QUIESCENT SV40 VIRUS TRANSFORMED 3T3 CELLS IN CULTURE

Dieter Paul

The Salk Institute for Biological Studies
Armand Hammer Center for Cancer Biology
San Diego, California 92112

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SUMMARY

Serum counteracts low nutrient concentrations in the culture medium in SV40 virus transformed 3T3 (SV3T3) cells. The transport of [^{3}H]-leucine into TCA soluble material in SV3T3 cells is stimulated by serum and inhibited by But_2-cAMP. When SV3T3 cells are cultured in low leucine concentrations ($\leq 8 \times 10^{-6}$ M), the cell's morphology is similar to the one of cells incubated in complete medium in the presence of But_2-cAMP and cells become quiescent. Cells become arrested throughout the cell cycle. The results suggest that the mechanism by which But_2-cAMP inhibits growth of SV3T3 cells is by inhibiting the transport of leucine in SV3T3 cells.

INTRODUCTION

Serum is essential for survival and growth of most mammalian cells in tissue culture (1). The growth rate and the final cell density of untransformed 3T3 mouse cells and of malignant SV40 virus transformed 3T3 (SV3T3) cells are proportional to the serum concentration in the culture medium (2,3). Different serum fractions have been isolated and partially purified which control the growth rate or the final cell density of SV3T3 cells (4,5) or the final cell density of 3T3 cells (3). When essential serum growth factors have been depleted by growing 3T3 cells, they become quiescent. In contrast, SV3T3 cells never become quiescent under the usual culture conditions but die due to unknown reasons after the final cell density characteristic for a given serum concentration has been attained (4).

Recently it was suggested that the levels inside the cells of essential nutrients required for growth might regulate growth in mammalian cells (6). This hypothesis was strongly supported by the finding that quiescent fetal rat liver hepatocytes cultured in arginine deficient medium (7-9) can be induced to synthesize DNA approximately 16 hours after addition of fresh serum or ornithine or arginine to the cultures (10). This finding led to the concept that the intracellular levels of either these low molecular weight compounds or their proximal metabolites might be involved in controlling the initiation of

DNA synthesis in cultured hepatocytes. Results which lead to similar conclusions were found by Holley and Kiernan (11) and others (12-15).

On the basis of these results studies were undertaken to determine whether the growth of malignant SV3T3 cells might be reversibly arrested by limiting certain essential nutrients in the culture medium.

MATERIALS AND METHODS

Materials: [^{3}H]-thymidine and [^{3}H]-leucine (spec. act. 30-40 c/mM) were obtained from New England Nuclear Co. But$_2$-cAMP and theophilline were purchased from Sigma.

Cells and growth media: SV3T3 cells were obtained from Drs. Marguerite Vogt and Renato Dulbecco. The cells were routinely cultured in Dulbecco's and Vogt's modification of Eagle's medium in the presence of 10% calf serum. The cells were transferred using 0.5% trypsin in Ca^{++} and Mg^{++}-free Tris-saline solution (pH 7.4). After centrifugation, cells were washed twice in medium and plated at the desired density in 55mm (NUNC) plastic dishes. Cultures were incubated at 37°C in a humidified 10% CO_2 incubator.

Transport studies: After cultures were incubated in the presence of [^{3}H]-leucine, the medium was removed, the cultures washed 3 times with 5 ml of ice cold Tris-saline solution (pH 7.4) (5-10 sec), trypsinized, and the resulting cell suspension then added to 1 vol of 10% trichloroacetic acid (TCA). After centrifugation (3000 rpm, 5 min), the TCA soluble radioactivity in supernatant aliquots was determined in a Beckman scintillation counter using Toluene/Triton X-100/PPO/dimethyl-POPOP scintillation fluid.

Microfluorometric analyses: SV3T3 cells were plated in the appropriate culture medium supplemented with 5% dialyzed calf serum. At desired times after plating cells were removed from the dish with crystalline trypsin (0.01%), washed several times, fixed in 20% formalin/saline solution and incubated overnight at 3°C. Cells were then treated with 4N HCl (0°C), washed and stained in a 0.005% acriflavin solution. After several washes, cells were analyzed in a flow microfluorimeter (laser beam:λ:448nm) for DNA content in individual cells.

RESULTS AND DISCUSSION

When the concentration of amino acids and vitamins in the culture medium was reduced, cells grew when high serum concentrations were present in the culture medium but they did not survive in the presence of low serum concentrations (Fig. 1). These results indicate that serum counteracts low nutrient concentrations in the medium and suggest that serum might be involved in the

TABLE 1

Uptake of [^{3}H]-leucine into TCA soluble material of SV3T3 cells under different culture conditions

Culture conditions	TCA soluble cpm per culture after incubation with [^{3}H]-leucine for 30 min.	60 min.	120 min.	240 min.	360 min.
Experiment 1 (serum step-up)					
0% serum	1800	2800	-	9000	-
0.15% serum	1800	2900	-	13000	-
0.5% serum	2000	2750	-	14000	-
1% serum	2200	4000	-	19300	-
10% serum	2600	5300	-	25800	-
Experiment 2 (serum step-down)					
0% serum	6500	12500	24000	-	-
0.15% serum	6350	12000	25500	-	-
0.5% serum	6400	12300	27500	-	-
1% serum	8500	16500	34500	-	-
10% serum	9600	19200	37500	-	-
Experiment 3					
5% serum	-	2500	-	14200	24400
5% serum plus dibutyryl-cAMP/ theophilline	-	1560	-	7200	11400
0.5% serum	-	1300	-	7100	11000

Table 1: In experiments #1 (serum step-up), #2 (serum step-down), and #3, SV3T3 cells were plated in 0.15%, 10%, and 10% calf serum, respectively. In experiment #3, But_2-cAMP (10^{-4} M) and theophilline (10^{-3} M) were added to the cultures. Sixteen hours after plating, the medium was removed, the cultures washed 3 times with warm leucine-free medium supplemented with 0.15% calf serum dialyzed against $\geq$ 100 vol of isotonic saline with 3 changes of the dialysate. The leucine concentration in dialyzed serum is $\leq 8 \times 10^{-7}$ M. Cells were then incubated in the appropriate leucine-free medium for 2 hours as indicated. Then [^{3}H]-leucine (5 µC/dish, final concentration 4×10^{-6} M) was added for the desired incubation times. The TCA soluble radioactivity was determined as indicated in Materials and Methods.

control of nutrient transport into the cells (16-19). That this might be the case is illustrated by the results summarized in Table 1. It was observed that the uptake of [^{3}H]-leucine into TCA soluble material of SV3T3 cells was stimulated by serum (Table 1). In two types of assays (serum step-up and serum step-down experiments) the [^{3}H]-leucine transport was found to be increased at higher serum concentrations (Table 1). But_2-cAMP in the culture medium exerted a striking inhibitory effect on the uptake of [^{3}H]-leucine by SV3T3 cells (Table 1).

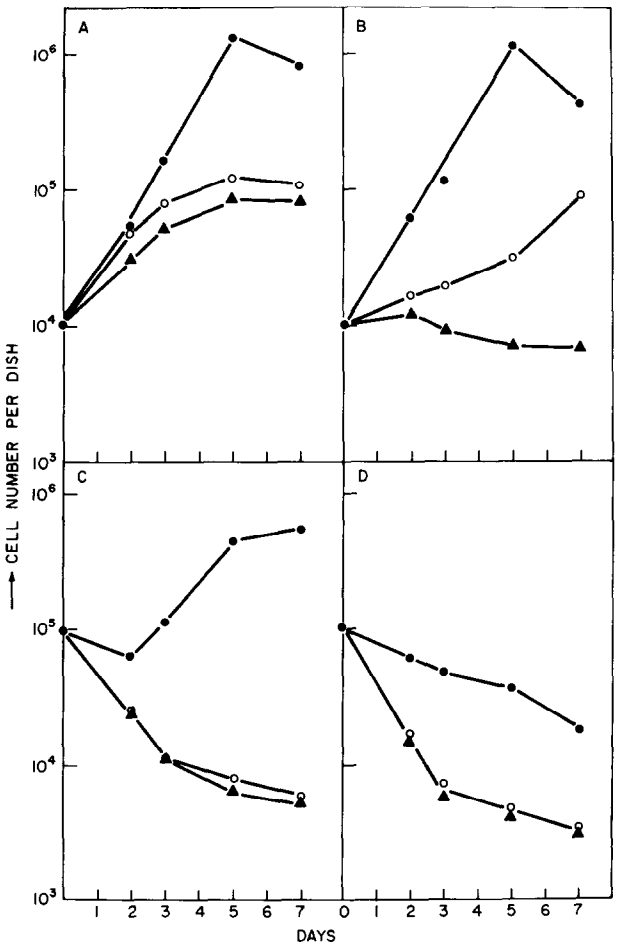

Fig. 1: SV3T3 cell growth in culture media of different composition.
SV3T3 cells (10^5 per dish) were cultured in different media containing varying concentrations of nutrients (amino acids and vitamins) and of fetal calf serum dialyzed against isotonic salt solution (see Materials and Methods). Cell number was determined with a Coulter Counter at different periods after start of the experiment. (A) Complete medium; (B) medium containing 20% of amino acids and vitamins present in complete medium; (C) medium containing 10% of amino acids and vitamins present in complete medium; (D) medium containing 5% of amino acids and vitamins present in complete medium.
●---●: 10%; o---o: 1%; ▲---▲: 0.5% serum. Abscissa: time (days). Ordinate: cell number per dish.

When the concentration of certain individual essential amino acids (leucine, histidine, isoleucine, phenylalanine, arginine) was reduced in otherwise complete medium, the morphology of SV3T3 cells changed drastically. The shape of cells cultured in low concentrations of leucine ($\leq 8 \times 10^{-6}$ M) (Fig. 2) is similar to the morphology of SV3T3 cells incubated in complete medium in the presence of But_2-cAMP/theophilline (20). Since serum enhances the uptake of leucine (Table 1), SV3T3 cells cultured in medium containing low concentrations

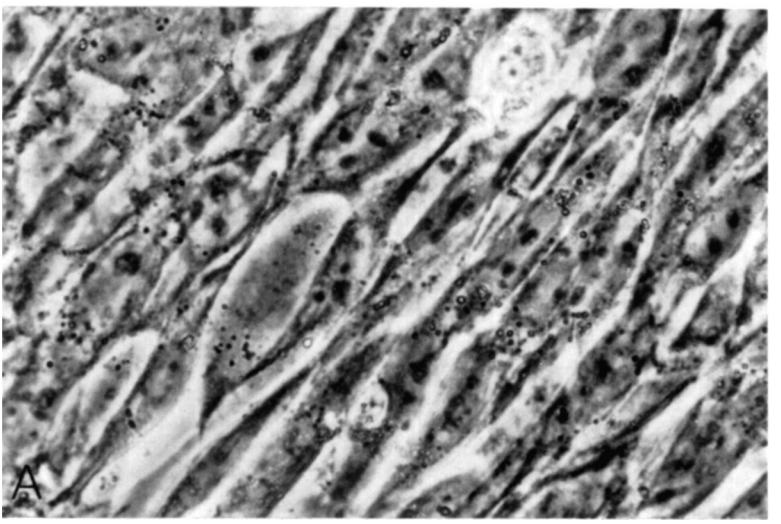

Fig.2

of leucine only survived in the presence of high concentrations of serum (data not shown).

The growth rate and the maximal density of SV3T3 cultures attained at a given serum concentration (5% dialyzed calf serum) depends on the leucine concentration in the culture medium (Fig. 3). It can be seen that at high leucine concentrations (8×10^{-4} M in complete medium) cells grow to high densities but DNA synthesis never ceases and cells start to die after a few days. In medium containing very low leucine concentrations (2×10^{-7} M) in the presence of 5% dialyzed calf serum cells grow very little if at all and die soon after the start of the experiment. At an intermediate leucine concentration, however, cells grow to a low density which remains stationary, DNA synthesis ceases and cells survive for at least 18 days without significant changes in cell number per dish (Fig. 3). It seems as if the capacity of the cells to stop synthesizing DNA and to survive is a function of a complex balance between the concentrations of serum and of leucine in the culture medium.

Addition of leucine (8×10^{-4} M) to cultures which were incubated for 11 days in 4×10^{-6} M leucine in the presence of 5% dialyzed calf serum (Fig. 3) led to a reversion of the cell's shape (Fig. 2c) within 24 hours and cells resumed growth (Fig. 3). Thus, under these conditions cells do not die at low leucine concentration but survive and are able to initiate DNA synthesis after the leucine is added back to the cultures in the appropriate concentration. Therefore, it seems possible that the growth inhibitory effect of But_2-cAMP/ theophilline on SV3T3 cells (20-28) which is accompanied by a gross morpho-

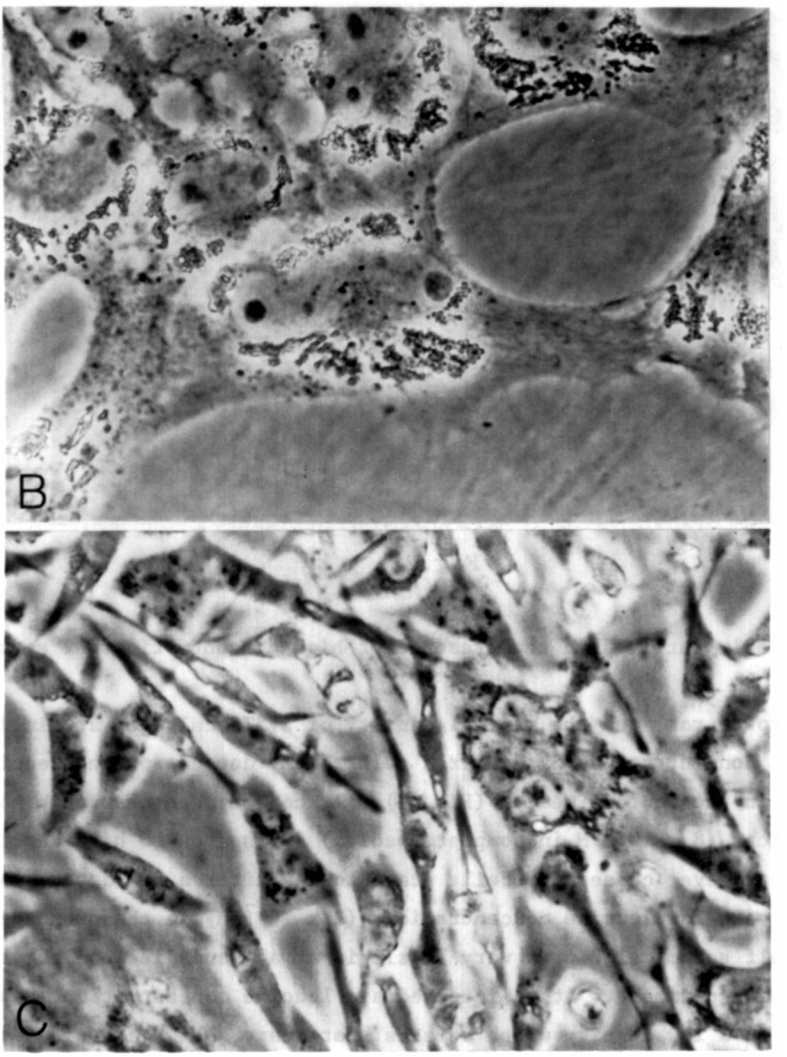

Fig. 2: Micrographs of SV3T3 cells cultured in the presence of low leucine concentrations in the culture medium.

SV3T3 cells (10^5 per dish) were plated in the appropriate medium and incubated for different periods of time. Phase-contrast microscopy, 400x. (A) Cells in complete medium (8×10^{-4} M leucine) supplemented with 5% dialyzed calf serum (day 3); (B) cells in medium containing 4×10^{-6} M leucine supplemented with 5% dialyzed calf serum (day 3); cultures were essentially unchanged at day 18; (C) cells cultured for 11 days in medium containing 4×10^{-6} M leucine supplemented with 5% dialyzed calf serum. At day 11 8×10^{-4} M leucine was added to the cultures; micrograph (2C) was taken 24 hours after leucine addition.

logical alteration of the cells might be the consequence of an inhibitory activity of the drug on the leucine uptake by SV3T3 cells (Table 1).

When a quiescent culture of SV3T3 cells kept for 11 days in medium containing 5% dialyzed calf serum and 4×10^{-6} M leucine was analyzed using a

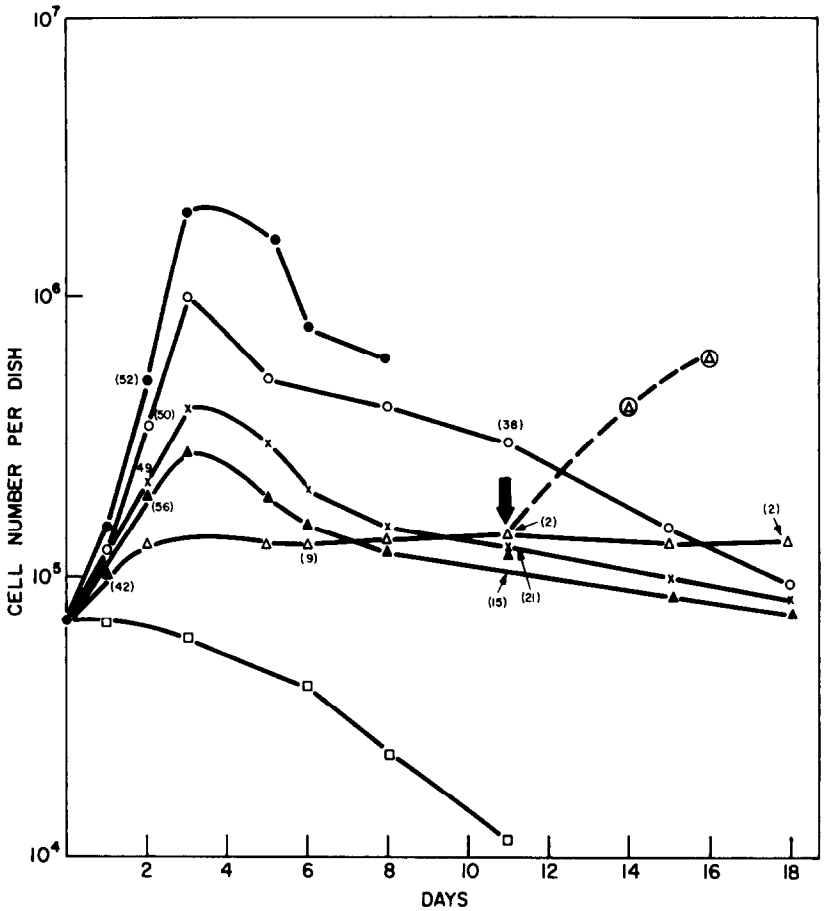

Fig. 3: Growth curves of SV3T3 cells in media supplemented with 5% dialyzed calf serum containing varying leucine concentrations.

SV3T3 cells (7 x 10^4 cells per dish) were plated in the appropriate culture media (see legend to Table 1). Cell number was determined every day. In parallel cultures, autoradiograms were prepared (50 min [^{3}H]-thymidine pulses, spec. act. 20 Ci/mM, 5 µCi/dish). Then the medium was removed, the cultures washed twice with Tris-saline solution (pH 7.4) and fixed in formaline/saline solution. Cultures were then stripped with Kodak AR-10 autoradiographic film and exposed for ≥ 7 days before being developed. Numbers in parentheses on curves indicate percentage of nuclei labelled. At day 11 (arrow) leucine (8 x 10^{-4} M) was added to the cultures which were previously incubated in 4 x 10^{-6} M leucine for 11 days. Cell number was determined at days 14 and 17.

●---●: Complete medium (8 x 10^{-4} M leucine); o---o: 3 x 10^{-5} M leucine; x—x: 1.6 x 10^{-5} M leucine; ▲--▲: 8 x 10^{-6} M leucine; Δ--Δ: 4 x 10^{-6} M leucine; □--□: 2 x 10^{-7} M leucine; Ⓐ-Ⓐ: 8 x 10^{-4} M leucine addition at day 11 to cultures containing 4 x 10^{-6} M leucine. Abscissa: time (days). Ordinate: cell number per dish.

flow microfluorimeter, results were obtained which suggested that quiescent cells are not only arrested in G1 (or G0) but also in other phases of the cell cycle (Fig. 4). The histogram shown in Fig. 4b is similar to the one obtained when SV3T3 cells cultured in the presence of But_2-cAMP/theophilline were

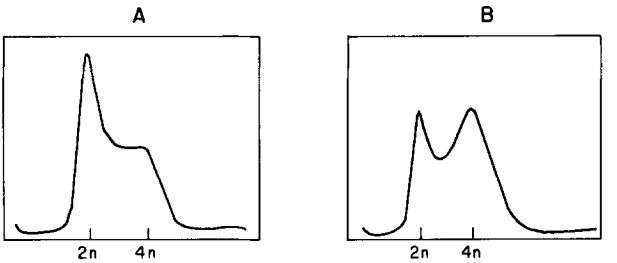

Fig. 4: Histograms obtained by microfluorometric analysis of SV3T3 cells under different culture conditions.

Cells were cultured and prepared for microfluorometry as indicated in Materials and Methods. (A) SV3T3 cells in complete medium harvested at day 2 when growing exponentially; (B) quiescent SV3T3 cultured in 4 x 10^{-6} M leucine, harvested at day 11 (see Fig. 3). Abscissa: relative amounts of DNA per cell (arbitrary units). Ordinate: cell number.

analyzed for DNA content in individual cells (27). In both instances a pronounced shift toward DNA levels of premitotic (G2) cells was observed (27) (Fig. 4).

It was previously shown that the increased cAMP content in quiescent 3T3 cells as compared with growing cells is not the result of cell-cell contact but presumably the consequence of the depletion of an essential serum growth factor which controls final cell density in 3T3 cells (29). Therefore, if cAMP is indeed involved in the regulation of growth in some mammalian cells in culture, its metabolism is probably linked to serum factors. The data reported in the present communication suggest that a link might exist between the mechanism of leucine transport and the cAMP metabolism in SV3T3 cells, both being controlled by serum constituents.

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REFERENCES

1. Eagle, H., Science, 122, 501 (1955).
2. Holley, R. W., and Kiernan, J. A., Proc. Nat. Acad. Sci. USA, 60, 300 (1968).

3. Holley, R. W., and Kiernan, J. A., in Ciba Foundation Symposium Growth Control in Cell Cultures (edit. by Knight, J., and Wolstenholm, G. E. W.), 3 (J. & A. Churchill, London, 1971).
4. Paul, D., Henahan, M., and Walter, S., manuscript in preparation.
5. Paul, D., Lipton, A., and Klinger, I., Proc. Nat. Acad. Sci. USA, 68, 645 (1971).
6. Holley, R. W., Proc. Nat. Acad. Sci. USA, 69, 2840 (1972).
7. Leffert, H., and Paul, D., J. Cell Biol., 52, 559 (1972).
8. Paul, D., Leffert, H., Sato, G., and Holley, R. W., Proc. Nat. Acad. Sci. USA, 69, 374 (1972).
9. Leffert, H., and Paul, D., J. Cell. Physiol., 81, 113 (1973).
10. Paul, D., and Walter, S., Proc. Soc. Exp. Biol. Med., in press.
11. Holley, R. W., and Kiernan, J. A., unpublished.
12. Ley, K. D., and Tobey, R. A., J. Cell Biol., 47, 453 (1970).
13. Enger, M. D., and Tobey, R. A., Biochemistry, 11, 269 (1972).
14. Rubin, H., Proc. Nat. Acad. Sci. USA, 69, 712 (1972).
15. Pohjanpelto, R., and Raina, A., Nature New Biol., 235, 247 (1972).
16. Isselbacher, K. J., New Engl. J. Med., 286, 929 (1972).
17. Sefton, B. M., and Rubin, H., Proc. Nat. Acad. Sci. USA, 68, 3154 (1971).
18. Cunningham, D. D., and A. B. Pardee, Proc. Nat. Acad. Sci. USA, 64, 1049 (1969).
19. Foster, D. O., and Pardee, A. B., J. Biol. Chem., 244, 2675 (1969).
20. Sheppard, J. R., Proc. Nat. Acad. Sci. USA, 68, 1316 (1971).
21. Peery, C. V., Johnson, G. S., and Pastan, I., J. Biol. Chem., 246, 5785 (1971).
22. Johnson, G. S., Morgan, W. D., and Pastan, I., Nature, 235, 54 (1972).
23. Otten, J., Johnson, G. S., and Pasten, I., Biochem. Biophys. Res. Comm., 44, 1192 (1971).
24. Sheppard, J. R., Nature New Biol., 236, 14 (1972).
25. Burk, R., Nature, 219, 1272 (1968).
26. Rozengurt, E., and Pardee, A., J. Cell. Physiol., 80, 273 (1972).
27. Smets, L. A., Nature New Biol., 239, 123 (1972).
28. Paul, D., Nature New Biol., 240, 179 (1973).
29. Seifert, W., and Paul, D., Nature New Biol., 240, 281 (1973).