The Role of Precipitation During Activation Treatments of Growth Factor for *Caenorhabditis briggsae* (34297)

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Axenic cultures of *Caenorhabditis briggsae*, (1) a free living hermaphroditic nematode, have been maintained in chemically defined media (2) supplemented with a proteinaceous growth factor (3). It has been found that this factor was increased in effectiveness as much as thirtyfold by treatment with a variety of specific processes termed "activation" (2, 4). An explanation for the mechanism of this increase has been sought.

It had previously been observed that the complete medium containing Ficoll-activated growth factor lost activity upon filtration (2) suggesting that a partially precipitated material might be of importance in nutrition of the nematode. It had also been observed that when other methods of activation were used there was a noticeable increase in turbidity. We therefore attempted to determine if precipitation in the medium occurred in all activation processes, and if so, whether the precipitated portion of the protein carried the biological activity. The specificity of growth factor from this point of view was examined.

Materials and Methods. The response of *C. briggsae* to its culture medium is assayed by the rate of maturation of newly hatched larvae (5). This is expressed as the number of days required for a reproductive cycle (F_1 time).

The chemically defined portion of the medium (2), *C. briggsae* maintenance medium (CbMM), was obtained from Grand Island Biological Company, Grand Island, N.Y. It was compounded at 2X concentration and pH 6.0.

Growth factor, the proteinaceous supplement, was prepared from lamb liver (3, 6). For most of the experiments reported below, the preparation was clarified immediately before use by passage through a Millipore PH filter of 0.3μ porosity. The protein concentration was 5.5 mg/ml. For most of the assays reported here, growth factor was added to the defined medium at 250 μg/ml. At this protein level, the pH of the complete medium was 6.5. For some experiments hydrochloric acid was added so that the final pH of the complete medium was 5.5.

Several other proteins were subjected to activating procedures and assayed for biological activity. These were: the residual material which, during the preparation of growth factor, is not adsorbed by hydroxylapatite at a potassium phosphate molarity of 0.15, pH 7.0; bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.); Fetuin (Grand Island Biological Co.); β-lactoglobulin (Calbiochem, Los Angeles, Calif.); γ-globulin (Cohn Fraction II, Cutter Laboratories, Berkeley, Calif.); and Cohn Fraction IV-4 (a gift of Dr. A. Pappenhagen of Cutter Laboratories). Fraction IV-4 was precipitated with 70% ammonium sulfate and resuspended in 0.1 *M* potassium phosphate, pH 7.0.

Activation was carried out by the previously established methods: freezing of the complete medium (4); inclusion of 10% Ficoll in the medium (2); heating of growth factor at 53° (7), either alone or in the presence of defined medium, to the point of turbidity; incubation of growth factor at 37° (8); or lowering of the pH of the complete medium to 5.5 (2), the approximate isoelectric point of growth factor.

In the new methods of activation described in this paper, particles are added to the culture medium. This type of activation was accomplished by one of the following methods: (i) γ-globulin at 500 μg/ml was frozen in CbMM. A portion of the protein precipitated. This precipitate was resuspended and

an equal volume of CbMM containing 500 μg/ml of growth factor was added so that in the assay each protein was 250 μg/ml. (ii) A stable micell of γ-globulin and cardiolipin (CL) (Applied Science Laboratories, State College, Pa.) was formed according to the method of Marinetti and Pettit (9) and added to the defined medium or to growth factor so that the concentrations were 100 μg/ml γ-globulin, 16 μg/ml CL, and 250 μg/ml growth factor. (iii) Growth factor at 2.0 mg/ml was incubated for 1 hr at 20° with polystyrene latex beads (PSL) (Dow Chemical Co., Midland, Mich.) 250 mμ in diameter. The bead suspension had been diluted to opalescence and sterilized by filtration through a Millipore PH membrane. After incubation, the mixture was resuspended and added to CbMM to give a final protein concentration of 250 μg/ml.

The increase in turbidity due to activation was determined by measuring the absorbance at 550 mμ (9). When additives were included, the increase in turbidity was the difference between the absorbance of the complete medium as used in the assay and the sum of the absorbances of growth factor alone in CbMM and additive alone in CbMM. Removal of the precipitates was usually done by centrifuging at 10,000*g* for 15 min.

Protein concentrations were determined by the absorbance method of Warburg and Christian (10). This method was applicable in experiments involving activation by heat or by γ-globulin + CL micells because here activation occurred in stock solutions without presence of interfering materials. Ficoll, polystyrene beads, and the amino acids of the defined medium in which growth factor is diluted for freeze activation interfere with measurements of absorbance. In these instances the protein remaining in solution after removal of precipitates was determined biologically after freeze activation.

Electrophoresis was done on Millipore Phoroslides using a Veronal buffer of ionic strength 0.06 and pH 8.5, applying 100 V for 17 min.

Solutions were initially sterilized by filtration and combined with aseptic precautions. All assays were monitored to detect contaminating bacteria. Centrifugation and lyophilization of growth factor were done under aseptic conditions.

Growth factor is usually assayed at concentrations of 10–100 μg/ml. For most of the work, however, the level was raised to 250 μg/ml to bring the absorbance at 550 mμ to a conveniently measurable level.

Results. Growth factor is a high molecular weight globulin which has a marked tendency to aggregate (11) and precipitate. It is opalescent; this opalescence persists after sterilization by filtration through a Millipore membrane of 0.3-μ pore size. Upon prolonged storage at 4°, the opalescence increases to turbidity and eventually a small amount of precipitate forms. Lyophilized preparations of growth factor may also contain precipitated protein after reconstitution. The tendency of growth factor to aggregate and precipitate is increased by the activation processes. The increases in absorbancy at 550 mμ of the complete medium are shown in Table I for several activation processes.

The precipitate causing this increase in absorbance was removed by centrifugation. The resuspended sediment was biologically active; the supernatant fluid was essentially inactive unless subjected to a further activation process.

In the system of activated growth factor and defined medium, maturation occurs in the range of between 50 and 10 μg/ml of protein concentration. Above 50 μg/ml the response plateaus at about 5 days F_1 time while below 10 μg/ml maturation rarely occurs. The level of 250 μg/ml was chosen to permit good turbidity measurements; however, it also allowed sufficient protein to remain in solution after the first activation for the supernatant fluid to show a second activation.

The results of an experiment with Ficoll-activated growth factor are shown in Table II. The top layer was withdrawn and the lower layer was reconstituted to its original volume with CbMM. The activity of the lower layer was equivalent to that of the original mixture before centrifugation. The withdrawn top layer had very low activity; how-

TABLE I. Precipitation of Growth Factor Accompanying Activation.[a]

Method of activation	Increase in absorbance at 550 mμ	F_1 time (days)
(a) None		
GF freshly filtered	0	21, 21
GF frozen, thawed	0.004	13, 13
(b) Without additives		
GF frozen in CbMM	0.026	5.5, 5.6
GF heated in CbMM	0.072	6.5, 6.5
GF in CbMM, pH 5.5	0.076	4.9, 4.9
GF lyophilized	0.053	5.7, 5.1
(c) With additives		
10% Ficoll	0.118	4.8, 4.9
γ-Globulin, frozen	0.020	5.7, 5.5
γ-Globulin + cardiolipin	0.082	5.6, 5.6
Polystyrene latex beads	0.172	5.5, 5.5

[a] Growth factor (GF) was included in the defined medium (CbMM) at a protein concentration of 250 μg/ml. In the absence of GF, neither CbMM alone nor CbMM and any of the additives shown above supported maturation of *C. briggsae*. The increase in absorbance at 550 mμ for (a) and (b) is the difference between the activated and unactivated complete medium. For (c), the increase is the difference between the absorbance of the complete mixture as used in the assay, and the sum of the absorbances of GF alone in CbMM and additive alone in CbMM.

ever, when this supernatant fluid was subsequently freeze-activated, activity was considerably increased. Media activated with PSL beads and with γ-globulin + CL were also centrifuged, and, as with Ficoll, the supernatant fluids were inactive unless subjected to a further activation procedure.

Association of biological activity with the precipitate was also shown in heat activation. The experiment is summarized in Table III. Approximately half of the protein was removed in the pellet. The activity resided completely in the resuspended precipitate, whereas the supernatant at an equivalent protein concentration was totally inactive. The activity of the resuspended precipitate was somewhat lower than that of the original mixture, possibly because of failure of some of the closely packed pellet to redisperse.

When a stock solution of the growth factor is activated by controlled heating, an increase from opalescence to turbidity can be seen; in fact, with experience, the exact degree of heating required for activation can be determined visually. After dilution to microgram levels in defined medium, loosely aggregated protein particles can be seen microscopically at 20× magnification. At a higher magnification of 900× with phase contrast, the precipitate appeared as irregular masses of approximately 10 μ, composed of loose aggregates of particles about 0.5 μ in size. Still larger loose aggregates were seen in the precipitate from growth factor activated with PSL beads or γ-globulin + CL.

As the final step in the preparation of growth factor, it is adsorbed onto hydroxylapatite from a 0.15 *M* phosphate buffer solution and then eluted with 0.5 *M* phos-

TABLE II. Association of Activity with the Precipitate Formed by Inclusion of Ficoll in the Medium.[a]

Medium	F_1 time (days)
(a) Before centrifugation	4.8, 4.9
(b) After centrifugation	
lower layer	4.8, 4.8
upper layer	20, 20
upper layer, freeze activated	10, 12

[a] Growth factor at 250 μg/ml was added to CbMM containing 10% Ficoll. An aliquot of the mixture was centrifuged for 15 min at 10,000*g*. The upper layer was withdrawn and assayed before and after freeze activation. The lower layer was brought to the original volume with CbMM.

TABLE III. Association of Activity with the Precipitate Formed by Heat Activation.[a]

Medium	F_1 time (days)
(a) Before centrifugation	5.5, 5.7
(b) After centrifugation	
supernatant, 109 μg/ml	nonmaturing
pellet, 100 μg/ml	6.5, 6.5

[a] Growth factor at 1 mg/ml was heat activated for 12 min at 53° and assayed at 100 μg/ml. An aliquot of the activated protein was diluted to 400 μg/ml and centrifuged for 15 min at 10,000*g*. The supernatant was diluted with an equal volume of 2× CbMM; the pellet was resuspended in 1× CbMM.

phate. Adsorption from the 0.15 *M* buffer is not complete; the discarded solution contains, in addition to other proteins, a small amount of the same two electrophoretic bands in the β-globulin region shown by growth factor. This 0.15 *M* fraction is less opalescent and more difficult to precipitate than growth factor. In routine assays fractions are activated by freezing at 100 μg/ml. The F_1 time of growth factor is 5.9 days while the F_1 time of the 0.15 *M* fraction is 16 days. By increasing the heat-activation time of the 0.15 *M* eluate from 2 to 3 days at 37°, the opalescence approached that of heat-activated growth factor. When this was done, the F_1 time of *C. briggsae* in the 0.15 *M* fraction at 100 μg/ml was 6.5 days compared with 5.5 days in growth factor.

Proteins other than growth factor were tested under several activating conditions. Albumin precipitated neither with the usual heat activation nor with more prolonged heating. Precipitates formed by heating at a higher temperature were inactive. In the defined medium albumin remained soluble even at pH 4.8, its isoelectric point. Fetuin precipitated when added to water, but redissolved when added to the defined medium. It could not be precipitated at its isoelectric point in the defined medium. When added to the γ-globulin + CL micell, both serum fraction IV-4 and γ-globulin showed a small increase in turbidity at 550 mμ but neither was biologically active. These materials had previously been tested at milligram levels and had shown no activity as growth supplements. Only the 0.15 *M* eluate showed considerable increase in turbidity at 550 mμ and had biological activity.

Discussion. The results indicate that activation is the controlled precipitation of growth factor into particles of approximately 0.5- μ size. This increases its availability to the nematode and explains the apparent increase in specific activity. In the presence of the nematodes, precipitate in the medium gradually disappears. Particles can be seen in the intestine of the nematodes and are apparently digested there, since we have been unable to discover stable solubilizing enzymes in the medium.

After sedimentation of activated medium, the growth factor remaining in solution was biologically ineffective, although a sufficient level remained for the supernatant fluid to show activity after a subsequent activation process. This suggests that soluble protein at low levels is not used by the nematode, and that only the insoluble form is effective. Since activity resides in the precipitate, previous attempts to explain activation (11, 12) by measurements carried out on the protein remaining in solution appear to be less pertinent.

Since *C. briggsae* is a bacterial feeder, it was thought that particles might have a special physiological role, perhaps in stimulating ingestion or regulating the passage of material along the intestine. An attempt was therefore made to substitute biologically inert particles. The polystyrene latex beads and the stable micells of γ-globulin+ CL appeared microscopically similar to activated growth factor and were within the size range of bacteria. The inability of either to support maturation when added to chemically defined medium suggests that the effect of particles is not simply a mechanical one.

The contribution of growth factor to the nutrition of the nematode is not yet understood, and it is possible that its activity is due to components other than its protein content. Growth factor contains small amounts of lipid and carbohydrate and possibly as yet unidentified small molecules. Any of these, highly concentrated, might account for its activity.

The 0.15 *M* eluate obtained during extraction of growth factor comprises a range of proteins including a portion with the electrophoretic mobility of growth factor. Good biological activity was shown when the material was effectively precipitated by heat activation or by γ-globulin + CL micells. However, routine assay after freeze activation showed poor activity. The differences in biological activity obtained by these activation methods presents a problem which must be taken into account in following specific activity during fractionation.

The specificity of growth factor may not rest solely on its chemical composition but

may also depend upon its tendency to aggregate. Other proteins tested for biological activity were ineffective at high assay levels or at lower levels after activation treatments. γ-Globulin can be precipitated in a suitable form; however, it is not effective as a growth supplement. This suggests that it lacks the essential components of growth factor, and that the precipitated growth factor is not merely a concentrated source of amino acids.

Other proteins, β-lactoglobin and serum albumin, are not precipitated by the methods used to activate growth factor. Still other proteins active as growth supplements in other biological systems e.g. Fetuin (13) and Serum IV-4 (14), are poorly precipitated, or as with Fetuin, the precipitate formed by heating redissolves when added to the defined medium.

Growth factor is a complex heterogeneous protein whose precipitation renders it peculiarly suitable for ingestion by *C. briggsae*. The precipitate of γ-globulin + CL can also be engulfed and digested by the nematode and is chemically defined. If such a precipitate can act as a nucleus for adsorption of specific molecules, the chemical basis for the specificity of growth factor could be investigated.

Summary. Activation of growth factor for cultures of *Caenorhabditis briggsae* is accompanied by formation of a fine, flocculent precipitate which can be measured by increased absorbance at 550 mμ. Biological activity resides in the precipitate after separation by sedimentation. By precipitation the growth factor is made available to the nematode in a concentrated and particulate form. If the level of soluble protein in the medium is insufficient, concentration may be essential for growth. The particulate form may have a specific physiological role for bacteria-feeding nematodes. Other proteins do not support growth of *C. briggsae* even when used at high levels or subjected to activation procedures.

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1. Dougherty, E. C., Hansen, E. L., Nicholas, W. L., Mollet, J. A., and Yarwood, E. A., Ann. N. Y. Acad. Sci. **77**, 176 (1959).

2. Buecher, E. J., Hansen, E. L., and Yarwood, E. A., Proc. Soc. Exptl. Biol. Med. **121**, 390 (1966).

3. Sayre, F. W., Hansen, E. L., Starr, T. J., and Yarwood, E. A., Nature **190**, 1116 (1961).

4. Hansen, E. L., Sayre, F. W., and Yarwood, E. A., Experientia, **17**, 32 (1961).

5. Lower, W. R., Hansen, E. L., and Yarwood, E. A., J. Exptl. Zool. **161**, 29 (1966).

6. Lower, W. R., Hansen, E. L., and Perez-Mendez, G., Federation Proc. **27**, 254 (1968).

7. Sayre, F. W., Hansen, E. L., and Yarwood, E. A., Exptl. Parasitol. **13**, 98 (1963).

8. Hansen, E. L., Silverman, P. H., and Buecher, E. J., J. Parasitol. **52**, 137 (1966).

9. Marinetti, G. V. and Pettit, D. Chem. Phys. Lipids **2**, (1968) 17.

10. Warburg, O. and Christian, W., Biochem. Z. **310**, (1942) 384.

11. Sayre, F. W., Lee, R. T., Sandman, R. P., and Perez-Mendez, G., Arch. Biochem. Biophys. **118**, 58 (1967).

12. Sayre, F. W., Fishler, M. C., Humphreys, G. K., and Jayko, M. E., Biochem. Biophys. Acta **160**, 63 (1968).

13. Fisher, H. W., Puck, T. T., and Sato, G., J. Exptl. Med. **109**, 649 (1959).

14. Geiman, Q. M., Siddiqui, W. A., and Schell, J. V., Science **153**, 1129 (1966).

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