Glycogen as a Supplement in Media for Axenic Cultivation of Nematodes (35786)

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Free-living nematodes have been cultured continuously under axenic conditions for several years (1). The culture medium consists of a chemically defined portion (2) and an organic supplement. All supplements reported thus far, such as liver growth factor (3) and serum γ-globulin (4) are proteinaceous and heat-labile. We now report the use of a polysaccharide, glycogen, as an effective heat-stable supplement.

Materials and Methods. The chemically defined medium, *Caenorhabditis briggsae* maintainance medium (2) (CbMM), was obtained from Grand Island Biological Company, Grand Island, New York.

Samples of purified beef liver glycogen were obtained from Nutritional Biochemical Corporation (NBC), Cleveland, Ohio; Calbiochem, Los Angeles, California; and Fischer Scientific Company, New York, New York. They were prepared for use by dry sterilization at 115° for 4 hr or by autoclaving an aqueous suspension of 200 mg/ml for 20 min at 121°. In some cases the complete culture medium was autoclaved.

Hemin chloride (5) (Calbiochem) and cytochrome *c* (5) (NBC) were prepared as 1 mg/ml solutions and sterilized by Millipore filtration. They were added to a concentration of 1.2×10^{-2} m*M* in the assay; *i.e.*, hemin, 8 μg/ml, or cytochrome *c*, 160 μg/ml.

The glycogen samples were checked by the method of Lowry *et al.* (6) to determine if there was a possibility of small residual amounts of protein being present. A high degree of turbidity, measured by absorbancy at 550 mμ (7), prevented valid measurement of Lowry color at 750 mμ. An approximate measurement, however, was made by visual comparison. Similarly, any direct measurement of possible protein by absorbancies at 280 and 260 mμ were invalidated by the turbidity. This turbidity persisted even after centrifugation at 48,000*g* for 30 min.

As a further check ponceau S and nigrosin were used to estimate the amount of staining material in glycogen as compared with diluted samples of human serum. Ten μl of serial dilutions were applied to Millipore Phoroslides; they were stained for 10 min in ponceau or overnight in nigrosin.

Biological activity of the medium was determined as previously described (8) using *Caenorhabditis briggsae* as the assay organism. The response of the nematode is expressed both by the number of days required for a complete reproductive cycle (generation time) and the population after a given period.

Results and Discussion. Glycogen proved to be an effective supplement to CbMM containing hemin in promoting the growth and reproduction of *C. briggsae* (Table I). Either dry heat or autoclaving could be used for sterilization. Although the highest populations were obtained with NBC and Calbiochem samples, the Fischer sample was more active at low concentration. Autoclaving the complete medium, a convenient method of preparation, decreased activity. The medium was inactive in the absence of hemin, but this could be replaced by cytochrome *c* of equal molarity. The medium was not improved by the addition of cholesterol at 5 μg/ml.

Solutions of glycogen from the three sources differed. Samples obtained from Calbiochem and NBC produced solutions that were turbid and distinctly yellow, while the solution of Fischer glycogen was clearer and almost colorless. At $OD_{550m\mu}$ the absorbancies of autoclaved samples at 200 mg/ml in water

TABLE I. Response of *C. briggsae* to Defined Medium with Hemin Supplemented with Glycogen.

Glycogen (mg/ml)	Source[a]	Method of sterilization[b]	Generation time (days)		Maximum population at 30 days (nematodes/ml)
100	N	A	8	11	200
	C	A	7.5	8.5	3200
	F	A	12	12	800
	N	D	7.5	8.5	3200
	F	D	8	8.5	330
	F	A[c]	15	15	270
20	N	A	13	17	240
	N	D	10	11	800
	F	D	9	9	310
4	N	A	18	18	80
4[d]	N	A	18	18	80
4	N	D	12	15	240
	F	D	11	12	85
0.8	N	A	23	25	40
	N	D	17	20	48
0.2	N	D	40	nm[e]	12
	F	D	17	27	30

[a] Source of glycogen samples: N = Nutritional Biochemical Corp., F = Fischer; C = Calbiochem.

[b] A = autoclaved; D = dry heat.

[c] Complete medium autoclaved; hemin at 20 μg/ml.

[d] Supernatant fluid of glycogen centrifuged at 48,000*g* for 30 min prior to addition to medium.

[e] Nonmaturing.

were: Calbiochem, 0.92; NBC, 0.80; Fischer, 0.41. At 100 mg/ml in CbMM + hemin, using CbMM + hemin as a reference blank, the difference was even more striking; absorbancies at $OD_{550m\mu}$ were: Calbiochem, 0.82; NBC, 0.77; and Fischer, 0.27.

As with growth factor, the activity of glycogen is associated with the precipitated portion of the medium (9). This was shown by centrifuging an aliquot of the complete medium containing autoclaved glycogen. The medium was assayed before centrifugation; following which both the supernatant fluid and the resuspended pellet were assayed. The results are shown in Table II. On centrifuging glycogen autoclaved alone a small amount of yellow material was removed, but there was no discernible loss of turbidity, Lowry color, or biological activity (Table I, footnote *d*). The precipitate required by *C. briggsae* appears therefore to be formed by an interaction between the glycogen and medium components.

Assay of the Calbiochem sample was reported as 0.1% N. Data could not be obtained for the other samples. A visual estimate of the Lowry color formed in the turbid samples

TABLE II. Association of Activity with the Precipitated Portion of the Medium.[a]

Medium	Generation time (days)	Maximum population at 20 days (nematodes/ml)
a. Before centrifugation	10 15	150
b. After centrifugation		
Supernatant fluid	Nonmaturing	12
Pellet	6.5 6.5	400

[a] The complete medium containing CbMM, hemin 10 μg/ml and autoclaved glycogen (Fischer) 100 mg/ml. An aliquot of the complete medium was centrifuged at 30,000*g* for 30 min at 4° under aseptic conditions. The supernatant fluid was assayed as recovered; the pellet was resuspended in CbMM to the original volume and then assayed.

indicated a maximum protein content of 0.6% in the Calbiochem and NBC materials and 0.1% in the Fischer sample. Staining tests with ponceau and nigrosin indicated the same approximate value. It should be pointed out, however, that these tests are not specific for protein. Further attempts at purification of glycogen by precipitating with alcohol from an acid solution did not change the Lowry color; enzymatic treatment with pronase followed by dialysis did not decrease the staining reaction.

Our conclusion is that glycogen provides nutrient in the proper particulate form. The possibility of protein in trace amounts in the glycogen could not be proved but it is unlikely that it would account for the biological activity since the maximum possible levels are too low. With our best protein supplement, activated growth factor, a minimum of 1.2 μg/nematode was required for maturation. At 200 μg/ml with Fischer glycogen the maximum possible protein that could be present is less than 2% of this minimum requirement.

C. briggsae has been maintained through 10 serial subcultures in CbMM containing glycogen, 100 mg/ml, and hemin, 8 μg/ml. Mass cultures (10) of *C. elegans* under aeration in peptone-yeast basal medium (1) supplemented with glycogen, 10 mg/ml, and hemin, 10 μg/ml, increased 600-fold to 187,000 nematodes/ml in 3 weeks.

Media containing glycogen were tested with other nematodes. *Turbatrix aceti* reproduced in 8 days in a medium containing 199 mg/ml of glycogen, 8 μg/ml of hemin, and 5% ethanol, but failed to reproduce when subcultured. Maturation of *Neoaplectana carpocapsae* and *Aphelenchus avenae* was not supported.

Attempts to substitute other carbohydrates, corn starch, rice starch, rice flour, and trehalose, in the medium were unsuccessful.

Summary. Glycogen is an effective heat-stable supplement for the culture of some free-living nematodes. Activity is associated with the precipitated portion of the complete medium.

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