Kinetics of Early Changes in Phosphate and Uridine Transport and Cyclic AMP Levels Stimulated by Serum in Density-Inhibited 3T3 Cells

(fibroblasts/membrane/tissue culture/growth regulation)

LUIS JIMENEZ DE ASUA, ENRIQUE ROZENGURT, AND RENATO DULBECCO

Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A, England

ABSTRACT   A kinetic study of the early changes in uridine- and phosphate-transport rates and 3':5'-cyclic AMP levels induced by the addition of serum to quiescent 3T3 cells revealed that the increase in phosphate uptake and the decrease in the intracellular concentration of 3':5'-cyclic AMP immediately follow the addition of serum. In contrast, the increase in uridine transport occurred after a lag of several minutes and was clearly preceded by the other changes. The activation of phosphate transport involved an increase in V_{max}, while a more complex pattern was observed for uridine transport. A possible role of phosphate in connection with the other early events was also studied. The addition of phosphate to quiescent cells produced a time-dependent decrease in 3':5'-cyclic AMP levels and this ion was required for full activation of uridine transport by serum. The results show that the increase in phosphate transport is a primary event in the reinitiation of growth and suggest that the increase in phosphate transport may be connected with subsequent metabolic steps.

Addition of serum to density-inhibited 3T3 cells rapidly stimulates several changes in membrane-dependent properties (1) and subsequently promotes reinitiation of DNA synthesis and cell division (2). Within minutes of serum addition, phosphate- and uridine-transport systems are increased several fold (3), and 3':5'-cyclic AMP (cAMP), which is strongly implicated in the regulation of growth and morphology of cultured fibroblasts, decreases (4–6). cAMP may play an important role in the changes of several transport systems (7), and it strongly affects the early activation of uridine transport by serum or insulin (8). However, the stimulation of phosphate transport by serum is affected only slightly by changes of cAMP concentration (8), suggesting that this activation is achieved by a cAMP-independent mechanism. Therefore, phosphate transport is of particular interest because it may be a primary event in the regulation of cell growth. This possibility is also suggested by the observation that the saturation density (9) and the migration of 3T3 cells (10) are influenced by the concentration of this ion in the medium.

In this paper, we report on the activation of phosphate transport in density-inhibited 3T3 cells by serum, defining its relationship to other early events such as changes of cAMP levels and of uridine transport activity.

MATERIALS AND METHODS

BALB/c/3T3 cells (Clone A_{31}) (17) were maintained in reinforced Eagle's medium supplemented with 10% serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in Nunc petri dishes. Transport was measured at 37° on cells attached to 30-mm dishes. Cultures of confluent cells were usually used 3 days after the last change of medium. In order to measure uptake of labeled substrates into acid-soluble pools, the cells

were washed four times with ice-cold isotonic saline and extracted for 30 min at 4° with 5% trichloroacetic acid. Radioactivity was measured in an aliquot counted in Triton-toluene scintillation fluid.

cAMP determinations were carried out by the method of Gilman (11) using cAMP-binding protein and protein kinase inhibitor from bovine muscle. The preparation of the samples for cAMP determinations was carried out as described by Manganiello and Vaughan (12). For each determination, the contents of two dishes were pooled and the cAMP was measured in triplicate. Protein was determined by the method of Lowry (13) after washing the attached cells five times with phosphate-buffered salt solution.

Isotopically labeled compounds were obtained from the Radio-chemical Center.

RESULTS

The changes in phosphate and uridine transport and in cAMP concentration after the addition of serum to density-inhibited 3T3 cells are shown in Fig. 1. The rates were determined by exposing the cells to [³H]uridine and [³²P]phosphate simultaneously for 2.5 min. During this period the uptake of either substance is linear with time. The rate of phosphate transport increased rapidly and linearly after the addition of serum and reached a maximum 10 min later; then it stayed constant. The curve has been extrapolated back to the origin. The absence of detectable lag strongly suggests that phosphate uptake is directly stimulated by serum. A rapid increase in phosphate transport (i.e., detectable within 5 min) was also observed when serum was added to secondary mouse embryo fibroblasts that had been serum-starved indicating that this fast transport increase is not restricted to 3T3 cells.

In sharp contrast with the results obtained with phosphate, the rate of uridine transport started to increase after a lag of 5–7 min, suggesting that additional steps mediate the activation by serum of this transport system. After the lag, the rate of uridine transport increased linearly to a maximum. Although the experiment shown in Fig. 1 was carried out at low phosphate concentration (in order to use the double-labeling technic described in the figure legend), a similar lag was seen in other experiments performed in the presence of phosphate at the concentration present in the regular medium.

The cAMP level dropped rapidly without an evident lag. A significant reduction was observed 3 min after serum addition and minimal values were reached after about 30 min. This result confirms previously published results (4–6).

Without serum addition there was no change of transport or of cAMP level (Fig. 1, broken lines).

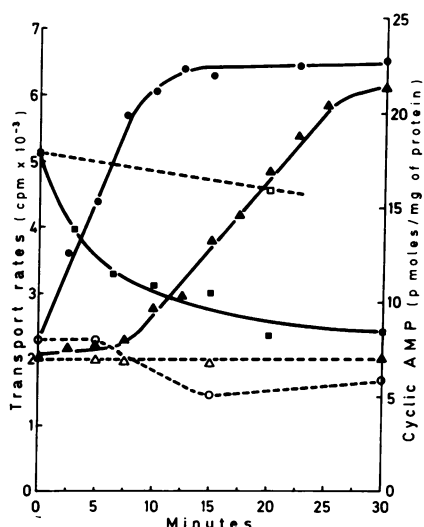Phosphate and uridine transport systems display important

FIG. 1. Kinetics of the serum effect on phosphate (●———●) and uridine- (▲———▲) transport rates and on cAMP levels (■———■) in density-inhibited 3T3 cells. At time 0, quiescent BALB/c/3T3 cells grown in 30-mm dishes were washed with phosphate-free medium and incubated in the absence (broken lines) or in the presence (solid lines) of 25% dialyzed serum. (The numbers on the left ordinate have been multiplied by 10^{-3}.) In the presence of serum, transport rates were determined every 2.5 min as indicated. The cultures were labeled with [^{32}P]phosphate (50 μCi/μmol, 5 μCi/ml) and [^{3}H]uridine (29 mCi/μmol, 5 μCi/μml) for 2.5 min and then the uptake into acid-soluble pools was measured. The amount of isotope incorporated by the cell in a pulse is plotted at the end of the pulse. Each point represents the average of two determinations. cAMP was measured in cells grown in 90-mm dishes. At the beginning of the experiment the medium of some dishes was rapidly replaced by cold 5% trichloroacetic acid (time 0) and other dishes received medium containing 25% serum. The incubation was terminated at different times, as indicated, by replacing the medium with cold 5% trichloroacetic acid. cAMP was measured as described in *Materials and Methods*.

kinetic differences. The activation of phosphate transport by serum only involves a change in the V_{max}, while the activation of uridine transport follows a more complex pattern: serum causes a shift from a two-slope kinetics, as previously reported (14), to a single-component kinetic with an increased V_{max} (Fig. 2).

Since the increase in phosphate transport occurs immediately after serum addition and is almost insensitive to changes in cAMP levels (8), we studied the possibility that phosphate affects cAMP levels and uridine transport. Addition of 2 mM phosphate to density-inhibited 3T3 cells grown in the presence of a regular phosphate concentration (0.9 mM) produced a decrease in cAMP level, which was slower than that brought about by 25% serum (Fig. 3). Furthermore, the same concentration of phosphate added to the medium together with a small amount of serum (1%) caused a decrease in cAMP level comparable to that produced by the addition of 25% serum alone.

Since the addition of phosphate decreased the levels of cAMP and this nucleotide was shown to be involved in the regulation of uridine transport (8), we also studied the influence of different concentrations of phosphate on uridine-transport rate (Fig. 4). Phosphate clearly enhanced the increase in uridine transport produced by serum. These results suggest that the activation of phosphate uptake by serum
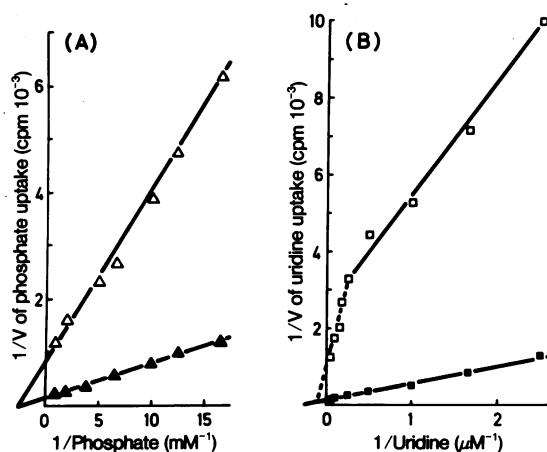

FIG. 2. Double-reciprocal plots of transport rates as a function of phosphate (A) or uridine (B) concentration. (The numbers on the ordinates have been multiplied by 10^{-3}.) At the beginning of the experiment, the medium of these cultures was replaced by serum-free medium (open symbols) or by medium containing 25% of dialyzed serum (solid symbols); 20 min later, the cultures were exposed to different concentrations of [^{3}H]uridine and of [^{32}P]phosphate for 5 min. Determination of uptake into acid-soluble pools and other experimental details were as described in *Materials and Methods*.

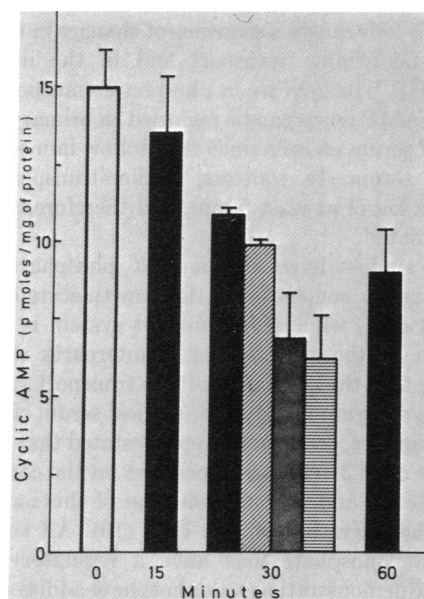may have a positive effect on the subsequent activation of uridine transport.

FIG. 3. Effect of phosphate, serum, or both on cAMP levels of density-inhibited BALB/c/3T3 cells. The cultures were grown to confluence in 90-mm dishes as described in *Materials and Methods*. At the beginning of the experiment the medium of some dishes was rapidly replaced by 5% cold trichloroacetic acid to determine the cAMP content as time 0. Other dishes received 2 mM phosphate (solid bars) at pH 7.3 and cAMP was measured 15, 30, and 60 min later, as indicated. Other dishes received 1% serum (bars with oblique cross-hatching), 1% serum + 2 mM phosphate (dotted bars), and, for comparison, 25% serum (bars with horizontal cross-hatching); cAMP levels were measured 30 min later. All the additions were made directly into the depleted medium. Other experimental details were as described in *Materials and Methods*.
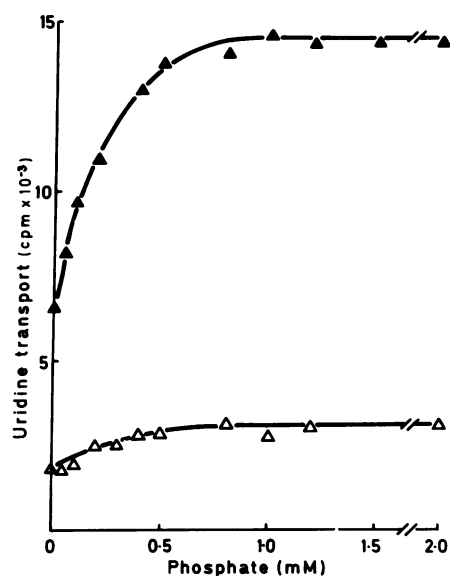
FIG. 4.   Effect of phosphate concentration on uridine transport in the absence (open symbols) or in the presence (solid symbols) of 25% serum. (The numbers on the ordinate have been multiplied by 10^{-3}.) Quiescent 3T3 cells were washed with phosphate-free medium and incubated with or without serum in the presence of different phosphate concentrations. After 20 min, the cultures were exposed to [^{3}H]uridine for 5 min and the transport rates determined as described in *Materials and Methods*.

DISCUSSION

The present results show that the addition of serum to density-inhibited 3T3 cells causes a sequence of changes in the rates of phosphate and uridine transport and in the intracellular levels of cAMP. The increase in phosphate transport and the decrease in cAMP levels can be regarded as primary events in the action of serum on cells since they follow immediately the addition of serum. In contrast, uridine-transport changes begin after a lag of at least 5 min, and therefore, may be indirectly mediated.

Previous studies have shown that phosphate-transport activity is tightly coupled with the growth state of untransformed 3T3 cells, while this transport system is constantly switched on in the transformed counterparts (3–15). Our results show that the activation of this transport system upon the addition of serum is one of the earliest serum effects so far described. Further, preliminary reports stated that the saturation density of 3T3 cells was dependent on the concentration of phosphate (9) and that the addition of this ion markedly increased the migration of 3T3 cells (10). All these results suggest that phosphate ions have a regulatory effect on growth. The demonstration that phosphate addition decreases the steady-state level of cAMP provides a possible explanation for these effects. Thus, the stimulation of cell migration by phosphate (10) is consistent with a role of cAMP in control of movement (16) and with the effect of phosphate addition on cAMP levels reported here.

Rapid decreases in cAMP levels have been reported after exposure to proteolytic enzymes or to insulin (4–6) and are consistent with a cAMP role in growth regulation. The fact that in our experiments the decrease in cAMP level preceded the change in uridine-transport rate is consistent with cAMP-dependent steps in the activation of this transport system (8). In addition, the saturation kinetics of the uridine and phosphate-transport systems are also affected in a different way by serum, since for uridine there is a change from a two-slope to a single-slope kinetics, whereas for phosphate the kinetics are always of the single-slope type. All these results support the conclusion that these transport systems are activated by different mechanisms.

Considering the early change in phosphate transport, the effect of phosphate on cAMP concentration and uridine transport, and the previously shown role of cAMP in the activation of uridine transport, it is tempting to speculate that these events are ordered in a causal sequence in which a change in phosphate transport is the primary event. However, other interpretations are also open at present, including the possibility that different serum factors independently affect phosphate transport rates and cAMP levels. Our study offers experimental approaches for distinguishing between these alternatives in order to unravel the complex regulatory circuits that operate when the growth of resting cells is reinitiated.

1. Pardee, A. B. & Rozengurt, E. (1974) in *Biochemistry of Cell Walls and Membranes*, ed., Fox, C. F. (Medical and Technical Publ. Co., London), in press.
2. Todaro, G. J., Matsuiya, Y., Bloom, S., Robbins, A. & Green, H. (1967) in *Growth Regulating Substances for Animal Cells in Culture*, eds, Defendi, V. & Stoker, M. (Wistar Inst. Press, Philadelphia, Pa.), pp. 87–98.
3. Cunningham, D. D. & Pardee, A. B. (1969) *Proc. Nat. Acad. Sci. USA* 64, 1049–1056.
4. Otten, J., Johnson, G. S. & Pastan, I. (1972) *J. Biol. Chem.* 247, 7082–7087.
5. Sheppard, J. R. (1972) *Nature New Biol.* 236, 14–16.
6. Burger, M. M., Bombik, B. M., Breckenridge, B. M. & Sheppard, J. R. (1972) *Nature New Biol.* 239, 161–163.
7. Kram, R., Mamont, P. & Tomkins, G. M. (1973) *Proc. Nat. Acad. Sci. USA* 70, 1432–1436.
8. Rozengurt, E. & Jimenez de Asua, L. (1973) *Proc. Nat. Acad. Sci. USA*, in press.
9. Holley, R. W. (1972) *Proc. Nat. Acad. Sci. USA* 69, 2840–2841.
10. Bürk, R. R. (1974) in *Control of Proliferation in Animal Cells*, eds. Clarkson, B. Baserga, R. (Cold Spring Harbor), in press.
11. Gilman, A. G. (1970) *Proc. Nat. Acad. Sci. USA* 67, 305–312.
12. Manganiello, V. & Vaughan, M. (1972) *Proc. Nat. Acad. Sci. USA* 69, 269–273.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275.
14. Weber, M. J. & Rubin, H. (1971) *J. Cell. Physiol.* 77, 157–167.
15. Weber, M. J. & Edlin, G. (1971) *J. Biol. Chem.* 246, 1828–1833.
16. Johnson, G. S., Morgan, W. D. & Pastan, I. (1972) *Nature* 235, 54–56.
17. Aaronson, S. A. & Todaro, G. (1968) *Cell. Physiol.* 74, 141–148.