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REGULATION OF URIDINE UPTAKE BY SERUM AND INSULIN IN DENSITY-INHIBITED 3T3 CELLS

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Summary

Stimulation of nucleoside uptake in quiescent 3T3 cells by insulin and serum is preceded by a substantial lag phase. Our findings point to the length of the lag phase as a major target for regulation. The length of such phase varies markedly with the concentration of insulin (10^{-9}—10^{-6} M) or serum (0.5—10%) but it is not eliminated by high, saturating levels of the activating agents. Further, variations in the temperature at which the stimulation process occurs (24—39°C), addition of compounds like prostaglandin E_1 (1—5 μg/ml) or theophylline (0.4 mM) and differences in the age of the cultures primarily affect the length of the lag time while the final uptake rates achieved are remarkably constant. Analysis of the temporal order of the events in the lag phase reveals that there is a discrete temperature-sensitive period located in the early and middle part of the lag, while the prostaglandin E_1-sensitive step(s) appear to be toward the end of the lag. The transition from the basal to the stimulated rate of uptake is abrupt. Indeed, the kinetics of activation does not fit a simple exponential law but a high power of an exponential, suggesting that the switching mechanism involves cooperative steps. Since the transition is abrupt, the nucleoside uptake system exists largely in two alternative states either switched off or on. The regulation of the lag period is by the control of the time at which this switch occurs. On the basis of the data presented here, we propose a working hypothesis of uptake stimulation.

Introduction

'Normal' fibroblasts in culture accumulate in the G_1 or G_0 phase of the cell cycle under conditions of serum depletion [1—6]. Addition of serum to such cultures induces rapid (within minutes) metabolic changes [6] and subse-

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quently (9—15 h accelerates the entry into DNA synthesis and cell division [1—5]. The early events include an increase in the transmembrane flux of ions like K^+ [7] and P_i [8—10], in the uptake of nucleosides [8—10] and glucose [11,12] and a decrease in cellular cyclic AMP [6,9,10,13—15]. Although the possibility that alterations in uptake may play an important role in the control of cell growth has received considerable attention [16—18], the mechanisms of regulation of uptake by serum or peptide hormones remain poorly understood.

The stimulation of uridine uptake by external signals provides a useful model system to explore basic mechanisms of regulation of uptake in general. Such stimulation is apparently preceded by a lag period [10], is inhibited by compounds that elevate the endogenous level of cyclic AMP [9], is cycloheximide-insentitive [12,18] and is manifested kinetically by an increase in the V of the system [19]. Furthermore, the nucleoside uptake system can also be stimulated by the addition of insulin [9,18].

To obtain a deeper insight into the mechanism of activation of uptake by growth factors and hormones, we have investigated the detailed kinetics of stimulation of uridine uptake. The data substantiates the existence of a lag in the stimulation process. We have studied the effect of serum and insulin concentration, the influence of temperature and that of compounds like prostaglandin E_1 and theophylline that elevate cyclic AMP levels on both the lag period and the final activity of the system. Our findings point to the lag period as the phase primarily sensitive to regulatory influences. The transition from the basal to the stimulated level of uptake is abrupt and possibly involves a cooperative process. A striking implication of such a regulatory behaviour is that the uptake system is essentially in two alternative states of activity either basal or fully stimulated. On the basis of the data presented here, we propose a working hypothesis of the stimulation of uridine uptake.

Materials and Methods

Stock cultures of Swiss 3T3 [20] cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of CO_2/air (10 : 90, v/v) at 37° C. The cultures were maintained at subconfluent densities to avoid the selection of spontaneous transformants and replaced every 6—8 weeks by new ones recovered from frozen stocks of cells. Cells were subcultured to 30-mm Nunc petri dishes with medium (2 ml) containing 6% serum. The medium was replaced once usually 2 days after plating and the cultures were allowed to reach the confluent state. Since the lag period of transport stimulation was found to vary with the age of the cultures (see Results) the actual age of the cultures used in each experiment is stated in the legends of the figures. The cultures of 3T3 cells used throughout this study were confluent monolayers arrested in the G_1 phase of the cell cyle. After labelling with [*Me*-^{3}H]thymidine (1 μCi/ml) for 26 h, less than 1% of the nuclei become radioactively labelled as seen after radioautography. Addition of 10% fetal calf serum induced DNA synthesis in over 85% of the population.

Serum or insulin stimulation was carried out at 37° C (unless otherwise indicated) taking great care to maintain the pH of the medium in the range 7.1—7.3.

Control cultures were treated in the same manner except that saline solution was used instead of serum. Serum was routinely dialyzed against several changes of saline solution. The cells were incubated with labelled [5-^{3}H]uridine (1 μCi/ml; 1 μM) for variable periods of time (as indicated in each experiment). Uptake of uridine into acid-soluble uridine metabolites was measures as previously described [7,9]. Briefly, after exposing the cells to radioactive substrate for the desired period of time, the medium was rapidly removed, the monolayers were washed five times with ice-cold isotonic saline (the whole process occupying some 20 s) and the cultures extracted with 1.5 ml of 5% trichloroacetic acid at 4°C for 20 min. An aliquot (1 ml) was mixed with 10 ml of Triton/toluene scintillation fluid and the radioactivity measured in a Packard scintillator counter. Chromatographic analysis of acid-soluble uridine metabolite was performed on samples that were prepared as for the uptake studies. The trichloroacetic acid-soluble material was extracted with ether, the aqueous phase lyophilized, re-suspended in a small volume of water and chromatographed in ethanol/ammonium acetate pH 3.8 (as described in ref. 21) on Whatmann 3MM paper.

Protein content was determined by the method of Lowry et al. [22] after the attached cells were washed five times with saline solution (pH 7.2).

Results

At 1 μM, the uridine concentration used throughout the present studies, uptake proceeds mainly by facilitated diffusion followed by phosphorylation of the nucleoside by intracellular uridine kinase [23]. Considerable evidence indicates that both the activity of uridine kinase as measured in homogenates [23,24] and the levels of ATP [25] in 3T3 cells and other cell lines, are not rate limiting in substrate phosphorylation, although control mechanisms in intact cells may modify the nucleoside phosphorylation. Fig. 1 shows that the chromatographic profile of acid-soluble [^{3}H]uridine derivatives is similar in quiescent and serum-stimulated cells. Thus fibroblasts take up uridine by transport followed by a rapid phosphorylation [26,27]. Since the experiment shown in Fig. 1 does not distinguish between transport or the subsequent phosphorylation as the rate-limiting step, in what follows overall uridine uptake (transport plus phosphorylation) is taken as the biological response that is measured. To emphasise this point, we shall everywhere use the neutral word "uptake" to describe this overall process, since the interpretation of the experiments to be described does not depend on which step is rate limiting for the uptake of uridine.

Kinetics of stimulation of uridine uptake

The rate of uridine uptake by quiescent 3T3 cells after the addition of serum demonstrates a substantial lag period. The rate then increases until it reaches a maximum and constant slope (Fig. 2A). As an objective measure of the process that we are considering, we take the time at which the straight line for the unswitched system meets the straight line for the fully switched system as defining the "lag time" for the particular set of experimental data analysed. The two required lines are drawn through the experimental data as in Fig. 2A.

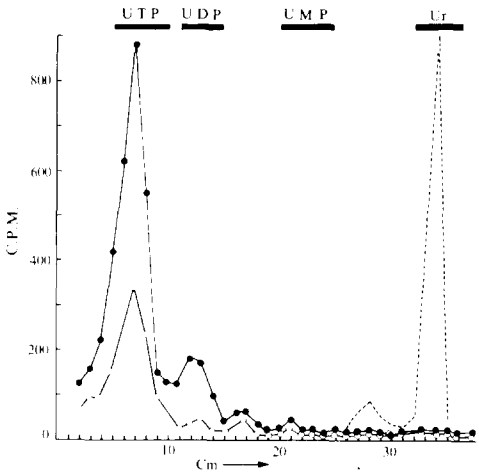

Fig. 1. Chromatographic analysis of acid-soluble pools. Quiescent 3T3 cells cultured in 9-cm dishes, were exposed to medium with (●——●) or without (○——○) 10% serum for 20 min, and then labelled with [^{3}H]uridine for 5 min. The samples were prepared and analysed by descending paper chromatography as described in Materials and Methods. The chromatograms were cut into 1-cm segments and the segments eluted with 0.1 MHCl. The radioactivity of the eluate was determined by scintillation counting. The ordinate of the graph represents the radioactivity in each segment and the abcissa represents the distance from the origin of each segment. The dashed line shows the radioactivity of commercial [^{3}H]uridine from a similar chromatogram.

Later, we show that if the switching process is a simple exponentail one, the lag time so defined gives exactly the reciprocal of the time constant λ for an exponential process.

That the lag observed when uridine uptake is stimulated by serum or insulin is not an artefact produced by experimental manipulation of the cells is shown by two separate types of experiments. First, if serum (Fig. 2B) or insulin (results not shown) is added to the cultures for a time sufficient to stimulate maximally the system and only then is the labelled substrate added, the lag is no longer observed. Secondly, if the cells are exposed to [^{3}H]uridine and $^{86}Rb^+$ simultaneously, serum addition activates cation uptake without a substantial lag (Fig. 2D) while nucleoside uptake in the same cells is switched after a lag of at least 5 min (Fig. 2C). This differential effect suggests that the lag period does not reflect simply a time required for the binding of serum factors. The lag was essentially unchanged when the concentration of uridine was varied 100-fold (0.2—20 μM). All these results substantiate the existence of a lag as part of the process of stimulation of nucleoside uptake by serum or insulin.

Effect of the concentration of insulin and serum on the nucleoside uptake by resting 3T3 cells

Is the length of the lag phase of activation of uridine uptake a target for regulation? To explore this possibility we have determined the time-course of uridine uptake by quiescent cultures of 3T3 cells incubated in the presence of different concentrations of insulin (Fig. 3, upper panel). The hormone, added at low levels markedly stimulated nucleoside uptake; at the lowest concentration tested (5 ng/ml) the maximal rate of uptake was not achieved even after

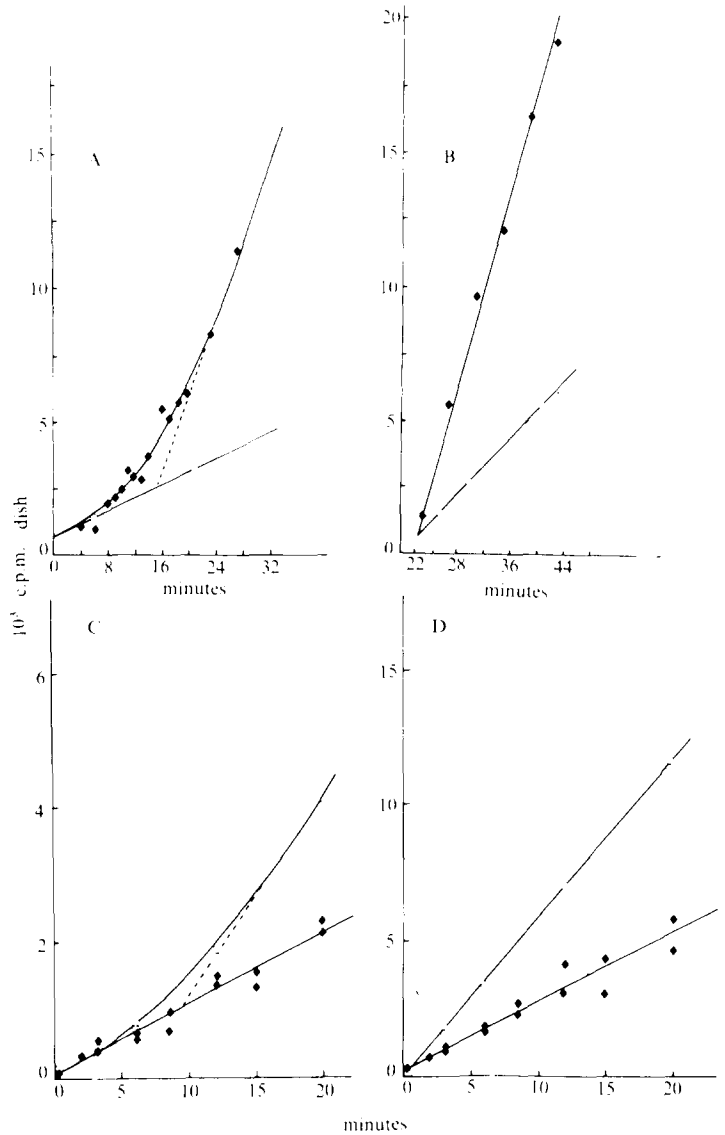

Fig. 2. Uptake of uridine and rubidium as a function of time at 37°C. Cultures were exposed to uridine at 1 μM, 1 μCi/ml (curves A, B, C) or RbCl at 0.1 mM, 2.5 μCi/ml (curve D) in medium containing either 10% fetal calf serum, (upper curves in each of A, B, C and D) or saline (lower curves in each of A, B, C and D). C and D were performed simultaneously by a dual label technique. In curves A, C and D, the radioactive label was added together with the serum or saline medium. In B it was added 22 min later. At the times indicated, cultures were removed for assay of label, taken up. The cultures were 13, 15, 9 and 9 days after last change of medium for curves A, B, C and D, respectively.

60 min of exposure. To determine the final rate of uptake parallel cultures were exposed to insulin for a total period of 120 min. After 60 min of incubation the media of such cultures were replaced by fresh ones to counteract any hormone degradation [28]. At the end of the incubation, the rates of uptake were measured by pulse labelling the cultures for 10 min. These rates (insert to

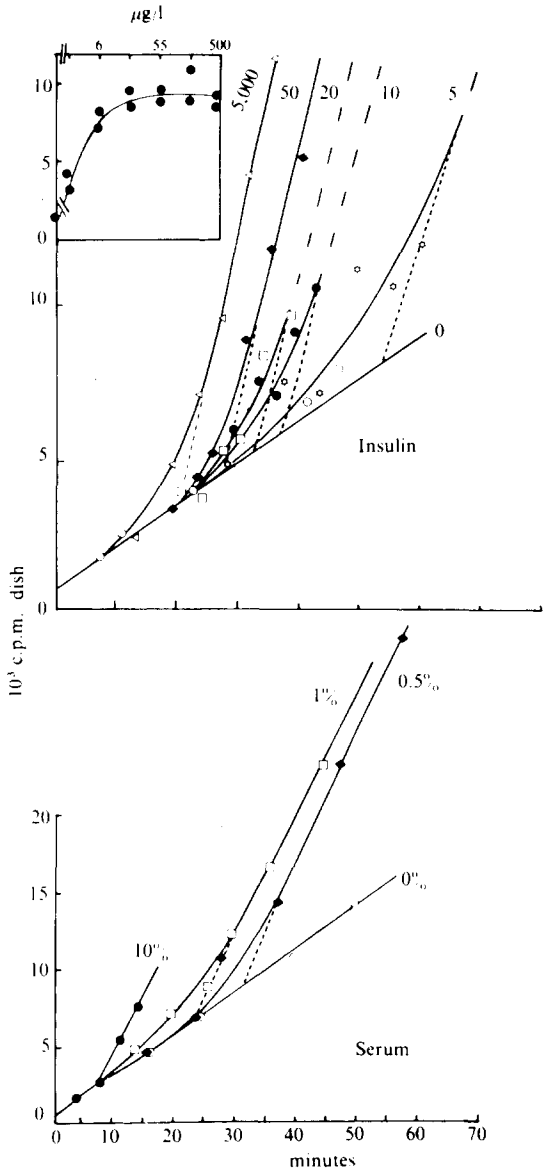

Fig. 3. Activation of uridine uptake at different insulin concentrations (upper curves) and serum concentrations (lower curves). Cultures were exposed at 37°C to medium containing 1 μM uridine, 1 μ Ci/ml and either insulin at the concentration shown (in ng/ml) or foetal calf serum (%, v/v) and removed at intervals indicated for measurement of uridine taken up. The inset shows the uptake of uridine during a 10 min pulse after the cultures had been kept at 37°C for 120 min in the presence of insulin at the concentration plotted (logarithmically), in μg/1. The broken lines in the upper curve (being the terminal slopes of these curves) are taken from the inset curve by interpolation. Cells were 24 days from last change of medium for the upper curves, 13 days for the lower curves.

Fig. 3) serve to define the final slopes (large dash lines) of the uptake curves at the low (5, 10 and 20 ng/ml) concentrations of insulin shown in Fig. 3, upper panel. The dose vs. response of the cell to the hormone, is manifested by a gradual decrease in the lag period from some 55 to 20 min, when the concen-

tration of insulin is increased from 5 to 5000 ng/ml, while the maximal rate of uptake only shows a marginal changes in this range of concentration. When the values of the lag period are plotted against the reciprocal of the insulin concentration, a reasonally straight line is obtained (now shown); from such a relationship, it becomes clear that the lag is not eliminated at infinite hormone concentration. Thus, the lag in nucleoside uptake is altered in response to nanomolar concentrations of hormone. Such insulin concentrations are in the range over which the hormone binds to surface receptors in fibroblasts (K_d, 3 nM) [29,30]. The lag period also changes dramatically when different levels of serum are used to stimulate nucleoside uptake (Fig. 3, lower panel). When the serum level is decreased from 10 to 0.5%, the lag increases from 9 to 31 min, while the final rate of uptake only shows a marginal change.

Effect of temperature on the activation of uridine uptake

Previously, we found that the dependence of V on temperature in non-stimulated cells was not substantially different from that of fully activated cells [19]. The influence of the temperature on the activation process, rather than on the activated uptake system is shown in Fig. 4. The experiment consisted in defining the time-course of serum stimulation of uridine uptake at temperature ranging from 24 to 39°C. Rates and lag values derived from this experiment as well as the effect of temperatures on the basal uptake are summarized in Table I. The striking feature of this experiment is the marked increase in the length of the lag, when the temperature was reduced from 39 to 24°C. The variations in the lag period contrast with the small change in the activity of the fully switched system, particularly between 39 and 30°C. This indicates that a temperature-sensitive step is part of the events leading to the activation of this transport system.

Effect of prostaglandin E_1 and theophylline on the activation process

Previously, it was found that prostaglandin E_1 and theophylline prevented the stimulation of uridine uptake by both serum and insulin [9]. Although other mechanisms cannot be completely excluded, the major effect of these drugs is almost certainly to increase the level of intracellular cyclic AMP, since

TABLE I

EFFECT OF TEMPERATURE ON URIDINE UPTAKE AND ITS ACTIVATION

Temperature (°C)	Activation lag [a] (Min)	Activated rate [b] (cpm/dish per min)	Basal rate [c] (cpm/dish per min)
39	9.5	690	—
34	13	580	140
30	20	510	140
28	24	360	120
24	44	250	115

[a] Data from Fig. 4. Lag measured as intercept of extrapolated straight line portions of the time course.

[b] Data from Fig. 4. Rate measured as the slope of the final (straight line) portion of the curves.

[c] Data measured in parallel with that of Fig. 4 (but not shown for clarity of presentation). Rate measured as the best straight line through the (linear) time-course of uptake in the absence of added serum.

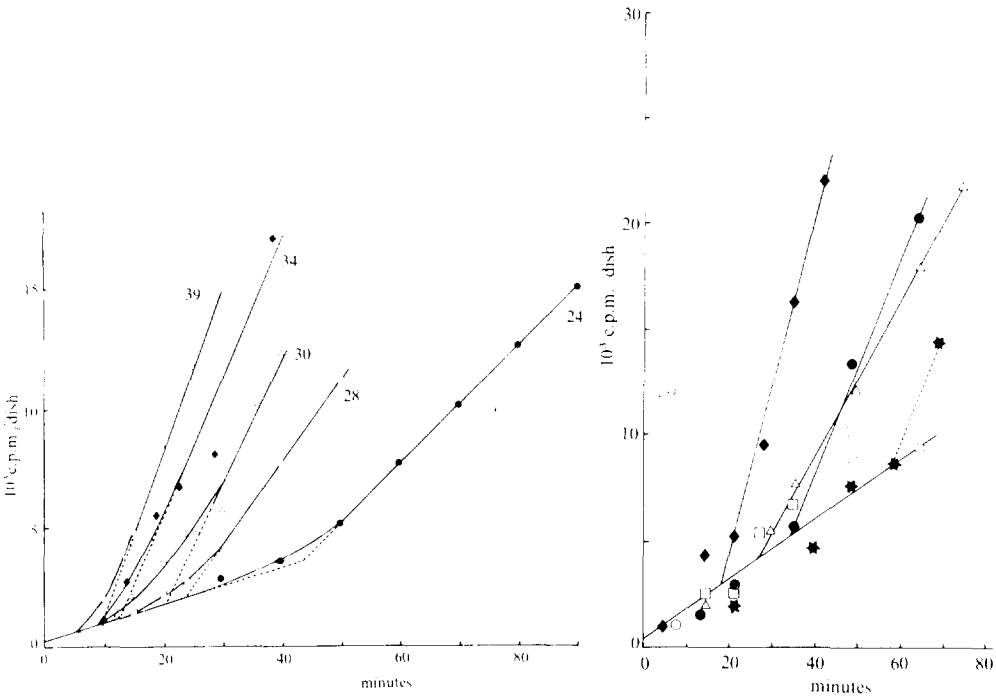

Fig. 4. Time-course of activation of uridine uptake at different temperatures. At zero time at each temperature, all cultures received a change of medium to one containing 10% fetal calf serum in medium together with uridine at 1 μM, 1 μCi/ml, at the indicated temperature. Parallel cultures (not shown) received the same medium but with saline replacing the serum. At the indicated times, cultures were removed for measurement of uridine uptake, (cells 9 days after last change of medium). Slopes and lag time collected in Table I.

Fig. 5. Effect of theophylline and prostaglandin E_1 on activation of uridine uptake by 50 ng/ml of insulin at 37°C. All cultures received labelled uridine (1 μM, 1 μCi/ml) at time zero. Open circles were medium alone, all other symbols received insulin also. Open triangles received 2.5 μg/ml prostaglandin, stars and open squares received 1 μg/ml prostaglandin. Stars and solid circles received 0.4 mM theophylline. (Stars received both theophylline and prostaglandin.) Cultures were 22 days since last change of medium.

it has been established that fibroblast cyclic AMP levels are elevated by prostaglandin E_1 [9,13,14,31—33] and by prostaglandin E_1, synergistically with theophylline and other phosphodiesterase inhibitors [9,32]. We have now studied the effect of these drugs on the lag phase of the response of 3T3 cells in insulin. We examined the time-course of stimulation in the presence of low levels of prostaglandin E_1, theophylline, or both. Such an experiment (Fig. 5) reveals that low levels of theophylline (0.4 mM) prolong the lag for many minutes (from 20 to 35 min) while the final activity in its presence was similar to that of cells which did not receive the inhibitor. Prostaglandin E_1 added at 1 or 2.5 μg/ml also increases preferentially the lag period. When theophylline and prostaglandin E_1 are added together, there is a marked increase in the length of the lag. This preferential effect of prostaglandin E_1 and theophylline on the lag rather than on the final rate makes very unlikely the possibility that these compounds interfere with the uptake process itself.

Effect of short periods of exposure to low temperature and to prostaglandin E_1

As an approach to dissecting the events leading to the switching of the

nucleoside uptake system, we have attempted to define the temporal order of the steps sensitive to temperature and prostaglandin E_1. We asked whether the temperature-sensitive step(s) are distributed throughout the lag period or confined to a discreet segment of it. To test this possibility, cultures of 3T3 cells were exposed (Fig. 6A) at 26°C for short periods (8 min) namely, between 0 and 8 min, 8 and 16 min and 4 and 12 min. The lag period of these cultures was previously measured to be 16 min. The transitions of temperature were rapidly performed by replacing the medium with an equivalent one equlibrated at the required temperature. The cultures kept continuously at 37°C also received changes of medium, but at that temperature. At 16 min all the cultures were exposed to radioactive substrate at 37°C, so the temperature of the cultures at the time of the uptake assay were everywhere the same. If the temperature-sensitive step extends throughout the lag period, all the treatments, being of equal duration, should equally delay the onset of the serum stimulation, no matter when the cultures were exposed to 26°C. Conversely, if the temperature-sensitive step is confined to a discrete segment of the lag, one of the treatments should produce a bigger delay than the others. The results support the second alternative. Clearly, the delay is more pronounced when the cultures were exposed to 26°C at approximately the middle (4—12 min) part of the lag. In order to further substantiate the existence of a defined temperature-sensitive period in the lag phase of activation we have analysed the effect of reduced temperature on the lag phase in cultures displaying a somewhat shorter lag (14 min). The intervals chosen here were 0—7, 3.5—10.5 and 7—14 min. The last treatment has very small delaying effect while the other two produced a pro-

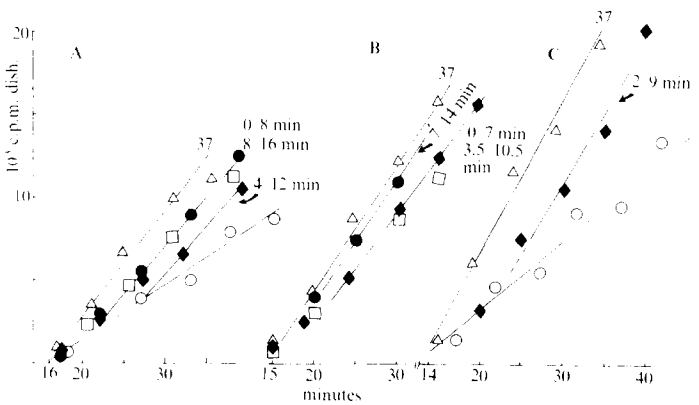

Fig. 6. Effect of periods of low temperature on the activation of uridine uptake by serum. All cultures received at zero times either medium with 10% saline (open circles) or 10% foetal calf serum (all other symbols) and were then subjected to various regimes of high (37°C) or low (26°C) temperature. In A, B and C, triangles and open circles were at 37°C throughout. In A cultures were at 26°C for: solid circles from the 0th to 8th min, open squares from 8th to 16th min. Solid diamond from 4th to 12th min, and at all other times at 37°C. Radioactive uridine was added at the 16th min. In B, cultures were at 26°C for: open squares 0th to 7th min, solid circles 7th to 14th min, solid diamonds 3.5 to 10.5th min: and at all other times at 37°C. In C, the solid diamonds were at 26°C from the 2nd to the 9th min and at 37°C at all other times. B and C received labelled uridine at the 14th min. The label was 1 μM, 1 μCi/ml. The cultures were 16, 12 and 12 days since last change of medium for A, B and C, respectively. (B and C were on the same set of cultures on the same day).

nounced delay of equal duration (Fig. 6B). We reasoned that such equal delays can reflect the existence of a discrete temperature-sensitive period between 2 and 9 min, so, the early and middle periods of exposure chosen in the experiment, described in Fig. 6B (0—7 and 3.5—10.5 min) overlap partly with such a putative temperature-sensitive period. This interpretation receives support from the experiment shown in Fig. 6C carried out in parallel cultures to those used in Fig. 6B; exposure to 26°C between 2 and 9 min caused a substantial delay in the onset of stimulation which was bigger than the delays produced by the other treatments. The experiments clearly illustrate that the temporal order of the events underlying the activation of uridine uptake by external signals is amenable to experimental dissection.

Similar experiments have been done to define what part of the lag is maximally sensitive to the additon to prostaglandin E_1. Such a dissection of the effect is feasible because the addition or removal of prostaglandin E_1 from the medium rapidly changes the intracellular levels of cyclic AMP in fibroblasts [9,31]. The experiment shown in in Fig. 7 indicates that there is a period sensitive to prostaglandin E_1 at the end of the lag time.

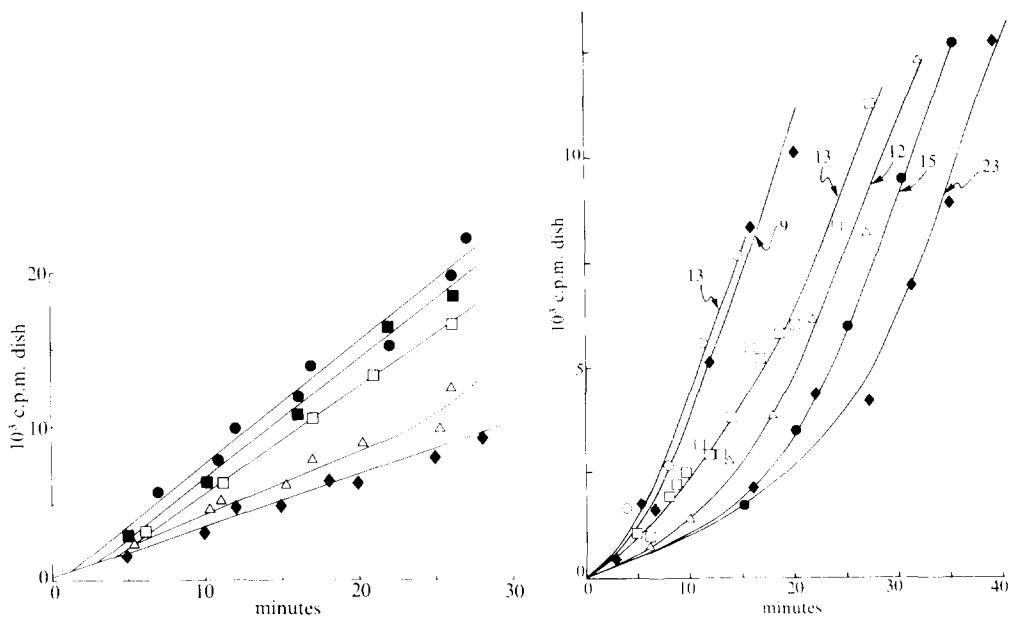

Fig. 7. Effects of periods of exposure to prostaglandin E_1 (5 μg/ml) on the stimulation of uridine uptake by insulin (50 ng/ml). All the cultures received at zero time either medium with 10% saline (—◆—) or insulin containing medium (all other symbols) and were subjected to various periods of exposure to prostaglandin E_1 except some cultures which only received insulin (●———●). The periods of exposure to prostaglandin E_1 were: ■———■, 0—8 min; □———□, 8—16 min; △———△, throughout. All the cultures received labelled uridine at 17 min. The abscissa represents the time after the addtion of [^{3}H]uridine. All cultures were washed three times after the prostaglandin E_1 or before radioactivity.

Fig. 8. Variabiltiy of activation of uridine uptake by 10% calf serum. A summary of various experiments with cultures that had been (reading from left to right in the figure) 13, 9, 13, 12, 15 and 23 days since the last change of medium. Uridine everywhere was 1 μM, 1 μCi/ml, at 37°C.

Effect of the age of the culture on the duration of the lag period

In the course of the present experiments, we noticed that the length of the lag is somewhat variable (Fig. 8). It seemed that a factor of importance in defining the length of the lag might be the time that the cultures had remained arrested in G_1. To test this possibility, we performed an experiment in which the time-course of stimulation of uptake for cultures of different age was obtained under identical conditions. The lags were 10 and 21 min for cultures that had been kept in the incubator for 8 and 21 days respectively.

Kinetic analysis of the stimulation process

If the rate of metabolite uptake is directly and simply related to the concentration of hormone-receptor complexes present, the time-course of stimulation should follow a simple exponential law. Such a relationship has been already demonstrated with adipocyte cells, in which insulin stimulates glucose uptake [34,36]. Since our results suggest a mechanism considerably more complex than this, it seemed of interest to ask whether the stimulation of uridine uptake follows an exponential law as given by:

$$y' = y'_{\infty} (1 - e^{-\lambda t}) \tag{1}$$

where y' is the increment in transport rate at time t, while y'_{∞} is its value at infinite time, i.e. in a fully switched system and λ is the time constant for the stimulation process. The integrated form of Eqn. 1 is given by:

$$y = y'_{\infty} \left(t + \frac{e^{-\lambda t}}{\lambda} - \frac{1}{\lambda} \right) \tag{2}$$

where y is the amount of uridine (stimulated) that has accumulated by the time

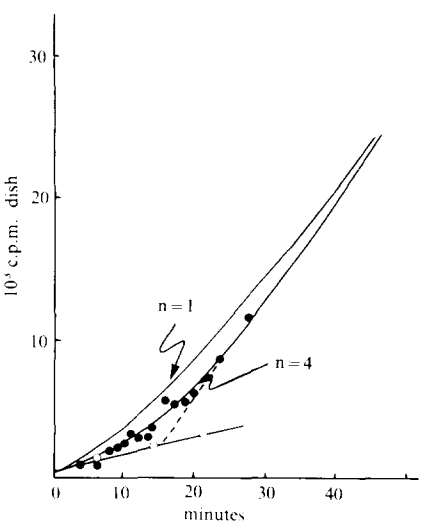

Fig. 9. The curves are theoretical curves, defined by Eqn. 2 (upper curve) or the integrated form of Eqn. 3 with $n = 4$ (lower curve) with λ calculated so that the curves intercept the straight line for the unswitched curve at a time of 15 min, the lag period for the data of Fig. 2 with the basal uptake being added in. The final slope is that derived from the data of Fig. 2A as is the initial slope. The points from Fig. 2A are drawn onto the present figure. The better fit is clearly to the coperative model (lower curve).

t. The lag, defined as the time at which the extrapolated straight line portion of the curve meets the unstimulated line, gives exactly $1/\lambda$. (Eqn. 2 approaches the straight line $y = y'_\infty\ (t - 1/\lambda)$ which itself intercepts the time axis at $t = 1/\lambda$). Using this value of λ and the experimentally determined initial and final rates, we can generate the theoretical curve for uptake. Such a curve is drawn in Fig. 9, together with the experimental data, previously seen in Fig. 2A. Clearly, the simple exponential fails to fit the data. The experimental points leave the straight line (describing the basal uptake) later than does the simple exponential curve and they approach the straight line (describing the fully stimulated uptake) more rapidly than does the simple exponential curve. The equation obtained by integrating

$$y' = y'_\infty(1 - e^{-\lambda t})^\eta \qquad (3)$$

does provide a good fit to the data (Fig. 9). We do not claim, however, that this equation is necessarily the correct one to describe the system. One might suggest, however, that the stimulation of uridine uptake follows the kinetics of a cooperative phenomenon rather than that of a simple exponential law.

Discussion

The present study demonstrates that the lag in the uptake of nucleosides when quiescent 3T3 cells respond to serum or insulin is a phenomenon which is under cellular control. Diverse conditions such as changes in the concentration of serum and insulin (Fig. 3), variations in the temperature at which the stimulation process occurs (Fig. 4), addition of compounds that elevate cyclic AMP (Fig. 5) and ageing of the cultures (Fig. 8) primarily affect the length of the lag period. In contrast, the final uptake rates are remarkably constant. Since the transition from the basal to the stimulated rate of uptake is abrupt, the activity of the nucleoside uptake system largely exists in two alternative states, either switched off or on. The regulation of the lag phase of activation is the control of when this switch occurs. For this reason, the following discussion focuses on the nature of the lag.

On the basis of the present, as well as previous findings, some possible explanations for the biochemical nature of the lag phase of stimulation are made very unlikely. The possibility that the lag is an artifact of the experimental manipulation is very unlikely since other actions of serum like the stimulation of Rb^+ uptake (Fig. 2D and ref. 7) and P_i uptake [10] occur without detectable lag; further, if the cells are maximally stimulated by serum and only then labelled, no lag is observed (Fig. 2B). It is unlikely that serum or insulin stimulate the formation of a uridine derivative that, in turn, switches uridine uptake, the lag is the same whether uridine is present or absent during the stimulation (results not shown). The lag period does not reflect a time required for the "de novo" synthesis of carrier molecules or any cytoplasmic protein that activates uptake since cyloheximide added at concentrations that severely inhibit protein synthesis does not block the uptake increase [12–18]. Finally, the lag phase of stimulation is not a peculiar feature of 3T3 cells, since it is also seen in serum-stimulated resting cultures of secondary mouse embryo fibroblasts (Rozengurt, E., unpublished results).

A plausible explanation for the lag might be in the time taken for the activating agent to reach equilibrium with a membrane receptor. Indeed, the stimulation of uridine uptake by insulin occurs at concentrations which are in the range expected for hormone interaction with surface receptors, having a dissociation constant of 2—4 nM, as has been reported with fibroblasts [29,30] and other tissues [35,36]. Although direct binding of insulin to surface receptors has not been measured in our studies, several lines of evidence indicate that the lag is not given by the binding process. When the biological response (uptake stimulation) is a simple function of receptor occupancy, the half time ($T_{1/2}$) of hormone binding and action should be given by the expression [36]:

$$T_{1/2} = \frac{\ln 2}{k_{-1} + k_{+2}[\mathrm{H}]}$$

where k^{-1} and k_{+1} are the rate constants of dissociation and association, respectively. Hence, at limiting high concentrations of hormone the time taken for binding vanishes and the lag in the biological response disappears. Such elimination of the lag has been clearly demonstrated when the glucose uptake system of adipocytes is stimulated by high levels of insulin [34—36]. In contrast, our studies on uridine uptake in the 3T3 model system shows that although the length of the lag varies markedly with the concentration of insulin or serum it is not eliminated by high, saturating levels of the activating agents (Fig. 3). In addition, the simple binding interpretation fails to explain the dramatic effect of temperature on the lag phase seen in our experiments (Figs. 4—6), since hormone binding is affected only slightly by moderate changes in temperature [36]. We conclude that the lag phase reflects events largely distal to hormone binding.

Analysis of the effect of temperature on the lag phase (Fig. 6) indicates that a temperature-sensitive step exists as an early event in the lag, and is located considerably before onset of the bulk of the stimulation of uptake. It appears that a decrease in the temperature inhibits the transmission of the signal from the receptor to those effectors which control the increase in uridine uptake. A large body of recent evidence supports the notion that most membrane proteins including receptors are able to move laterally in the plane of the membrane [37—39], that such mobility may contribute to the generation of biological responses [40] and that it is greatly depressed by reduced temperature [41—43]. It seems reasonable to suppose that the early critical event of the lag phase affected by reduced temperature might be the lateral diffusion of newly formed hormone-receptor complexes. The possibility that changes in the concentrations of cyclic AMP are involved in the stimulation of nucleoside uptake has been suggested previously. Essentially, serum and insulin which increase uridine uptake decrease the intracellular concentration of cyclic AMP [6,9,10,13—15,33], the fall of cyclic AMP precedes the stimulation of uridine uptake [10], and the addition of compounds like prostaglandin E_1 or theophylline, which prevent the drop in cyclic AMP induced by serum or insulin, prevent the increase in nucleoside uptake [9]. The novel feature contributed by the present results is that low levels of prostaglandin E_1, theophylline, or both (Fig. 5), delay the onset of the stimulation, but alter only slightly the final rate of uptake. These findings suggest that the cyclic AMP-elevating com-

pounds effect preferentially the switching process rather than the uptake itself. It is interesting that the addition of serum [44] or insulin [45—47] appears to increase the high affinity, membrane-bound, cyclic nucleotide phosphodiesterase activity. This activation is consistent with the very short lag obtained in Fig. 7 on removal or prostaglandin E_1 after cells have been exposed to both prostaglandin E_1 and insulin; here, the phosphodiesterase activity is presumably already activated by the insulin.

When present and previous experiments are taken together, the regulation of uridine uptake by external signals emerges as a complex and intricated mechanism. A working hypothesis that can unify the diverse observations as well as suggest further experimental work and that is consistent with the fluid properties of the membrane [37—40] and with recent proposals on the mechanism of action of peptide hormones [8] and choleratoxin [9] is reported in Fig. 10. Insulin or growth factors (F) bind to receptors (R) which are supposed to be mobile and not permanently associated with the effector. Once formed, the hormone-receptor complex (FR) diffuses laterally in the plane of the membrane, interacts with an effector (e) and changes the activity of the effector to E. According to this scheme, a reduction in the concentration of insulin should decrease the concentration of FR and prolong the time required for the formation of the productive complex FRE; as a result, the lag period increased. Only when FR and hence FRE is very small, will the maximaly biological response be reduced. This explains why the effects are largely on lags and much less on final rates in a certain range of hormone concentration (Fig. 3). Since the rate of lateral diffusion of membrane proteins depends on the fluid state of the lipid bilayer and such a state is altered by temperature [41—43], it is clear that, according to the scheme of Fig. 10, a decrease in the temperature of incubation should have a profound effect on the lag as is, indeed, shown in Fig. 4. Further, as required by the working hypothesis, the temperature-sensitive step occurs early in the stimulation process (Fig. 6). As a result of the formation of the productive complex, cyclic AMP falls; when it reaches a threshold level, the uptake system switches on. This threshold phenomenon can explain the delay in stimulation seen in the presence of theophylline, prostaglandin E_1 or both (Figs. 5 and 7) and can generate the cooperative behaviour consistent with a high power of an exponential (Fig. 9). Alternatively, cyclic AMP might modulate the formation of the productive complex, since the position of receptors on the cell surface is controlled partly by the state of the internal cytoskeleton (microtubules, microfilaments) [39,40] and such structures are rapidly altered by changes in the levels of cyclic AMP [50,51]. An attractive feature of a model involving mobile receptors is that they can interact potentially with several independent effectors [48] suggesting a unified interpretation for the

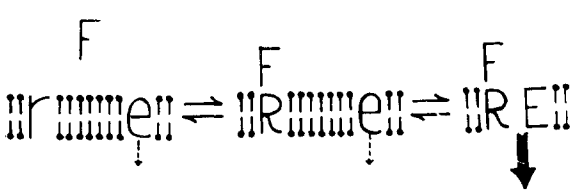

Fig. 10. See text for explanation.

dissociability of the complex array of early events seen in growth stimulation [52]. Regardless of the actual mechanism involved, the findings described in the present study point to the lag phase of stimulation of uridine uptake as the period where the critical molecular events that switch this system occur and open up this phenomenon to experimental analysis.

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