Growth Factor Activity Modifiers in Human Blood Serum (35621)

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A protein has been isolated from mammalian tissues and bacteria which enables the cultivation of nematodes (*Caenorhabditis briggsae*) through their entire life cycle when it is added in small amounts (10 μg/ml) to a chemically defined medium (1, 2). It exists in two *interconvertible* forms—one of which promotes the growth of the nematodes and one which does not (3). The protein has been described in some detail, previously (3, 4, 5). For the purpose of this paper, activated growth factor is the form that promotes growth of the nematodes: non-activated growth factor is the form that will not promote nematode growth. Activation of growth factor was previously accomplished in the laboratory by nonphysiological means (3). If the observed differences in activity of the two forms of growth factor have physiological meaning, one can predict that there would be physiological moderators to accomplish their interconversions. Thus investigations were made dealing with physiological moderators of growth factor activity found in human and other mammalian sera.

Methods and Materials. Growth factor preparations were obtained from lamb liver by ammonium sulfate precipitation followed by fractionation on hydroxylapatite and sephadex columns as previously described (2, 4). All protein solutions were sterilized by filtration through Millipore filters of mean pore size 0.30 μ(PH).

The protein content of solutions was determined by the micromethod of Lowry *et al.* (6).

Ultracentrifugal characteristics were ascertained by means of the Spinco Model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, California) using sedimentation velocity methods (7).

Biological activities were determined according to previously described methods (1, 2). Three freshly hatched larvae of *C. briggsae* were inoculated into sterile 10 $\times$ 75 mm culture tubes containing 0.25 ml of defined medium and other additives at the specified concentrations. The number of days required for the cultures to go through a total life cycle and produce new larvae (F_1 time) was determined for the test conditions to the nearest half day. A detailed statistical analysis of reproducibility of controls and repeat assays established validity and reliability of the assay method (8) within the limits shown.

Samples of serum were drawn by a clinical laboratory from selected volunteer patients and normal subjects. Dilutions of serum in defined medium were bioassayed for growth factor after holding overnight at 4° (which does not produce activation), and after holding overnight at —25° (which does produce freeze-activation).

The growth factor content of individual sera was estimated by diluting sera with defined medium to the point where growth factor activity was no longer demonstrable. Activity was routinely determined in controls without added serum at 4° (normally nonactivated) and after freezing at —25° (normally activated for comparison).

Preliminary separations of biological activator and inhibitor activities were obtained by fractionation with ammonium sulfate. All fractions were dialyzed free of ammonium ion and sterilized by filtration through Millipore filters prior to assay.

Inhibitor was applied to a Sephadex G-200 column in 0.1 *M* potassium phosphate

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TABLE I. Growth Factor and Modifier Activity of Human Blood Serum.[a]

Additive (% whole serum)	Maturation time (days)			
	75 μg/ml added growth factor		No added growth factor	
	4°[b]	Frozen[c]	4°[b]	Frozen[c]
20	7½, 9	10, 10	7½, 8½	n.m.[d]
10	1½, 9	10, 7½	n.m.	n.m.
5	7½, 7½	12, 8½	n.m.	n.m.
1	13, n.m.	n.m., n.m.	n.m.	n.m.
None	n.m., n.m.	5½, 5½[e]	n.m.	n.m.

[a] Serum from a normal man was tested at the concentrations indicated for its ability to replace growth factor, and for its ability to modify known growth factor activity. These results are typical of those obtained with serum from 24 normal subjects, 11 female and 13 male. Similar results were obtained with pools of normal serum from three to five normal adults. Duplicate assay results are shown in terms of the number of days to produce F_1 generations.

[b] Samples held overnight at 4°, which does not produce activation.

[c] Samples were activated by freezing overnight before inoculation.

[d] Abbreviation n.m. means nonmaturing.

[e] Minimal time of maturation possible is 5½ days under these conditions, when growth factor is completely activated.

buffer, pH 7.0. The column was eluted with the same buffer, and samples collected by an automatic fraction collector. Column effluent was monitored fluorometrically with a Turner 111 Fluorometer using a microflow cell.

Results. Natural activator and natural inhibitor of growth factor as well as the growth factor itself, have been found in the serum of man, lamb, rat, and mouse. The presence of activator and inhibitor was detected by the ability of dilutions of added serum to modify the expected biological response to known amounts of added growth factor in a nematode maturation assay. At adequate concentrations serum supported reproduction of nematodes, indicating the presence of growth factor in the activated form. The presence of activator was indicated by the ability of serum, at appropriate concentrations, to activate added exogenous growth factor known to be in the nonactivated state. If only growth factor (without activator or inhibitor) were present in serum, one would expect nematode growth and reproduction to be supported by serum only if the serum growth factor were in the activated state. If growth factor were present, but not in the activated state, nematode growth and reproduction would be supported only after activation (*e.g.* by freezing the serum in defined medium).

Evidence for the presence of natural inhibitor in serum was as follows: at appropriate concentrations, added serum depressed or completely blocked the expected biological response of standard amounts of exogenous growth factor in the nematode maturation assay system even following activation by freezing. Controls without added serum exhibited normal freeze-activation of growth factor in the defined medium. Failure of 20% serum to support maturation after freezing (Table I) is not completely understood. It has, however, frequently been observed that optimally activated growth factor becomes irreversibly inactivated when it is subjected to a second activation procedure such as freezing (3).

Activator activity was found in a fraction precipitating from serum between 0 and 40% saturation with ammonium sulfate (Table II). This fraction of serum contained no growth factor activity, but could activate a known added exogenous growth factor. Attempts at further fractionation of activator were unsuccessful in that all fractions obtained were without effect (either positive or negative) upon the activity of added ex-

TABLE II. Inhibitor, and Activator Fractions from Human Blood Serum.[a]

Material added to defined medium		Maturation time (days)	
Growth factor	Additive	4°[b]	Frozen[c]
75 μg/ml	None	n.m.[d]	5½, 6½
75 μg/ml	0–40% serum fraction (75 μg/ml)	6, 7	5½, 5½
75 μg/ml	Over 90% serum fraction (250 μg/ml)	n.m.	n.m.
None	0–40% serum fraction (1000, 250, and 75 μg/ml)	n.m.	n.m.
None	Over 90% serum fraction (250 μg/ml)	n.m.	n.m.

[a] Fractions separated with ammonium sulfate from the serum of a normal man were tested for their ability to modify the biological activity of a known amount of growth factor. Isolated fractions of inhibitor and activator have been obtained from the serum of three different normal male subjects (one of these upon two different trials), and from three different pools of serum from normal subjects. Assays were done in the usual way, without activation, and after activation by freezing. Results of a duplicate assay are shown in terms of the number of days required to produce F_1 generations.

[b] Samples held overnight at 4°, which does not produce activation.

[c] Samples were activated by freezing overnight before inoculation.

[d] The abbreviation n.m. means nonmaturing.

ogenous growth factor.

The fraction remaining in the supernatant fluid at 90% saturation of ammonium sulfate prevented activation of added exogenous growth factor activity even after freeze activation (Table II). Inhibitor was eluted from a column of Sephadex G-200 as a single symmetrical peak which after dialysis against distilled water, showed streaming birefringence upon stirring, indicating a needle-shaped protein with estimated length of 1–2 Å.

A specific interaction between growth factor and physiological inhibitor was shown by the following experiments. An aliquot of the inhibitor fraction, prepared as above and dissolved in 0.10 *M* phosphate buffer, sedimented as a symmetrical peak in the Analytical Ultracentrifuge with an $S_{20,w}$ of 3.9. When the above inhibitor fraction was allowed to react with lamb liver growth factor and then centrifuged, another single symmetrical peak was obtained with $S_{20,w}$ of 4.6 (Fig. 1). The area beneath the peak of the combined growth factor and inhibitor was essentially the sum of the individual materials at the same concentrations: actually the sum was 5% less and this difference may be due to a mismatch of the proportions of the reacting proteins.

Other evidence for specific interactions between growth factor, activator, and inhibitor was obtained by studying the chemical reactivity of the sulfhydryl groups of growth factor following exposure to activator and inhibitor, separately and together (5).

Discussion. The growth factor, which has been isolated from mammalian tissues, constitutes a normal portion of the α-2 globulin fraction of serum proteins. The protein growth factor (from animal or bacterial sources) appears essential to the growth and reproduction, although not to the survival, of the nematode, in a chemically defined medium (1, 2, 4). No other purified protein has replaced this requirement although such mixed tissue proteins as minced liver or serum (at appropriate concentrations) support the nematode's growth and maturation. The evidence indicates that growth factor is a biologically active protein and that for growth promoting activity it must have a certain structure (3, 4) which we have called the activated form. Growth factor also shows biolog-

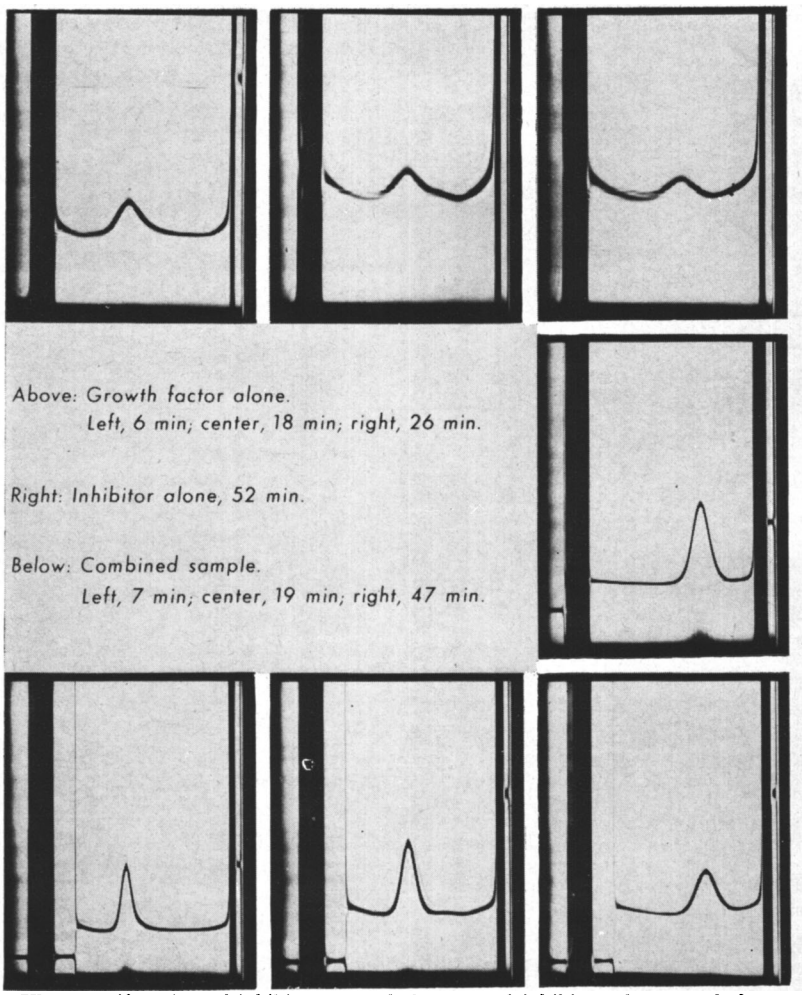

FIG. 1. Ultracentrifugation of inhibitor, growth factor, and inhibitor plus growth factor. Samples were in 0.1 *M* potassium phosphate buffer at pH 7.0. Pictures were taken at the indicated times after reaching two-thirds final speed, of 59,780 rpm, at 20°. Inhibitor = 4.0 ml/ml: growth factor, 5 mg/ml: inhibitor 4 mg/ml plus growth factor, 4 mg/ml. Sedimentation is from left to right.

ical activity in culture of McCoy strain epithelial cells where activated growth factor can replace the serum requirement (1, 2, 9), however nematodes provide by far the most convenient quantitative assay of growth factor and of its *state of activation* (3).

Activation and deactivation of growth factor by nonphysiological procedures have been studied extensively and exhibit evidence of accompanying structural changes within the molecule (5). Nonphysiological activation procedures may mimic physiological activators in producing activation of the protein. Two simple and consistent activation procedures (1, 10) were used for comparative purposes.

Physiological modifiers of growth factor activity (for nematode growth) were obtained from human serum—which also contains the growth factor protein. One of these modifiers converted the growth factor to the active form for nematode growth and the other prevented its conversion to the active form. The modifiers were both found in all samples of serum examined (well over 100 samples): the biological effectiveness (for nematode

growth) of the growth factor in serum appears to be the result of the relative balance of the two modifiers.

Summary. The serum of man and other animals has been found to contain a growth factor of protein nature, and chemical modifiers of its biological activity. The growth factor, in one of its two readily interconvertible forms, will support the growth and reproduction of nematodes in defined culture; the other form is without growth promoting activity in the cultures. Distinct materials have been separated from serum which either convert the growth factor into the activated form or prevent its conversion. These have been called activator and inhibitor, respectively. Although growth factor has growth promoting activity in tissue cells in culture as well as nematodes, nematodes provide the most convenient quantitative assay of growth factor and of its chemical state of activation. Its biological activity in all systems depends on which form it is in. Specific chemical interaction has been demonstrated between growth factor and its natural modifiers. The balance of physiological activator and inhibitor appears to regulate its biological effectiveness.

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