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THE KINETIC DISSECTION OF TRANSPORT FROM METABOLIC TRAPPING DURING SUBSTRATE UPTAKE BY INTACT CELLS

URIDINE UPTAKE BY QUIESCENT AND SERUM-ACTIVATED NIL 8 HAMSTER CELLS AND THEIR MURINE SARCOMA VIRUS-TRANSFORMED COUNTERPARTS

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Summary

1. We present a theoretical analysis of the tandem processes of transport and metabolic trapping which together constitute uptake of a substrate by intact cells.

2. Transport is assumed to occur by means of a simple carrier here analysed in its general form. Trapping is assumed to occur by a simple enzymic reaction.

3. We show how to obtain the separate parameters of the two steps by analysing uptake data over a range of uptake times and substrate concentrations.

4. We present uptake data for uridine and cytosine-β-D-arabinoside entering Nil 8 hamster fibroblasts, normal and murine sarcoma virus transformed, in the quiescent condition and after stimulation by added serum. We analyse the data in terms of the theory for tandem processes.

5. Transport is characterised by a system having a high K_m and a high V for entry. The data for cytosine-β-D-arabinoside suggest that the cytosine-β-D-arabinoside system is not far from a symmetric one. The data for uridine transport do not differ when quiescent and serum-activated cells are compared. Transformed cells transport uridine at half the maximum velocity of normal cells, with or without added serum.

6. Trapping of cytosine-β-D-arabinoside is insignificant. Trapping of uridine occurs by a system with both V and K_m at least an order of magnitude smaller than are these parameters for transport. Trapping of uridine by non-

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transformed cells activated by serum, has twice the V of such cells in the quiescent state.

7. In the virus-transformed cells, the control of uridine trapping by added serum is lost, along with control of growth by this stimulant.

Introduction

A number of recent studies have been concerned with the uptake of various metabolisable substrates by cells in cell culture, and with the effect of the condition of the cells (for instance, whether the cells are growing or non-growing, normal or virus transformed) on such rates of uptake [1—5]. The measure of 'uptake' has often been the total amount of labelled material that has entered the cell at defined times, regardless of whether or not the labelled metabolite has undergone metabolic conversion after it entered the cell.

It has become important to assess whether it is the transport step or rather a subsequent metabolic step that is affected when the state of the cell is altered. Plagermann and his co-workers [6] in a pioneering study, showed that the kinetics of deoxythymidine uptake in Novikoff hepatoma cells could be dissected into a transport and a subsequent phosphorylation step.

We have recently performed in our laboratory a preliminary dissection of the uptake system for uridine and shown that the transport step for uridine has rather similar kinetic features in many cell lines [7]. We have also shown that in 3T3 cells, it is the phosphorylation of uridine which is affected when quiescent cells are activated by the addition of serum, and not the transport across the plasma membrane [8]. In the present paper, we extend these earlier studies by providing a theoretical treatment for such 'tandem' processes, where the transport step accords with the kinetics of the simple carrier [9] (i.e. a facilitated diffusion system), while the subsequent metabolic step accords with the kinetics of a simple enzyme reaction (the Michaelis-Menten equation). We show how the kinetics of the two steps can be derived separately using in situ methods on intact cells, by making uptake measurements over a wide range of concentrations of substrate and uptake times. We apply this analysis to data on uptake of uridine and of a poorly phosphorylated uridine analogue by the Nil 8 strain of hamster fibroblasts and also by a murine sarcoma virus-transformed strain of these same cells. In these cells we find that (as in 3T3 cells) the transport step is not affected when quiescent cells of the parent line are activated by addition of serum. The phosphorylation step is, however, accelerated. The transformed line transports uridine at a lower rate than the parent line, and neither transport nor the subsequent phosphorylation is increased when serum-starved cells of the transformed line are given a medium rich in serum.

Materials and Methods

The cell lines used were gifts from (the late) Dr. I. MacPherson of the Imperial Cancer Research Fund laboratories, London. The normal line was the Nil 8 clone of golden hamster fibroblasts. The transformed line was derived

from this Nil 8 clone by infection with a murine sarcoma virus and is known as the Nil 8 HSV line. Frozen stocks of cells were thawed at six-weakly intervals, and grown and maintained at 37°C in a CO_2-enriched, humidified atmosphere in Dulbecco's modified Eagle's medium (Biolab, Jerusalem), to which 10% of fetal or newborn calf serum (Biolab, Jerusalem) had been added.

Uptake experiments. The cells were left for 72—96 h in a low-serum (0.25%) medium, and then activated or sham-activated, by replacement of the low-serum medium by Dulbecco's modified Eagle's medium containing either 10% newborn calf serum or else 10% phosphate-buffered saline (for the controls). The cells were left for 90 min at 37°C in this medium and then washed twice with saline buffered with phosphate at pH 7.4, 20°C. At time zero, 0.5 ml of a solution of the desired concentration of uridine in the buffered saline, labelled with radioactive [^{3}H]uridine (Israel Atomic Energy Agency, Isotope Laboratory, Nuclear Research Center, Negev, Dimona, Israel) at 2 μCi/ml, was added to each dish and the dish kept on a water-bath at 20°C for the desired time. Termination of the uptake of uridine was achieved by five washes with 2 ml of the buffered saline, ice-cold. With the dishes on ice, 0.75 ml of 5% trichloroacetic acid in water was added and left for at least 20 min to extract acid-soluble material. 0.5 ml of this extract was taken for liquid scintillation counting. Zero time samples were obtained by commencing the washing procedure as soon as possible after adding the labelled uridine solution, the dishes being placed on ice prior to the addition of the labelled uridine. The uptake of cytosine-β-D-arabinoside and 3-*O*-methylglucose was measured as for uridine, both radiochemicals being obtained from the Radiochemical Centre, Amersham, England.

Wash-out experiments. The radioactively labelled medium was removed by suction after the 10 min uptake period and replaced by a large excess of phosphate-buffered saline free of radioactivity. The wash-out was continued for the desired time and five rapid washes in ice-cold buffered saline were used to terminate the flow of uridine.

Chromatographic analysis of the labelled material taken up by the cells. 0.5 ml of 0.6 N perchloric acid was added to the dishes after the uptake experiment instead of the 5% trichloroacetic acid, and left for 20 min in the cold. The extract was neutralised with 5 N KOH and a sample applied to thin-layer cellulose chromatography plates. The plates were developed in a solvent containing 3 parts of 1 M ammonium acetate (pH 5.0) with 7 parts of 95% ethanol, for 2—3 h. This solvent system effectively separates nucleosides from nucleotides, the latter remaining near the origin. 1 cm strips of the chromatogram were cut, scraped off the plate into scintillation vials containing 0.5 ml water plus 3.5 ml toluene-Triton scintillation mixture, and counted as before. Authentic samples of nucleosides and nucleotides were run in parallel as markers.

Phosphorylation of uridine in cell extracts [10]. Cells were scraped off the dishes (on adding 15 mM potassium chloride) using a rubber spatula, then fragmented by seven successive cycles of freezing and thawing, followed by 15 min of sonication in a bath sonicator (Ladd Research Industries), with the cell extracts being maintained at 0°C. For the assay 25 μl of the cellular extract was added to a 50 μl assay volume containing the desired concentration of

uridine labelled with radioactive [^{3}H]uridine at 25 μCi/ml, 100 mM Tris-HCl, 300 mM KCl, 10 mM ATP and 10 mM $MgCl_2$. The mixture was left at 37°C for the desired time and enzyme activity stopped by boiling the mixture for 30 s. 40 μl of this boiled extract was taken and spotted onto a 1.2 cm disc of Whatman DE81 ion-exchange paper. The paper disc was dried and then twice washed with 20-ml portions of 1 mM ammonium formate, twice with 20 ml water and finally twice with 20 ml ethanol. The disc was finally dried and the radioactivity remaining on the disc counted by liquid scintillation. To measure the extent of the phosphorylation of the labelled uridine, aliquots of the reaction mixture were spotted on to the discs and counted without going through the washing procedure.

Theory

We will assume that the uptake of metabolite occurs by a facilitated diffusion system, obeying the kinetics of the simple carrier [9], this transport step being followed by metabolic conversion of the substrate to a 'trapped' form (in which it cannot leave the cell). This conversion is assumed to obey the kinetics of a simple Michaelis-Menten reaction. Now, if S_1 is the concentration of substrate outside the cell and S_2 is the concentration of free substrate within the cell, the net rate of the transport step (see ref. 9) is given by v_t and that of the phosphorylation step by v_p in the following equations:

$$v_t = \frac{K(S_1 - S_2)}{K^2 R_{00} + K R_{12} S_1 + K R_{21} S_2 + R_{ee} S_1 S_2} \tag{1}$$

$$v_p = \frac{V S_2}{\Phi + S_2} \tag{2}$$

where K is a measure of the affinity of the transport system for the substrate, while the terms in R are the resistances experienced by the various forms of the carrier as these diffuse across the membrane or break down to yield free substrate. These resistances determine the maximum velocity of transport in various types of experiments. The interpretation of the K and R parameters in terms of the rate constants of the simple carrier model is given in the paper of Lieb and Stein [9]. Table I of the present paper reproduces part of Table IV of ref. 9 and gives the maximum velocities and half-saturation concentrations of various transport experiments in terms of the K and R parameters.

V in Eqn. 2 is the maximum velocity of the enzymic reaction leading to trapping of the substrate, while Φ is the half-saturation concentration for that reaction. Here it is the internal concentration of substrate S_2 that is relevant.

When the uptake experiment commences, S_2 is zero, v_p is also zero while v_t is the initial value defined for the zero trans experiment, when one side of the membrane is devoid of substrate. As uptake continues and S_2 rises v_t, being a net flow, is reduced as substrate accumulates and begins to leave the cell, while v_p rises. At some value of S_2 (defined by the kinetic parameters and S_1) v_p and v_t will coincide. From that point on, the net rate of entry of substrate by transport just balances the consumption of substrate by the metabolic reaction and a steady-state is reached. The rate of accumulation of metabolite is now

TABLE I

INTERPRETATION OF TRANSPORT PARAMETERS V AND K_m IN TERMS OF THE BASIC PARAMETERS OF THE SIMPLE CARRIER

Taken from Table IV of Lieb and Stein [9]. These interpretations can be obtained by appropriate substitutions into Eqn. 1 of ref. 9. The infinite cis experiment is one where net flows are measured with the substrate concentration at one face of the membrane kept saturatingly high, that at the other being varied. The equilibrium exchange experiment is one where tracer flux is measured while the substrate concentration is kept the same at both faces of the membrane. The parameter K measures the affinity of the carrier for substrate averaged over both faces of the membrane, the R parameters are relevant resistances to flow or breakdown of the carrier or carrier-substrate complex. The subscripts 1 and 2 refer to the two faces of the membrane so that, for instance, V_{12}^{zt} is the maximum velocity of the zero trans flow measured from face 1 to face 2 of the membrane.

Experimental procedure	V	K_m	K_m/V
Zero trans	$V_{12}^{zt} = 1/R_{12}$	$K_{12}^{zt} = K\frac{R_{00}}{R_{12}}$	KR_{00}
	$V_{21}^{zt} = 1/R_{21}$	$K_{21}^{zt} = K\frac{R_{00}}{R_{21}}$	KR_{00}
Infinite cis	$V_{12}^{ic} = 1/R_{12}$	$K_{12}^{ic} = K\frac{R_{12}}{R_{ee}}$	—
	$V_{21}^{ic} = 1/R_{21}$	$K_{21}^{ic} = K\frac{R_{21}}{R_{ee}}$	—
Equilibrium exchange	$V^{ee} = 1/R_{ee}$	$K^{ee} = K\frac{R_{00}}{R_{ee}}$	KR_{00}

constant and the uptake curve is linear with time. It is very important to note that for such tandem processes, the uptake curve against time may be (apparently) linear at two different phases: (i) apparently linear, at the very initial period of uptake when the internal concentration is so low that outward transport is negligible, and (ii) truly linear, when the internal concentration is just sufficient for net transport to equal metabolic consumption.

The uptake curve will depart clearly from the initial apparent linearity when the internal concentration of free substrate reaches some 20—30% of that of the external substrate. Linearity of a reaction is generally considered as sufficient evidence that a true initial rate is being measured. This may well be true when enzyme mechanisms are being considered, but in the case of the tandem processes of transport and metabolic trapping, we can now see that only the first, fleeting linear phase can be so interpreted. The second, persistent linear phase merely indicates that the process is now sufficiently far from the initial rate for a balanced situation to have been attained. Clearly, one must distinguish very sharply between these two linear phases if one is not to fall into the trap of falsely identifying an initial rate.

The way to distinguish the two regions of linearity is as follows: Extrapolate the data on uptake as a function of time back to zero time. If the zero-time uptake obtained by this extrapolation coincides with a directly determined zero-time value, one can begin to suspect that the true initial rate is being measured. In addition this zero-time value should be that predicted from the amount of substrate trapped between the cells, substantially lower than that expected for the amount of substrate that would be present within the cells at

transport equilibrium. Finally, the extrapolated zero-time values must be exactly proportional to the external substrate concentration over the whole of a wide range of substrate concentrations.

On extrapolating to zero data obtained after fairly substantial periods of time, error in the extrapolated value may be far larger than even the value expected within the cells at transport equilibrium let alone the value to be expected between the cells. To avoid this, data must be taken at sufficiently early times on the linear portion of the curve for the extrapolation to zero to be meaningful.

Returning now to our discussion of Eqns. 1 and 2, we note that when different values of the external substrate concentration S_1 are chosen, a particular value of the steady-state internal concentration S_2 will be reached for each S_1, depending on the kinetic parameters of the system. The slope of the uptake curve with time, which is the rate of trapping v_p in Eqn. 2, will vary with this S_2. The maximum velocity reached as S_1 is varied will be exactly the maximum velocity V of Eqn. 2, but the half-saturation concentration Φ is correctly defined only when expressed in terms of the unknown intracellular concentration S_2, rather than the extracellular value S_1, set by the experimenter. We shall now proceed to show how S_2 can be found from the experimental data.

Method 1

We note that when the uptake curve becomes linear with time, $v_t = v_p = v$, where v is the constant rate of metabolite accumulation. This v is a measured quantity. We can equate it with the righ-hand side of Eqn. 1 and solve for S_2 as:

$$S_2 = \frac{KS_1 - vKR_{12}(KR_{00}/R_{12} + S_1)}{K + vR_{ee}(KR_{21}/R_{ee} + S_1)} \tag{3}$$

$$S_2 = \frac{S_1 - v/V_{12}^{zt}(K_{12}^{zt} + S_1)}{1 + v/V_{21}^{zt}(1 + S_1/K_{21}^{ic})} \tag{4}$$

on substituting into Eqn. 3 from Table I. Eqn. 4 is exact, and enables S_2 to be found from S_1 at any value of v, providing that the transport parameters V_{12}^{zt}, K_{12}^{zt}, V_{21}^{zt} and K_{21}^{ic} are available. The first two represent no real problem, since they will be available from studies, at short times, of the uptake rate as a function of S_1. The latter two parameters need to be ascertained by measurement of the net efflux of metabolite from saturating concentrations within the cell, as a function of S_1. However, if the system is such that v_p saturates well below the concentration at which the transport system saturates, S_1 will be small in comparison with the K terms and can be neglected within the brackets in Eqn. 4. Finally, if the transport maximum velocity V_{21}^{zt} can be assumed to be large in comparison with v, Eqn. 4 simplifies to:

$$S_2 = (\text{approx.})\, S_1 - vK_{12}^{zt}/V_{12}^{zt} \tag{5}$$

from which S_2 can be calculated. In particular, we can do this calculation at that value of S_1 for which v is half maximal, and hence estimate Φ.

Method 2

Once again we set $v = v_t = v_p$ at the steady-state. Eliminating S_2 between Eqns. 1 and 2, we obtain:

$$\left(\frac{\Phi}{K} VR_{21} + \frac{\Phi S_1}{K^2} VR_{ee} - VR_{00} - \frac{S_1}{K} VR_{12}\right)\left(\frac{v}{V}\right)^2 +$$

$$\left(\frac{S_1}{K} VR_{12} + \frac{\Phi}{K} + VR_{00} + \frac{S_1}{K}\right)\left(\frac{v}{V}\right) - \frac{S_1}{K} = 0 \qquad (6)$$

This is an explicit solution for the steady-state velocity v in terms of the kinetic parameters and the external substrate concentration S_1. A useful special case of Eqn. 6 is where the external concentration reaches a limitingly low value. We solve Eqn. 6 for v/S, and take the limit of v/S_1 as S_1 (and, hence, v/V) tends to zero, obtaining:

$$\lim_{S_1 \to 0}\left(\frac{v}{S_1}\right) = \Pi = \frac{1}{\frac{\Phi}{V} + KR_{00}} = \frac{1}{\frac{\Phi}{V} + \frac{K_{12}^{zt}}{V_{12}^{zt}}} \qquad (7)$$

since KR_{00} is the limitingly low value of $(v_t/S_1)^{-1}$ and is given by K_{12}^{zt}/V_{12}^{zt} (see Table I and Lieb and Stein [9]). The values of K_{12}^{zt} and V_{12}^{zt} are available from the uptake measurements at short times, while V is the known maximum velocity of trapping at high substrate concentrations. Thus from Eqn. 7 and the limiting value of v/S_1, we can derive the only unknown Φ, the half-saturation concentration of the trapping reaction, in terms of the internal concentration of substrate.

A useful transformation of Eqn. 7 is in terms of the slopes and intercepts of the often-used plots of S/v against S, where S is the substrate concentration (here S_1) and v is the velocity of reaction (here v_t or v_p). The plot of S/v_t against S for the transport step (i.e. for the data at short times) gives as the intercept on the y-axis the value of K_{12}^{zt}/V_{12}^{zt}. Similarly, the intercept on the y-axis of the plot of S/v against S in the case of the data for trapping (the data at long times) gives the value of $1/\Pi$ in Eqn. 7. The slope of this latter plot gives the reciprocal of the maximum velocity of trapping, V. Transforming Eqn. 7 in terms of these slopes and intercepts gives the required half-saturation concentration as Φ in:

$$\Phi = \frac{y\text{-cept}_{trapping} - y\text{-cept}_{transport}}{\text{slope}_{trapping}} \qquad (8)$$

If the effect of transport were ignored, the half-saturation concentration of the trapping step (or 'uptake', since we ignore the distinction between transport and trapping) would have been calculated as the ratio of the y-cept to the slope for the trapping plot only, and would have been , more or less grossly, over-estimated.

Finally, we record here the result of integrating Eqn. 1 for the case where the trapping reaction is insignificant, i.e where transport only is occurring by a simple carrier. We set $v_t = dS_2/dt$ in Eqn. 1 and integrate by parts obtaining, for $S_2 = 0$ at $t = 0$, and $= S_2$ at $t = t$:

$$(K^2R_{00} + K(R_{12} + R_{21})S_1 + R_{ee}S_1^2)\ln(1 - f) + (KR_{21} + R_{ee}S_1)S_1 f = -Kt \qquad (9)$$

where f is the fractional uptake S_2/S_1. If we plot $\ln(1-f)/t$ against f/t and find the slope and y-intercept (y-cept) of this straight line, we obtain

$$KR_{00} + R_{12}S_1 = -(1 + \text{slope})/y\text{-cept} \tag{10}$$

A replot of the quantity on the right hand side of Eqn. 10 against S_1 has slope R_{12} from which the maximum velocity of uptake can be found (Table I) and has slope/intercept equal to the half-saturation concentration for uptake. In addition, the quantity $((1/-\text{slope})-1)\cdot S_1$ is equal to

$$K_{12}^{ic}\frac{(S_1 + K_{12}^{zt})}{(S_1 + K_{21}^{ic})}$$

If S_1 is sufficiently high, in comparison with the relevant half-saturation concentrations in the bracketed terms, the half-saturation concentration at the inner face of the membrane, K_{12}^{ic}, can be found.

Results and Discussion

Fig. 1a shows the results of an experiment in which the time course of uptake of the uridine analogue, cytosine-β-D-arabinoside into Nil 8 HSV cells, was studied at a number of different external cytosine-β-D-arabinoside concentrations, at 20°C.

At concentrations below 0.3 mM, the uptake curve clearly reaches a plateau value by 15 min, while at 0.3 mM the 15 min value closely approaches this plateau.

In this experiment, the external solution contained in all cases radioactivity to the amount of $1\cdot 10^6$ cpm/ml. The radioactivity present on each dish at 15 min of uptake was 5458, 5550, 5830 and 5500 cpm, when the cytosine-β-D-arabinoside concentration was 0.03, 0.05, 0.1 and 0.3 mM, respectively. The amount of cell water associated with each dish (assuming that the cytosine-β-D-arabinoside has reached equilibrium across the cell membrane) is, therefore, betweem 5.46 and 5.83 μl for these cases. Since the cell count here was $2.2\cdot 10^6$ cells/dish, the cell water can be calculated to be some 2.4 pl, equal to the value determined directly from uptake data for the non-metabolized sugar, 3-*O*-methylglucose. The plateau value of counts is, therefore, independent of the concentration of cytosine-β-D-arabinoside present outside the cells and is consistent with a value to be expected for diffusion equilibrium. The simplest interpretation of these results and of the data of Fig. 1a is that we are witnessing here a simple transport process, uncomplicated by any subsequent metabolism of the cytosine-β-D-arabinoside. Consistent with this interpretation are the data recorded in Table II. Here cells were loaded with cytosine-β-D-arabinoside for 10 min, from external concentrations of 10, 100 and 1000 μM. After this 10 min period, the external medium was changed to one free of cytosine-β-D-arabinoside and the time course of the wash-out of cytosine-β-D-arabinoside followed. After a 10 min wash-out period only about 10% of the radioactivity present after the original incubation remained within the cells (or was trapped between the cells). Most of the radioactive cytosine-β-D-arabinoside is free to leave the cells between the 10 and 20 min of wash-out at 20°C and little is trapped by a metabolic step subsequent to transport.

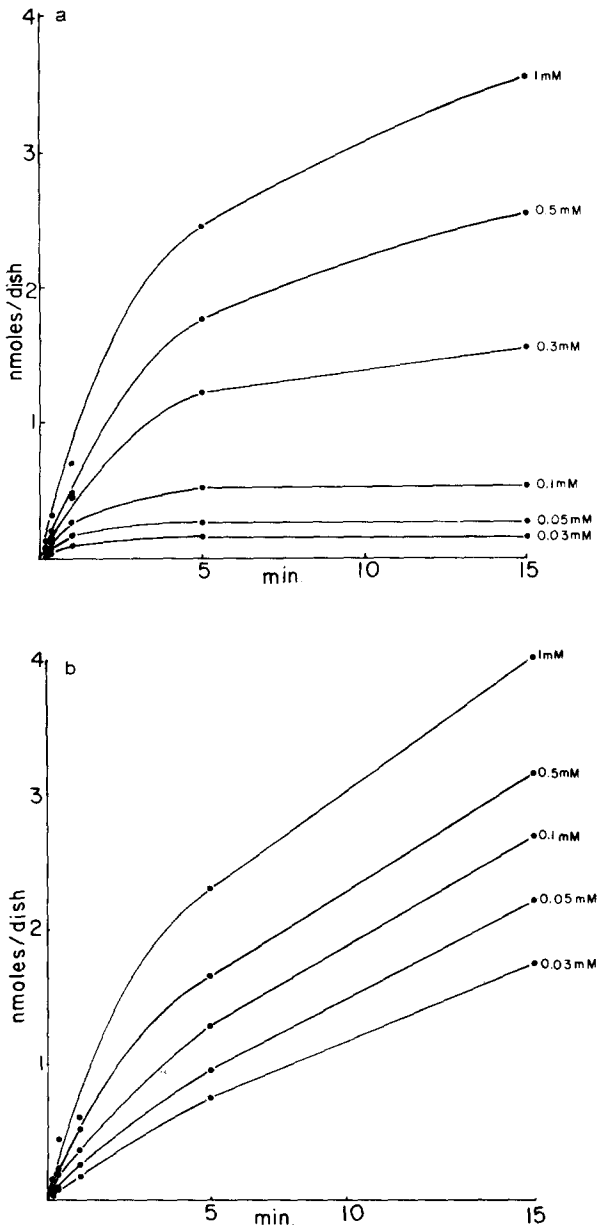

Fig. 1. (a) Uptake of cytosine-β-D-arabinoside by Nil 8 HSV cells as a function of time and concentration. The ordinate represents nmol of substrate that have entered a 3 cm dish of cells at the times indicated on the abscissa (in min). Uptake is at 20°C, pH 7.4, phosphate-buffered saline. Concentration of substrate indicated next to each uptake curve. A dish contained some $2.3 \cdot 10^6$ cells. (b) Uptake of uridine by Nil 8 HSV cells. Conditions as in the parallel experiment depicted in (a).

This behaviour in our hands is in contrast to the reports that cytosine-β-D-arabinoside undergoes metabolic conversion in a number of cell types [11] to a form in which it is an effective chemotherapeutic agent. One can reconcile these divergent findings if one assumes that 15 min is not enough time at the

TABLE II

EFFLUX OF LABELLED CYTOSINE ARABINOSIDE (CAR) AND OF URIDINE FROM CELLS PRELOADED WITH NUCLEOSIDE, AS A FUNCTION OF EFFLUX TIME AT 20°C

Cells were loaded with nucleoside for 10 min with external concentrations of nucleoside of 10, 100 and 1000 μM. Data are % of zero time.

Initial concn. (μM)	CAR				Uridine			
	0 s	20 s	60 s	10 min	0 s	20 s	60 s	10 min
10	100	55	30	11	100	97	92	83
95	100	69	37	8	100	84	76	68
600	100	97	83	8	100	92	80	59

low temperature of 20°C to allow for a substantial conversion of the intracellular cytosine-β-D-arabinoside.

In contrast to these results with cytosine-β-D-arabinoside, Fig. 1b shows the uptake of labelled uridine in an experiment performed in parallel with that of Fig. 1a. Here the uptake curves do not plateau at any uridine concentration. Indeed the uptake curve seems to approach asymptotically a constant upward slope, this slope being noticeably greater at the higher uridine concentrations. At 15 min of uptake, the cpm/dish were 58 300, 44 900, 27 200, 6400 and 4200 when the external uridine solution (labelled with $1 \cdot 10^6$ cpm/ml) was at 30, 50, 100, 500 and 1000 μM, respectively. In the parallel experiment of Fig. 1a, where cytosine-β-D-arabinoside reached diffusion equilibrium, some 5500 cpm were associated with each dish. Thus, by 15 min the uridine is about 10 times above the diffusion-equilibrium value for an external concentration of 30 μM, and is well above the equilibrium value also for 50 and 100 μM. This is consistent with the interpretation that uridine is being trapped within the cell. The data on the wash-out of uridine are recorded in Table II. Here, in an experiment performed in parallel with that for cytosine-β-D-arabinoside, the data show that 10 min of wash-out leave within the cell between 60% to 85% of the radioactivity as trapped counts. In an additional experiment to confirm this result, the cells were incubated with radioactive uridine for 15 min, as for Table II, and the cells subsequently extracted on the dishes with 0.6 N perchloric acid. The extract was chromatographed by thin-layer chromatography as described in Materials and Methods. The data show that the free uridine formed some 2—5% of the total counts when the external uridine was 10 μM, some 30—40% from 100 μM and 55—75% from 1000 μM, consistent with the data of Table II.

Table II and a comparison of Figs. 1a and b bring out very clearly the different character of uptake by a system that can trap substrate within the cell (Fig. 1b) and by one that does not, under the given experimental conditions (Fig. 1a). With these differences established we can proceed, along the lines of the theory developed above, to a detailed kinetic analysis of the uptake curves.

We calculate for each time point of the data of Fig. 1a, the quantity f, the cpm/dish at that time divided by the cpm/dish at equilibrium, and plot, as required by Eqn. 9, $\ln(1-f)/t$ against f/t (Fig. 2A, B and Table III). Fig. 2C is a replot of (1 + slope)/y-cept from these plots, against the external concentra-

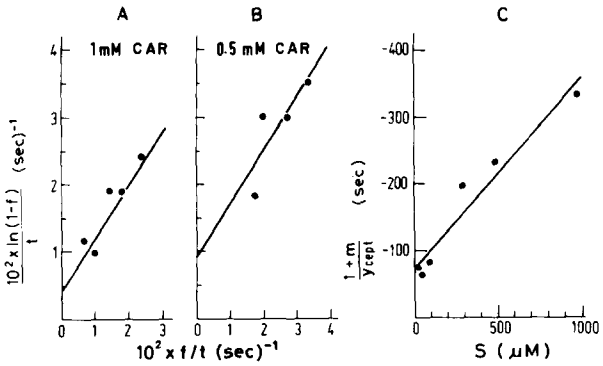

Fig. 2. Data in Fig. 1a analysed by the integrated rate equation method (Eqn. 9 of text). A and B uptake curves for 1000 μM and 500 μM cytosine-β-D-arabinoside (CAR) plotted as $\ln(1-f)/t$ on ordinate against f/t on abscissa, where f is the fraction of the equilibrium uptake value that is reached by time t. C is the replot of data derived by linear regression of plots such as A and B, where $-(1 + \text{slope})/y\text{-cept}$ of these linear regressions is plotted on the ordinate against the external concentration S_1, as in Eqn. 10, on the abscissa. The lines are the linear regressions.

tion of cytosine-β-D-arabinoside. From the slope and intercept of this replot (Eqn. 10) the half-saturation concentration for cytosine-β-D-arabinoside at the outer face of the membrane in a zero trans experiment, K_{12}^{zt}, is calculated to be approx. 250 μM, while the maximum velocity of cytosine-β-D-arabinoside uptake, V_{12}^{zt}, is calculated to be approx. 500 pmol/1 · 10^6 cells per min. To obtain the kinetic parameters for cytosine-β-D-arabinoside at the inner face of the membrane we need the value of $-(1 + \text{slope})/\text{slope}$ in the plots of $\ln(1-f)/t$ against f/t. From Table III, we see that the value of the slope here is not significantly different from zero for those cytosine-β-D-arabinoside concentrations below 500 μM. In such cases, no meaninful value for the half-saturation concentration at the inner face can be derived. For 500 μM and 1000 μM, however, the slope is non-zero and from the data, (Figs. 2A and 2B), the value of the required function ($-(1 + \text{slope})/\text{slope}$ multiplied by the external concentration, see Theory) gives a value of 133 μM and of 172 μM at external cytosine-β-D-arabinoside concentrations of 500 μM and 1000 μM, respectively. To the extent that at these concentrations the transport system is approaching saturation at the external face of the membrane, Eqn. 10 allows us to equate the values so derived with the desired term K_{21}^{ic}.

TABLE III

RESULTS OF LINEAR REGRESSION OF $\ln(1-f)/t$ AGAINST f/t FOR THE DATA ON THE UPTAKE OF CYTOSINE-β-D-ARABINOSIDE (CAR) BY Nil 8 HSV CELL LINE, DEPICTED IN FIG. 1

External CAR concentration	Slope	y-cept · 10^{-2}
30	0.61 ± 0.77	−2.12 ± 0.89
50	0.20 ± 0.25	−1.76 ± 0.31
100	−0.18 ± 0.61	−1.02 ± 0.63
300	−0.13 ± 0.36	−0.438 ± 0.148
500	−0.79 ± 0.43	−0.089 ± 0.11
1000	−0.85 ± 0.175	−0.043 ± 0.028

Thus the system half-saturates at the inner face of the membrane at about 150 μM cytosine-β-D-arabinoside. If one considers that the half-saturation concentration for entry is some 250 μM, the system seems essentially symmetric. There seems no reason to suppose that cytosine-β-D-arabinoside uptake by the Nil 8 HSV cell line is in any way more complex than a simple (possibly even symmetric) carrier system.

The data of Fig. 1b cannot be treated in the above fashion since we have shown that the uptake of uridine does not stop at the diffusion equilibrium level, but follows rather the kinetics of a trapping system. The theory for the 'tandem' process must therefore be applied.

Fig. 3 shows the uptake curves for a typical experiment with uridine performed at short incubation times. The initial velocity of uridine uptake, obtained by linear regression, is plotted against the external concentration of uridine in Fig. 4. This figure includes corresponding data for cells in different states of growth or transformation, obtained in the same way, Table IV records the slopes and intercepts of such uptake curves, the latter representing the zero-time values.

At long uptake times, the slope of the uptake curve reaches an asymptotic

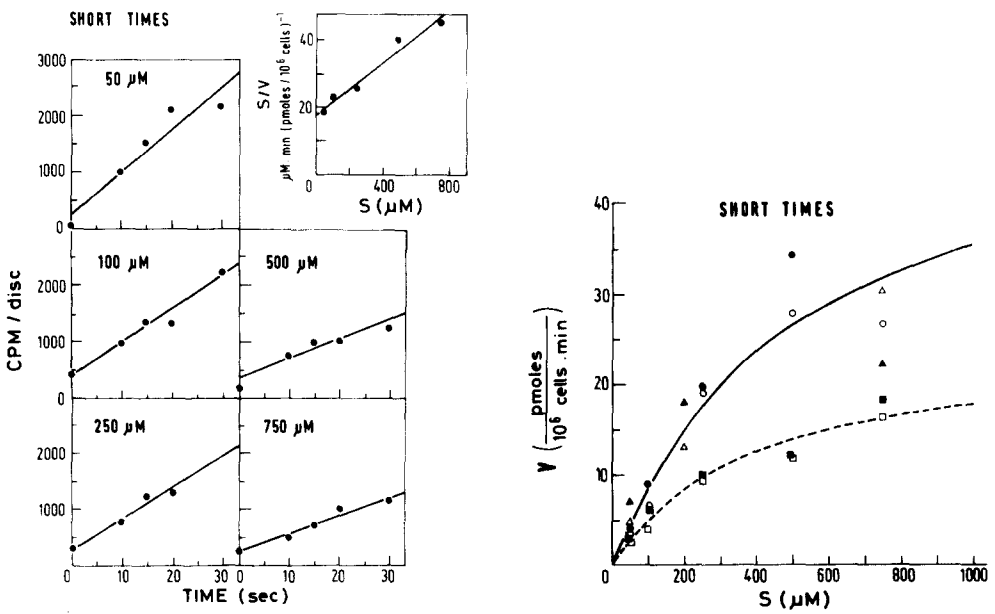

Fig. 3. Uptake of uridine by Nil 8 HSV cells at short times at various substrate concentrations. Conditions as in the legend of Fig. 1a. The reciprocal of the slopes obtained by linear regression at each substrate concentration are plotted in the upper right panel for the linear form S/v against S. All lines are linear regressions.

Fig. 4. Velocity of uptake of uridine at short times by normal and transformed Nil 8 cells rendered quiescent or serum activated, plotted as a function of the uridine concentration. △, Normal cells — serum, Exp. 1; ○, normal cells — serum, Exp. 2; ▲, normal cells + serum, Exp. 1; ●, normal cells + serum, Exp. 2; ■, transformed cells + serum; □, transformed cells — serum. ———, Normal cells; - - - - -, transformed cells. Velocities were derived from slopes of linear regression in Fig. 3 and a similar experiment. The lines drawn, are calculated from the Michaelis-Menten parameters obtained from a linear plot (S/v against S) of the data.

TABLE IV

COMPARISON OF EXTRAPOLATED AND DIRECTLY MEASURED ZERO-TIME VALUES FOR URIDINE UPTAKE BY NORMAL AND TRANSFORMED Nil 8 CELLS, AND DATA ON 3-*O*-METHYLGLUCOSE UPTAKE BY THESE CELLS

Direct measurement in brackets, extrapolate given along side ± S.E. All data in cpm/dish. External solution is everywhere 9000—11 000 cpm/μl of solution. (—), no determination performed.

Cell type and protocol	Serum added	Uridine concentrations (μM)									
Short times:		50		100		250		500		750	
Normal	+	(53)	71 ± 60	(53)	77 ± 53	(—)	65 ± 28	(—)	38 ± 22	(—)	79 ± 31
	0	(—)	97 ± 38	(—)	112 ± 65	(62)	58 ± 39	(43)	70 ± 52	(—)	55 ± 16
Transformed	+	(334)	78 ± 323	(40)	113 ± 76	(240)	368 ± 167	(232)	337 ± 146	(270)	309 ± 71
	0	(29)	267 ± 272	(459)	410 ± 155	(304)	313 ± 112	(207)	353 ± 124	(298)	286 ± 79
Long times:		2		5		10		20		50	
Normal	+	(172)	1 550 ± 2 460	(—)	−325 ± 2 740	(—)	900 ± 738	(—)	134 ± 334	(—)	403 ± 190
	0	(—)	478 ± 2 470	(153)	974 ± 1 840	(104)	−447 ± 1760	(—)	636 ± 386	(—)	167 ± 174
Transformed	+	(611)	10 050 ± 15 200	(—)	18 100 ± 15 700	(—)	3900 ± 4200	(—)	6810 ± 5580	(—)	5640 ± 3790
	0	(—)	10 040 ± 8 410	(733)	4 400 ± 6 400	(1,305)	6400 ± 9020	(—)	7030 ± 7000	(—)	−266 ± 3450

	3-*O*-Methylglucose	
	Zero time	40 min
Normal	41	530
Transformed	231	3036

value. Data points from this portion of such curves were analysed by linear regression (Table IV) and the resulting slopes from a number of different experimental systems are depicted in Fig. 5.

In Table V, we record the parameters obtained by a linear regression of the data of Figs. 4 and 5, using the linear transformation S/v against S of the Michaelis-Menten equation (S being the external substrate concentration; v, the velocity).

Taking first the uptake data at short times, we see from Table IV that the zero-time extrapolation for these data are consistent with the values obtained by direct measurement, are independent of the uridine concentration and are far below the equilibrium value found for the uptake of 3-*O*-methylglucose, a substance that is known to reach diffusion equilibrium. Thus, one can argue with some force that the data of the initial step of uptake represent the transport event itself. Kinetic parameters derived from the relevant data are collected in the first six rows of Table V. Since the intercepts and slopes for the data are not significantly different in the presence or absence of serum we can conclude that serum addition does not affect the transport step, either in normal cells or in the transformed counterpart. We have, therefore, lumped together the data obtained with and without serum and derived the kinetic parameters of the transport step, separately for normal and transformed cells. The half-saturation concentration for these two cell lines is about 400—500 μM, and is not different for the two lines. The maximum velocity of transport is some 3300 and 1500 pmol/min per $1 \cdot 10^6$ cells for normal and transformed cells, respectively, significantly lower for the transformed line.

We take now the data at long times of uptake. The zero-time intercepts,

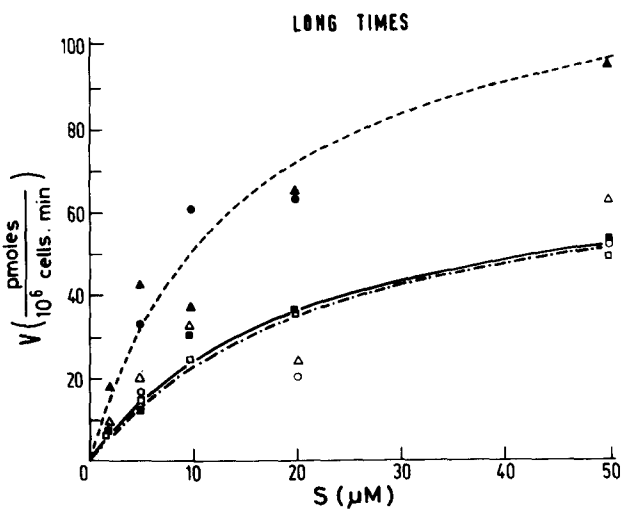

Fig. 5. Velocity of uptake of uridine, measured at long times, by normal and transformed Nil 8 cells with and without prior treatment with 10% serum, after serum starvation. ▲, Normal cells + serum, Exp. 1; △, normal cells — serum, Exp. 1; ■, transformed cells + serum; □, transformed cells — serum; ●, normal cells + serum, Exp. 2; ○, normal cells — serum, Exp. 2, - - - - -, Normal activated cells; · — · — · normal cells (quiescent); ———, transformed cells. Velocities were derived from slopes of linear regressions of data obtained at long periods of time (more than 57 min) but otherwise as in Fig. 4. The lines drawn are calculated from the derived Michaelis-Menten parameters.

TABLE V

Linear regression analysis of the data on uptake of uridine by Nil 8 cells (transformed and non-transformed) at short and long times of uptake, after addition or not of serum. Data taken from Figs. 5 and 6.

Cell type	Serum	Intercept (min)	1/slope (pmol/min per $1 \cdot 10^6$ cells)	K_m (μM)	V (pmol/min per $1 \cdot 10^6$ cells)
Short times					
Non-transformed	—	0.189 ± 0.046	2.50 ± 1.01 *		
Non-transformed	+	0.116 ± 0.005	1.90 ± 0.16 *		
Non-transformed	combined	0.143 ± 0.032	2.77 ± 0.92 *	516 ± 199	3600 ± 1170
Transformed	—	0.300 ± 0.020	6.41 ± 0.70 *		
Transformed	+	0.230 ± 0.050	6.78 ± 1.28 *		
Transformed	combined	0.260 ± 0.030	6.59 ± 0.74 *	400 ± 64	1500 ± 170
Long times					
Non-transformed	—	0.300 ± 0.120	1.37 ± 0.43 **	21.8 ± 10.7	73.0 ± 23
Non-transformed	+	0.120 ± 0.020	0.80 ± 0.08 **	14.9 ± 3.1	125 ± 13
Transformed	—	0.300 ± 0.037	1.41 ± 0.15 **		
Transformed	+	0.270 ± 0.035	1.34 ± 0.14 **		
Transformed	combined	0.280 ± 0.025	1.38 ± 0.25 **	20.4 ± 2.3	72.5 ± 5.3

* $\times 10^4$.
** $\times 10^2$.

collected in Table IV, contain too much error to be interpretable. But we have already argued that the short times represent transport. Hence the long times may well represent a subsequent step of trapping. Kinetic parameters derived from plots of S/v against S using the slopes obtained at long times are collected in Table V (bottom five rows). For these plots, the intercepts at zero-substrate concentration do not differ much from the similar parameters derived at short times, but the slopes of the lines of S/v against S differ by at least an order of magnitude as between the early and late time data. The processes at short and long times clearly have different kinetic behaviours and thus represent a tandem situation. The data for normal cells at long times differ significantly in intercept and also in slope when cells with and without added serum are compared. The K_m and V parameters have therefore been separately calculated for these two cases. The data reveal that the maximum velocity of trapping is significantly greater for cells stimulated by serum as compared with the controls, but the K_m values do not differ significantly. The data for transformed cells show no difference between cells in the presence or absence of serum. The latter data were therefore combined for the calculation of K_m and V. The parameters obtained for the transformed cells in the presence or absence of serum are indistinguishable from those for normal cells in the absence of serum. We note that 'K_m' here is in terms of the substrate concentration at the outer face of the membrane, although it is the concentration within the cell that is relevant for an intracellular trapping system.

If the data at long times indeed represent the trapping of uridine by an intracellular process, it should be possible to confirm this by studies of uridine phosphorylation in cell extracts. With this in mind, we prepared extracts of intracellular material from Nil 8 cells, by freeze-thawing followed by sonication. We measured (as described in Materials and Methods) the rate of transformation of uridine to a form which could be bound to an ion-exchange paper

disc at pH 7.4, presumably a phosphorylated derivative of uridine. This rate was measured as a function of the uridine concentration added to the enzyme assay mixture (Fig. 6). The data, plotted in Fig. 6 in the form of S/v against S, yield for uridine a half-saturation concentration of 16.1 ± 2.7 μM in this cell-free preparation. This value is consistent with the parameter derived from the slopes of the uptake curves at long times, as measured in whole cells.

We proceed to consider how the intracellular K_m of trapping can be derived from the uptake data. We use, first of all, Method 1 of the Theory section. Consider Eqn. 4. Since we already know the value of the transport parameter K_{12}^{zt}, we are sure that at the half-saturation concentrations for the trapping process, S_1 can be neglected in comparison with K_{12}^{zt}. We do not know K_{21}^{ic}, but since this refers to the same side of the membrane and the same transport site as K_{12}^{zt}, we are almost certainly justified in neglecting S_1 in comparison also with this term. Now, when the trapping rate is half-maximal, the value of v is half of the V for trapping. We can now substitute in Eqn. 4 and calculate S_2 at the value of S_1 when the trapping rate is half-maximal. (We need also to know the maximum velocity of efflux V_{12}^{zt}. We will assume that the transport system is approximately symmetric on the basis of the analog with the system that transports cytosine-β-D-arabinoside. In any event, v is so low in comparison with the transport maximum velocity for entry that, unless the system is very highly asymmetric in the direction favouring uridine entry, the term v/V_{21}^{zt} will be only a second order correction.) We substitute into Eqn. 4, using the data for v and S_1 and V_{12}^{zt} collected in Table IV. We thus obtain for S_2 (the half-

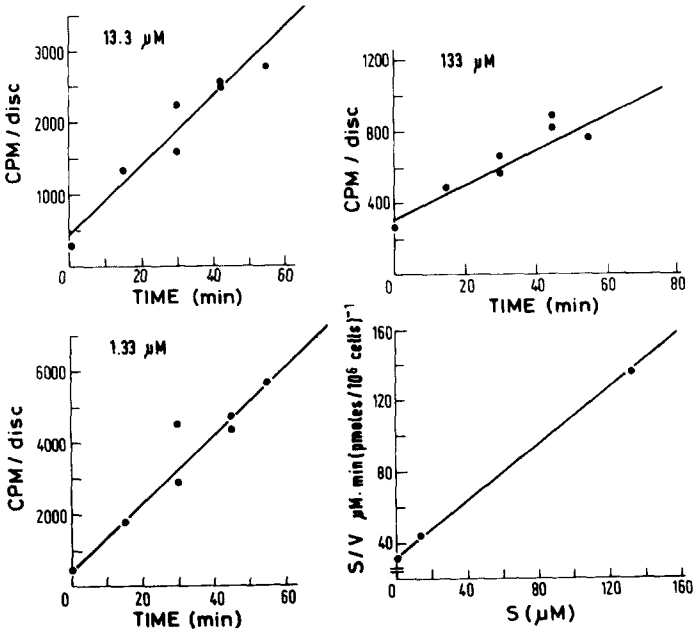

Fig. 6. Phosphorylation of uridine by fragmented preparations of Nil 8 HSV cells at 37°C, 7 mM ATP. Counts trapped on ion-exchange paper are plotted on the ordinate as a function of incubation time on the abscissa, at three uridine concentrations. The fourth panel is the plot of the reciprocal of the slope of these lines, (proportional to S/v) against the substrate concentration, S. All lines are linear regressions.

saturation concentration for the trapping of uridine with respect to the intracellular uridine levels) 15.8 ± 11.0 μM, 4.9 ± 5.0 μM, and 10.6 ± 3.0 μM for, respectively, non-transformed cells in the absence of serum, non-transformed cells in the presence of serum, and transformed cells (with and without serum). A value of some 10 μM for the half-saturation concentration with reference to the intracellular uridine level seems to be indicated. The intracellular uridine level is, on the basis of the recorded parameters for transport and trapping, some 10 μM below the extracellular level when the external is about 20 μM.

To use Method 2, we apply Eqn. 8 of the Theory Section. This is an exact method of obtaining the half-saturation concentration of trapping, Φ, but depend on the difference between two numbers often of similar size (the y-cepts of the linear plots), and thus depends very much on the success of the data-fitting procedures. If we substitute in Eqn. 8 using the data of Table IV, taking into account the error in the values of the intercepts, we obtain for Φ (the half-saturation concentration for the trapping of uridine with respect to the intracellular uridine level) values of 8.1 ± 8.7 μM, −1.8 ± 7.9 μM, and 2.6 ± 2.9 μM, respectively, for non-transformed cells in the absence of serum, for non-transformed cells in the presence of serum and for transformed cells. The data show a disappointing degree of error but are consistent with those obtained by Method 1, suggesting that the value of Φ is of the order of 10 μM but may be less.

We note that the values obtained for the trapping step, for the half-saturation concentration for uridine are perfectly consistent with the value of 16.1 ± 2.7 μM found for conversion of uridine to a highly charged form in extracts from Nil 8 cells (Fig. 6). The trapping of uridine as measured by uptake of uridine into intact cells at long times does not have very different kinetic characteristics from that found for cell extracts, especially if it is so considered that the data on cell extracts were obtained at 37°C, rather than at 20°C for the intact cells, and in the presence of high concentrations of ATP (7 mM).

The kinetic methods applied here thus present a clear picture of uridine uptake's being a result of the tandem action of a transport of high capacity (V) and low affinity (high K_m), followed by a trapping step of low capacity and high affinity. At very low uridine concentrations the two processes have very similar rates (given by the reciprocal of the intercepts on the y-axis, of the data collected in Table IV), so that much of the uridine that enters is trapped immediately. The trapping system soon saturates as the external uridine concentration is raised so that uridine levels of about 1 mM, most of the uridine that enters is free for long periods of time. These conclusions account for the similarities and differences between the uptake curves for cytosine-β-D-arabinoside and uridine (Figs. 1a and b). At low substrate concentrations, the uridine curve reaches an asymptotic slope rapidly, and soon far surpasses the cytosine-β-D-arabinoside curve. As the substrate levels are raised externally, the slopes of the curves for the two substrates begin to approach one another, as trapping of the uridine becomes relatively insignificant. (It is always so for cytosine-β-D-arabinoside.) By 1000 μM, the curves for cytosine-β-D-arabinoside and uridine are practically superimposable for some 10 min.

Finally we note that it is the trapping of uridine and not the transport step that is stimulated by serum in non-transformed cells. The transformed cells

show no stimulation by serum of either trapping or transport and the uptake is indeed lower in the transformed line. In these Nil 8 cells, the loss of growth control that is associated with the transformation by virus is accompanied by the loss of control by serum of uridine uptake.

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