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TRANSPORT OF ANTIVIRAL AGENT 9-(S)-(2,3-DIHYDROXYPROPYL) ADENINE TO ANIMAL CELLS

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Summary. — Transport properties of 9-(S)-(2,3-dihydroxypropyl) adenine (DHPA) in cell cultures were studied. Transport of DHPA into chick embryo (CE), ZP (a cell line derived from rabbit lungs) and HeLa cells reached equilibrium values after 10 min incubation. The concentration of intracellular DHPA varied from 30 to 50 per cent of that in the medium. DHPA transport was only slightly affected during the lag phase of vaccinia virus replication. The opinion that DHPA is transported into the cell by facilitated diffusion, is supported 1) by the data on DHPA transport as a function of temperature and extracellular concentration, 2) by evidence of countertransport, 3) by temperature-dependent exit of DHPA, 4) by specific inhibition of DHPA transport in the presence of adenosine and deoxyadenosine and 5) by the fact that intracellular concentration of DHPA in equilibrium does not reach the concentration of DHPA in the medium. V and K_M values varied in the range of 2—17 pmoles/min per 10^6 cells and 4—7 μM, respectively.

Key words: *9-(S)-(2,3-dihydroxypropyl) adenine*; *animal cell transport*; *intracellular concentration*; *antimetabolite*

Introduction

9-(S)-(2,3-dihydroxypropyl) adenine (DHPA) inhibits replication of several RNA and DNA viruses (De Clercq et al., 1978; Rada and Holý, 1980). DHPA is an adenosine analogue, in which the ribose moiety is replaced by an aliphatic chain, resembling to the lower part of ribose (C_1', C_2' and C_3'). It was observed that DHPA is not transported into procaryotic cells and its level inside the eucaryotic cells is rather low. The analysis of cellular pools showed that DHPA is metabolized neither in uninfected nor in virus infected cells (Čihák and Holý, 1978; Rada *et al.*, 1980; Holý and Čihák, 1981).

The aim of the present work was to characterize the time course of DHPA transport into the cell, to determine its actual concentration in the cell and to

investigate the mode of the DHPA transport. In addition, experiments were performed to search for possible changes in the transport of the analogue after virus infection.

Materials and Methods

Chemicals. 8-[^{3}H]-9-(S)-(2,3-dihydroxypropyl) adenine ([^{3}H]-DHPA), specific activity 18.5 GBq/nmol was prepared by Dr. J. Černý, Isotope Laboratories of Czechoslovak Academy of Sciences, Prague. [^{14}C]-Adenosine (14.4 GBq/nmol) and [^{14}C]-deoxyadenosine (13.3 GBq/nmol) (both universally labelled) were purchased from the Institute for Research, Production and Application of Radioisotopes, Prague. Adenosine was purchased from Koch-Light, Ltd. (England), guanosine, uridine, 2'-deoxyuridine, 2'-deoxyadenosine and 2'-deoxyguanosine from Calbiochem (U.S.A.), cytidine from Reanal (Hungary) and thymidine from Lachema (Czechoslovakia).

Cells. All experiments were performed in suspension cultures. Cells in concentration of 5×10^7/ml were infected with vaccinia virus at multiplicity 7—10 PFU/cell. After adsorption at 37 °C for 1 hr, the cells were washed twice with medium to remove unadsorbed virus and resuspended at the indicated density (Salzman *et al.*, 1963).

Chick embryo (CE) cells were used in the second passage, the primary one was performed in Roux bottles. After trypsinization, the cells were diluted in medium 199 containing 10% calf serum at a density $7—11 \times 10^6$ cells/ml.

HeLa cells were cultured in spinner modified minimal Eagle's medium (Difco Labs. U.S.A.) containing 5% calf serum and 2% glutamine; for the experiments, cells were concentrated to $4—6 \times 10^6$ cells/ml.

ZP cells [a cell line derived from rabbit lungs by Szántó (1960)] were grown in Roux bottles containing Earle's medium supplemented with 0.1% TC-yeastolate, 0.45% glucose and 10% calf serum. Since the volume of these cells was larger than that of HeLa cells, in suspension they were concentrated to $2—5 \times 10^6$ cells/ml.

Virus. Vaccinia virus, strain WR was obtained from Dr. N. P. Salzman, National Institute of Allergy and Infectious Diseases, Bethesda, U.S.A. Stock virus prepared in monolayers of HeLa cells titered 2×10^9 PFU; it was stored at —60 °C.

Transport determination. Cell suspension was supplemented with [^{3}H]-DHPA as indicated in appropriate experiments. At various intervals, duplicate or triplicate 1 ml samples of suspension were centrifuged at 4 °C and washed twice with cold phosphate buffered saline (PBS). Then the cells were suspended in 0.4 ml of 5% (w/v) trichloroacetic acid and both supernatants were combined. The radioactivity was determined in these combined supernatants (acid-soluble material) and in sediment (acid-insoluble material). Total radioactivity was determined as a sum of both fractions, or directly from an aliquot of washed cells degraded in trichloroacetic acid. Values of transported DHPA were expressed in pmoles per 10^6 cells. The counting efficiency was 63%.

Removal of DHPA. The high radioactivity bound to the cell after short-time incubation (see Fig. 1-II) was suggestive for adsorption of DHPA to cell surface. HeLa cells labelled with [^{3}H]-DHPA were washed several times with ice-cold PBS to remove DHPA from the cell surface Radioactivity was determined in washed cells (Fig. 1-I). A considerable drop of cell-bound radioactivity occurred after the first wash (from 70 to 25 pmoles per 10^6 cells); this was followed by a smaller decrease after the second wash (to 15 pmoles per 10^6 cells), while after the 3rd—5th washes the radioactivity had decreased very slowly (to 12 pmoles per 10^6 cells). In the cultures of CE and ZP cells the effect of washing was similar. The observed loss of radioactivity after the washes was corrected for decrease of the cell number which had always occured during washing. It is noteworthy that after first wash, values were obtained which were close to the equilibrium between extra- and intracellular DHPA concentration. On the basis of these results, in all following experiments the cells were washed twice after DHPA application.

Determination of cell volume. Cell suspensions ($200—500 \times 10^6$ cells) rinsed twice with cold saline were centrifuged at low speed. The pellet was resuspended in a precise volume of cold saline were the cell suspension was measured as whole. The cell volume was determined from the difference of both volumes based on the fact that extracellular space in the cell pellet does not exceed 15% of the pellet volume (Plagemann *et al.*, 1978).

Results

Time course of the DHPA transport

Suspension cultures of CE, ZP or HeLa cells were incubated in the presence of [^{3}H]-DHPA (185 kBq/ml). Radioactivity was determined in 1 ml samples at various time intervals. DHPA transport had a saturable pattern, maximal radioactivity was observed approximately after 10 min incubation (Fig. 1-II). About 30—40% of maximal radioactivity had been cell-bound already within 1 min incubation; at the extended incubation time, the radioactivity inside cells remained constant or slightly decreased. The maximum values of DHPA transport (about 40 pmoles per 10^6 cells) were found in ZP cells; in HeLa cells the values varied within a range of 15—25 pmoles per 10^6 cells, while the transport efficiency in CE cells was by 3-10-fold lower than in ZP cells (4—15 pmoles per 10^6 cells). These values are very low in comparison with those observed for natural nucleosides, none the less, the intracellular levels of DHPA are similar to those found for 6-azauridine (Dragúň and Rada, unpublished data).

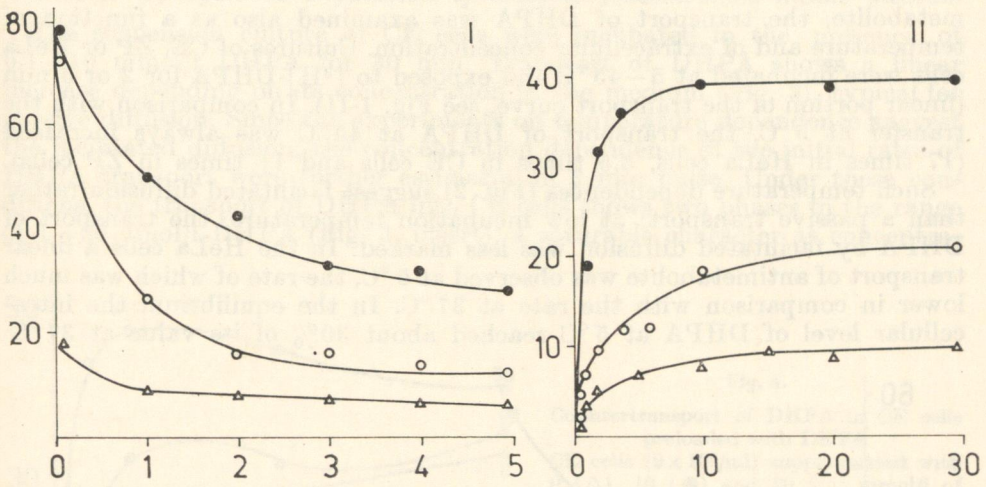

Fig. 1.

Effect of DHPA removal from surface of CE, ZP and HeLa cells and the time course of DHPA transport

I — 1 ml samples were supplemented with [^{3}H]-DHPA (185 kBq/ml) and incubated for 30 min at 37 °C; △ = CE cells (11×10^6/ml); ○ = HeLa cells (4×10^6/ml) and ● = ZP cells (5×10^6/ml).
Abscissa: wash number; ordinate: pmoles of DHPA per 10^6 cells.

II — Cell suspensions were supplemented with [^{3}H]-DHPA (185 kBq/ml) and incubated at 37 °C. At indicated intervals 1 ml samples were twice washed and analysed for radioactivity; △ = CE cells (10^7/ml), ○ = HeLa cells (6×10^6/ml) and ● = ZP cells (4×10^6/ml).
Abscissa: incubation time (min); ordinate: pmoles of DHPA per 10^6 cells.

Table 1. Characteristics of DHPA transport into CE, ZP and HeLa cells

Cells	V* (pmoles/min per 10^6 cells)	K_M* (μM)	Cell volume** (μl/10^6 cells)	$\frac{DHPA_i}{DHPA_e}$***
CE	2.4	5.8	1.5—3.0	0.3
ZP	16.7	7.1	10—15	0.5
HeLa	3.4	4.0	3.5—5.0	0.4

* Mean values from at least 3 experiments; ** Extreme values from more than 5 determinations.*** The ratio of intracellular and extracellular DHPA equilibrium concentration (intracellular concentration after two washes was calculated from specific cell radioactivity related to the cell volume; extracellular concentration of DHPA is represented by that added to cells)

Intracellular concentrations of DHPA were determined from the maximum transport values taking into account the volume of respective cells. Table 1 shows that in CE, ZP and HeLa cells, the intracellular level of DHPA reached 30—50% values of its concentration in the medium.

Effect of incubation temperature

To distinguish between passive and facilitated diffusion of the antimetabolite, the transport of DHPA was examined also as a function of temperature and of extracellular concentration. Cultures of CE, ZP or HeLa cells were incubated at 5—45 °C and exposed to [^{3}H]-DHPA for 2 or 3 min (linear portion of the transport curve, see Fig. 1-II). In comparison with the transfer at 5 °C, the transport of DHPA at 45 °C was always increased (17 times in HeLa cells, 5.5 times in CE cells and 11 times in ZP cells).

Such temperature dependences (Fig. 2) suggest facilitated diffusion rather than a passive transport; at low incubation temperature, the transport of DHPA by facilitated diffusion was less marked. In the HeLa cells a linear transport of antimetabolite was observed at 5 °C, the rate of which was much lower in comparison with the rate at 37 °C. In the equilibrium the intracellular level of DHPA at 5 °C reached about 30% of its value at 37 °C.

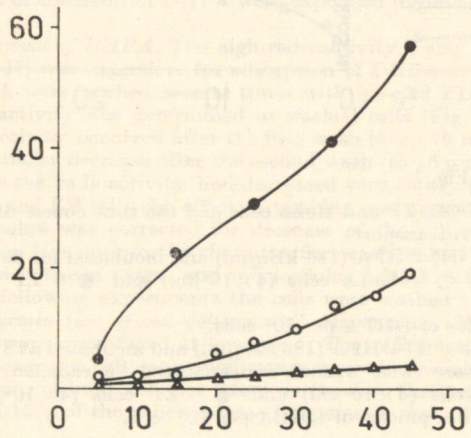

Fig. 2.

Effect of temperature on the transport of DHPA in CE, ZP and HeLa cells Suspension cultures of CE (10^7/ml, △), ZP (3×10^6/ml, ●) and HeLa cells (7×10^6/ml, ○) were equilibrated at indicated temperatures and then supplemented with 185 kBq of [^{3}H]-DHPA/ml. After 2 min (CE and HeLa cells) or 3 min (ZP cells), respectively, 1 ml samples were analysed for radioactivity. Abscissa: temperature (°C); ordinate: pmoles/10^6 cells.

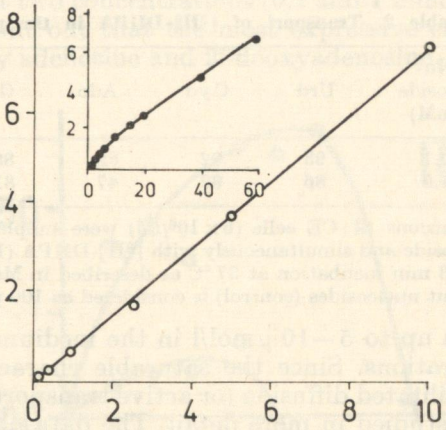

Fig. 3.

Effect of DHPA concentration on its transport to CE cells

Suspensions of CE cells (10^7/ml) were supplemented with 185 kBq of [^{3}H]--DHPA/ml and unlabelled DHPA to indicated concentrations. After 30 min incubation (in insert 3 min) at 37 °C, duplicates of 1 ml samples were analysed for total cell radioactivity.

Abscissa: concentration of DHPA in mmol/l (insert in μmol/l; ordinates: nmoles.min^{-1} per 10^6 cells (insert in pmoles . min^{-1} per 10^6 cells).

After extended incubation (more than 3 hr) at 37 °C, the cell-bound radioactivity decreased (not shown).

DHPA transport as a function of DHPA concentration in the medium

The suspension culture of CE cells were incubated in the presence of 0.1—10 mmol/l DHPA for 30 min. Transport of DHPA shows a linear increase depending on its concentration in the medium (Fig. 3), typical for passive diffusion. Since the experiments on temperature dependence suggest the facilitated diffusion, the concentration dependence of the initial rates of DHPA transport were further estimated in 3 min pulse. Under these conditions, the transport of DHPA into CE cells shows two phases in the range of 1—60 μmol/l DHPA (Fig. 3, insert): a saturable character at concentra-

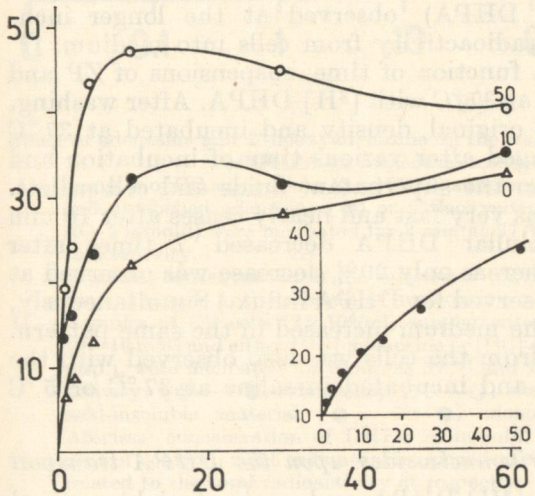

Fig. 4.

Countertransport of DHPA in CE cells preloaded with DHPA

CE cells (9×10^6/ml) supplemented with 0 (△), 10 (●) and 50 (○) mmol/l of unlabelled DHPA were incubated at 37 °C for 1 hr.

When resuspended at the same density in fresh medium (37 °C) containing 100 μmol/l [^{3}H]-DHPA (28 counts.min^{-1}.pmol^{-1}) the suspensions were incubated at 37 °C and analysed for total radioactivity. Insert: countertransport of DHPA at 37 °C by 2 min.

Abscissa: time (min); abscissa of the insert: concentration of DHPA in mmol/l; ordinates: counts × 10^3.min^{-1}.ml^{-1}.

Table 2. Transport of [^{3}H]-DHPA in the presence of 2′-deoxy- and ribonucleosides

Nucleoside (mM)	Urd	Cyd	Ado	Guo	dTrd	dCyd	dAdo	dGuo
0.1	93	92	62	89	96	101	75	79
1.0	86	99	47	81	81	87	47	89

Suspensions of CE cells (6×10^6/ml) were supplemented with either 0.1 mmol/l or 1 mmol/l nucleoside and simultaneously with [^{3}H]-DHPA (185 kBq/ml). Cell radioactivity was determined after 3 min incubation at 37 °C as described in Materials and Methods. Radioactivity in culture without nucleosides (control) is considered as 100 per cent.

tions up to 5—10 μmol/l in the medium and a linear process at higher concentrations. Since the saturable character of the transport again suggests a facilitated diffusion (or active transport), this concentration range of DHPA was studied in more detail. The data obtained with CE, ZP and HeLa cells are presented in Table 1. It is remarkable that maximal value of DHPA transport (V) was proportional to the volume of the cells studied.

Countertransport and efflux of DHPA

The transient intracellular accumulation of a radioactive substrate probably resulted from a rapid isotope dilution of the transported labelled substrate by the unlabelled substrate present inside of cells (Plagemenn and Richey, 1974).

Fig. 4 illustrates that preloading of cells (60 min) with unlabelled DHPA (10 and 50 mmol/l) results in an accelerated transport of [^{3}H]-DHPA (0.1 mmol/l) and its accumulation up to 3.5 times against a concentration gradient (Fig. 4, insert). The rate of countertransport increased with the concentration of DHPA used for preloading of the cells and was several times higher than that for the low DHPA concentrations. The decrease of intracellular radioactivity (50 mmol/l DHPA) observed at the longer incubation time reflects an efflux of radioactivity from cells into medium.

To study the DHPA efflux as a function of time, suspensions of ZP and HeLa cells were treated for 60 min at 37 °C with [^{3}H]-DHPA. After washing, the cells were resuspended to the original density and incubated at 37 °C or 5 °C. The samples were centrifuged after various time of incubation and the radioactivity estimated both in the supernatant fluids and cell pellets. The efflux of radioactive DHPA was very fast and nearly ceases after 10 min incubation. The level of intracelullar DHPA decreased 7 times after 30—60 min incubation at 37 °C, whereas only 20% decrease was observed at 5 °C. (The same time course was observed for DHPA influx.) Simultaneously, the extracellular radioactivity in the medium increased in the same pattern. A similar course of DHPA efflux from the cells was also observed with the cells pretreated with [^{3}H]-DHPA and incubated in saline at 37 °C or 5 °C (not shown).

The effect of 2′-deoxyribo- and ribonucleosides upon the DHPA transport

The simultaneous transport of [^{3}H]-DHPA and each of eight natural

nucleosides into CE cells was tested at two concentrations (0.1 and 1 mmol/l). The data summarized in Table 2 point out that the most expressive effect upon DHPA transport is exhibited by adenosine and 2'-deoxyadenosine. The

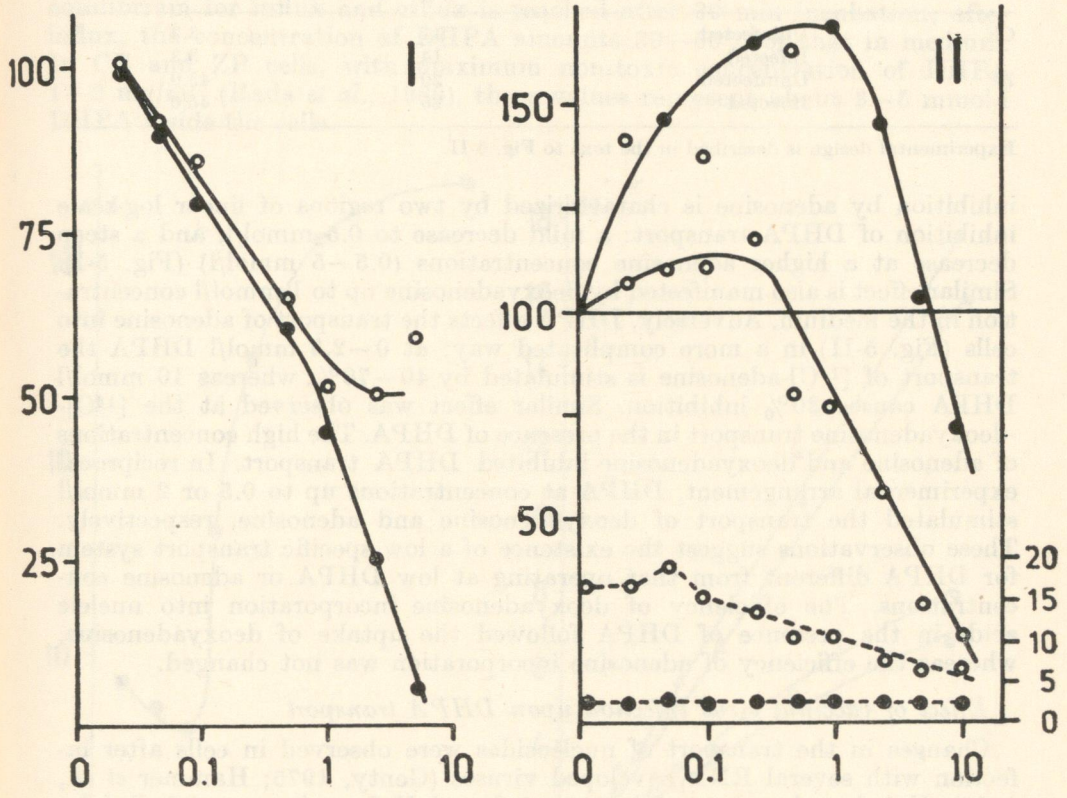

Fig. 5.

Effect of adenosine and 2'-deoxyadenosine on the transport of ^{3}H-DHPA into CE cells and that of DHPA on nucleoside transport and incorporation

I — Samples of CE suspensions (7×10^{6}/ml) supplemented with with 185 kBq of [^{3}H]-DHPA/ml and unlabelled adenosine (●) or 2'-deoxyadenosine (○) to the indicated concentrations (0—5 mmol/l) were incubated for 3 min at 37 °C and 1 ml samples were analysed for total radioactivity.
Abscissa: concentration of the nucleoside (in mmol/l); ordinate: DHPA in the cell (% of control sample in the absence of nucleoside).

II — Samples of CE cells (7×10^{6}/ml) supplemented with indicated concentrations of DHPA (0—10 mM) and either [^{14}C]-adenosine or [^{14}C]-deoxyadenosine (7.4 kBq/ml and3.7 kBq/ml, resp.), were incubated for 5 min at 37 °C and 1 ml samples were analysed for total radioactivity: (●——●, adenosine, ○——○, deoxyadenosine) or for radioactivity of the acid-insoluble material (●— — —●, adenosine, ○— — —○, deoxyadenosine).
Abscissa: concentration of DHPA in mmol/l; left ordinate: nucleoside transported (per cent of control); right ordinate: incorporation of the nucleoside into acid-insoluble material related to the total radioactivity at respective DHPA concentrations (%).

Table 3. Kinetic parameters of DHPA transport into uninfected and vaccinia virus-infected cells

Cells	Infection	K_M (μM)	V (pmoles/min per 10^6 cells)
CE	Uninfected	11	3.3
	Infected	14	3.3
ZP	Uninfected	20	45.0
	Infected	25	45.0

Experimental design is described in the text to Fig. 6-II.

inhibition by adenosine is characterized by two regions of linear log-scale inhibition of DHPA transport: a mild decrease to 0.5 mmol/l, and a steep decrease at a higher adenosine concentrations (0.5—5 mmol/l) (Fig. 5-I). Similar effect is also manifested by deoxyadenosine up to 1 mmol/l concentration in the medium. Adversely, DHPA affects the transport of adenosine into cells (Fig. 5-II) in a more complicated way: at 0—2.5 mmol/l DHPA the transport of [^{14}C]-adenosine is stimulated by 40—70%, whereas 10 mmol/l DHPA causes 30% inhibition. Similar effect was observed at the [^{14}C]--deoxyadenosine transport in the presence of DHPA. The high concentrations of adenosine and deoxyadenosine inhibited DHPA transport. In reciprocal experimental arrangement, DHPA at concentrations up to 0.5 or 2 mmol/l stimulated the transport of deoxyadenosine and adenosine, respectively. These observations suggest the existence of a low specific transport system for DHPA different from that operating at low DHPA or adenosine concentrations. The efficiency of deoxyadenosine incorporation into nucleic acids in the presence of DHPA followed the uptake of deoxyadenosine, whereas the efficiency of adenosine incorporation was not changed.

Effect of vaccinia virus infection upon DHPA transport

Changes in the transport of nucleosides were observed in cells after infection with several RNA enveloped viruses (Genty, 1975; Hammer *et al.*, 1976). Uninfected and vaccinia virus infected HeLa cells were labelled by [^{3}H]-DHPA for 5 min in 30 min intervals during the latent period of the virus replication, i.e. 2—6 hr after infection. In this period a limited if any inhibition of the drug transport was observed. Infected and uninfected ZP or CE cells exposed to [^{3}H]-DHPA for 3 min in the middle of latent period (Fig. 6-I) did not show any essential changes in the transport rate of DHPA either. The total DHPA transport into infected cells was only moderately inhibited (10—25%) (Fig. 6-II). The kinetic parameters of the transport are summarized in Table 3.

Discussion

The temperature dependence of DHPA transport into eucaryotic cells in suspension suggests a mechanism of facilitated diffusion (Q_{10} is close to 2 in the range of 25—45 °C). The transport is linear only during 2—3 min at 37 °C in comparison with 30—60 min at 5 °C; the maximum level of DHPA

at 5 °C is about 30% of that at 37 °C. Despite of the fact that no substantial metabolic conversion of DHPA occurs (Rada et al., 1980), the drug accumulates inside the cell and its concentration gradient from the medium to the cell rapidly decreases. Independently on the DHPA concentration used, equiiibrium for influx and efflux is reached after 30 min incubation; after influx, the concentration of DHPA amounts 30—50% of that in medium. In CE and ZP cells, with maximum non-toxic concentration of DHPA 1—2 mg/ml (Rada *et al.*, 1980), these values represent about 2—5 mmol/l DHPA inside the cells.

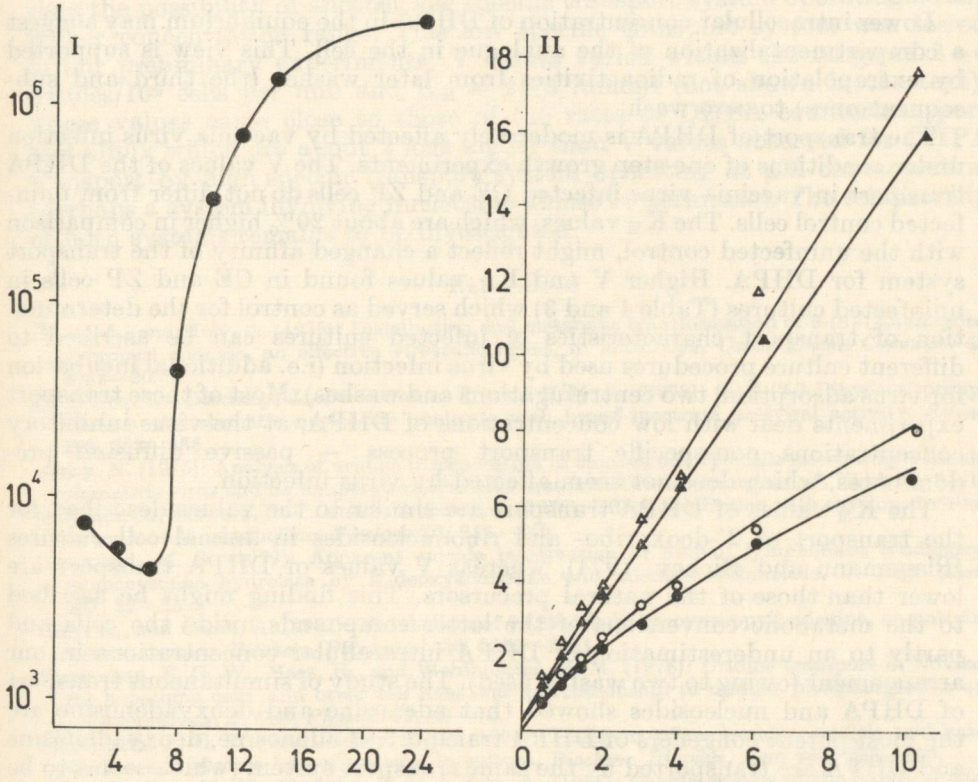

Fig. 6.
Multiplication of vaccinia virus and DHPA transport to infected cells

I — Virus replication. Multiplicity of the virus added was 7 PFU/cell; adsorption period 60 min; unadsorbed virus was removed by repeated washing.
Abscissa: hr; ordinate: PFU/ml

II — Transport of DHPA into vaccinia virus infected CE and ZP cells. Infected and uninfected cultures were exposed to [^{3}H]-DHPA (0.5—10 μmol/l, i.e. 310 counts . min^{-1} . pmol^{-1}) for 3 min at 37 °C in the middle of lag period (3.15 hr after infection of ZP cells or 2.30 hr after infection of CE cells), then 1 ml samples were analysed for radioactivity; (△) uninfected and (▲) infected ZP cells (2×10^6/ml); (○) uninfected and (●) infected CE cells (5×10^6/ml). Abscissa: concentration of DHPA in μmol/l; ordinate: counts × 10^3 . min^{-1} . ml^{-1}.

The first two washes of labelled cultures resulted in marked removal of the radioactivity from cells. The extracellular space represents about 10—15% of the cell pellet volume (Plagemann *et al.*, 1978) and the exit of DHPA from the cells at low temperature (5 °C) does not exceed 10—15% of cell-bound radioactivity during one wash (60 min incubation results only in a 20% decrease of intracellular radioactivity). These calculations lead to the conclusion that about 40—60% of total cell-bound radioactivity is removed from cell surface after the first wash. The decrease of cell-bound radioactivity during the second and subsequent washes can be ascribed mainly to the net efflux.

Lower intracellular concentration of DHPA in the equilibrium may suggest a compartmentalization of the analogue in the cell. This view is supported by extrapolation of radioactivities from later washes (the third and subsequent ones) to zero wash.

The transport of DHPA is moderately affected by vaccinia virus infection under conditions of one-step growth experiments. The V values of the DHPA transport in vaccinia virus infected CE and ZP cells do not differ from uninfected control cells. The K_M values, which are about 20% higher in comparison with the uninfected control, might reflect a changed affinity of the transport system for DHPA. Higher V and K_M values found in CE and ZP cells in uninfected cultures (Table 1 and 3) which served as control for the determination of transport characteristics of infected cultures can be ascribed to different culture procedures used by virus infection (i.e. additional incubation for virus adsorption, two centrufugations and washes). Most of these transport experiments deal with low concentrations of DHPA, at the virus inhibitory concentrations non-specific transport process — passive diffusion predominates, which does not seem affected by virus infection.

The K_M values of DHPA transport are similar to the values described for the transport of 2'-deoxyribo- and ribonucleosides in animal cell cultures (Plagemann and Richey, 1974), whereas V values of DHPA transport are lower than those of the natural precursors. This finding might be ascribed to the metabolic conversions of the latter compounds inside the cells and partly to an underestimation of DHPA intracellular concentrations in our arrangement (owing to two washes used). The study of simultaneous transport of DHPA and nucleosides showed that adenosine and deoxyadenosine are the most potent congeners of DHPA transport. If adenosine, deoxyadenosine and DHPA are transported by the same transport system (which seems to be probable), reciprocal effects can be expected at the cell membrane. Indeed, DHPA at lower concentrations stimulated the transport of adenosine and deoxyadenosine and inhibited the process at higher concentrations. Votruba and Holý (1980) studied inhibition of S-adenosyl-L-homocysteine hydrolase purified from rat liver. They have found marked inhibition of hydrolysis with $K_i = 3.5 \times 10^{-6}$ mol/l what is the value close to those found for DHPA transport in our experiments. As a consequence of the inhibition of hydrolytic reaction is the decrease of the level of adenosine inside the cell. This can be related to the stimulation of adenosine transport by DHPA. This is in accordance with the finding of Hershfield (1979) that 2'-deoxyadenosine and

adenine arabinoside are potent inactivators of S-adenosyl-L-homocysteine hydrolase, which they tightly bind to. It is attractive to hypothesize that DHPA would act similarly. Different DHPA effects upon the incorporation of adenosine and deoxyadenosine into nucleic acids may be connected with influencing extra- and intracellular gradients.

However, it is surprising that the coupled effects of adenosine, deoxyadenosine and DHPA functioning at the cell membrane could be observed at much higher concentrations (mM) than expected from their transport characteristics ($K_M \sim 10$ μmol/l) (Plagemann and Richey, 1974). This finding rises the possibility of another low specific transport system operating at the higher concentration range. This low specific transport system was tested in our preliminary experiments; V values varied within the range of 2—4 nmoles/10^6 cells per min and $K_M = 2-3$ mmol/l (not shown in the text). These values came close to those of the rates of DHPA countertransport and efflux which are about 2 logs higher than V values observed for DHPA transport by high specific transport system operating at μM concentration (perhaps a system involving nucleoside kinase or permease). The relationship of both systems remains to be investigated.

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