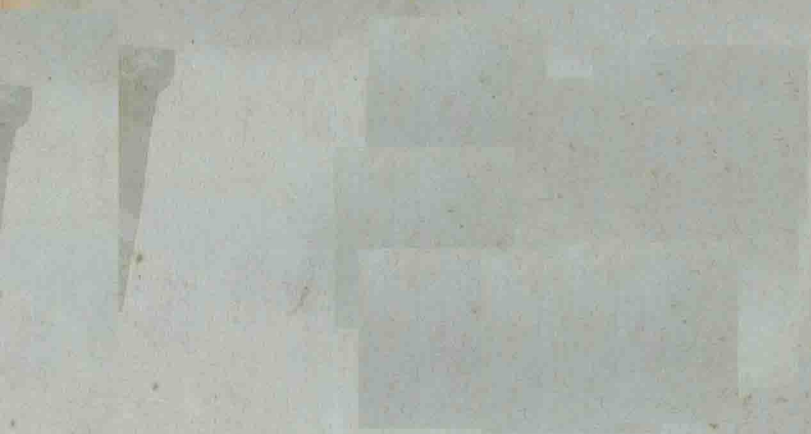
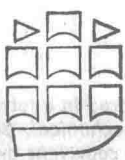
Growth Control in Cell Cultures



GROWTH CONTROL IN CELL CULTURES

A Ciba Foundation Symposium

Edited by
G. E. W. WOLSTENHOLME
and
JULIE KNIGHT



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GROWTH CONTROL IN CELL CULTURES

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The Ciba Foundation

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The Foundation's many activities are controlled by a small group of distinguished trustees. Within the general framework of biological science, interpreted in its broadest sense, these activities are well summed up by the motto of the Ciba Foundation: *Consocient Gentes*—let the peoples come together.

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CHAIRMAN'S INTRODUCTION

M. G. P. Stoker

It is nearly two years since Professor Franz Bergel initiated a symposium here on homeostatic mechanisms (*Homeostatic Regulators* 1969), and although we didn't come to any clear views about the principles involved, and certainly not the mechanisms, it was an extremely stimulating meeting and opened up a number of facets and views and a very wide-ranging discussion. After it, some of us felt that it would next be useful to have a meeting about regulation in the more easily controlled cell culture systems. Unfortunately, there is so far no such thing as real homeostasis in any cell culture system; in effect we give cells all the substances we believe they need and then ask naively why they sometimes fail to grow. In other words, we study restriction of growth rather than homeostasis, but it is the nearest we can get at present in cell culture systems that can be readily manipulated.

Interest in restriction of growth in cultured cells was greatly stimulated by the discovery of viral transformation *in vitro*, which removes many of the restrictions. The dramatic change in the growth capacity of virally transformed cells led very quickly to simple assumptions about cell contact as a regulator of growth, despite continual protests from Professor Michael Abercrombie that "contact inhibition" referred to movement and not to growth. The role of cell contact in growth regulation has been in and out of favour ever since, generally alternating with serum factors, and my own approach and interpretation have fluctuated as much as anyone's. During the last ten years or so of study a lot of knowledge has accumulated, and it has led to more meaningful experiments, but there has been little progress towards an understanding of the situation, except perhaps in the last year or so.

In this meeting, we shall be discussing new data and we shall try to formulate theories about mechanisms. I hope that at least we shall end up with some good ideas about the experiments to do next.

REFERENCE

Ciba Foundation Symposium (1969) *Homeostatic Regulators*. London: Churchill.

STUDIES OF SERUM FACTORS REQUIRED BY 3T3 AND SV3T3 CELLS

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OUR studies of certain mammalian cell growth factors began as an investigation of "contact inhibition" of cell division in 3T3 cells (a cell line derived from mouse embryos). Under normal culture conditions, 3T3 cells stop growing after they have formed a monolayer, which has approximately 3×10^4 cells per cm^2. The work of Todaro, Lazar and Green (1965) had shown that the addition of serum to a monolayer of resting 3T3 cells leads to initiation of DNA synthesis in some of the cells. We looked into this effect of serum since it seemed that this might give some clue to the nature of "contact inhibition" of cell division. In our studies we were impressed by the fact that the cell density at which "contact inhibition" takes place depends on the amount of serum added to the culture medium (Holley and Kiernan 1968). This is shown in Fig. 1*a*.

The interaction between serum concentration and cell density of 3T3 cells is shown in a different way in Fig. 1*b*. Here a "steady-state" concentration of serum is maintained by growing the cells on a small coverslip in a large dish, with daily fluid changes. Again the final cell density is dependent on serum but a considerably higher cell density is obtained at a given concentration of serum when the cells are not allowed to deplete the medium.

The results shown in Figs. 1*a* and *b* are consistent with each other, since assay of depleted 10 per cent serum medium after 3T3 cells have stopped growing—that is, the conditions of Fig. 1*a*—indicates that the depleted medium still contains growth activity for 3T3 cells equivalent to 1–2 per cent serum. This is approximately the "steady-state" concentration of serum that gives the same cell density (Fig. 1*b*). Depletion of the medium by growing or resting 3T3 cells poses many interesting questions, but definitive studies of depletion are complicated by the fact that there seem to be several serum factors, as is discussed below.

The virally transformed SV3T3 cell does not exhibit "contact inhibition" of cell division. An explanation for this is furnished by the fact that SV3T3 cells have a very low serum requirement for growth. At very low serum concentrations the growth of SV3T3 cells is limited by serum (Fig. 1*a*). (That the low number of SV3T3 cells found at low serum concentrations is not due to loss of cells from the surface but is due to a lengthened generation time has been confirmed by Ingrid Klinger in this laboratory by time-lapse photography.) The serum factor that is required by SV3T3 cells is not the same factor that is most limiting of the

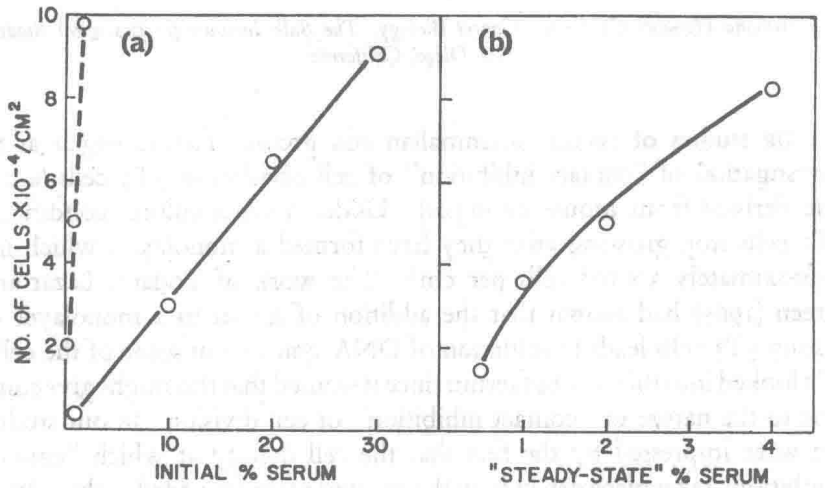

FIG. 1. Dependence of cell density on serum concentration. In Fig. 1*a* (*left*), the cell density is that attained after growth had largely ceased (4 days) in media with the serum concentration shown. ———, 3T3 cells; – – –, SV3T3 cells. In Fig. 1*b* (*right*), the cell density is that attained by 3T3 cells on a 12-mm coverslip after growth had ceased (5 days) in 10 ml medium, fluid being changed daily, with the serum concentration shown.

growth of 3T3 cells, since 10 per cent serum medium depleted of growth factor by 3T3 cells still assays as 10 per cent serum with SV3T3 cells, although it assays as only 1–2 per cent serum with 3T3 cells.

The observation that the cell density attained before 3T3 cells become "contact inhibited" varies with the amount of serum can, of course, be interpreted in various ways. One interpretation is that contact between cells is of primary importance in inhibiting growth and the effect of serum is secondary; in some way serum "antagonizes" contact between the cells. Alternatively it could be that the growth of 3T3 cells is controlled primarily by growth factors in the serum, and contact and crowding affect growth by limiting the uptake or utilization of the serum factors.

Although these two interpretations are in some respects operationally indistinguishable, we have chosen the interpretation that emphasizes serum factors because it is the simpler hypothesis and it encourages a direct experimental attack—the isolation and study of the serum factors.

Much of our effort therefore has been devoted to the fractionation of calf serum and rat serum with the aim of isolating purified growth factors. Rat serum has been used in many experiments because its greater activity (approximately 2·5 times the activity of calf serum) makes it easier to assay fractions.

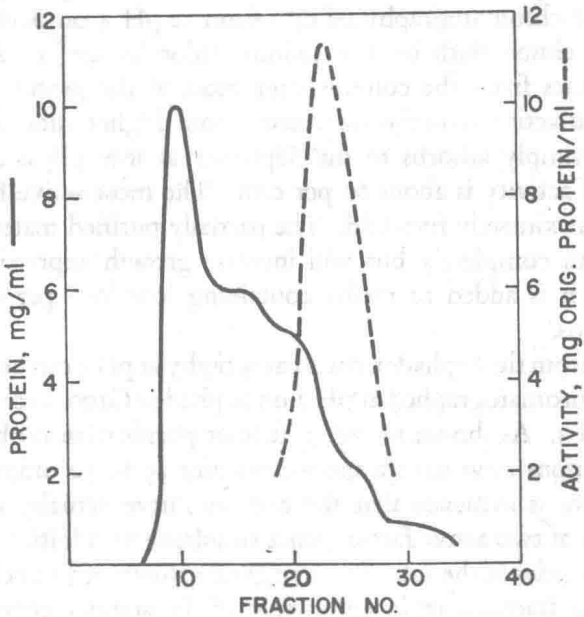

FIG. 2. Chromatography of rat serum, pH 4, on Sephadex G100 at pH 4 in 0·01 N-sodium chloride, pH 4. ———, protein; – – –, activity for 3T3 cells.

As might have been expected from the experience of others, isolation of a highly purified growth factor from serum has encountered technical difficulties. Activity is lost readily and the activity that remains often appears in various fractions. For example, chromatography of serum on DEAE-cellulose columns gives poor recovery of activity and the activity that is recovered is spread across most of the fractions. Also it seems that after many fractionation procedures combinations of fractions are somewhat more active than individual fractions. For example, ammonium sulphate gives some fractionation but the fractions seem to

give better growth when they are recombined. Frequently none of the fractions obtained has a higher specific activity than the starting serum, because of losses of activity.

Recently we have avoided some of these difficulties by working with serum at low pH. Much of the activity of serum survives pH 3 or pH 2. In agreement with the findings of Tritsch, Bell and Grahl-Nielsen (1968) for other serum growth factors, 3T3 and SV3T3 growth activity is largely removed from serum by charcoal at pH 3 but not at pH 7. This suggested that the growth factors might be dissociating from carriers at low pH, and, therefore, gel filtration of serum was tried at low pH. Fig. 2 shows the results of chromatography of rat serum at pH 4 on Sephadex G100 packed and eluted with 0·01 N-sodium chloride, pH 4. Activity for 3T3 cells elutes from the column after most of the protein has eluted. Whether the active material dissociates from higher molecular weight material or simply adsorbs to the Sephadex at low pH is not known. Recovery of activity is about 60 per cent. The most active fractions are purified approximately five-fold. The partially purified material will not replace serum completely but will increase growth approximately five times when it is added to media containing low (0·6 per cent) serum concentrations.

Fractions from the Sephadex chromatography at pH 4 can be lyophilized and then rechromatographed at pH 3 on Sephadex G100, in 0·1 N-sodium chloride, pH 3. As shown in Fig. 3, further purification is obtained, and the best fractions now have a specific activity 20 to 50 times that of rat serum. There is evidence that the activity curve actually represents a combination of two active factors, since simultaneous addition of fractions from the two sides of the activity curve gives growth of 3T3 cells to higher densities than fractions from either side of the activity curve alone. A serious difficulty with this partially purified preparation is the apparent lack of stability when it is neutralized. This has limited studies of further purification. The cause of the instability is unknown.

On the Sephadex G100 columns, activity for SV3T3 cells separates from activity for 3T3 cells. Paul, Lipton and Klinger (1971) in this laboratory have found that the best separation takes place at pH 2, with SV3T3 activity appearing in two peaks, one before and one after the 3T3 activity peak. These workers also found that activities for the two cell lines can be separated partially by electrophoresis of rat serum, for example, on Pevikon at pH 7·6 in Veronal buffer.

Our most promising results have been obtained very recently with commercial bovine serum fractions. Chromatography of commercial β-globulin (Cohn fraction III) at pH 3 on Sephadex G100 in 0·1 N-sodium

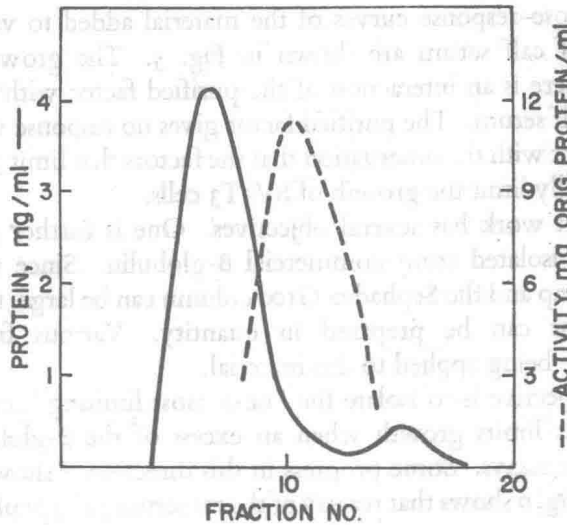

FIG. 3. Rechromatography of lyophilized fractions 25–30 from Fig. 2 at pH 3 on Sephadex G100 in 0·1 N-sodium chloride, pH 3. ———, protein; – – –, activity for 3T3 cells.

chloride, pH 3 gives the results shown in Fig. 4. Activity separates well from most of the protein. The best fractions have specific activities a few hundred times the activity of calf serum. The addition of 1 μg of this material per ml of medium gives a significant growth response with

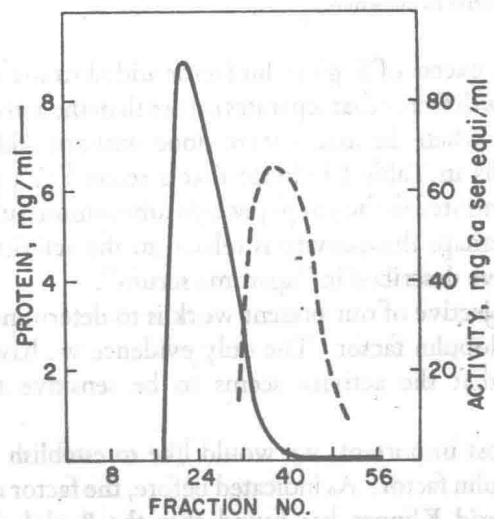

FIG. 4. Chromatography of commercial bovine β-globulin (Cohn fraction III) at pH 3 on Sephadex G100 in 0·01 N-sodium chloride, pH 3. ———, protein; – – –, activity for 3T3 cells.

3T3 cells. Dose–response curves of the material added to varying concentrations of calf serum are shown in Fig. 5. The growth response shows that there is an interaction of the purified factor with some other factor(s) in calf serum. The purified factor gives no response with SV3T3 cells, consistent with the observation that the factors that limit 3T3 growth do not normally limit the growth of SV3T3 cells.

Our present work has several objectives. One is further purification of the factor isolated from commercial β-globulin. Since the starting material is cheap and the Sephadex G100 column can be large, the partially purified factor can be prepared in quantity. Various fractionation procedures are being applied to this material.

Another objective is to isolate the "next most limiting" serum factor, the factor that limits growth when an excess of the β-globulin factor is added to the assays. Some progress in this direction is shown in Fig. 6 and Table I. Fig. 6 shows that reassay of the rat serum pH 4 Sephadex G100

TABLE I

ASSAY OF 3T3 GROWTH ACTIVITY IN AMMONIUM SULPHATE FRACTIONS OF CALF SERUM

Ammonium sulphate fraction	*Protein concentration, mg/ml*	*Specific activity,* assayed alone*	*Specific activity,* assayed with added β-globulin factor*
0–35%	48	0·2	0·6
35–50%	46	0·6	2
50–70%	18	0·4	4

* Activity per mg, relative to calf serum.

fractions with an excess of β-globulin factor added to the assays discloses an additional growth factor that separates earlier than the activity previously observed (Fig. 2) when the assays were done without added β-globulin factor. The results in Table I indicate that a second 3T3 growth factor seems to be concentrated in the 50–70 per cent ammonium sulphate fraction of calf serum. Perhaps this activity is related to the activity Jainchill and Todaro (1970) have described in "agamma serum".

Still another objective of our present work is to determine the chemical nature of the β-globulin factor. The only evidence we have at present is the observation that the activity seems to be sensitive to proteolytic enzymes.

Finally, and most important, we would like to establish the biological role of the β-globulin factor. As indicated before, the factor cannot replace serum. Also, Ingrid Klinger has found that the β-globulin factor has little or no activity in stimulating "crawling" of 3T3 cells from the edge of a wound; the "crawling" phenomenon must therefore be dependent