, 1977; Castillo, Chin & Roberts, 1982). We report here an effective method for mass surface sterilization of *R. culicivorax* preparasites.

Most of the published *in vitro* culture attempts with *R. culicivorax* have used a variety of combinations of tissue culture media and many defined and undefined supplements (Sanders, Stokstad & Malatesta, 1973; Finney, 1977; Castillo, Chin & Roberts, 1982). *R. culicivorax* cannot be cultured completely in any of the media/supplement combinations that have been tried (Roberts & Van Leuken, 1973; Sanders, Stokstad & Malatesta, 1973; Finney, 1977; Castillo, Chin & Roberts, 1982). Finney (1977) reported the culture of *R. culicivorax* parasites to 12-14 mm in length with a survival rate of 30 % after six weeks in Grace's insect tissue culture medium (GITC) supplemented with 10 % heat inactivated fetal bovine serum (HIFBS). These are the best published results for the *in vitro* culture of *R. culicivorax*. Unfortunately, we have not been able to reproduce the results of Finney (1977) and have found the basal medium of Castillo, Chin and Roberts (1982) with GITC, 20 % HIFBS and 1.5 % (7.5 %) bovine albumin (BA) to be better for the evaluation of substitutions or deletions.

Many of the physical and biochemical properties of the hemolymph of the larvae of *Culex pipiens* and of *in vivo* cultured postparasites of *R. culicivorax* have now been characterized (Schmidt & Platzer, 1980; Giblin & Platzer, 1984; Powers, Platzer & Bradley, 1984; Womersley & Platzer, 1984; Giblin & Platzer, 1986). Since GITC is patterned after the hemolymph of the silkworm, *Bombyx mori* (Wyatt, 1956; Grace, 1962) and large differences exist in the hemolymph composition between dipterans and lepidopterans (Buck, 1953) a reasonable approach to the culture of *R. culicivorax* would be to systematically modifiy GITC to approximate the nutrient parameters of mosquito hemolymph. We report here attempts at the *in vitro* culture of *R. culicivorax* in modified GITC supplemented with 20 % HIFBS and 1.5 % (7.5 %) BA.

Materials and methods

Mass surface sterilization of *R. culicivorax*

R. culicivorax was reared *in vivo* in *Culex pipiens* mosquito larvae as described by Platzer and Stirling (1978). Preparasites were collected and surface sterilized as follows : 1) a storage pan was flooded for 1 hr with dechlorinated tap water (the yield of one pan ranged from 10^5 to 10^6 preparasites); 2) preparasites were passed through a 60 mesh screen; 3) preparasites were concentrated on a 20 µm nitex filter (HD3-20, Tetko, Inc.) supported by a fritted glass funnel; 4) the nitex screen with trapped preparasites was placed in a 100 × 15 mm sterile plastic Petri dish and covered with 2 or 3 ml of 1 % low temperature gelling agarose (Type VII, Sigma Chemical Co.) which had been autoclaved and mixed with the antibiotic-antimycotic mixture of Castillo, Chin and Roberts (1982) at 27°; 5) the agarose was covered with a single layer of sterile Kimwipe®, previously cut to 40 mm diameter, and cooled at 4-5° for 1 min; 6) and additional 30 ml of the 27° agarose + the antibiotic-antimycotic mixture was poured over the filter; 7) 3 to 4 ml of sterile distilled water were aseptically placed on the surface of the congealed agarose and the plate was placed directly under a fluorescent light for 30 min; 8) preparasites were collected aseptically with a plastic serological pipette and dispensed into an autoclaved plastic Millipore® filter apparatus with a 3 µm nitex filter (HD3-5); 9) rinsed three times with a total of 250 ml of sterile distilled water; 10) concentrated on the filter; 11) steps 4-10 were repeated and 12) the clean preparasites were suspended in a measured volume, counted three times in diluted aliquots (volume < 50 µl) and dispensed aseptically into 13 mm × 100 mm sterilized borosilicate glass test tubes. All steps from 4-12 were done in a sterile enclosure (Integrated Air Systems®) and sterile technique was practiced. The average yield of preparasites from a pan with an initial population density of 10^6 nematodes was 3.5×10^4 or 3-4 % of the initial population. The media itself was used for assessing breaks in sterility.

Preparation of media

Grace's insect tissue culture (GITC) medium was formulated as described by Grace (1962) and modified by Gibco Lab. (1982) from frozen stock solutions of salts, amino acids, sugars, organic acids, and vitamins. Antibiotics were not included in any of the media tested. All salts used in test media were from Mallinckrodt® and all other defined media components were of the

Table 1

Composition of defined portion of test media

Test medium	*Code*	*Salts*	*Amino Acids*	*Sugars*	*Vitamins*	*Reduc-tant*	*Organic acids*	*Buffer*
	BB	Gr[2]	Gr	Gr	Gr	—[3]	Gr	H[4]
	BBo	Gr	Gr	Gr	0[5]	—	Gr	H
	CCg	C[6]	Cp[7]	Gr	Gr	g[8]	Gr	H
	CCGo	C	Cp	Gr	0	g	Gr	H
	Bc	C	Gr	Gr	Gr	—	Gr	H
	Bco	C	Gr	Gr	0	—	Gr	H
	Bcg	C	Gr	Gr	Gr	g	Gr	H
	BCgo	C	Gr	Gr	0	g	Gr	H
	BCgh	Ch[9]	Gr	Gr	Gr	g	Gr	H
	BCght	Ch	Gr	Tr[10]	Gr	g	Gr	H
	BCghn	Ch	Gr	ns[11]	Gr	g	Gr	H
	RCg	C	R[12]	Gr	Gr	g	Gr	H
	RCgo	C	R	Gr	0	g	Gr	H

[1] Each test media was supplemented with 20 % heat inactivated fetal bovine serum (Gibco, lots 27K4440, 30N6833, or 31K1435) and 1.5 % of a 7.5 % stock solution of bovine albumin (Sigma).
[2] Gr = Class of nutrients was in the proportion reported for Grace's insect tissue culture medium (GITC) (Grace 1962) as modified in Gibco (1982).
[3] — = not added.
[4] H = Hepes (N-2-hydroxethylpiperazine-N[1] -2-ethanesulfonic acid) was added (25 mM (5.98 g/l) at final volume).
[5] O = Choline chloride and isoinositol deleted.
[6] C = salt concentration based upon analysis of hemolymph of fourth instar *Culex pipiens* (Powers, Platzer & Bradley, 1984).
[7] Cp = amino acid series based upon proportion of free amino acids in hemolymph of fourth instar *C. pipiens* (Schmidt & Platzer, 1980; see Tab. 2).
[8] g = reduced glutathione added (200 mg/l).
[9] Ch = C salts + $NaHCO_3$ (350 mg/l).
[10] tr = trehalose dihydrate (29.98 g/l) was substituted for sucrose in GITC.
[11] ns = sucrose deleted.
[12] R = amino acid series based upon hydrolyzed postparasitic females of *R. culicivorax* (Giblin & Platzer, 1986; see Tab. 2).

highest purity available from Sigma. The test media compositions are reported in Table 1. The *Culex pipiens* salt ratio (C) was based upon the values reported by Powers, Platzer and Bradley (1984) and contained 500 mg/L $CaCl_2.2H_2O$, 1 120 mg/L KCl, 920 mg/L $MgSO_4.7H_2O$, 1 013 mg/L $NaH_2PO_4.H_2O$, and 6 000 mg/L NaCl. The amino acid concentrations used in GITC or to modify GITC are presented in Table 2. Two amino acid concentration series were substituted for GITC amino acids. Namely, the Cp series which was based upon Schmidt and Platzer's (1980) report of the free amino acid composition of fourth instar *C. pipiens* hemolymph (unparasitized by *R. culicivorax)* and the R series which was based upon the amino acid composition of female postparasites of *R. culicivorax* which were reared in *C. pipiens* as reported by Giblin and Platzer (1986). Each test media was made up from frozen concentrated stock solutions. Immediately prior to the final volume adjustment, calcium chloride dihydrate, sodium bicarbonate, tyrosine and cystine (solubilized in 2 and 1N HCl, respectively) Hepes [25 mM at (5.98 g/L) final volume] (N-2-hydroxethylpiperazine-N[1]-2-ethanesulfonic acid), and glutathione were added as required. The media was brought to final volume with sterile distilled water and was filtered through a 0.22 µm filter in a Falcon® disposable filter unit under vacuum. Heat inactivated fetal bovine serum (HIFBS) from Gibco Lots # 27K4440, 30N6833, or

Table 2

Amino acid concentrations for the defined portion of test media and fetal bovine serum (FBS) (mg/l)[1].

Amino acids	*Graces' insect tissue culture medium (Gr)*	Culex pipiens *hemolymph (Cp)*	Romanomermis culivorax *postparasitic females (R)*	*FBS (Gibco)*[2]
L-Arginine	579	533	697	—
L-Aspartate	350	63	433[3]	10
L-Asparagine	350	—	433	—
L-Alanine	225	611	352	111
β-Alanine	200	124	—	5
L-Cystine[4]	22	—	312	—
Glutamate	600	195[5]	609[5]	186
L-Glutamine	600	195	609	37
L-Glycine	650	208	372	54
L-Histidine	2 500	1 575	262	13
L-Isoleucine	50	201	384	22
L-Leucine	75	235	631	36
L-Lysine	500	215	731	37
L-Methionine	50	184	233	4
L-Proline	350	727	471[6]	7
L-Phenylalanine	150	191	394	23
DL-Serine	1 100	2 549	423	30
Taurine	—	68	—	21
L-Tyrosine[7]	50	223[8]	332	17
L-Tryptophan	100	24[8]	100[9]	5
L-Threonine	175	279	542	17
L-Valine	100	274	453	44
Total amino acids (mg/l)[10]	8 776	8 674	8 773	679

[1] Note that in the supplemented media the amino acids contributed by the defined portion are multiplied by 0.785 and the contributions from FBS are multiplied by 0.200 per unit total volume.

[2] Based on free amino acid determinations for FBS (Gibco) (Mitsuhashi, 1982).

[3] The ASX value reported by Giblin and Platzer (1986) for *R. culicivorax* was divided in half and equal amounts of asparagine and aspartic acid were used.

[4] Dissolved in 1N HCl.

[5] The glutamic acid value reported by Schmidt and Platzer (1980) for *Culex pipiens* and the GLX value reported by Giblin and Platzer (1986) for *R. culicivorax* were divided in half and equal amounts of glutamine and glutamic acid were used .

[6] The proline value represents proline + hydroxyproline from the ratio reported by Giblin and Platzer (1986).

[7] Dissolved in 2N HCl.

[8] Tyrosine and tryptophan values for the Cp series were based upon HPLC analysis of the hemolymph of fourth instar *C. pipiens* (Powers & Platzer, unpubl.).

[9] Tryptophan was not quantified in the hydrolysis of *R. culicivorax* postparasites (Giblin & Platzer, 1986). We used the GITC value.

[10] Total amino acids for each of the ratios was adjusted to the total for GITC.

31K1435 was stored at — 20° in the dark until used. A 7.5 % stock Bovine albumin (BA) solution was prepared by slowly mixing BA fraction V powder (catalog # A4503) Sigma in water and filter sterilizing it through a 0.45 µm filter. The defined portion of the test media was supplemented with 20 % v/v HIFBS and 1.5 % v/v of the 7.5 % BA solution. The pH of the supplemental media was adjusted to 6.5 ± 0.5 with 0.22 µm filtered 2N NaOH; pH was measured with Merck® pH strips and only the RCg media required the addition of NaOH (< 0.6 % v/v). Osmolarity of each test media was measured with a Wescor® vapor osmometer (model 5100B) and recorded. No attempts to modify osmolarity were made. One ml of test media was pipetted into a test tube with surface sterilized preparasites. The tube was capped and double sealed with Parafilm®. Tubes were

incubated at 27 ± 1° for 21 days in the dark. Observations for general growth, appearance and breaks in sterility were made weekly or biweekly. Contaminated media appeared cloudy or fungal mycelia were present. Media testing was replicated at least three times. Growth was assessed at the end of each replicated trial by chosing one tube and randomly selecting twenty parasites which were rinsed with *Aedes* saline and transferred to test tubes for NaOH digestion and protein analysis. Digestion and protein analysis were performed as described by Giblin and Platzer (1986) using the Bradford technique with crystalline bovine albumin fraction V (Sigma) as the standard. Protein determination from *R. culicivorax* grown *in vivo* in *C. pipiens* days 0-6 postinfection (PI) (Giblin and Platzer, 1986) were used to generate a equation to compare *in vitro* protein values to *in vivo* protein values to determine relative growth [measured in *in vivo* developmental time (PI)]. The equation generated was $Log_{10}y = 0.4899\ x + 0.9386$, where x = *in vivo* developmental time (PI) and y = ng protein/parasite. The correlation coefficient (r) for this equation was 0.98. *In vitro* growth of *R. culicivorax* was statistically compared after a square root transformation of the data with a general linear models procedure with a Student Newman Keuls multiple range test on SAS.

Table 3

Growth quantification by protein determination of *in vitro* cultured *Romanomermis culicivorax* [2].

	Protein/Parasite [4]			*Parasites*	*Relative* in vivo	
Test Medium[3]	*Mean*	*S.D.*	*Osmolarity* [5]	*Tube*[6]	*Growth*[7]	*N* [8]
BB	344.3	23.0 CD[9]	357	159 ± 25	3.3	3
BBo	262.4	66.6 D	337	109 ± 22	3.0	3
CCg	269.7	13.8 D	410	136 ± 28	3.0	3
CCgo	340.1	17.2 CD	426	124 ± 55	3.3	3
BC	613.9	104.7 BC	417	170 ± 53	3.8	3
BCo	559.9	84.7 BC	426	124 ± 56	3.7	3
BCg	702.3	48.3 AB	425	113 ± 42	3.9	3
BCgo	558.5	45.6 BC	426	122 ± 40	3.7	5
BCgh	745.7	56.1 AB	441	188 ± 100	3.9	3
BCght	772.1	47.6 AB	442	184 ± 54	4.0	3
BCghn	1 004.5	115.2 A	369	134 ± 63	4.2	3
RCg	944.4	32.1 AB	441	157 ± 6	4.2	3
RCgo	1 077.7	145.9 A	445	142 ± 57	4.3	5
RCgo[10]	386.5	53.0 CD	445	46 ± 16	3.4	7

[1] Growth was quantified using a pooled sample of 20 randomly chosen *R. culicivorax* parasites that were NaOH digested and the total protein determined with a Bradford total protein assay. (See text for method).

[2] Surface sterilized preparasites of *R. culicivorax* were cultured in 1 ml of media in borosilicate tubes (13 mm × 100 mm) which were capped and double parafilm sealed and incubated at 27 ± 1° for 21 days.

[3] See Table 1 for legend.

[4] ng total protein/nematode.

[5] mosm/kg, S.D. not greater than 10 for supplemented medium.

[6] Mean ± S.D.

[7] The mean protein value for parasites grown *in vitro* in a designated test medium was used to calculate the *in vitro* growth in *in vivo* developmental time (days postinfection [PI]). See materials and methods for the equation used.

[8] Number of replicates.

[9] Means followed by different capital letters in a column are significantly different ($P < 0.05$).

[10] Parasite density was less than 71 per culture.

Results

Contamination was observed in only four of the 239 test cultures run in this study (1.7 %). This demonstrates that the mass surface sterilization technique reported here is an effective alternative to the tedious task of individually transfering preparasites through antibiotic washes. Surface sterilized preparasites were infective to second instars of *C. pipiens*. Two migrations by preparasites through the agar was essential for the low rate of contamination. A single migration through the agar yielded greater numbers of *R. culicivorax* preparasites but an unidentified mycobacterium frequently produced a 50-100 % contamination rate.

The results of the growth quantifications for axenically grown *R. culicivorax* are presented in Table 3. Most of the parasites grown in the media series BC, BCg, BCgh and RC were moving at 21 days. Some cuticular abnormalities were noted and size was variable, but the parasites appeared healthy with relatively little internal vacuolation and continued to grow and survive for longer than 42 days. Conversely, nematodes grown in the BB and CCg media series were highly vacuolated and appeared lethargic. Molting was not observed and the genital primordium remained undifferentiated in all of the media that were evaluated. Axenic development of *R. culicivorax* appeared to follow Castillo, Chin and Roberts' (1982) description for their *in vitro* culture attempts. However, visual observations of the development of parasites were not routinely made and all axenic growth was quantified relative to the *in vivo* protein growth equation (see materials and methods). The best culture results occurred in media RCg and RCgo where the GITC salt concentration was altered to that reported for the hemolymph of fourth instar *C. pipiens* (C), the amino acid series was changed to that based upon hydrolyzed female postparasites of *R. culicivorax* (R), and reduced glutathione was added. Many of the parasites in media RCg, RCgo, and BCghn achieved lengths of greater than 6.0 mm which would be equal to growth of 5-6 days PI *in vivo*.

There was no consistent trend to the performance of parasites with or without choline chloride and isoinositol. However, there were many more cuticular abnormalities in parasites grown in media without chloride and isoinositol. There was a slight improvement in the mean growth pf parasites exposed to reduced glutathione *versus* those that were not (see BC *vs* BCg in Tab. 3). A significant decrease in the mean growth ($P < 0.05$) was noted when parasite densities per tube were less than 71 (Tab. 3). The addition of bicarbonate did not significantly alter the growth of parasites in media with C salts ($P < 0.05$) (Tab. 3). Substitutions of trehalose for surcrose or the omission of sucrose from GITC did not significantly affect the growth of *R. culicivorax in vitro* ($P > 0.05$). Growth was poorest in media BB and CCg which suggests that the C salt concentrations improves performance over the GITC salt concentration and that the R and GITC amino acid concentrations improve parasite growth over the Cp series.

Discussion

There are a wide variety of techniques available for the surface sterilization of nematodes (see Platzer, 1977 for review). Unfortunately, most of these techniques were unacceptable for the mass surface sterilization of *R. culicivorax*. Our technique takes advantage of the active swimming behavior as well as the increased migration of preparasites in the presence of light and/or heat. The use of low temperature gelling agarose prevents the breakdown of antibiotics and antimycotics used and does not add risk of heat damage to the nematodes. The migration by preparasites through the agarose may help to mechanically clean them. When this technique was used concurrently with the *in vivo* mass rearing procedure for *R. culicivorax* (Platzer & Stirling, 1978) large numbers of surface sterilized nematodes were easily procured for *in vitro* media testing.

Results from this study show significant improvements in the growth of *R. culicivorax* in GITC medium modified with a salt concentration which approximated larval *C. pipiens* hemolymph (Tab. 3). This was not surprising since the GITC salt concentration was high in Mg^+ and K^+ and relatively low in Na^+ as compared with *C. pipiens* hemolymph (Grace, 1962; Powers, Platzer & Bradley, 1984).

The CCg medium, which contained the salt and amino acid ratios from *C. pipiens* was one of the poorest performing media in this study. This shows that *R. culicivorax* is sensitive to altered amino acid concentrations and confirms the preliminary work by Platzer and Giblin (1984). Examination of the amino acid concentrations used in our experiment (Tab. 2) shows that the aspartate, asparagine, glutamate, glutamine, cystine, lysine, and tryptophan levels are much lower for the Cp amino acids than those for the GITC or R series. All of our experiments were done in static conditions with no media changes throughout the 21 day trial. Thus, the only supplemental source for these amino acids would be from free amino acids and from the breakdown of proteins in the FBS or BA supplements. The free amino acid composition of FBS (Gibco) is listed in Table 2 and their contribution to the total media concentration can probably be considered negligible. The static nature of our experiments contrasts greatly with the dynamic nature of the hemolymph of the mosquito host. Protein stores in the hemolymph of *C. pipiens* were depleted by 80 % during parasitism by *R. culicivorax* while amino acid levels were not significantly affected (Schmidt & Platzer, 1980). This suggests a high turnover rate for amino acids in the hemolymph with the parasite acting as a nutritional sink.

Thus, use of the free amino acid ratio from mosquito hemolymph may have failed because of the static nature of the medium and the relatively low levels of the aforementioned amino acids.

Of those amino acids in low availability in the Cp series, aspartate, asparagine, glutamine, glutamate were considered " non-essential " and lysine and tryptophan were considered " essential " to the nematodes, *Caenorhabditis briggsae, Neoaplectana glaseri, Aphelenchoides rutgersi* and *Rhabditis maupasi* (Vanfleteren, 1973; Jackson, 1973; Myers & Balasubramanian, 1973; Brockelman & Jackson, 1978). Interestingly, asparagine has been demonstrated to be an essential amino acid for *C. pipiens* (Dadd, 1978). It would not be surprising to expect differences in the " essential " amino acids between the obligate insect parasitic *R. culicivorax* and the bacterial feeding *C. briggsae, N. glaseri,* and *R. maupasi,* and the fungal feeding *A. rutgersi.* Comparisons of media BCg and RCg, where the differences were the GITC and R amino acid series respectively, showed that the R series gave better growth performance but the difference was not significant ($P > 0.05$). The major difference between the GITC and R amino acid series was that the levels of histidine and serine were lower with concomittant increases in most of the other amino acids in the R series. Histidine was " essential " and serine " non-essential " for growth in *C. briggsae, N. glaseri, R. maupasi,* and *A. rutgersi* (Vanfleteren, 1973; Jackson, 1973; Myers & Balasubramanian, 1973; Brockelman & Jackson, 1978). Histidine produced population increases with *A. rutgersi* with increasing titers to 125 mg/l where reproduction leveled off until 500 mg/l where histidine became inhibitory to reproduction (Myers & Balasubramanian, 1973). Histidine was present at levels of over 1 500 mg/l in both Cp and GITC series but only at 262 mg/l in the R series. This suggests that inhibitory concentrations of certain amino acids might be contributing to differences in the performance of the three amino acid series used here. Deletion work with either the GITC or R amino acid concentrations as a basal medium would seem appropriate for future work.

Even though trehalose is the most prevalent carbohydrate in the hemolymph of larval *C. pipiens* (9 240 mg/l) (Schmidt & Platzer, 1980) there is no evidence to support that disaccharides are available as nutrients for *R. culicivorax.* The mermithid nematode, *Mermis nigrescens,* was able to take-up ^{14}C-glucose but not ^{14}C-trehalose, suggesting that dissacharides cannot be taken up transcuticularly by mermithids (Rutherford & Webster, 1974). Sucrose is the most abundant nutrient in GITC but neither it nor trehalose significantly improved growth of *R. culicivorax.* In fact, the best results were obtained when disaccharides were omitted (Tab. 3). The slight difference in parasite performance may be due to the difference in osmolarity between the media (Tab. 3).

Choline and isoinositol are precursors to phosphatidylcholine and phosphatidylinositol, respectively, which can serve as components of membranes and transport lipoproteins in animals (Lehninger, 1975). Their exact role in *R. culicivorax* is unknown but together or separately they appear to be important in preventing cuticular abnormalities. Both of these components were present in M-10 medium for *A. rutgersi,* Cbmm medium for *C. briggsae,* EM medium for *Turbatrix aceti,* and *Neoplectana glaseri* medium (Platzer, 1977). Choline is considered a requirement for insects while isoinositol is considered " essential " for most insects. Both choline and isoinositol are considered " essential " for *C. pipiens* (Dadd, 1976).

Reduced glutathione (ca 200 mg/l) has been added to the following defined media, M-10, Cbmm, and EM for the axenic culture of nematodes (Platzer, 1977). In addition, certain thiols and disulfides have been shown to promote replication in mouse lymphoid cells *in vitro* (Broome & Jeng, 1973). Platzer and Giblin (1984) reported an improvement of the *in vitro* performance of *R. culicivorax* with 200 mg/l of reduced glutathione. Reduced glutathione improved growth slightly but not significantly in some of the media tested in this paper (Tab. 3). Oxidized glutathione (200 mg/l) and 2-mercaptoethanol (0.01 mM) hampered and improved the mean growth of parasites *in vitro,* respectively. However, these results were not significantly different ($P > 0.05$) from BC medium supplemented with 200 mg/l reduced glutathione (Giblin, unpub. obs.). The role of reduced glutathione or 2-mercaptoethanol in *in vitro* growth of *R. culicivorax* is not clear at this time. Broome and Jeng (1973) showed that under their culture conditions the half lives of added thiols were less than 8 h with less than 7.5 % of the initial thiol remaining in solution after 24 h. Obviously, more work on the effects of reduced glutathione and 2-mercaptoethanol on the *in vitro* culture of *R. culicivorax* is needed.

Most previous studies of the *in vitro* culture of *R. culicivorax* involved less than 25 preparasites per culture (Sanders, Stokstad & Malatesta, 1973; Finney, 1977; Castillo, Chin & Roberts, 1982). This was due to the difficulty in obtaining larger amounts of surface sterilized inoculum. Platzer and Giblin (1984) reported that densities of less than 25 preparasites per culture performed poorly compared with cultures with higher preparasite densities (> 50). Results from Table 3 confirm this apparent density dependent effect in RCgo medium. This effect may be due to detoxification of some medium component(s) by larger numbers of preparasites, production of growth stimulatory factors by large numbers of preparasites, or could be an artifact due to damage incurred during surface sterilization procedures. The latter effect can probably be ruled out since the infectivity of surface-sterilized preparasites for *C. pipiens* was unaffected (see Results).

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