Biochem. J. (1983) **214**, 695–702
Printed in Great Britain

Phosphate and the regulation of DNA replication in normal and virus-transformed 3T3 cells

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(*Received 7 March 1983/Accepted 19 May 1983*)

3T3 cells were cultured in media with different phosphate concentrations and the effects on DNA synthesis were examined. Even a modest phosphate depletion markedly inhibited DNA synthesis and cell multiplication in proliferating cultures. Furthermore, the decrease in the proportion of DNA-synthesizing cells observed after phosphate starvation followed the same time-course as the decrease seen after serum starvation. Cells starved to quiescence in a medium with a 100-fold decrease in phosphate concentration remained viable but non-proliferating for up to 3 weeks, i.e. they had entered a state of quiescence comparable with that seen after serum starvation. Addition of phosphate to phosphate-depleted cultures restored DNA synthesis within 24 h. Furthermore, the kinetics of [^{3}H]thymidine labelling after phosphate addition were nearly identical with the labelling kinetics following addition of serum to serum-depleted cultures. In contrast, phosphate deprivation had no inhibitory effects on DNA synthesis in simian-virus-40-transformed 3T3 cells. Furthermore, the inhibitory effects on DNA synthesis in such cells caused by a complete removal of serum could not be further enhanced by decreasing the phosphate concentration in the culture medium.

Phosphate ions have for some time been suggested to play an important role in the regulation of cell proliferation *in vitro*. One role suggested for phosphate has been to mediate the regulatory effects of serum and growth factors on DNA synthesis and cell division (Cunningham & Pardee, 1969; Weber & Edlin, 1971; Holley, 1972; Jimenez de Asua *et al.*, 1974; Holley & Kiernan, 1974; Hilborn, 1976; Greenburg *et al.*, 1977; Barsh *et al.*, 1977; Engström, 1981; Becker & Rosengurt, 1982; Zetterberg *et al.*, 1982). Experimental evidence for this suggestion was given by Cunningham & Pardee (1969), who showed that the cellular uptake of phosphate decreases when non-transformed cells become density-inhibited. In contrast, the phosphate uptake does not decrease when transformed cells grow to a confluent state. Furthermore, addition of fresh serum to density-inhibited cells causes a rapid increase in the cellular uptake of phosphate. In contrast, no change in phosphate uptake was observed after serum addition to virus-transformed cells. These results, taken with the demonstration by Holley & Kiernan (1974) that 3T3 cells can be arrested in the G_1 (or G_0) phase of the cell cycle by decreasing the phosphate concentration in the culture medium 100-fold, and that a subsequent re-addition of phosphate leads to a re-initiation of DNA synthesis, point to the phosphate ion as a potential mediator of mitogenic messages.

The aim of the present study was to characterize the role of phosphate in the regulation of cell proliferation. Special interest was focused on the kinetic effects on initiation of DNA synthesis after phosphate depletion and re-addition, as well as on the relationship between phosphate and serum for the initiation of DNA synthesis.

Abbreviation used: DMEM, Dulbecco's modified Eagle's medium; SV40, simian virus 40.

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Materials and methods

Cell culture

Normal and SV40-transformed Swiss 3T3 mouse embryo fibroblasts (Flow Laboratories, McLean, VA, U.S.A.) were maintained in NUNC tissue-culture bottles. The stock cultures were grown under humidified CO_2/air (3:47) in DMEM, supplemented with 10% (v/v) foetal-calf serum (Vogt & Dulbecco, 1960), 50 units of penicillin/ml and 50μg of streptomycin/ml. The cells were removed from the dish before transfer by treatment with 0.25% trypsin in

Tris-buffered 0.9% NaCl containing 0.5 mM-EDTA. The line was maintained by seeding approx. 3000 cells/cm^2 of culture-bottle area. These cells were transferred every third day, and never allowed to reach confluent growth. Cells used for experimental purpose were grown in NUNC 10 ml plastic tissue-culture bottles or in Petri dishes, which contained a glass coverslip on the bottom.

Normal 3T3 cells were starved to quiescence by washing the proliferating cells twice in Eagle's balanced salt solution and then placing them in medium supplemented with 0.5% foetal-calf serum for 48 h.

Growing and quiescent cells were exposed for various lengths of time to media of different serum and phosphate contents and then analysed with respect to DNA synthesis and cell multiplication. Sterile water was supplemented with all components forming DMEM (Morton, 1970) except phosphate. (All media were produced at our laboratory.) Dialysed foetal-calf serum at concentrations as indicated in the Figure legends was added together with the desired concentration of phosphate to the media. Low-molecular-weight compounds, including phosphate, were removed from serum on a Sephadex G-50 column (Piez *et al.*, 1960) or by membrane dialysis against 0.9% NaCl (50 ml of serum against 2 litres of NaCl) for at least 48 h (the NaCl was replaced three times).

Autoradiography

The cell cultures were labelled with [^{3}H]-thymidine (25 Ci/mol) either continuously (0.5 μCi/ml of culture medium) for a period of 24 h before fixation or with a pulse (5 μCi/ml of culture medium) for 1 h before fixation. At the end of the experiment, these cultures were washed twice in 0.9% NaCl solution, fixed in 95% (v/v) ethanol for 1–24 h and thereafter maintained in an air-dried state until the autoradiography was performed. Before the film (Kodak AR-10 stripping film) was applied, these cells were treated in 5% (v/v) trichloroacetic acid at 4°C for 5 min and then washed in cold running water for 20 min to remove non-incorporated [^{3}H]-thymidine. After an exposure period of 7–10 days, the autoradiographs were developed with Kodak D 19b developer (3.5 min at 18°C), fixed in Kodak acid X-ray fixative with hardener (5 min at 18°C), washed in cold running water (20 min at 4°C), routinely stained in haematoxylin and eosin, and finally dehydrated in ethanol. The percentage of labelled cells was determined by light-microscopic counting of at least 1000 cells per slide (Zetterberg & Killander, 1965).

Flow cytophotometry

The cells cultivated in bottles were washed with EDTA solution (0.2 mM) briefly at room temperature. The EDTA was removed and the cells were then exposed to 0.25% trypsin solution at 37°C until the cells were detached from the glass surface (normally 2–5 min). The cells were then suspended in medium containing 10% foetal-calf serum. After 5 min centrifugation at approx. 180 *g*, the supernatant was removed. The cells were then washed once in 0.2 M-Tris-buffered saline and fixed by adding of 1 ml of 95% ethanol at 0°C, drop by drop with vigorous stirring. The suspension was kept in a refrigerator (4°C) until further analysis was completed.

The cells were stained in a buffer solution containing 0.14 M-NaCl, 0.11 M-Tris/HCl (pH 7.0), and 50 mg of ethidium bromide per litre of buffer solution; 50 μl of ribonuclease (100000 units/ml in 0.9% NaCl solution) was added (at 37°C) to every 5 ml of stain solution.

The cellular DNA content was determined in a flow cytophotometer (Phywe) (Zetterberg & Engström, 1981).

Results

Effects of phosphate deprivation on DNA replication in normal 3T3 cells

The effects of phosphate deprivation on DNA synthesis in exponentially growing 3T3 cells are shown in Fig. 1. A 10-fold decrease in the phosphate concentration in a culture medium supplemented with 10% serum caused a decrease in the proportion of cells synthesizing DNA from 43% to 17% as measured by [^{3}H]thymidine labelling between 71 and 72 h after the change of medium. The proportion of labelled cells fell further, to less than 10%, if the concentration was instead decreased 100-fold or 1000-fold. If phosphate was completely removed, only a minor proportion of cells (4%) synthesized DNA between 71 and 72 h after onset of phosphate starvation. Only a small difference in the inhibitory response to phosphate starvation was observed between the cultures supplemented with 2% and with 10% serum. The inhibitory effects of phosphate deprivation on DNA synthesis therefore appeared to be independent of the serum concentration in the culture medium.

Fig. 2 shows the effects of phosphate starvation for various times on DNA synthesis in growing 3T3 cells. A marked inhibitory effect on DNA synthesis was observed after a 10-fold decrease in the phosphate concentration. This inhibitory effect could be slightly enhanced after a 100-fold or 1000-fold decrease in the concentration of phosphate. The major inhibitory effect caused by phosphate depletion was exerted during the first 24 h after the change of medium. The inhibitory effect was only

slightly enhanced by a prolonged phosphate starvation. The proportion of S-phase cells decreased with the same kinetics when 10% serum or 2% serum was added to the culture medium. Again the inhibitory effects of phosphate deprivation appeared to be independent of the serum concentration in the culture medium. Furthermore, Fig. 2 shows an important similarity between effects of phosphate deprivation and serum starvation on DNA synthesis in growing cells. The time courses for the decrease in percentage of [^{3}H]thymidine-labelled cells were almost identical for both 'starvation' situations.

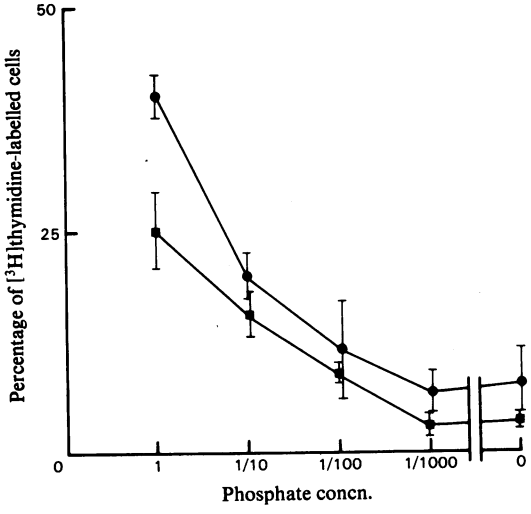

Fig. 1. *Quantitative effect of deprivation of phosphate ions on DNA synthesis in exponentially growing 3T3 cells*
These cells were allowed to grow in complete DMEM supplemented with 10% (●) or 2% (■) foetal-calf serum for 24h and were thereafter briefly washed with 0.9% NaCl. The cells were then exposed to media in which the concentration of phosphate had been lowered to 1/10, 1/100 or 1/1000 of that in DMEM or from which it had been totally excluded (0), for 72h. These media were supplemented with 10% (●) or 2% (■) foetal-calf serum. The cells were exposed to 5μCi of [^{3}H]-thymidine/ml of medium during 1h before fixation. The proportion of cells synthesizing DNA during that period was determined by autoradiography. The results are means ± S.D. for four different experiments.

Table 1 shows the long-term effects of phosphate starvation on proliferation in 3T3 cells. Control cultures grown in a complete DMEM reached confluency already after 2 days (i.e. in total 3 days). If the phosphate concentration was decreased to one-tenth of that in DMEM, the cells continued to proliferate, but at a lower rate, and reached confluence 3 days after change of medium. In contrast, when the phosphate concentration was decreased to one-hundredth of that in DMEM, the cells ceased to proliferate at a subconfluent stage. The cells remained viable but quiescent for up to 3 weeks, after which morphological signs of cellular detachment and cell death were observed. If the phosphate concentration was further decreased to one-thousandth of that in DMEM, or completely removed, toxic effects were observed already 7–8 days after change of medium.

Figs. 3(*a*)–3(*c*) show the effects on DNA synthesis of phosphate re-addition to exponentially growing cells previously depleted of phosphate. Fig. 3(*a*) shows that phosphate re-addition to cells exposed to media in which the phosphate concentration had been lowered to one-tenth of the normal value almost restored the proportion of DNA-synthesizing cells from 22% to 37% [i.e. to a value comparable with that in exponentially growing cells (45%)].

The stimulatory effect of phosphate addition to phosphate-depleted cells appeared to be independent of the duration of the phosphate starvation, since the stimulatory responses were almost identical if phosphate was added to cells previously deprived of phosphate for 24, 48 or 72h. Significant stimulatory

Table 1. *Long-term effects of phosphate starvation on the viability of continuously proliferating 3T3 cells*
These cells were grown in tissue-culture bottles containing complete DMEM supplemented with 10% serum for 24h and were thereafter briefly rinsed in 0.9% NaCl. The cells were then exposed to media with normal (1) phosphate concentration, to media in which the concentration of phosphate has been lowered to 1/10, 1/100 or 1/1000 of that in DMEM or to media from which phosphate had been excluded (0) for 21 days. These media were supplemented with 10% dialysed foetal-calf serum and were changed every second day. Ten defined areas of each bottle were checked daily with an inverted microscope. The cells were classified as subconfluent and viable (S), confluent and viable (C) or as dead cells (+) as judged by the morphological examination.

Day ...	1	2	3	4	7	8	9	10	11	12	13	14	15	16	21
[Phosphate]															
1	S	S	C	C	C	C	C	C	C	C	C	C	C	C	C
1/10	S	S	S	C	C	C	C	C	C	C	C	C	C	C	C
1/100	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(+)
1/1000	S	S	S	S	S	(+)	+	+	+	+	+	+	+	+	
0	S	S	S	S	+	+	+	+	+	+	+	+	+	+	+

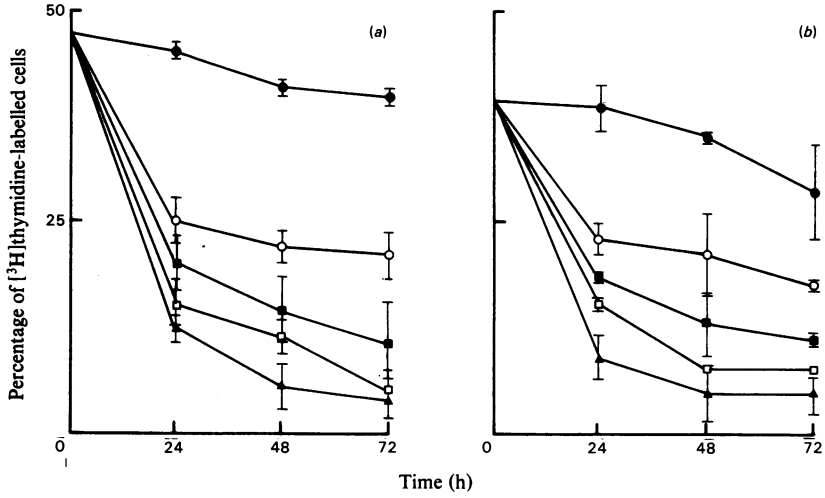

Fig. 2. *Kinetic effect of deprivation of phosphate ions on DNA synthesis in exponentially growing 3T3 cells*
These cells were allowed to grow in complete DMEM supplemented with 10% (*a*) or 2% (*b*) foetal-calf serum for 24 h and were thereafter briefly washed in 0.9% NaCl. The cells were then exposed to media with normal phosphate concentration (●), to media in which the concentration of phosphate had been lowered to 1/10 (○), 1/100 (■) or 1/1000 (□) of that in DMEM for various length of time. These media were either supplemented with 10% (*a*) or 2% (*b*) dialysed foetal-calf serum. Exponentially growing cells exposed to a complete medium supplemented with 0.5% serum were used as controls (▲). The cells were exposed to 5 μCi of [^{3}H]thymidine/ml of medium during 1 h before fixation. The proportion of cells synthesizing DNA during that period was determined by autoradiography. The results are means ± S.D. for three different experiments.

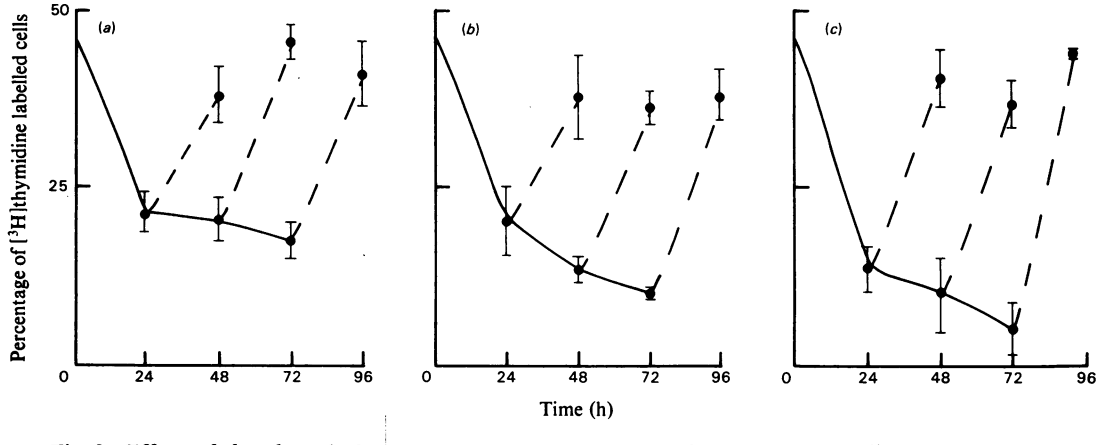

Fig. 3. *Effects of phosphate depletion and re-addition on DNA synthesis in exponentially growing 3T3 cells*
These cells were allowed to grow in complete DMEM supplemented with 10% foetal-calf serum for 24 h and were thereafter briefly rinsed with 0.9% NaCl. The cells were then exposed to media in which the concentration of phosphate had been lowered to 1/10 (*a*), 1/100 (*b*) or 1/1000 (*c*) of that in DMEM for up to 72 h. These media had been supplemented with 10% dialysed foetal-calf serum. To elucidate the effects of phosphate re-addition, 0.9 mM-phosphate was added to cultures previously deprived of phosphate, and cells were fixed 24 h after phosphate re-addition (dashed lines). All cell cultures were exposed to [^{3}H]thymidine/ml of medium during 1 h before fixation. The proportion of cells synthesizing DNA during that period was determined by autoradiography. The results are means ± S.D. for three different experiments.

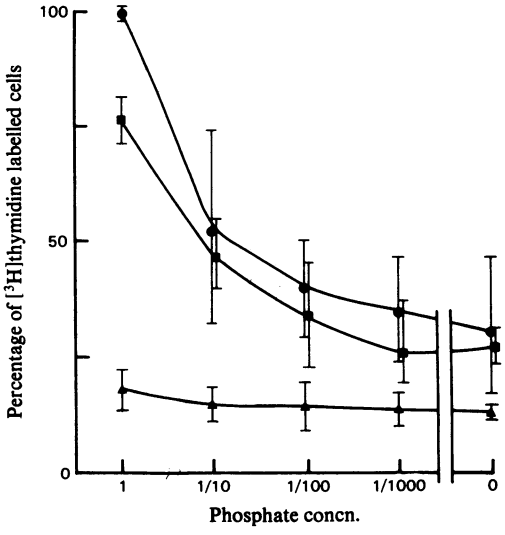

Fig. 4. *Effect of phosphate deprivation on DNA synthesis after serum stimulation of quiescent cells*

Quiescent serum-starved cells were exposed to media with normal phosphate concentration (1), to media in which the concentration of phosphate had been lowered to 1/10, 1/100 or 1/1000 of that in DMEM or to media from which phosphate had been excluded (0) for 24h. These media were supplemented with either 10% (●) or 2% (■) dialysed foetal-calf serum. Quiescent cells exposed to media with 0.5% dialysed foetal-calf serum (▲) were used as controls. To each dish 0.5μCi of [^{3}H]-thymidine/ml of medium was added, and it was present during the whole 24h assay period. The proportion of cells that had initiated DNA synthesis during the 24h period was determined by autoradiography. The results are means ± S.D. for four different experiments.

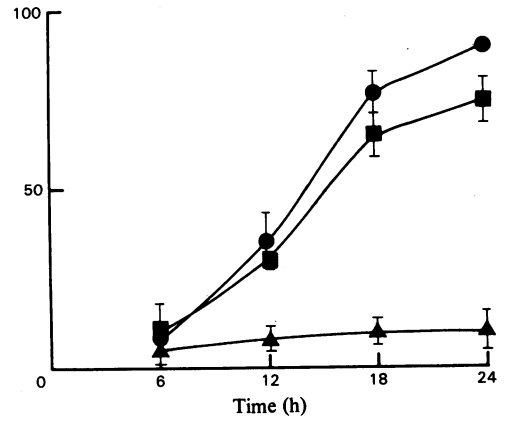

Fig. 5. *Effect of phosphate and serum addition on DNA synthesis in 3T3 cells*

Cells were starved to quiescence in 0.1% serum (●) or in 10% serum and low phosphate concentrations (■) for 48h. After this period of time 10% serum (●) or 1mM-phosphate (■) was added back to the cultures. Cells continuously maintained in 0.1% serum were used as controls (▲). The cells were then fixed after various periods of time. The proportion of cells that had initiated DNA synthesis at various times was determined by autoradiography after continuous labelling with 0.5μCi of [^{3}H]thymidine/ml of medium. The results are means ± S.D. for four different experiments.

effects could also be achieved by adding phosphate to cells exposed to media in which the phosphate concentration had been previously lowered to one-hundredth (which increased the [^{3}H]thymidine labelling percentage to 34–36%) or to one-thousandth (34–44%) of that in DMEM (Figs. 3*b* and 3*c*). In all these situations the stimulatory effects were achieved within 24h after phosphate re-addition.

Fig. 4 shows that the stimulatory response of serum addition to serum-starved quiescent cells was markedly inhibited if phosphate was depleted from the culture medium. A 10-fold decrease in the concentration of phosphate decreased the proportion of cells that had initiated DNA synthesis 24h after serum addition from 99% to 45%. This inhibitory effect became further enhanced if the phosphate concentration was decreased 100- or 1000-fold. However, the inhibitory effect caused by phosphate deprivation was equally pronounced when 2% or 10% dialysed serum was added to the culture medium. Thus the counteractive effect that was caused by phosphate depletion on serum addition to serum-starved cells appeared to be independent of the serum concentration in the culture medium.

The kinetics of [^{3}H]thymidine labelling after addition of phosphate to phosphate-depleted cells were nearly identical with the labelling kinetics after stimulation of serum-starved cells by addition of 10% serum (Fig. 5). In neither of these stimulatory situations was there any increase in the proportion of [^{3}H]thymidine-labelled cells during the first 6h after the onset of stimulation. After 12h a proportion of cells had entered S-phase, and after 18h most of the cells that responded to growth stimuli had initiated DNA synthesis in both situations. Only a minor increase in the proportion of cells that went through the S-phase was observed between 18 and 24h.

Effects of phosphate deprivation on DNA replication in SV40-transformed 3T3 cells

The effects of phosphate deprivation on DNA synthesis in normal 3T3 cells compared with SV40-transformed 3T3 cells are shown in Fig. 6. The diagrams represent the intercellular relative distribution of DNA contents in growing 3T3 cells and SV40-transformed 3T3 cells immediately before

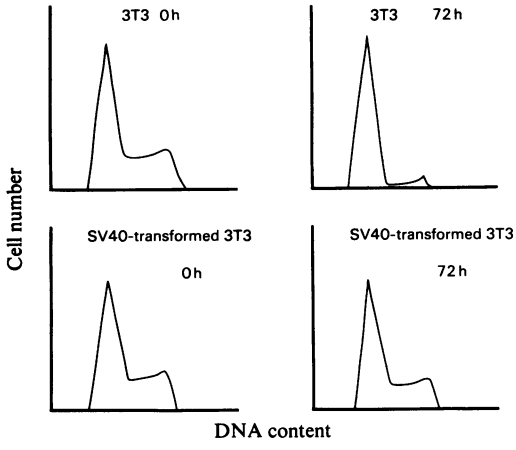

Fig. 6. *Effect of phosphate starvation on DNA synthesis* Normal and SV40-transformed 3T3 cells were allowed to grow in a complete DMEM supplemented with 10% foetal-calf serum and were thereafter briefly rinsed with 0.9% NaCl. The cells were then exposed to media in which the concentration of phosphate had been lowered to 1/100 of that in DMEM for 72h. These media were supplemented with 10% dialysed foetal-calf serum. DNA distribution curves at the beginning of the phosphate starvation (0h) and 72h later (72h) were obtained by flow cytophotometry after staining with ethidium bromide.

(0h) and 72h after a 100-fold decrease in the phosphate concentration in the culture medium.

After 72h exposure to a phosphate-depleted medium, the overwhelming majority of the normal 3T3 cells contained post-mitotic amounts of DNA, indicating that the cells had been starved to quiescence in G_1 phase. In contrast, SV40-transformed 3T3 cells contained both post-mitotic intermediate and premitotic amounts of DNA, which indicates the existance of G_1-phase as well as S- and G_2-phase cells in the population.

The effects of phosphate depletion on DNA synthesis in SV40-transformed 3T3 cells are shown in detail in Figs. 7 and 8. There was no decrease in the proportion of cells synthesizing DNA after a 10-, 100- or 1000-fold decrease in the phosphate concentration. Nor did the phosphate concentration exert any further inhibitory effects on DNA synthesis when the serum concentration was lowered to 0.1 or 0%. Thus there does not appear to exist any synergistic effect between serum depletion and phosphate starvation on DNA synthesis in growing SV40-transformed cells.

Discussion

In the present study 3T3 cells were exposed to culture media with different phosphate concentrations, and the effects on DNA synthesis were

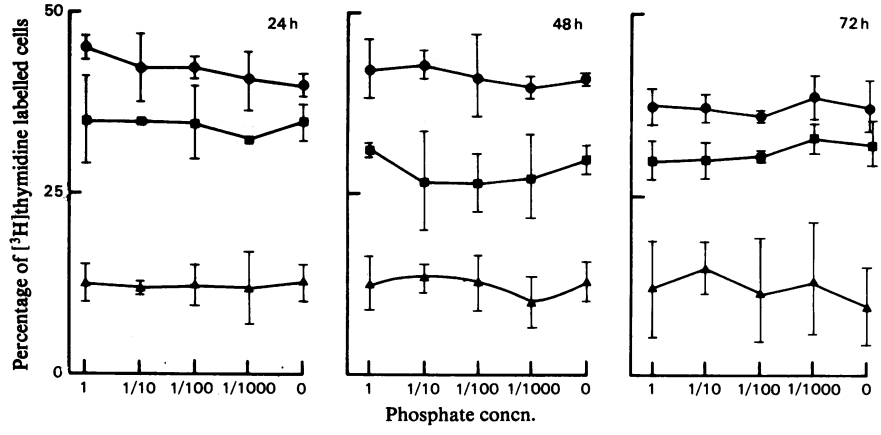

Fig. 7. *Effect of deprivation of phosphate ions on DNA synthesis in exponentially growing SV40-transformed 3T3 cells* These cells were allowed to grow in complete DMEM supplemented with 10% foetal-calf serum for 24h and were thereafter briefly washed with 0.9% NaCl. The cells were then exposed to media in which the concentration of phosphate had been lowered to 1/10, 1/100 or 1/1000 of that in DMEM or from which it had been totally excluded (0), for up to 72h. These media were supplemented with 10% (●) or 0.1% (■) foetal-calf serum or no serum at all (▲). The cells were exposed to 5 μCi of [^{3}H]thymidine/ml of medium during 1h before fixation. The proportion of cells synthesizing DNA during that period was determined by autoradiography. The results are means ± S.D. for three different experiments.

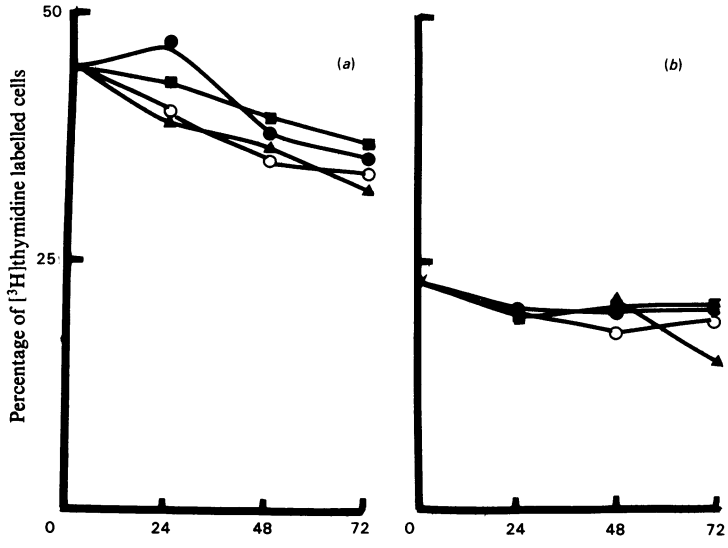

Fig. 8. *Kinetic effect of phosphate deprivation on DNA synthesis in exponentially growing SV40-transformed 3T3 cells* These cells were allowed to grow in complete DMEM supplemented with 10% foetal-calf serum for 24 h and were thereafter briefly washed in 0.9% NaCl. These cells were then exposed to media with normal phosphate concentration (●) or to media in which the concentration of phosphate had been lowered to 1/10 (■), 1/100 (○) or 1/1000 (▲) of that in DMEM for various lengths of time. These media were either supplemented with 10% (*a*) or no (*b*) dialysed foetal-calf serum. The cells were exposed to 5 μCi of [^{3}H]thymidine/ml of medium during 1 h before fixation. The proportion of cells synthesizing DNA during that period was determined by autoradiography. The results are means for three different experiments.

examined. Even a modest phosphate depletion markedly inhibited DNA synthesis and cell multiplication in proliferating cultures. Furthermore, the decrease in the proportion of DNA-synthesizing cells observed after phosphate starvation followed the same time-course as the decrease seen after serum starvation. Cells starved to quiescence in a medium with a 100-fold decrease in phosphate concentration remained viable but non-proliferating for up to 3 weeks, i.e. they had entered a state of quiescence comparable with that seen after serum starvation. Addition of phosphate to phosphate-depleted cultures restored DNA synthesis within 24 h. Furthermore, the kinetics of [^{3}H]thymidine labelling after phosphate addition were nearly identical with the labelling kinetics after addition of serum to serum-depleted cultures. In contrast, phosphate deprivation did not exert any inhibitory effects on DNA synthesis in SV40-transformed 3T3 cells. Furthermore, the inhibitory effects on DNA synthesis in SV40-transformed 3T3 cells caused by a complete removal of serum could not be further enhanced by lowering the phosphate concentration in the culture medium.

A plausible explanation for these results and those presented by Holley & Kiernan (1974) may be that the effects of serum on DNA replication and cell multiplication are mediated by altered fluxes of phosphate ions from the external medium into the cells. Holley & Kiernan (1974) showed that exponentially growing cells can be starved to quiescence in G_1 phase by decreasing either the serum content or the phosphate concentration of the culture medium. The present paper shows that the kinetics of the decrease in percentage of [^{3}H]thymidine-labelled cells after phosphate depletion is similar to that after serum starvation. Furthermore, Holley & Kiernan (1974) showed that phosphate addition to phosphate-depleted cells restored DNA synthesis. We demonstrate in the present paper that there is an important similarity between the time course of cellular entry into the S-phase after serum stimulation and that after phosphate addition to phosphate-depleted cells. This similarity indicates that serum depletion and phosphate depletion starve cells to quiescence at a similar stage in the G_1-phase of the cell cycle. Furthermore, the results suggest that both types of stimuli activate an equally long chain of reactions preceding initiation of DNA synthesis.

Alterations in the external supply of phosphate ions may affect the growth-regulatory response in several different ways, since phosphate is involved in several different metabolic pathways. Phosphate ions are utilized in phosphorylation reactions, energy metabolism, purine pyrimidine biosynthesis *de novo*

and as a constituent in phospholipids (see Rothblath & Cristofalo, 1972, for review). The mechanism by which phosphate ions play a critical role in the regulation of cell proliferation can be interpreted in at least two different ways.

(1) Phosphate ions regulate DNA synthesis primarily at an intracellular level. It is then plausible that variations in the intracellular concentrations of phosphate control the initiation of DNA replication.

(2) Phosphate ion acts primarily at the cell surface.

Experimental evidence for the first alternative was given by Cunningham & Pardee (1969), who reported that addition of fresh serum to density-inhibited 3T3 cells, which brings about initiation of DNA synthesis and cell division, causes increases in the cellular uptake of phosphate. Conversely, the phosphate uptake decreases when cells grow to a density-inhibited state of quiescence (Bladé *et al.*, 1966; Cunningham & Pardee, 1969; Weber & Edlin, 1971; Havel *et al.*, 1975; Jullien & Havel, 1976). On the other hand, it was shown that insulin addition to quiescent 3T3 cells stimulated phosphate uptake, but had little effect on DNA synthesis (Holley & Kiernan, 1974).

These contradictory considerations prompted Cunningham and co-workers to evaluate the possible involvement of a rapid increase in phosphate uptake as a causal event in the initiation of DNA synthesis after addition of serum to quiescent 3T3 cells. They clearly demonstrated that initiation of proliferation was not always accompanied by an increase in phosphate uptake, and further showed that the rapid increase in phosphate uptake that follows addition of serum is not necessary for the subsequent initiation of DNA (Greenburg *et al.*, 1977). They also demonstrated that an increase in the intracellular pool of phosphate is not a necessary prerequisite for initiation of proliferation (Barsh *et al.*, 1977). The second interpretation, that phosphate controls cell proliferation by an action on the cell surface, therefore appears more plausible. This suggestion is also in line with findings by Mastro (1979) that endogenous membrane phosphorylation increases in serum-stimulated 3T3 cells. This phosphorylation, which was apparently dependent on the supply of phosphate ions from the culture medium, was found to increase in early G_1-phase and to reach a maximum before DNA synthesis. Mastro (1979) therefore concluded that membrane phosphorylation was one of the biochemical steps in G_1-phase necessary for cells to move into S-phase.

It remains, however, to be clarified whether phosphate in fact plays a critical regulatory role by some action at the cell surface. It cannot be excluded at this stage that, in spite of the evidence given, the cellular uptake of phosphate is a critical prerequisite for cells to initiate DNA synthesis after mitogenic stimulation.

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