# Axenic Cultivation of *Caenorhabditis briggsae* (Nematoda: Rhabditidae). V. Maturation on Synthetic Media.* (22714)

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The free-living, soil-dwelling, hermaphroditic rhabditid nematode, *C. briggsae*, can mature under axenic conditions on a variety of media containing ingredients of complex, undefined composition—for example, certain liver fractions(1-3), plasma protein fractions (4), chick embryo juice(1), and extracts of equine pancreas, thymus, or spleen(5). Some of these media have also contained a large number of added known substances(5,6), but, until recently, all have included at high levels certain of the complex, crude substances mentioned. However, we have recently recorded (7) success in rearing *C. briggsae* from larva to larva on a defined medium containing only a trace of the standard liver medium used by us in maintenance of stock axenic cultures of this organism. Now we are able to describe limited success following elimination of even this trace, such that *C. briggsae* can be reported as passing one generation on certain media of synthetic composition.

*Materials and methods.* Experiments were conducted as follows: 1) young adult worms were selected from stock axenic cultures maintained in standard liver medium, washed through 4 changes of 1/15 M K phosphate buffer (pH 7) in small watch glasses, and left to lay eggs for approximately 24 hours; 2) larvae hatched by the end of this period were taken up singly or in groups of 2 or 3 by micropipette and inoculated into tubes, 10 mm wide by 75 mm long, each containing 0.2-0.3 ml of liquid medium to be tested—generally only one larva was placed in a tube, 3 tubes of each medium being used; 3) tubes were plugged with cotton, sealed with 2 layers of parafilm, and incubated upright in the dark at 20°C in a constant-temperature incubator; 4) each tube was inspected daily, or every 2 to 3 days during less critical periods, placed almost horizontally on a transparent plastic square (with plugged end of tube elevated slightly) and studied under the dissecting microscope at 19.5 x magnification; 5) with each observation a record was kept, of: a) approximate size of each worm during the first generation; b) time at which the first $F_1$ larva or larvae were seen; c) size range of $F_1$ larvae during the second generation; d) time of appearance of $F_2$ larvae; and e) nature of the ultimate population after several weeks of incubation, determined by total or aliquot counts. We succeeded in rearing worms through one generation on 3 synthetic media, 2 being simplifications of the third. The components of the most complex medium (GM-8) are listed in Table I; its compounding is explained in Table II. The reason for using a considerable number of stock solutions in preparing GM-8 was to permit ready preparation of such simplifications of the latter as are illustrated by GM-9 and -11. Their composition is indicated in footnotes to Table I. Recent experiments leading up to, and including, those in which GM-8, -9, and -11 were tested also included testing of other defined media, the details of which will be reported later. We also tested them supplemented with various levels of standard liver medium, consisting of equal parts of liver pro-

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TABLE I. Synthetic Medium* for *Caenorhabditis briggsae*.

| Components | mM | mg/l | Components | mM | mg/l |
|---|---|---|---|---|---|
| *Amino acids:* | | | *Vitamins and growth factors:* | | |
| ‡L-alanine | 12 | 1070 | N-acetylglucosamine | | 2.0 |
| L-arginine (free base) | 4 | 697 | D-alanine | | 1.0 |
| ‡L-aspartic acid | 10 | 1331 | *p*-aminobenzoic acid | | 1.0 |
| †‡L-cysteine (free base) | 3 | 363 | Biotin | | .5 |
| ‡L-glutamate (monosodium) | 15 | 2537 | Cyanocobalamine | | .5 |
| ‡Glycine | 5 | 375 | Folinate (calcium) | | .5 |
| L-histidine | 4 | 621 | Niacin | | 1.0 |
| L-isoleucine | 8 | 1049 | Niacinamide | | 1.0 |
| L-leucine | 13 | 1705 | Pantetheine | | .5 |
| L-lysinium acetate | 10 | 2062 | Pantothenate (calcium) | | 1.0 |
| L-methionine | 3 | 448 | Pteroylglutamic acid | | 1.0 |
| L-phenylalanine | 10 | 1652 | Pyridoxal | | .5 |
| ‡L-proline | 17 | 1957 | Pyridoxamine | | .5 |
| ‡L-serine | 11 | 1156 | Pyridoxine | | 1.0 |
| L-threonine | 7 | 834 | Riboflavine-5-phosphate (sodium) | | 1.0 |
| L-tryptophane | 2 | 408 | Thiamine chloride • hydrochloride | | 1.0 |
| ‡L-tyrosine | 1 | 81 | §DL-6-thioctate (sodium) | | .5 |
| L-valine | 11 | 1289 | ‡Ascorbic acid | 1 | 88 |
| (Total) | (146) | | | | |
| *Other "structural" compounds:* | | | *Salts:* | | |
| Choline diacid citrate | 2 | 590 | $KH_2PO_4$ | 6 | 817 |
| *i*-inositol • $2H_2O$ | 2 | 432 | KOH | 11 | 617 |
| Nucleic acid substituents | | | Mg • H • citrate • $5H_2O$ | 2 | 610 |
| Adenylic acid | 1 | 347 | $CaCl_2 \cdot 2H_2O$ | 1 | 147 |
| Guanylic " | 1 | 363 | $K_3$ • citrate | 1 | 324 |
| Cytidylic " | 1 | 323 | $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ | .1 | 39.2 |
| Uridylic " | 1 | 324 | $MnCl_2 \cdot 4H_2O$ | .075 | 14.8 |
| Thymine | 1 | 126 | $ZnCl_2$ | .05 | 6.8 |
| | | | $CuCl_2 \cdot 2H_2O$ | .025 | 4.3 |
| *Energy source:* | | | †‡$H_3BO_3$ | .01 | .62 |
| D-glucose | 73 | 13,150 | †‡$(NH_4)_2VO_4$ | .01 | 1.16 |
| | | | †‡$(NH_4)_2MoO_4 + H_2MoO_4$ | .01 | 1.77 |
| | | | †‡KI | .01 | 1.66 |
| | | | *Solvent (for certain vitamins):* | | |
| | | | Triethanolamine | .057 | 8.5 |

* GM-8. † Omitted for GM-9. ‡ Omitted for GM-11. Na • citrate • $2H_2O$ at 5 mM (1471 mg per l) and citric acid at 3 mM (576 mg per l) were added to replace $Na^+$ and acid. § Replaced in GM-9 and -11 by thioctic acid dissolved initially at 20,000× in 5% triethanolamine (TEA).

| Expressed as ions: | mM | | mM |
|---|---|---|---|
| $K^+$ | 20 | $NH_4^+$ | .24 |
| [1]$Na^+$ | 15 | $SO_4^=$ | .2 |
| [2]$PO_4^{\equiv}$ | 10 | $Fe^{++}$ | .1 |
| $Mg^{++}$ | 2 | $Mn^{++}$ | .075 |
| $Cl^-$ | 2 | $Zn^{++}$ | .05 |
| $Ca^{++}$ | 1 | $Cu^{++}$ | .025 |
| | | [3]$Co^{++}$ | .00037 |
| $MoO_4^=$, $BO_3^{\equiv}$, $I^-$, $VO_3^-$ (each) | | | .01 |

[1] As monosodium glutamate.
[2] As nucleotides + $KH_2PO_4$.
[3] As cyanocobalamine.

tein fraction (LPF-C)†, dissolved in 1/15M K • phosphate buffer, and an autoclaved liver infusion or extract (ALE)‡(4) prepared with the same buffer. It was added to the synthetic media at levels from 50% down to 0.032% of the level used for routine stocks, Finally we tested GM-8 supplemented separately with LPF-C and ALE at 50% and at 10% respectively. Tubes containing 100% liver medium without added ingredients were used as controls.

*Results.* GM-8, -9, and -11 by themselves permitted slow maturation of certain

† Dry wt 31.88 mg/ml; N content (by micro-Kjeldahl) 2.488 mg/ml. LPF-C prepared in Aug., 1954, and stored in frozen state.

‡ Dry wt, 49.00 mg/ml; N content, 2.519 mg/ml. ALE prepared on 5-23-55 and stored in frozen state.

TABLE II. Stock Solutions* for Medium GM-8.

| Stock solutions | Concentrations (relative to full-strength basal medium) |
|---|---|
| 1. "Essential" amino acids | 10× |
| 2. "Non-essential" amino acids† | 5× |
| 3. D-glucose | 20× |
| 4. $KH_2PO_4$ | 100× |
| 5. Nucleic acid bases (with KOH) | 20× |
| 6. Choline | 100× |
| 7. *i*-Inositol | 100× |
| 8. Ascorbic acid | 100× |
| 9. Vitamins: Group I | 1000× |
| 10. " II | 1000× |
| 11. " III | 1000× |
| 12. Salts‡: Group I | 50× |
| 13. " II | 1000× |

* All made up with triply distilled $H_2O$, which was also used to bring vol of medium to double strength. Latter then sterilized, usually in 10 ml amounts, by filtration through fritted glass. Stock solutions not obvious from Table I are as follows: 1. 10 amino acids essential to rat. 10. Pyridoxal • HCl, pyridoxine • 2HCl, calcium folinate, d-pantetheine. 11. Vitamins requiring initial dissolving in TEA for 20,000 × concentrated sub-stocks: pABA (5% TEA), PGA (5% TEA), niacin (1% TEA), biotin (1% TEA). 13. Salts of B, V, Mo, and I.

† Cysteine omitted; added as dry powder to final solution.

‡ $CaCl_2 \cdot 2H_2O$ omitted; added as dry powder to final solution. KOH omitted; used with nucleic acid bases.

inoculated larvae, with production of eggs by the resulting adults and hatching of $F_1$ larvae. On GM-11 limited growth of $F_1$ larvae beyond their size when newly hatched also occurred. With addition to these media of liver medium at 0.1%, maturation of the first generation (P) worms was somewhat faster, and some of the resulting $F_1$ larvae reached maturity and produced $F_2$ offspring. With liver medium at 50% the P and $F_1$ generations were passed rapidly, faster than in controls, but more slowly than under previously established conditions(5) in which *C. briggsae* was grown on nutrient agar slants in monoxenic association with *Escherichia coli.*

These trials, in which GM-8, -9, and -11 were tested with supplements of liver medium at 50% and 0.1% of the standard level, have been the only such experiments so far with 2 of the media—namely, GM-9 and -11. GM-8, however, was further tested with supplements at levels of 10%, 3.2%, 1%, 0.32%, and 0.032%. All worms (some 23) that were followed sufficiently long matured and produced $F_1$ larvae in GM-8, supplemented with liver medium at all levels down to 0.1%; but at 0.032% this was true for only one of 3 worms. At all levels except 0.032% some of the larvae of almost all progeny groups matured in turn and produced $F_2$ larvae. Generation I time tended to be progressively longer at progressively lower levels of supplementation with liver medium; and this was more strikingly true for Generation II time.

By contrast with liver medium, its components (LPF-C and ALE), when tested separately, proved far less active as supplements to GM-8. Starting with 3 P larva in each test, we obtained $F_1$ larvae with levels of both 50% [*i.e.*, the level in liver medium] and 10% for LPF-C and ALE respectively. The 50% level of LPF-C also supported the production of an $F_2$, but not the 10% level. By contrast, in the case of ALE the reverse was true, although only one out of the 3 tubes at 10% produced $F_2$ larvae and only in small numbers.

Additional quantitative data are summarized in Table III; and data from the experiments with GM-8 have been used to construct a graph expressing the activity of mixtures of it and successively smaller amounts of liver medium (Fig. 1). The lines shown were calculated by the method of least squares.

TABLE III. Typical Maturation Times in Different Media at 20°C.

| Media | Maturation times in days Generation I | Generation II |
|---|---|---|
| Synthetic media | | |
| GM- 8 | 24 | — |
| GM- 9 | 25 | — |
| GM-11 | 17 | — |
| Liver medium (LM) | 7 | 5 |
| GM-8 + .1% LM | 9 | 19 |
| " + 1.0% " | 9 | 12 |
| " + 50 % " | 4.5 | 4 |
| " + 50% LPF-C | 6 | 5 |
| " + 10% " | 12.5 | — |
| " + 50% ALE | 16 | — |
| " + 10% " | 25 | 27 |
| Associated with *E. coli* on nutrient agar | 3.5 | Not determined |

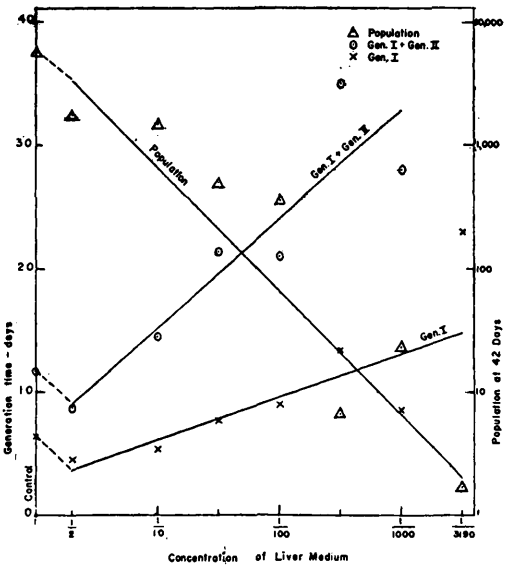


FIG. 1. Maturation time and population level as functions of concentration of liver medium in synthetic medium, GM-8.

*Discussion.* The foregoing results establish 3 important points: 1) it is possible to obtain slow maturation and reproduction of *C. briggsae* in certain synthetic media of known composition; 2) very small amounts of liver medium (0.032% being the lowest tested) are stimulatory to these synthetic media; but 3) liver medium must be present at much higher levels (approaching 50%) before the growth rate is equal to, or greater than, that in 100% liver medium alone.§

Even though GM-8, -9, and -11, when unsupplemented, are not adequate for indefinite cultivation of *C. briggsae,* the fact that they permit maturation and reproduction is a highly significant advance toward an adequate defined, synthetic medium. Numerous earlier attempts(5,6) with other defined media, including certain media used in tissue culture work (*e.g.,* medium 858 of Healy *et al.* (8)) had met with uniform failure; the P larvae grew very little if at all. It was not until media (GM 1-7, of which the ingredients are not given here) approaching GM-8 in composition were tried that significant growth was observed. Relatively little can be said at present as to the essentiality *vs.* non-essentiality of the various ingredients of GM-8, -9, and -11. An important point in this connection is, however, that GM-11, from which the 8 amino acids that are not required by the rat were omitted, supported better growth than GM-8, which had them.

The results with GM media, unsupplemented and supplemented with liver medium, admit of 3 interpretations: 1) the liver medium supplies fundamental growth factors that the organism cannot make, at least in adequate amounts; 2) it provides substances that have no basic metabolic role, but are needed to reverse certain toxic properties of the synthetic media; or 3) it acts in both ways.

Whatever the case may be, we feel it highly probable that a larva as originally inoculated contains a store of some highly potent substance or substances, which permit maturation of the P generation, but are insufficient to allow progress much beyond that point without the presence of liver medium. Moreover, the fact that traces of liver medium can support a relatively rapid Generation I time, yet provide for but slow growth of the $F_1$ larvae with a consequently much prolonged Generation II time, suggests that the original worm can efficiently utilize a trace of liver medium as a source of one or more needed substances, absent or inadequately present in the synthetic media, so long as it has an adequate store of the same or other substances carried over from the egg, but that, with the depletion of the latter store, it has difficulty in deriving its needs from the synthetic media supplemented with but traces of liver medium. Finally, the fact that the liver medium must be present at high levels to permit continued growth at a rapid rate beyond the appearance of $F_1$ larvae may be interpreted to mean that only high levels of liver medium can counteract the effect of depleting the original larva's store of an essential substance, or substances. It is impossible to say at the present time whether the liver medium is itself acting to supply one or more substances

§ We have found the standard liver medium to permit indefinite cultivation of *C. briggsae,* one stock of this organism now being in its fourteenth transfer after 20 months of axenic growth (July, 1956).

that the worm needs in trace amounts and that the liver medium can supply only when present at high levels, or whether some major substituent of the liver medium is needed at high concentrations, or whether both conditions obtain. In other words, it cannot as yet be said whether the liver medium is supplying one or more micronutrients, macronutrients, or both.

The results of separately supplementing the synthetic media with LPF-C and ALE provide important information. Since neither component by itself is nearly so potent as the standard liver medium, which combines the two in equal volumes, there would appear to be 2 requirements, or sets of requirements, involved—both present in each fraction, but one at relatively high levels in LPF-C and the other in ALE. The relative potency of these 2 requirements, or sets of requirements, has not yet been ascertained.

As regards *factor Rb,* a nutritional requirement for *C. briggsae* defined as protein-like and relatively heat-labile(1,7), it appears to be present in both LPF-C and ALE.

*C. briggsae* is the first nematode to be grown on a completely defined medium through an entire generation. Certain nematodes not belonging to the family Rhabditidae are known to be capable of axenic growth on complex media similar to our liver medium—for example, *Neoaplectana glaseri*(9); the vinegar eelworm, *Turbatrix aceti*(10) and the free-living larval stages of the dog hookworm, *Ancylostoma caninum*(11). Recently Weinstein(12) has succeeded in rearing the trichostrongyle, *Nippostrongylus muris,* of the rat to maturity on an even more complex though cell-free medium. All these species belong to the nematode order Rhabditida. It would be of great interest to test them for their capacity to grow on media similar to GM-8, with the ultimate goal (among others) of devising a rational nematocide. We ourselves have tried to grow a plant pathogen, *Ditylenchus dipsaci* (order Tylenchida), on a medium similar to GM-8, supplemented with liver medium at the 10% level, but we observed no growth of the larvae introduced into this medium.

*Summary.* 1. Newly hatched larvae of *Caenorhabditis briggsae* can grow to adulthood and $F_1$ larvae can be produced on a completely defined synthetic medium (GM-8) consisting of 18 L-amino acids, D-glucose, 4 ribonucleotides plus thymine, choline, *i*-inositol, ascorbic acid, 17 "trace" vitamins and growth factors, and a mixture of salts. 2. Media (GM-9 and -11) like GM-8 except for certain omissions (and minor additions to restore acid-base balance and salt levels) also support maturation and reproduction. 3. In the case of GM-11, omitted substances include the 8 "non-essential" amino acids. 4. The presence of a trace amount of the standard liver medium used for the indefinite axenic cultivation of *C. briggsae* is markedly stimulatory to the synthetic media, and some $F_1$ larvae mature and produce $F_2$ larvae when liver medium is present at only 0.032% of its standard strength. 5. For sustained growth, however, *C. briggsae* requires the presence of much higher levels of liver medium—between 10 and 50% of the standard level used for stock cultures. 6. Evidence is here presented that the liver medium contains 2 requirements, or sets of requirements, not present in the synthetic media, at least in adequate amounts or proportions.

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