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Ficoll Activation of a Protein Essential for Maturation of the Free-Living Nematode *Caenorhabditis briggsae.** (30786)

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Previous work with axenic culture of the free living nematode, *Caenorhabditis briggsae,* led to isolation of a heat labile fraction from liver which was essential for maintenance of these cultures in an otherwise chemically defined medium(1,2). Subsequently, this fraction has been further separated by chromatography on hydroxylapatite(1A). The globulin recovered has the interesting property of being "activated," that is, certain specific treatments increased the biological activity 30-fold. This phenomenon may have a bearing on the availability of proteinaceous materials in biological systems. This proteinaceous growth factor is present in most animal tissues as well as in bacteria. Preliminary studies show that the material is effective in a wide range of biological systems.

The first method of activation discovered was freezing the growth factor in the basal defined medium. This activation proved to be limited in stability and restricted to relatively acid media. The need to expand the cultural conditions led to reexamination of the activation process and to the development of other methods of producing this effect. Recently activation has been produced by dialysis of growth factor to low phosphate, by controlled periods of heating, by lowering the pH of the defined medium to 5.6, and by addition of proper levels of the sucrose polymer Ficoll (Pharmacia).

With several methods available for activation, the next step was a critical evaluation of the effects of each separately and in combination with each other. In this the outstanding results obtainable with Ficoll soon became evident and increased emphasis was given to this phase of the investigation.

Materials and methods. The free-living, self-fertilizing protandrous nematode *C. briggsae* was used under axenic conditions in all assays. Stock cultures were maintained in a medium containing liver extracts(2,3). Larvae used in assays were derived from eggs laid by young adults(1). Three larvae were inoculated into duplicate 10 × 75 mm tubes containing 0.25 ml aliquots of the medium to be tested.

Effectiveness of culture media was measured by time for maturation and reproduction(4,1). Time of maturation was determined by observations at ½ day intervals and calculated to 0.1 day based on the rate of appearance of F_1 larvae. Previous observations had shown that the rate of larvae produced at the 100 μg/ml level averaged one larva per adult every 2½ hours, *i.e.,* 0.1 day. The time at which the first larva hatched was thus calculated to 0.1 day. Worms were observed for 20 days, any worm not producing an F_1 by this time being classified as a non-maturer. Degrees of effectiveness of different media were compared by rates of maturation expressed as relative response. An F_1 time of 2.8 days, obtained when worms were grown with *E. coli* on agar slants(1), was considered the maximum response (=100).

The medium used to measure dose response consisted of two components(1), the basal medium of chemically defined ingredients and the proteinaceous growth factor preparation. The growth factor used for these experi-

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ments was additionally treated by precipitation with ammonium sulfate, then dissolved and dialyzed in 0.1 M potassium phosphate buffer, pH 7 to give a stock solution containing 10 mg/ml. It was sterilized by filtration, using Millipore pH size membranes. To study the effect of buffer strength, aliquots were redialyzed under aseptic conditions to lower molarity (20, 10 and 0.2 mM) potassium phosphate buffer, pH 7.0, and also to higher molarity (0.5 M). The protein level was held constant in the assays at 100 μg/ml for most experiments, lower levels being used only when a more sensitive test was required.

The composition of the published basal medium (4,3) has been modified: the level of amino acids has been lowered to 75% of that published; diaminopimelic acid and dl-alanine are now omitted; glutathione level is halved (to 0.66 mM); guanylic acid is included as the sodium salt (at 0.85 mM); pyridoxal is used as phosphate (at 15.2×10^{-3} mM); choline is used as the dihydrogen citrate (0.30 mM); inositol is lowered to 0.36 mM, and triethanolamine increased to 2.2×10^{-4} mM. This medium is designated No. 75. The pH of the medium is adjusted to 5.8-6.0. To investigate the effect of pH, other batches were compounded at pHs ranging 5.6 to 6.1, and further adjustment to wider ranges was made with sterile 0.1 N potassium hydroxide or acetic acid. Addition of growth factor solution increased the pH of the complete medium by 0.05; Ficoll introduced no change in pH.

The procedures used for activating growth factor were freezing at —23°C for 24 hours in the presence of the chemically defined medium(5), controlled heating of concentrated growth factor (0.1 M buffer) at 37°C for 24 hours(6), controlled heating of 100 μg/ml growth factor in defined medium at 37°C for 72 hours, dialysis against 0.2 mM potassium phosphate buffer, lowering the pH of the chemically defined medium to 5.6, and addition of the sucrose polymer Ficoll.

A stock solution of Ficoll was prepared by mixing 2.5 g of Ficoll with 5 ml of triple distilled water (38.5% solution, w/v). This was sterilized by autoclaving for 10 minutes at 121°C and stored at 4°C. Ficoll was used undialyzed after it was ascertained that a preliminary dialysis of the stock solution did not alter the effects in our media.

At the time of assay, a 15.4% w/v dilution of the highly viscous Ficoll stock was made to facilitate later pipetting. This was added to the basal medium prior to addition of growth factor; or, alternatively, growth factor was added to the Ficoll solution before it was added to the basal medium. The order of mixing appeared to have no influence on the activating effect. Mixing was performed with a "Vortex" mixer operated at low speed.

After the optimum ratio of growth factor to Ficoll was determined, other mixtures were made. A mixture of 400 μg/ml of growth factor with 15.4% Ficoll was stored and used as a convenient source. Mixtures of 200 μg/ml growth factor with 15.4% Ficoll were lyophilized.

Multiple activating procedures were observed: growth factor activated by dialysis was subjected to additional activating processes such as 37°C heating, freezing, lowering pH, and adding Ficoll.

Two isolated globulins [Fetuin (Lilly 236-415B-104) and Serum Fraction IV-4 (Hyland Laboratories)] that had been shown to be active in other biological systems were tested for "growth factor" effect and were also subjected to these activating procedures.

Results. Growth factor used at a concentration of 100 μg/ml in the basal medium was ineffective unless it had been subjected to specific treatments to induce activation. The degrees of activation observed with different procedures are compared in Table I.

The degrees of activity obtained with different amounts of Ficoll and growth factor are shown in Table II. The effective concentrations of Ficoll in the medium were found to be between 1.5% and 7.7% w/v. Below this range activation declined; stock solutions necessary to give higher concentrations were too viscous to handle accurately. With levels of growth factor lower than 50 μg/ml, Ficoll at 3.1% did not produce activation; these lower levels of growth factor, however, did show activity after freeze activation.

TABLE I. Activation of Growth Factor for Culture of *C. briggsae*.

Activator	F_1 time (days)	Relative response*
None	non-maturing	0
F/A	4.5, 4.7	61
H/A	5.0, 4.9	57
Dialysis	6.1, 4.7	52
pH	3.7, 4.0	73
Ficoll 3.9%	3.7, 3.9	74

* Maturation time of *C. briggsae* when associated with *E. coli* is 2.8 days (1) = 100; difference required for significance is 8.

F/A — complete medium frozen for 24 hr at −23°C.

H/A — growth factor held at 37°C for 24 hr.

Dialysis — growth factor dialyzed to 0.2 mM potassium phosphate buffer pH 7.0.

pH — acidity of medium adjusted to pH 5.6.

Except where specified above the growth factor used was from a stock solution of 10 mg protein/ml in 0.1 M phosphate buffer, pH 7.0. The chemically defined medium was pH 5.8 to 6.0; concentration of growth factor in medium 100 μg/ml.

Final activity could be increased by applying these several activating processes in sequence. The enhancement of activity that resulted from combining Ficoll with other activation procedures is shown in Table III. Certain combinations of procedures, however, led to decreased activity or total loss of activity (shown in Table III for H/A and F/A).

The activating effect of Ficoll in basal medium ranging from pH 4.6 to 6.9, and the effect of concomitant alterations in the molarity of potassium phosphate buffer contained in the growth factor solution are shown in Table IV. A high level of Ficoll was required for activation when the phosphate molarity was high and the medium relatively alkaline. With such media activation is not produced by either the heating or the freezing procedures.

Addition of Ficoll increased the stability of dilute solutions of growth factor. Solutions of 400 μg/ml of growth factor in 15.4% Ficoll stored at −23°C and 4°C for 2 weeks retained original activity, whereas solutions diluted in water showed a loss of activity and a variable response. Solutions with Ficoll retained activity after sterile filtration (membranes of 0.3 μ pore size); activity was lost if the filtration was performed on a solution

TABLE II. Maturation of *C. briggsae* in Chemically Defined Medium Supplemented with Growth Factor and Containing Added Ficoll.

Growth factor (μg/ml)	Ficoll (% in bioassay)	Maturation time (days)	Relative response
100	0	non-maturing	0
"	.4	8.5, 7.0	36
"	.8	5.8, 6.0	47
"	1.5	4.5, 4.5	62
"	3.1	4.4, 4.1	66
"	7.7	3.9, 4.2	70
50	1.5	10.6, 10.7	27
"	3.1	4.6, 4.5	62
25	1.5	non-maturing	0
"	3.1	"	0
0	7.7	"	0

Medium pH 6.0; growth factor from 10 mg/ml stock solution in 0.1 M phosphate buffer.

TABLE III. Increased Activity by the Addition of Ficoll.

Activators*	Growth factor (μg/ml)	Relative response 0% Ficoll	Relative response 3.9% Ficoll
Dial + pH 6.1	14	0	53
pH 5.6	9	0	32
Dial + pH 5.6	9	29	52
Idem + F/A	9	37	48
" + H/A	9	38	65
" + H/A + F/A	9	0	46

* See Table I for description.

TABLE IV. Increased Range of Activity by Addition of Ficoll.

Growth factor at 100 μg/ml

pH of basal medium	Growth factor in pot. phosph. buffer (molarity)	Relative response (%) 0% Ficoll	3.1-3.9% Ficoll	7.7% Ficoll
6.9	.5 M	non-mat.	—	58
	.2 mM	13	—	—
6.1	.5 M	non-mat.	non-mat.	65
	.1	"	56	—
	.02	"	78	—
6.0	.5 M	non-mat.	22	75
	.1	"	65	—
	.02	"	73	—
	.2 mM	14	—	—
5.8	.5 M	non-mat.	23	75
	.1	64	72	70
	.01	54	65	68
	.2 mM	53	—	—
5.6	.5 M	14	72	74
	.1	52	72	—
	.01	70	—	—
	.2 mM	50	67	—
4.6	.5 M	non-mat.	—	35

of growth factor in basal medium.

A lyophilized mixture of 7.7% Ficoll and 200 μg growth factor was stable at −10°C; it could be reconstituted without difficulty and was active thereafter.

Addition of Ficoll also reduced the variability of *C. briggsae* responses between duplicate tubes. A Wilcoxon matched pair test on 29 samples of duplicate tubes was highly significant: P, .003.

Fetuin and Serum Fraction IV-4 were tested by all of the "activating" procedures, but showed no "growth factor" effect.

Discussion. The proteinaceous material required for culture of *C. briggsae* undergoes activation in terms of enhanced biological activity following specific physical treatments. The mechanism of this action is not, as yet, understood. Data we now have suggests that activation is due to change in shape of the protein molecule, probably to a more compact form. For example, sulfhydryl determined as one per 100,000 in the unactivated protein cannot be detected after heat activation. The physical and chemical changes accompanying activation will be studied further as methods appropriate to this multimeric protein are developed.

The physical procedures used produce activity that varies in respect to degree, reliability, and stability. Comparison of the activating treatments showed that the sucrose polymer Ficoll provided the most reliable and stable activation over a wide range of physiological conditions. Ficoll also permitted greater latitude in handling and storage. These characteristics allow for assay of growth factor through a broad range of biological systems: one such use has been in culture of the parasitic stages of *Haemonchus contortus*(7).

Ficoll is stated to be nontoxic and inert. Reported uses include the determination of specific gravity of amoeba(8) and the isolation of cells for culture(9,10). When used in our culture medium, Ficoll was not toxic but it was not inert; rather it caused activation of the proteinaceous growth factor. It did not, however, activate other isolated biologically effective materials added to our basal medium. Thus, the growth factor appears to be biologically distinct.

An examination of the effect of this polymer on growth factor should give further insight into the phenomenon of enhanced biological activity. This information may have bearing on the effectiveness of proteinaceous supplements.

Summary. The sucrose polymer Ficoll at the proper concentration activates the proteinaceous growth factor necessary for continuous cultivation of the free-living nematode, *C. briggsae,* in a chemically defined medium. It increases the activity that is produced by other activating procedures. It increases uniformity of maturation, allows greater stability at 37°C, and produces a mixture with growth factor that can be readily lyophilized. Its use thus greatly extends the usefulness of the growth factor.

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