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STUDIES ON THE MECHANISM OF SERUM STIMULATION OF URIDINE UPTAKE IN SERUM-LESS MOUSE CELLS

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SUMMARY

Mouse embryo cells cultured several days in serum-less medium respond to serum by a prompt increase in [^{3}H]uridine uptake. The serum glycoprotein fetuin as well as adenosine, guanosine, ATP and adenosine 3',5'-cyclic monophosphate stimulate uridine uptake in the absence of serum. The active adenine derivatives do not stimulate adenosine, thymidine or phenylalanine uptake in 3–6 h and do not potentiate serum-stimulated thymidine uptake and DNA synthesis in 24 h. Pyrimidine ribonucleosides inhibit rather than stimulate uridine uptake. Stimulation by serum is partially inhibited by F^- but not by dinitrophenol or azide. Oligomycin rapidly inhibits the uridine-uptake system, implicating a high energy, phosphorylated intermediate in the process. Phloridzin and fluorodinitrobenzene, compounds which interact with the membrane, inhibit the pre- and post-stimulation uptake of uridine, providing probes with which to study the cellular alterations induced by serum.

INTRODUCTION

Serum has long been known to be important in the maintenance of cell division in a variety of normal and neoplastic cell systems *in vitro*[1–9], although its role is not clear[10]. Of the serum components, the glycoprotein fetuin appears to be most important as the mediator of growth regulation[7].

CUNNINGHAM AND PARDEE[11], as well as WIEBEL AND BASERGA[12], recently demonstrated that the addition of fresh serum to contact inhibited, non-neoplastic mouse and human cells rapidly stimulated the uptake of uridine. Since the functional and antigenic properties of normal and polyoma-transformed neoplastic cell membranes have been a subject of interest in this laboratory[13–16], an investigation of uridine uptake in serum-depleted mouse embryo cells permissive for polyoma virus replication was begun. This was to confirm and extend the previous observations[11,12] and to clarify possible mechanisms by which serum alters the physiologic state of mammalian cells in culture prior to and following neoplastic transformation by oncogenic viruses.

Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

MATERIALS AND METHODS

Mouse embryo cells were planted at $1 \cdot 10^6$ cells per 60 mm plastic petri dish (Falcon) in EAGLE's[17] minimum essential medium with 5 % fetal calf serum for 24 h, at which time the medium was removed and replaced with minimum essential medium without serum. After an additional 72 h, the medium was replaced with fresh serum-free medium and the cultures studied between 24 and 72 h later by the addition of serum directly to the medium, thus avoiding perturbations due to medium change.

Uptake of labeled compounds (New England Nuclear) was studied as described previously[13]. Following the labeling period, the dishes were chilled, washed rapidly three times with iced Tris-buffered saline and extracted in 2.0 ml of 10 % trichloroacetic acid. The cells were then washed twice with iced 10 % trichloroacetic acid and dissolved in 0.5 M KOH overnight at 37° to solubilize the acid-insoluble fraction. The first trichloroacetic acid extract and the KOH-soluble fraction were counted in BRAY's[18] scintillation mixture using Omnifluor. Duplicate dishes were used at each experimental point and duplicate samples were counted from each dish. The coefficient of variation of the mean in the experiments reported was less than 10 % in most cases.

Adenosine, adenosine triphosphate (ATP), adenosine 3',5'-cyclic monophosphate (cyclic AMP), the other nucleosides and the inhibitors 2,4-dinitrophenol, 1-fluoro-2,4-dinitrobenzene (FDNB) and phloridzin were obtained from Calbiochem. Oligomycin (Sigma) and FDNB were dissolved in absolute ethanol and diluted just prior to use in Tris-buffered saline. Ethanol controls showed no effect on uptake of labeled compounds at the final concentrations found in the treated cultures. A purified preparation of fetuin was kindly supplied by Charles Waldren and Theodore Puck of the University of Colorado Medical Center, which was reported by them to give 100 % plating efficiency of mammalian cells at a concentration of 5.0 μg/ml (personal communication).

RESULTS

Stimulation of uridine uptake by serum and fetuin

Fig. 1 illustrates the changes in [^{3}H]uridine uptake during a 5-min pulse period at 25° into an acid-soluble and insoluble fraction at different times after addition of 0.5 ml of fetal calf serum to 5.0 ml of serum-free medium. The results are expressed as the stimulation factor (experimental uptake/unstimulated control).

Uptake of [^{3}H]uridine rose progressively following addition of serum although no difference was detected after only 5 min. The relative increase in incorporation, as compared to the control, was greater into the acid-soluble fraction throughout the time period studied. The overall uptake stimulation factor (acid-soluble + insoluble) more closely resembles changes in the acid-soluble component since this latter fraction usually makes up 85 % of the total intracellular ^{3}H-labeled compounds using these extraction techniques.

The role of fetuin, the principle glycoprotein of fetal serum, in the stimulation of uridine uptake was then evaluated. Purified fetuin was compared with fetal and agamma calf serum (North American Biologicals). Table I shows the results of such an experiment. Fetal serum was slightly more active than agamma calf at comparable concentrations, the latter demonstrating a plateau at the 5–10 % concentrations level.

Other experiments have also shown the stimulating effect of fetal serum was dose-dependent up to 5–10 % at which point little further stimulation occurred up to 20 % serum, the highest level tested.

The stimulating effect of fetuin was also concentration dependent with 100 μg/ml being comparable to 1 % agamma calf and slightly less than 1 % fetal calf serum. It is of interest that fetuin at 10 μg/ml stimulated uridine uptake and, therefore, is active in this system at a concentration similar to that capable of producing a high cloning efficiency of mammalian cells in culture[7].

Stimulation by adenine ribonucleosides and several phosphorylated derivatives.

While studying the mechanism by which serum enhanced uridine incorporation, it was found that adenosine, ATP and cyclic AMP stimulated this process in the absence of serum and potentiated the stimulation when used together with serum. Stimulation by one of these compounds, cyclic AMP, is shown in Fig. 2 in which [^{3}H]uridine uptake over a 30-min period at 37° was measured 6 h after serum repletion. The standard deviation of the mean at each point was calculated and is shown

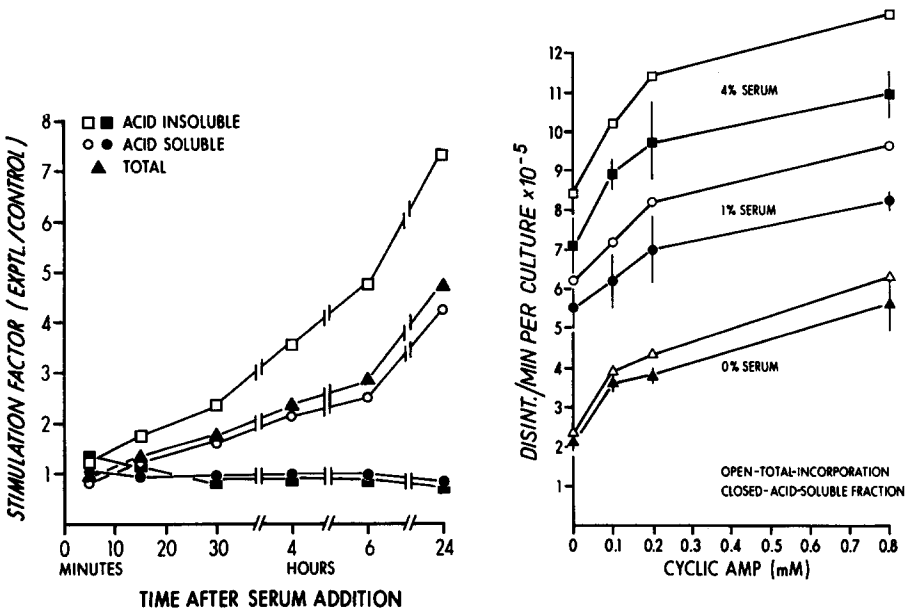

Fig. 1. Stimulation of [^{3}H]uridine uptake into an acid-soluble and insoluble fraction of mouse embryo cells cultured in serum-less medium for 4 days following additions of serum at time 0. The stimulation factor is the ratio of incorporation into the acid soluble or insoluble fraction of cells with and without serum at various times after serum repletion divided by the uptake into unstimulated, control cells at time 0. Uptake was measured over a 5-min period at 25° using 4 μC of [^{3}H]uridine (spec. act. 21.7 C/mmole) in 2.0 ml of Tris-buffered saline. ●, acid-soluble fraction in control with no serum; ■, acid-insoluble fraction in control with no serum; ○, acid-soluble fraction after serum addition; □, acid-insoluble fraction after serum addition; ▲, total incorporation (acid-soluble + insoluble) after serum addition.

Fig. 2. The stimulation of [^{3}H]uridine uptake into an acid-soluble and a total cell fraction (acid-soluble + acid-insoluble) of serum-deficient mouse embryo cells 6 h after repletion of serum with and without cyclic AMP. The standard deviation at each point on the curve of uptake into the acid-soluble fraction is indicated by the bar.

TABLE I

COMPARISON OF FETAL AND AGAMMA CALF SERUM WITH PURIFIED FETUIN IN THEIR ABILITY TO STIMULATE URIDINE UPTAKE IN SERUM-LESS MOUSE EMBRYO CELLS

Mouse embryo cells maintained on serum-less medium for 5 days were treated with serum or fetuin for 3 h and labeled 30 min at 37° with 3 μC of [^{3}H]uridine (21.7 C/mmole) in 2.0 ml of Tris-buffered saline. The cell monolayer was washed rapidly 3 times with iced Tris-buffered saline and extracted with 10% trichloroacetic acid to remove the acid-soluble fraction. The cells were then rinsed with trichloroacetic acid (2 times) to remove residual acid-soluble material and the acid-insoluble fraction solubilized in 0.5 M KOH overnight. Total uptake is the sum of the two fractions. Results are given as mean counts/min per petri dish ± 1 S.D.

	Total uptake (counts/min per dish)	*Percent of total*
No treatment	8 400 ± 860	100 ± 10
Fetal calf, 5%	25 420 ± 1270	303 ± 10
1%	17 260 ± 1050	206 ± 10
Agamma calf, 10%	25 430 ± 1270	303 ± 10
5%	24 720 ± 1470	294 ± 10
1%	14 700 ± 1200	175 ± 10
Fetuin, 100 μg/ml	14 260 ± 700	170 ± 10
10 μg/ml	10 340 ± 1140	123 ± 11
1 μg/ml	8 540 ± 530	102 ± 10
0.1 μg/ml	7 960 ± 650	95 ± 10

for only one set of curves in the interest of clarity. The level of incorporation into the acid-soluble fraction, as well as total uptake is influenced in an additive fashion by both serum and cyclic AMP. With 4% serum, however, the increase in total incorporation with 0.8 mM cyclic AMP is somewhat greater than would be expected from the sum of uptake in the presence of either alone.

It should also be noted in Fig. 2 that with increasing serum concentration, the difference between the curves of uptake into the acid-soluble fraction and total incorporation also increased, suggesting that there was stimulation of incorporation into the acid-insoluble fraction (RNA). In contrast, this difference between the two curves in the cyclic AMP-treated cultures was relatively small. These data would suggest that cyclic AMP stimulated uptake into a pool with little influence on RNA synthesis, while serum altered the uptake not only into the pool, but into RNA as well. This possibility was also suggested by the data in Fig. 1.

Stimulatory activity of other nucleosides

The fact that cyclic AMP, ATP and adenosine were active stimulators suggested that the action of the adenine ribonucleotides was mediated by adenosine following their conversion to the nucleoside at the cell membrane. Experiments were then carried out to determine the nucleoside specificity of uridine uptake stimulation in serum-less cells and one experiment is shown in Table II. The compounds were compared at 0.1 mM, a concentration found to be an optimum stimulating level for adenosine and ATP. The effects on uridine uptake fell into 3 categories, *i.e.* stimulatory, inhibitory, and neutral. Adenosine and ATP were most active in enhancing

TABLE II

ACTIVATION OR INHIBITION OF URIDINE UPTAKE IN SERUM-LESS MOUSE EMBRYO CELLS BY VARIOUS NUCLEOSIDES AND NUCLEOTIDES

Serum-less cells were treated for 3 h with the compounds shown by addition directly to medium, following which the medium was removed, the cells rinsed once with Tris-buffered saline and labeled with [^{3}H]uridine as in Table I. Total uptake is the sum of acid-soluble and -insoluble fractions ± 1 S.D.

Compound (*0.1 mM*)	*Total uptake* (*counts/min per dish*)	*Percent of control*
None	12 900 ± 1150	100 ± 9
Adenosine	47 700 ± 2670	370 ± 9
Cyclic AMP	18 060 ± 990	140 ± 9
ATP	54 180 ± 1880	420 ± 9
Deoxyadenosine	16 150 ± 720	125 ± 9
Guanosine	20 450 ± 1370	160 ± 9
Deoxyguanosine	16 390 ± 1240	127 ± 9
Thymidine	11 130 ± 620	86 ± 9
Uridine	4 260 ± 240	33 ± 9
Cytidine	2 190 ± 102	17 ± 9
Deoxycytidine	13 290 ± 800	103 ± 9

uptake, while cyclic AMP and guanosine were much less stimulatory. The deoxyribonucleosides of guanosine and adenosine were only slightly active.

In contrast to the stimulatory activity of purine ribonucleosides, the pyrimidine ribonucleosides uridine and cytidine were strongly inhibitory while the pyrimidine deoxyribonucleosides thymidine and deoxycytidine were either slightly inhibitory or showed no effect. The inhibitory effect of cytidine has been found to persist for up to 5 h after removal of the nucleoside. These results clearly demonstrate that the stimulatory activity is associated with the purine base and ribose sugar while inhibition is found with the pyrimidine base and the ribose moiety. The presence of the deoxyribose residue eliminates both types of activity.

The ability of purine ribonucleosides to stimulate uridine incorporation was somewhat paradoxical in view of the reports by STECK *et al.*[19] and PLAGEMANN[20] which showed that at high concentrations (10 mM), many nucleosides, including adenosine, inhibited the uptake of other labeled nucleosides in cells grown in serum-containing medium. The effect of adenosine (1 mM) on the simultaneous uptake of [^{3}H]uridine in cells activated 2 h by 0.1 mM adenosine was, therefore, tested. As shown in Table III, 1 mM adenosine strongly and rapidly inhibited the concurrent uptake of [^{3}H]uridine, demonstrating that a nucleoside capable of activating the uptake of uridine in serum-less cells, also competes with the same nucleoside, most likely at the level of translocation[20], when both compounds are presented simultaneously to the cell.

Specificity of the activated uptake state

This study has thus far demonstrated that serum, fetuin and purine ribonucleosides are capable of stimulating the uptake of uridine. Experiments were performed to determine the specificity of this activated state for other substrates such

as adenosine, thymidine and phenylalanine. As shown in Table IV, [^{3}H]adenosine incorporation was moderately inhibited by both adenosine and ATP but not by cyclic AMP, while in the same experiment, [^{3}H]uridine uptake was significantly enhanced following a 3-h exposure to these substances prior to labeling.

Table V shows the results of several experiments designed to examine the effect of serum and cyclic AMP on phenylalanine (Expt. A) and thymidine (Expt. B) uptake. Cyclic AMP was chosen for these experiments since it had been shown to have a wide variety of activities in other cell systems, some of which have been reviewed recently by RASMUSSEN[21]. Cyclic AMP alone had no significant effect on either uptake system in spite of the fact that control [^{3}H]uridine incorporation was stimulated in both

TABLE III

INHIBITION OF URIDINE UPTAKE BY THE SIMULTANEOUS EXPOSURE TO ADENOSINE IN SERUM-ACTIVATED MOUSE EMBRYO CELLS

Serum-less cells were stimulated with 0.1 mM adenosine for 2 h, rinsed and labeled at 37° with [^{3}H]uridine containing adenosine (final concentration of 1 mM) or Tris-buffered saline to give a final concentration of 4 μC of [^{3}H]uridine in 2.0 ml of Tris-buffered saline. At the end of the indicated uptake period, the dishes were rapidly cooled on ice and the acid-soluble and insoluble fractions extracted and counted.

	Total uptake (counts/min per dish) of [^{3}H]uridine with time		
	4 min	*8 min*	*16 min*
Control	51 100	91 400	164 200
Adenosine (1 mM)	9 300	15 600	34 700
Percent of control	18	17	21

TABLE IV

COMPARATIVE EFFECT OF ADENINE DERIVATIVES ON [^{3}H]ADENOSINE AND [^{3}H]URIDINE UPTAKE IN SERUM-LESS CELLS

Serum-less cells were treated with three adenine derivatives for 3 h, then rinsed and labeled 30 min at 37° with 3 μC in 2.0 ml of Tris-buffered saline of either [^{3}H]uridine (21.7 C/mmole) or [^{3}H]adenosine (23.6 C/mmole). The acid-soluble and -insoluble fractions were extracted and are reported as mean total uptake per petri dish in counts per minute $\pm$ 1 S.D. The percent of total uptake in the acid-insoluble fraction is reported as well as the total uptake after treatment as a percent of the control, untreated uptake rate.

Treatment	*Total uptake (counts/min per dish)*	*Percent of total uptake in acid-insoluble fraction*	*Percent of total*
	[^{3}H]adenosine		
None	14 800 $\pm$ 840	3.2	100
Adenosine (0.1 mM)	11 420 $\pm$ 910	2.2	77
ATP (0.1 mM)	10 490 $\pm$ 450	2.3	71
Cyclic AMP (0.1 mM)	13 430 $\pm$ 920	3.4	91
	[^{3}H]uridine		
None	9 200 $\pm$ 1050	13.4	100
Adenosine (0.1 mM)	30 350 $\pm$ 2500	15.6	330
ATP (0.1 mM)	30 260 $\pm$ 1510	20.6	329
Cyclic AMP (0.1 mM)	12 720 $\pm$ 1050	14.3	138

TABLE V

COMPARATIVE EFFECTS OF SERUM AND CYCLIC AMP ON URIDINE, PHENYLALANINE AND THYMIDINE UPTAKE IN SERUM-DEFICIENT MOUSE CELLS

Serum-less cells were treated with serum with and without cyclic AMP for the time periods indicated after which the dishes were rinsed and labeled for 30 min at 37° with [^{3}H]uridine (3 μC/2 ml, 21.7 C/mmole), [^{3}H]phenylalanine (92 μC/2 ml, 8.3 C/mmole) or [^{3}H]thymidine (3 μC/2 ml, 8.2 C/mmole). The acid-soluble and -insoluble fractions were extracted as in Table I and counted. Results are expressed as Uptake stimulation factor which is the ratio of experimental uptake to the appropriate, untreated control uptake. A statistical analysis (Student's t test) showed that difference in the stimulation factor of 0.3 was not significant ($P = 0.15$) but differences of 0.6 or greater were significant ($P < 0.01$).

	Uptake stimulation factor					
	Total	*Acid soluble*	*Acid insoluble*	*Total*	*Acid soluble*	*Acid insoluble*
Expt. A	[^{3}*H*]*uridine*			[^{3}*H*]*phenylalanine*		
Serum-free medium, 6 h	1.0	1.0	1.0	1.0	1.0	1.0
Serum-free medium + cyclic AMP (0.1 mM)	1.3	1.3	1.6	0.9	0.8	1.0
Serum-free medium + cyclic AMP (0.4 mM)	1.8	1.8	2.4	1.0	1.0	1.0
Serum-supplemented medium, 6 h	2.6	2.5	3.3	0.9	0.4	2.0
Serum-supplemented medium + cyclic AMP (0.1 mM)	3.0	2.8	4.5	0.9	0.6	1.8
Serum-supplemented medium + cyclic AMP (0.4 mM)	3.6	3.3	6.2	0.9	0.6	1.9
Expt. B	[^{3}*H*]*uridine*			[^{3}*H*]*thymidine*		
Serum-free medium, 6 h	1.0	1.0	1.0	—	—	—
24 h	1.0	1.0	1.0	1.0	1.0	1.0
Serum-free medium + cyclic AMP (0.2 mM), 6 h	2.1	2.1	3.2	—	—	—
24 h	2.0	2.0	2.2	1.3	1.1	1.5
Serum-supplemented medium, 6 h	3.2	3.0	3.2	—	—	—
24 h	3.5	3.5	3.8	3.1	2.2	3.5
Serum-supplemented medium + cyclic AMP (0.2 mM), 6 h	4.7	4.4	8.8	—	—	—
24 h	4.1	3.7	7.8	3.3	2.9	4.0

experiments. Serum, however, stimulated incorporation of [^{3}H]phenylalanine into an acid-insoluble fraction at the expense of an intracellular pool, suggesting that transport had not been influenced while protein synthesis had been enhanced. In the case of [^{3}H]thymidine incorporation, serum alone enhanced uptake especially into the acid-insoluble (DNA) fraction at 24 h, an effect not significantly altered by the presence or absence of cyclic AMP. Other experiments demonstrated that adenosine and ATP were similar to cyclic AMP in their failure to influence thymidine or phenylalanine uptake.

Effects of metabolic inhibition

In view of the recent demonstration by KIMMICH[22] that active sugar transport into intestinal epithelial cells *in vitro* is strongly inhibited by oligomycin, the effect of this compound as well as phloridzin on uridine uptake was studied. Preliminary experiments demonstrated that oligomycin rapidly inhibited uptake at concentrations

TABLE VI

INHIBITION OF [^{3}H]URIDINE UPTAKE INTO ACID SOLUBLE AND INSOLUBLE FRACTIONS OF SERUM-STIMULATED MOUSE EMBRYO CELLS BY OLIGOMYCIN AND PHLORIDZIN ALONE AND IN COMBINATION

Serum-less cells were stimulated 3 h with 10% serum, rinsed and labeled 30 min at 37° with [^{3}H]uridine (3 μC/2 ml, 21.7 C/mmole) in the presence and absence of the respective inhibitors. The acid-soluble and insoluble fractions were extracted as in Table I and counted. The results are expressed as mean uptake ± 1 S.D. The percent of control is based on total uptake relative to untreated, control incorporation.

	[^{3}H]Uridine uptake (counts/min per dish)			
	Acid-soluble fraction	*Acid-insoluble fraction*	*Total (soluble + insoluble)*	*Percent of control*
No treatment	49 920 ± 3040	6340 ± 430	56 260 ± 3470	100
Oligomycin (0.5 μg/ml)	8 310 ± 186	1370 ± 150	9 680 ± 336	17
Phloridzin (1.0 mM)	11 210 ± 80	1620 ± 290	12 830 ± 370	23
Oligomycin (0.5 μg/ml) + phloridzin (1.0 mM)	1 340 ± 10	490 ± 100	1 830 ± 110	3

TABLE VII

EFFECT OF METABOLIC INHIBITORS ON URIDINE UPTAKE STIMULATION BY SERUM IN SERUM-LESS CELLS

Serum-less cells were treated with the appropriate inhibitor for 1 h, following which some were stimulated with 10% serum. The cells were then labeled 30 min at 37° with [^{3}H]uridine (5 μC/2 ml, 25.4 C/mmole) 3 h after onset of serum stimulation and 4 h after addition of inhibitors. Results are expressed as mean uptake per plate into acid-soluble *plus* insoluble fractions ± 1 S.D. Inhibitors were: 2,4-dinitrophenol, F^-, and FDNB.

Inhibitor	*Serum depleted cells, control*		*Serum supplemented cells, stimulation*	
	Total	*Acid-soluble fraction*	*Total*	*Acid-soluble fraction*
No treatment	26 600 ± 1500	23 500	61 600 ± 5800	53 900
Dinitrophenol (1 mM)	32 400 ± 800	28 100	60 900 ± 4800	52 200
F^- (1 mM)	24 900 ± 3100	21 100	39 300 ± 3400	32 300
FDNB (0.001 mM)	27 100 ± 1200	22 800	61 000 ± 5400	53 000
FDNB (0.005 mM)	4 700 ± 250	4 300	8 300 ± 850	7 500

as low as 0.1 μg/ml (0.0003 mM). Furthermore, it was found that phloridzin inhibited the process most efficiently if present during the uptake period showing that the inhibition was readily reversible thereby indicating an effect on the translocation step.

Table VI demonstrates the action of oligomycin and phloridzin separately and together on concurrent [^{3}H]uridine uptake in serum-stimulated cells. It is clear that both compounds alone inhibited uptake significantly. The combination of drugs was even more effective, the level of inhibition being similar to that expected for two compounds inhibiting independent processes.

Studies with other metabolic inhibitors were carried out to evaluate the role of energy metabolism in the process of uptake stimulation by serum. Previous work[11,20] had shown that several inhibitors of glycolysis and oxidative phosphorylation were inhibitory for uridine uptake in "stimulated" cells but only at levels which could produce significant cytotoxicity. As shown in Table VII, 1 mM dinitrophenol had no effect on the unstimulated control level of uridine uptake or its stimulation by serum. Fluoride (1 mM) significantly inhibited the process of stimulation but had little effect on control levels. FDNB markedly depressed uridine uptake as well as stimulation at 0.005 mM but not at 0.001 mM.

In other studies, neither azide (1 mM) nor iodoacetate (1 mM) affected either uptake or its stimulation by serum. It was important to adjust the pH of any compound to be studied to approx. 7.0–7.4 since low pH in particular inhibited uridine uptake in this system.

DISCUSSION

Serum stimulation of uridine uptake must be considered from two aspects. The first is the identity of the serum constituents and other compounds which are capable of acting as stimulators of uridine uptake. The second is the identity of the stimulated system itself.

The components of serum responsible for stimulation is difficult to establish. The fact that purified fetuin is capable of stimulating uridine uptake at concentrations known to enhance mammalian cell growth *in vitro*[7] supports the idea that serum macromolecules acting on the cell surface influence cellular function.

The ability of purine ribonucleosides to substitute for serum as uridine uptake stimulators suggests several possible mechanisms. The first is through the phenomenon of phosphorylase inhibition analogous to that shown for thymidine phosphorylase in bacteria[23,24]. Studies are in progress to evaluate the possible role of uridine phosphorylase in a similar mode of action. Preliminary results, however, fail to support this mechanism.

A second possibility is suggested by the fact that the adenine nucleosides and nucleotides which stimulate uridine uptake are those reported by COHN AND PARKS[25] to enhance pinocytosis in mouse macrophages. It is difficult to explain, however, the specificity of the stimulatory effect on uridine and not thymidine, adenosine or phenylalanine uptake by such a general, nonspecific mechanism as pinocytosis.

An interesting effect of adenine ribonucleotides which may bear a relationship to the phenomenon described here is the observation by TORPIER AND MONTAGNIER[26] that the membranes of hamster tumor cells induced by Rous or polyoma viruses fix larger quantities of ruthenium red when grown in the presence of adenosine mono-

phosphate but not guanosine, cytidine or uridine monophosphate. This would suggest an alteration in the cell membrane in response to the adenine derivatives, one manifestation of which may be increased uridine uptake.

Another mechanism by which adenosine and its derivatives might activate uridine uptake could be through an effect on cyclic AMP levels by interfering with the intracellular activity of cyclic 3',5'-nucleotide phosphodiesterase[27]. At this point, the direct role of cyclic AMP in the process is not clear and cannot be ruled out. The failure of added cyclic AMP to stimulate either phenylalanine or thymidine uptake in this system is not surprising since this compound has been shown not to enter intact cells to a significant extent[28]. The lack of an effect on phenylalanine uptake is at variance with the demonstration of its stimulation of amino acid uptake in bone and kidney cells[29]. Its inability to influence DNA synthesis in serum-less cells is also of interest since cyclic AMP has been implicated in the mitogenic activity of isoproterenol[30] and bradykinin[31].

Stimulation of energy metabolism in response to alterations in the level of ADP derived from exogenous adenosine is yet another possible mechanism. Several lines of evidence make this general mode of action plausible. The most striking finding is the rapid inhibition of uridine uptake by oligomycin at 0.0003–0.0015 mM, concentrations as low or lower than that found to inhibit sugar transport in intestinal cells[22] as well as microsomal $(Na^+ + K^+)$-dependent ATPase activity *in vitro*[32]. Secondly, that F^- inhibits stimulation without affecting baseline uridine uptake in serum-less cells and that dinitrophenol has no effect, suggests that the enhanced uptake is related to the synthesis of ATP by glycolytic pathways in this tissue culture system.

These findings suggest that an oligomycin-sensitive, high-energy phosphorylated intermediate is involved in nucleoside uptake as has been postulated by KIMMICH[22] for sugar uptake. The interconversions of membrane-bound $(Na^+ + K^+)$-dependent ATPase[32,33] would be one possible site of oligomycin action. How an oligomycin-sensitive step is linked to uptake is not clear, although it may be suggested that the phosphorylated intermediate acts as the phosphate donor for uridine phosphorylation by the kinase. At this time, there is no direct evidence for this possibility.

On the other hand, the work of PLAGEMANN[20,34] as well as PETERS AND HAUSEN[35] has demonstrated that permeation or translocation of uridine is the rate-limiting step in uptake. The present study does not contradict this conclusion, but rather adds a further dimension to uridine translocation and phosphorylation. Uridine uptake in Novikoff hepatoma cells[20,34] was studied under culture conditions which maintain the cells in the "stimulated" state. Phosphorylation may not then be rate limiting and translocation would assume a more critical role.

The role of a carrier molecule and its configuration must be considered in view of the previous work[20,34,35]. The current study showing that phloridzin reversibly inhibits uridine uptake and that FDNB inhibits its uptake in both pre- and post-stimulated cells also suggests that a membrane function is vital to the uptake of the pyrimidine nucleoside under the conditions of these experiments. Further studies with FDNB similar to those of KRUPKA[36,37] are in progress to determine whether or not dynamic configurational changes in the translocation steps can be shown to be associated with serum or adenosine stimulation of uridine uptake.

These considerations suggest a broader concept of nucleoside uptake by mammalian cells in which translocation and phosphorylation are integral parts of the

overall reaction as also suggested by SCHOLTISSEK[38] and KAY AND HANDMAKER[39]. What can not be answered at this point is the degree of coupling between the two reactions, a question discussed recently in relationship to thymidine phosphorylation[40]. The marked and persistent suppression of uridine uptake by cytidine described here may be due to the ability of cytidine triphosphate to inhibit uridine–cytidine kinase[41] implicating this enzyme in the uptake process in addition to the effect of cytidine on the nucleoside carrier[19,20]. It remains to be determined whether the phosphorylation reaction is closely coupled to the translocation step at the membrane or merely acts as an intracellular sump to maintain a low nucleoside concentration thereby encouraging facilitated diffusion. Additionally, the mechanism by which a uridine transport inhibitor[42] effects the translocation–phosphorylation system will be of interest in regard to cellular responses mediated through membrane alterations.

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