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The Growth-Promoting Activity of Several Lipid-Related Compounds in the Free-Living Nematode *Caenorhabditis briggsae*[1,2] (40168)

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During the past decade, much progress has been made in the search of growth factors for the free-living nematode, *C. briggsae*. In 1968 Hieb and Rothstein (1) showed a sterol requirement in *C. briggsae*. Hieb, Stokstad and Rothstein (2) further reported a requirement for heme in 1970. These discoveries have led to the use of heme and sterol supplements in a chemically defined medium, the *C. briggsae* Maintenance Medium (CbMM) (3), for the cultivation of *C. briggsae*. For many years another unidentified growth factor has been known to be required for *C. briggsae*. A variety of proteinaceous sources has been used to provide this unidentified growth factor(s), including commercially available products such as soy peptone, egg albumin (4), and casamino acids (5), "defined" proteins such as insulin and TMV-protein (6), and yeast ribosomes (7). However, attempts at isolating the unidentified growth factor(s) from the proteinaceous sources have not been successful as yet. Vanfleteren (8) has recently suggested that an acid-precipitated hemin could replace the proteinaceous growth factor in *C. briggsae* due to the stimulation of phagocytosis by the precipitates. Also, Pinnock, Shane and Stokstad (9) reported that a dipeptide (leu-phe) can partially replace the growth factor activity of casamino acids (acid hydrolysate of casein).

This paper reports a new approach to the study of the unidentified growth factor(s) in *C. briggsae*, in which the activity of several lipid-related chemical compounds was investigated. It was discovered that individual compounds such as Tween 80, Tween 85, sodium oleate, sodium stearate, ethanol, n-propanol, and potassium acetate can greatly stimulate population growth in *C. briggsae* in place of the "proteinaceous" source. These lipid-related compounds are found to be even more potent than casamino acids in the basal medium.

Materials and methods. The experimental medium (5 ml) contained single-strength CbMM (3) (obtained in double strength from the Grand Island Biological Co., Grand Island, NY), 50 μg/ml cytochrome c (Sigma Chemical Co., St. Louis, MO), 50 μg/ml β-sitosterol (Sigma), 1.3 mg/ml Tween 80 (Sigma), and growth factor supplements of various concentrations. Tween 80 was routinely used as the emulsifying agent in dissolving β-sitosterol. Cytochrome c and β-sitosterol were sterilized by millipore filtration (0.22 μm pore size, Millipore Filter Corp., Bedford, MA). In order to minimize the pipetting procedure and also eliminate pipetting variations, a preliminary mix was first prepared for each experiment. The preliminary mix contained the components which were used in all experimental media: CbMM, cytochrome c and β-sitosterol. Individual supplements at various concentrations were then combined with the preliminary mix to constitute 5 ml of media in each assay tube.

Individual supplements to the basal medium included Tween 80 (polyoxyethylene sorbitan mono-oleate, Sigma), Tween 85 (polyoxyethylene sorbitan tri-oleate, Sigma), sodium oleate (Sigma), sodium stearate (Sigma), sodium linoleate (Sigma), potassium acetate (Mallinckrodt Inc., St. Louis, MO), casamino acids (acid-hydrolyzed casein, Difco Laboratory, Detroit, MI), and short-chain carbon chemicals (methanol, ethanol, *n*-propanol, acetone, butanol and ethylacetate). Tween 80, sodium oleate, and sodium linoleate were dissolved in water and sterilized by millipore filtration. Tween 85 and sodium stearate were not completely soluble in water and therefore were sterilized by autoclaving at 121° for 15 min. Casamino acids

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and potassium acetate, although readily dissolvable in water, were routinely prepared in large quantity and sterilized by autoclaving. Analytical or reagent grades were used for all short-chain carbon chemicals (i.e., methanol, ethanol). Ethanol was redistilled before use. Being volatile in nature, these chemicals were added to a volume of presterilized water to constitute a 40 mg/ml working solution.

The free-living nematode *C. briggsae* was maintained in a stock medium (SP-YE-HLE) containing 4% soy peptone, 1% yeast extract and 10% heated liver extract (10). Nematodes from the stock medium were washed twice in deionized water, harvested by centrifugation (Precision, counter-top model) and then resuspended in single-strength CbMM as an inoculum. A modified mass culture bioassay based on procedures suggested by Tomlinson and Rothstein (10) was used in all the experiments of this study. *C. briggsae* were cultivated in 18 × 150 mm culture tubes. The culture tubes were then rotated on a tissue culture rotator at 1 rpm and 20°. The initial nematode population in each tube was approximately 500–900 nematodes(Nema)/ml. The maximum population was reached in approximately 4 weeks.

Results. As shown in Table I, when 5.0 mg/ml casamino acids were present in the basal medium the population growth was stimulated threefold. However, when casamino acids were replaced by Tween 80 in the basal medium at the same concentration (5.0 mg/ml, 3.8 m*M*), a fourfold increase in population growth was observed. Furthermore, a maximum of 8-fold increase in population growth was observed with 10 mg/ml (7.6 m*M*) Tween 80 in the basal medium, showing that Tween 80 was more potent than casamino acids in stimulating population growth. Table I also indicates that when sodium oleate at 1.0 mg/ml (3.3 m*M*) replaced casamino acids in the medium, population growth increased greatly to 21-fold. Thus, sodium oleate was even more active in stimulating population growth than Tween 80 on both weight and molar basis. These findings suggest that oleate was the active moiety for population growth in *C. briggsae*. It was also observed that sodium stearate at 1.0 mg/ml (3.3 m*M*) stimulated similar population growth to that of sodium oleate. However, sodium linoleate at 0.10 mg/ml (0.33 m*M*) stimulated only a fivefold increase in population growth. Linoleate was less active than oleate both on weight and on molar basis, and was toxic for *C. briggsae* at 0.50 mg/ml

TABLE I. EFFECT OF CASAMINO ACIDS, TWEEN 80, SODIUM OLEATE, SODIUM LINOLEATE AND SODIUM STEARATE ON POPULATION GROWTH IN *C. briggsae*.

Supplements	Concentrations (mg/ml)	Concentrations (m*M*)	Average population
			(10^3 Nema/ml, 28 Days)
None	0	0	6.6 (6.3, 7.0)
Casamino acids	5.0	—	22 (21, 23)
Tween 80	2.0	1.5	13 (11, 14)
	5.0	3.8	28 (27, 28)
	10	7.6	52 (54, 50)
	20	15	37 (41, 33)
Na-oleate	0.10	0.33	51 (49, 53)
	0.50	1.6	71 (71, 71)
	1.0	3.3	141 (139, 143)
	2.5	8.2	0 (0, 0)
Na-linoleate	0.10	0.33	35 (47, 23)
	0.50	1.7	11 (14, 8)
	1.0	3.3	0 (0, 0)
	2.5	8.3	0 (0, 0)
			(10^3 Nema/ml, 22 Days)
None	0	0	8.0 (8.1, 7.9)
Casamino acids	5.0	—	45 (41, 49)
Na-stearate	0.10	0.33	102 (93, 111)
	0.50	1.6	112 (111, 114)
	1.0	3.3	147 (150, 143)
	2.5	8.1	81 (80, 82)

(1.7 m*M*) or higher.

In order to verify the role of oleate in stimulating population growth in *C. briggsae*, the growth effect of Tween 80 and Tween 85 was compared with that of casamino acids in Table II. Both Tween 80 and Tween 85 were again more active than casamino acids at the same concentration (5.0 mg/ml). Tween 85, which contains 3 moles of oleate, was significantly more active than Tween 80 (containing 1 mole of oleate) at all concentration levels tested. These results confirmed the active role of oleate in stimulating population growth in *C. briggsae*.

A group of short carbon-chain compounds from C_1 to C_4 were tested for growth-promoting activity in the media containing casamino acids. The results are shown in Table III. It was observed that both ethanol and *n*-propanol were very active at 4.0 mg/ml (87 m*M*), stimulating a fivefold increase in population growth. A slight increase in population growth was also observed with acetone at 4.0 mg/ml (69 m*M*). However, butanol was found to be toxic at 4.0 mg/ml (67 m*M*) or higher. These findings indicate that simple alcohols, especially ethanol and n-propanol, can play an active role in stimulating population growth in *C. briggsae*.

To verify the findings of Table III, the growth-promoting activity of ethanol was further tested in the medium containing no casamino acids, as shown in Table IV. It was observed that ethanol at 4.0 mg/ml (87 m*M*) again stimulated a fourfold increase in population growth even in the absence of casamino acids in the medium. Also, ethanol at 4.0 mg/ml stimulated more population growth than casamino acids at 5.0 mg/ml. However, a drastic reduction in population occurred at 16 mg/ml (34 m*M*). The toxicity of ethanol at 16 mg/ml was more severe in the medium containing no casamino acids than in the medium containing 5.0 mg/ml casamino acids (see Table III).

The growth-promoting activity of acetate, an oxidized form of ethanol, was also tested in *C. briggsae*. Various concentrations of potassium acetate were tested in the medium containing 5.0 mg/ml casamino acids and in the medium containing no casamino acids, as shown in Table V. It was observed that in the medium containing no casamino acids, po-

TABLE II. EFFECT OF TWEEN 80 AND TWEEN 85 ON POPULATION GROWTH IN *C. briggsae*.

Supplements	Concentrations (mg/ml)	Concentrations (m*M*)	Average population (10^3 Nema/ml, 29 Days)
None	0	0	12 (11, 12)
Casamino acids	5.0	—	89 (87, 91)
Tween 80	1.0	0.76	43 (58, 27)
	5.0	3.8	117 (118, 115)
	10	7.6	123 (122, 124)
	20	15	130 (135, 124)
Tween 85	1.0	0.55	105 (105, 104)
	5.0	2.7	150 (130, 170)
	10	5.5	250 (260, 240)
	20	11	190 (180, 200)

TABLE III. EFFECT OF CERTAIN SHORT CARBON-CHAIN COMPOUNDS ON POPULATION GROWTH IN *C. briggsae* IN THE MEDIUM CONTAINING CASAMINO ACIDS.[a]

Supplements	Concentrations (mg/ml)	Concentrations (m*M*)	Average population (10^3 Nema/ml, 27 Days)
None	0	0	40 (36, 44)
CH_3OH	4.0	130	49 (48, 49)
	8.0	250	45 (47, 42)
	16	500	30 (28, 31)
C_2H_5OH	4.0	87	210 (210, 210)
	8.0	170	170 (170, 170)
	16	350	48 (50, 46)
C_3H_7OH	4.0	67	190 (180, 190)
	8.0	130	50 (48, 52)
	16	270	0.5 (0.5, 0.4)
$(CH_3)_2CO$	4.0	69	71 (68, 73)
	8.0	140	43 (42, 44)
	16	280	32 (31, 33)
$CH_3(CH_2)_2OH$	4.0	67	0.6 (0.6, 0.5)
	8.0	130	0 (0, 0)
	16	270	0 (0, 0)
$CH_3COOC_2H_5$	4.0	46	46 (51, 40)
	8.0	91	42 (45, 38)
	16	180	39 (40, 38)

[a] 5.0 mg/ml casamino acids.

TABLE IV. EFFECT OF ETHANOL ON POPULATION GROWTH IN *C. briggsae*.

Supplements	Concentrations (mg/ml)	Concentrations (m*M*)	Average population (10^3 Nema/ml, 27 Days)
None	0	0	8.7 (8.8, 8.5)
Casamino acids	5.0	—	28 (30, 26)
Ethanol	0.50	11	16 (14, 18)
	1.0	22	17 (19, 14)
	2.0	44	23 (20, 25)
	4.0	87	37 (38, 35)
	8.0	170	34 (31, 36)
	16	350	1.4 (1.4, —)

tassium acetate at 5.0 mg/ml stimulated an 11-fold increase in population growth. When casamino acids at the same concentration (5.0 mg/ml) were added to the basal medium (containing no acetate), an eightfold increase in population growth was observed. Thus, potassium acetate was more potent in stimulating population growth in *C. briggsae* than casamino acids. However, the presence of casamino acids in the medium further stimulated a slight population growth at 1.3–10 mg/ml potassium acetate.

Discussion. It is known that higher animals cannot synthesize certain essential polyunsaturated fatty acids. The metabolic blockage occurs between oleic acid (one double bond) and linoleic acid (two double bonds). Thus, linoleic acid is a dietary essential for most higher animals. Our experimental results (Tables I and II) strongly indicated that the fatty acid oleate (or stearate) was more active for population growth in *C. briggsae* while linoleate was less active on both weight and molar basis. These results clearly suggested that the free-living nematode *C. briggsae* can better utilize fatty acids with one double bond (or saturated fatty acids) than fatty acids with two double bonds required by higher animals.

Our results with oleate and acetate also suggested a nutritional interrelationship between these two nutrients in *C. briggsae*. It has been reported (11) that *C. briggsae* can synthesize oleic acid and polyunsaturated fatty acid from acetate-2-^{14}C. Our results indicated that when oleate alone was added to the medium, the population growth was significantly increased (Table I). This suggested that the endogenous acetate (i.e., breakdown products from glucose or amino acids) is not sufficient to meet its requirement in *C. briggsae*. It is also known that fatty acids can be metabolically broken down to acetate by β-oxidation. On molar basis, 1 mole of oleate, theoretically, can be broken down to 9 moles of acetate. Therefore, oleate at 3.3 m*M* is equivalent to approximately 30 mM acetate. Our results indicated that oleate at 3.3 m*M* (Table I) and acetate at 13–150 m*M* (Table V) both supported a maximum population growth in *C. briggsae*. These results suggested that oleate and acetate can be nutritionally mutually replaceable in *C. briggsae*.

TABLE V. EFFECT OF POTASSIUM ACETATE ON POPULATION GROWTH IN *C. briggsae* WITH AND WITHOUT CASAMINO ACIDS (CAA) IN THE MEDIUM.

K-acetate[a]		Average population	
(mg/ml)	(m*M*)	(10^3 Nema/ml, 27 Days)	
		−CAA	+CAA (5.0 mg/ml)
0	0	6.2 (6.6, 5.7)	52 (56, 48)
0.63	6.3	45 (45, 44)	74 (76, 71)
1.3	13	66 (68, 63)	74 (75, 73)
2.5	26	67 (58, 76)	77 (77, 76)
5.0	51	70 (68, 72)	98 (111, 84)
7.5	76	73 (80, 66)	96 (89, 102)
10	100	74 (77, 71)	80 (78, 82)
15	150	68 (80, 56)	59 (—, 59)
20	200	50 (47, 52)	44 (48, 40)

[a] The concentration of K^+ in CbMM is 530 μg/ml which was adequate for supporting the maximum population in *C. briggsae* (unpublished data).

Our findings with simple alcohols (ethanol and *n*-propanol) and potassium acetate also suggest that two-carbon fragments are required for population growth in *C. briggsae*. The two-carbon fragments may have played more than a simple role in stimulating population growth. Previous studies have shown evidence that *C. briggsae* possesses the glyoxalate pathway (12, 13) which converts acetate (or simple alcohols) to oxaloacetate, which could be further utilized to synthesize other cell components. The glyoxalate cycle is a very important pathway to some organisms which use acetate as the sole source of carbon. The two-carbon fragments may have provided the building blocks for the synthesis of some essential metabolites in *C. briggsae*. On the other hand, acetate may also serve as a more readily available energy source than glucose (1.3 mg/ml in CbMM) for the nematode.

It was found (14) that 3% acetic acid which was routinely included in the stock medium for the vinegar eel, *T. aceti*, was actually required for the population growth rather than just lowering the pH of the medium to 3.4. When the pH of the medium was adjusted to 3.4 with HCl, no significant growth was observed. However, when ethanol (2.5%) was added in the medium in place of acetic acid, a moderate growth was observed. This might well be the reason that *T. aceti*, in the presence of acetic acid, is not known to require a "proteinaceous" growth factor for population growth (15). However, the quantitative requirement of acetic acid in *T. aceti*

was never further studied.

Vanfleteren (8) reported that an acid-precipitated hemin had unknown growth factor activity due to the stimulation of phagocytosis by the precipitates. The author further claimed that the unknown growth factor in *C. briggsae* was not a particulate protein but a particulate hemin (16, 17). It should be noted, however, when a nutrient was precipitated in the medium, the concentration of the nutrient was locally increased in the solution. The growth-promoting activity of the precipitated hemin might be related to the more condensed form of hemin which became more available to the worms. Also, it might have adsorbed some other nutrients from the medium (e.g., Tween 80 used in sterols preparation), thus providing a more concentrated form of food for the nematode.

The results from our experiments with fatty acids and two-carbon fragments have made possible the development of a completely chemically defined medium for the continuous cultivation of *C. briggsae*. In the chemically defined medium containing sodium oleate (0.50 or 1.0 mg/ml sodium oleate + *C. briggsae* Maintenance Medium + 50 μg/ml cytochrome c + 50 μg/ml β-sitosterol), *C. briggsae* was cultivated through eight serial subcultures with an average population of 80,000–120,000 Nema/ml in 4 weeks. In the chemically defined medium containing potassium acetate (5.0 mg/ml potassium acetate + *C. briggsae* Maintenance Medium + 50 μg/ml cytochrome c + 50 μg/ml β-sitosterol), *C. briggsae* have been cultivated through four serial subcultures with an average population of 60,000–70,000 Nema/ml in 4 weeks. The nematode subcultures cultivated in these two chemically defined media appeared to be very healthy and the average size of the worms was much larger than those cultivated in the medium with casamino acids. Therefore, a proteinaceous factor is no longer necessary. It is our hope that the availability of this completely chemically defined medium will open many doors of nutritional and biochemical studies on the free-living nematodes.

Summary. The growth-promoting activities of a number of lipid-related chemical compounds were studied in *C. briggsae*. It was found that several such compounds, Tween 80 (20 mg/ml), Tween 85 (10 mg/ml), sodium oleate (1.0 mg/ml), sodium stearate (1.0 mg/ml), ethanol (4.0 mg/ml), *n*-propanol (4.0 mg/ml), and potassium acetate (5.0 mg/ml), greatly stimulated population growth in *C. briggsae* and were much more potent than a casein hydrolysate (casamino acids). These findings have led to the recognition of a lipid-related factor for *C. briggsae* and to the successful development of a completely chemically defined medium for the cultivation of *C. briggsae* without the presence of a proteinaceous factor.

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