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UPTAKE OF 5′-DEOXY-5′-*S*-ISOBUTYL THIOADENOSINE INTO CHICK EMBRYO FIBROBLASTS

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Abstract—The uptake of 5′-deoxy-5′-*S*-isobutyl thioadenosine (iBuS)$^{5'}$Ado into chick embryo fibroblasts is due to a mixed transport system: (1) a high affinity system with apparent K_m values of 0.022 mM and 0.055 mM for normal and Rous Sarcoma Virus transformed cells respectively; (2) a low affinity system with apparent K_m values of 0.800 mM and 0.720 mM for the normal and transformed cells. The drug enters the cells rapidly, and is found in the acid-soluble fraction. Only analogues of (iBuS)$^{5'}$Ado modified in the 5′-*S*-side chain are found to be inhibitors of the high affinity system. This indicates that an intact adenosine is required. 2′-Deoxyglucose and factors affecting the cell membrane reduce the (iBuS)$^{5'}$Ado uptake.

INTRODUCTION

5′-Deoxy-5′-*S*-isobutyl thioadenosine [(iBuS)$^{5'}$Ado]† has been reported to inhibit cell transformation induced by oncogenic RNA and DNA viruses [1–3], the growth of transformed mouse mammary cells [4] and the mitogen-stimulated blastogenesis of lymphocytes [5]. (iBuS)$^{5'}$Ado has also an antimalarial activity against *Plasmodium falciparum* in culture [6]. The mechanisms of its action may be associated with its inhibitory action of methylations [3, 7, 8] and polyamine biosynthesis [9].

As cellular uptake is a decisive factor in the biological and therapeutic effect, the present study is concerned with details of transport and intracellular binding of (iBuS)$^{5'}$Ado in chick embryo fibroblasts (CEF) normal or transformed with Rous Sarcoma Virus (RSV).

MATERIALS AND METHODS

Cells and viruses. Secondary cultures from 11 day old lymphomatosis free Brown Leghorn chick embryos were prepared and cultivated as described earlier [10]. The virus used was a clonal isolate (SR_4) of Schmidt-Ruppin strain of Rous Sarcoma Virus type D. Infection was carried out one day after seeding with adequate amounts of virus to obtain 80 per cent transformation in 4–5 days at 37°. Cells were cultivated in Eagle medium supplemented with glutamine, antibiotics and 5 per cent calf serum.

Chemicals. All chemicals used were of the highest purity available and came from the following sources: (iBuS)′5′Ado (Sefochem Fine Chemicals, Emek Hayarden, Israël); 5′deoxy-5′-*S*- [$^{14}C_1$]-isobutyl adenosine 9 mCi/mM: Commissariat à l'Energie Atomique (Saclay, France). All compounds listed in Table 3 were synthesized in our laboratory [11–14] by known methods [15–17] except L-SAH and adenosine which were from Sigma Chemical Co. (St. Louis, MO). (iBuS)$^{5'}$ 3-deaza-Ado [18] was a gift of Dr. P. K. Chiang (NIH, Bethesda, MD), 9-(S)-2,3-dihydroxypropyl adenine (S-DHPA) [19] was a gift of Dr. A. Holy (Academy of Sciences, Prague, Czechoslovakia) and sinefungin was from Dr. R. S. Gordee (Lilly Laboratories, Indianapolis, IN). Erythro-9-(2′-hydroxy-3 nonyl) adenine was a gift from Dr. T. W. North (Health Science Center, New York, NY) and 2′-deoxy coformycin was a gift from Dr. J. D. Douros (NCI, Bethesda, MD). 2′-Deoxyglucose and 3-*O*-methyl-D-glucose, insulin, ATP, putrescine, spermidine and spermine were purchased from Sigma Chemical Co. (St. Louis, MO).

(iBuS)$^{5'}$Ado uptake. All experiments were performed in duplicate using 3 cm dia. Petri dishes with 6–10 × 10^5 cells/dish. Various concentrations of labelled (iBuS)$^{5'}$Ado were added to the cultivation media (0.25–1.5 μCi/ml). After different exposure times the medium was discarded and cells washed twice with cold Phosphate Buffered Saline (PBS) and kept at −20° until use. Cells were then treated successively with 1 ml ice-cold TCA 5 per cent for 15 min, 1 ml TCA 10 per cent at 80–90° for 30 min and with 1 ml NaOH 0.5 N at 80–90° for 30 min. Each treatment was made twice. The supernatants from each treatment represent respectively the acid-soluble fraction, the acid-insoluble fraction containing nucleic acids and the protein fraction [20]. Radioactivity was counted in an aliquot of these fractions. Total uptake is the sum of the radioactivity found in the three fractions. Protein concentration was determined by the method of Lowry *et al.* [21] on the alkaline hydrolysate, using crystalline bovine serum albumin as standard.

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† Abbreviations: RSV, Rous Sarcoma Virus; PBS, Phosphate Buffered Saline; TCA, Trichloracetic acid; CEF, Chick embryo fibroblasts; SAM, *S*-Adenosyl Methionine; SAH, *S*-Adenosyl Homocysteine; (iBuS)$^{5'}$Ado, Designated as SIBA in previous papers in other Journals [1, 2, 7, 8].

Metabolism of (iBuS)$^{5'}$Ado. To evaluate the extent of the metabolism of the drug during the incubation the radioactivity was extracted from the cells, which have been incubated for 2 hr with 1 mM labelled (iBuS)$^{5'}$Ado, and washed twice with ice-cold PBS. To prevent any chemical hydrolysis of the drug, no acidification step was used. The cells were scraped off with a rubber policeman and immediately extracted with ethyl acetate methanol (9/1,v/v).

After centrifugation of the precipitated macromolecules the supernatant was analysed either by t.l.c. or by Sephadex G-10 chromatography as described elsewhere [22].

Interference of different compounds with the cellular uptake of (iBuS)$^{5'}$Ado. Cells were treated with various compounds for 15 min before the addition of labelled (iBuS)$^{5'}$Ado at 0.07 and 1 mM. After 10 min, the cells were treated as described above.

RESULTS AND DISCUSSION

Time course of the uptake of (iBuS)$^{5'}$Ado

Figure 1 shows the time course of uptake of radioactivity from (iBuS)$^{5'}$Ado into normal chick embryo fibroblasts, as a function of external concentrations of the drug. The uptake was rapid and a steady state was reached within 30–60 min. Similar results were obtained with infected and transformed fibroblasts as shown in Table 1: when (iBuS)$^{5'}$Ado concentration in the culture medium was varied from 0.025 to 0.100 mM no significant differences between normal, infected and transformed cells were noticeable. However, when higher concentrations were used (up to 1 mM) the amount of radioactivity taken up was lower in transformed cells than in normal cells, and intermediate values were observed in infected cells (Table 1).

An estimate of the drug concentration in the cytoplasmic side of the membrane may be obtained, assuming that the drug in the acid-soluble pool represents free drug, and that the volume of 10^6 cells is 1.3 μl and corresponds to 300 μg of protein for normal and transformed cells [23]. Results summarized in Table 2 show that after 5 hr treatment the intracellular concentration of (iBuS)$^{5'}$Ado was always higher than the concentration of the drug in the culture medium, for all concentrations studied. However the ratio C^i/C^e (intracellular to extracellular concentration) decreased with increasing extracellular concentration of the drug. The accumulation is rapid: after 5–10 min contact the intracellular concentration is higher than the external one and a plateau is reached after 30 min.

Kinetic parameters of (iBuS)$^{5'}$Ado uptake

The double reciprocal plots [24] of the initial rate of entry of (iBuS)$^{5'}$Ado into cell material as a function of drug concentration in the medium (Fig. 2) shows that above 0.1 mM (iBuS)$^{5'}$Ado the uptake rate deviated from the straight line in the direction of the origin, thus suggesting the existence of two apparently distinct transport systems. This phenomenon is observed in normal, infected and transformed cells. The apparent kinetic constants of (iBuS)$^{5'}$Ado

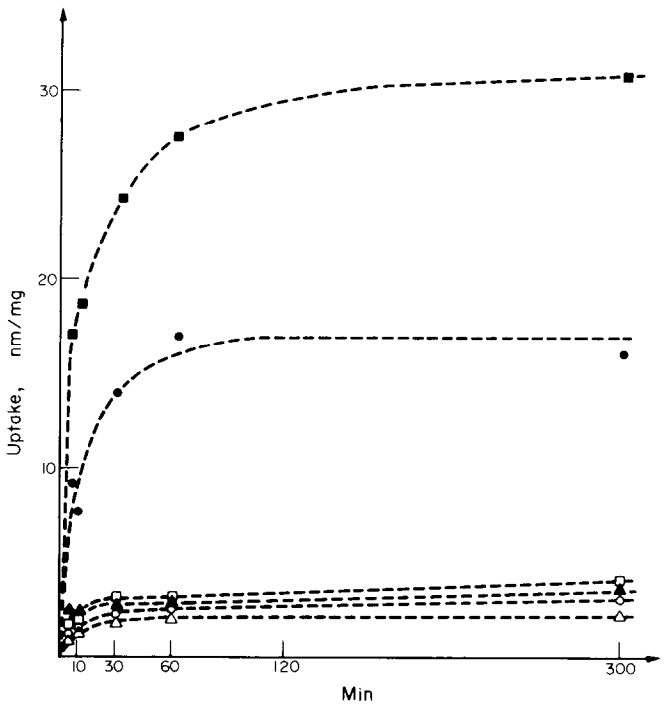

Fig. 1. Time course of the (iBuS)$^{5'}$Ado uptake into normal chick embryo fibroblasts. Cells were treated as described in Materials and Methods. The experiments were done at —△—△— 0.025 mM, —○—○— 0.050 mM, —▲—▲— 0.075 mM, —□—□— 0.100 mM, —●—●— 0.500 mM, —■—■— 1 mM. Results are expressed in nmoles of (iBuS)$^{5'}$Ado per mg of protein.

Table 1. Total uptake of (iBuS)[5′]Ado in normal, infected and transformed chick embryo fibroblasts*

(iBuS)[5′]Ado concentration in the medium (mM)	Time (min)	Normal cells	48 hr Infected cells	Transformed cells
0.025	5	0.750	0.882	0.811
	10	1.061	0.874	1.080
	30	1.715	1.647	1.663
	60	1.982	2.080	1.548
	300	2.168	2.555	1.552
0.050	5	1.277	1.292	1.306
	10	1.522	1.307	1.530
	30	2.109	2.289	2.395
	60	2.440	3.242	2.198
	300	3.292	3.044	2.149
0.075	5	1.383	1.800	1.183
	10	1.443	2.224	1.782
	30	2.948	2.370	2.514
	60	2.893	3.220	2.664
	300	4.132	4.316	2.472
0.100	5	2.166	2.190	1.744
	10	2.105	2.589	2.194
	30	2.715	2.943	2.388
	60	2.297	3.437	2.521
	300	3.741	5.394	3.200
0.500	5	9.188	6.577	4.104
	10	7.682	7.941	4.270
	30	14.013	11.709	5.368
	60	16.958	13.228	5.075
	300	16.219	12.299	5.482
	48 hr	21.611	11.979	7.197
1.000	5	17.150	13.104	18.042
	10	18.504	15.423	14.453
	30	24.525	18.525	20.015
	60	27.554	19.023	18.062
	300	31.033	19.925	18.437
	48 hr	37.288	36.920	26.546

* Results are expressed as nmoles of (iBuS)[5′]Ado/mg of protein.

uptake for the high affinity system were estimated to K_m 0.022 and 0.800 mM for the low affinity system. No significant differences were found in RSV transformed cells.

Interference of different compounds with the cellular uptake of (iBuS)[5′]Ado

(1) *Structural analogues.* Table 3 shows the structural requirement for (iBuS)[5′]Ado uptake. When experiments were performed at low (iBuS)[5′]Ado concentrations, the best inhibitory compounds were those having a short 1 to 4 carbon atom side chain such as methyl (compound 2), propyl (compound 3), allyl (compound 4) methyl-thio-methyl (compound 5), butyl (compound 6) 1-methyl-propyl (compound 7) and methyl-allyl (compound 9). The presence of a 2-hydroxyl group (compound 10) and a 2-hydroxy-propyl (compound 11) reduces the inhibitory activity. Products such as 5′-*S*-penicillamino adenosine (compound 14), *S*-adenosylhomocysteine (compound 15) and sinefungin (compound 16) having a polar 5′side chain are not inhibitory of (iBuS)[5′]Ado uptake, 5′-*S*-adenosyl-methionine (compound 17) being an exception which could partly be explained by hydrolysis to 5′-methyl-thioadenosine. Replacement of the adenine ring by uracil (compound 18), cytosine (compound 19) or hypoxanthine (compound 20) gave products which did not alter the (iBuS)[5′]Ado uptake. Similar results were obtained with modification of the adenine ring in the positions 6, 3 and 8 (compounds 20–24) as well as modification of the ribose moiety (compounds 25–27), thus indicating a requirement for intact adenosine.

In order to characterize the mechanism of action of these inhibitors we choose to study the effect of 5′methyl-thioadenosine (compound 2) on the kinetic parameters of (iBuS)[5′]Ado uptake. As shown in Fig. 2, 5′methyl-thio-adenosine inhibits both high and low affinity systems. The apparent k_i were estimated to 820 and 178 μM respectively for competitive and non competitive events.

(2) *Modification of cell membrane.* Treatment of

Table 2. (iBuS)$^{5'}$Ado accumulation in normal, infected and transformed cells as a function of (iBuS)$^{5'}$Ado concentration in the medium

Extracellular [(iBuS)$^{5'}$Ado] (mM)	Estimated intracellular [(iBuS)$^{5'}$Ado] (mM) after 5 hr labelling		
	Normal cells	48 hr Infected cells	Transformed cells
0.025	0.504 (20)*	0.590 (23)	0.360 (14)
0.050	0.770 (15)	0.710 (14)	0.537 (10)
0.075	0.961 (13)	1.003 (13)	0.574 (7)
0.100	0.870 (9)	1.254 (12)	0.744 (7)
0.500	5.025 (10)	2.785 (5)	1.673 (3)
1.000	8.671 (8)	8.586 (8)	6.173 (6)

* Values in brackets are the ratio C^i/C^e (intracellular to extracellular concentration of (iBuS)$^{5'}$Ado).

normal CEF with reagents blocking thiol groups impairs the (iBuS)$^{5'}$Ado uptake: thus after 15 min treatment with 0.4 mM *p*-chloromercuribenzoic acid or 1 and 20 mM iodoacetamide the (iBuS)$^{5'}$Ado uptake is reduced to 48, 69 and 28 per cent of the untreated cells respectively. Peptides such as insulin (0.001 mM) or glutathione (1 mM) and ATP which are known to alter cell permeability [25, 26] did not significantly affect (iBuS)$^{5'}$Ado uptake under the conditions used.

(3) *ATP depleted cells.* 2′-deoxyglucose which enters the cells, is phosphorylated to 2′-deoxyglucose-6-phosphate without further metabolism and thus serves as a trap for cellular ATP, contrary to 3-*O*-methyl glucose which is not phosphorylated [27]. Treatment of chick embryo fibroblasts with 2′-deoxyglucose (20 mM) reduces the uptake of (iBuS)$^{5'}$Ado by 40–50 per cent while 3′-*O*-methyl glucose treatment was ineffective, at the same concentration. Figure 3 shows that temperature and treatment of cells with 2′-deoxyglucose affects the low and high affinity systems.

(4) *Effect of some metabolic inhibitors.* Common metabolic inhibitors such as azide and 2–4 dinitrophenol have no significant effect on (iBuS)$^{5'}$Ado uptake. Polyamines such as putrescine, spermidine and spermine (1 mM) inhibit slightly the uptake when measured at high concentrations of (iBuS)$^{5'}$Ado.

(5) *Potent inhibitors of adenosine deaminase.* 2′-

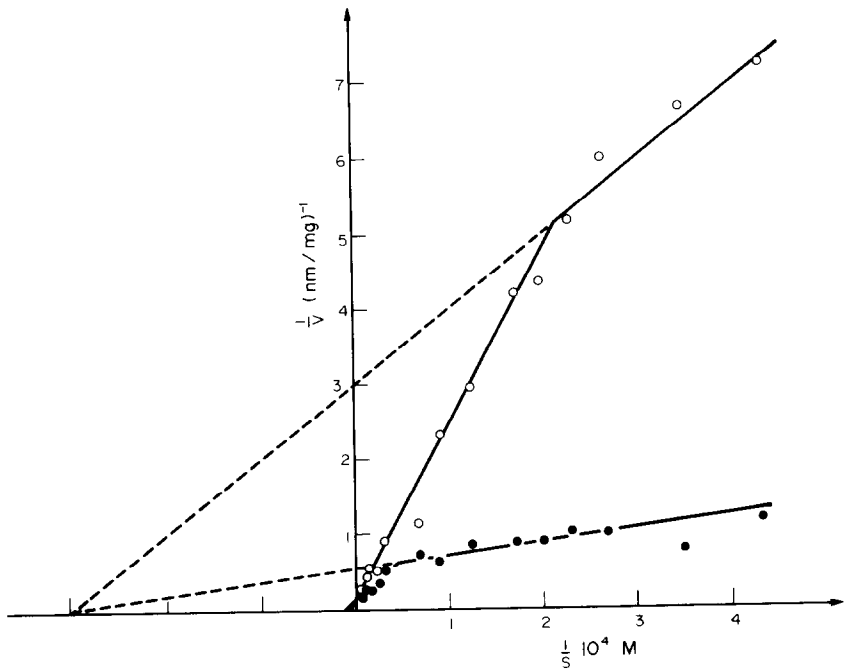

Fig. 2. Lineweaver–Burk plot of (iBuS)$^{5'}$Ado uptake in normal CEF in absence (●—●) and in presence of 0.8 nM 5′*S*-methyl-thioadenosine (○—○). Compound 2 was added to the culture medium 15 min prior to labelled (iBuS)$^{5'}$Ado. Uptake was carried out for 10 min then cells were treated as described.

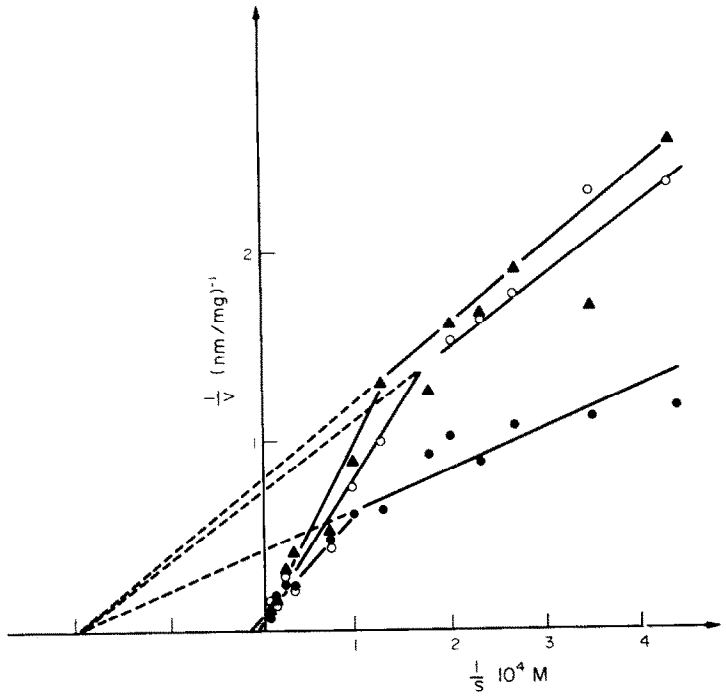

Fig. 3. Inhibition of (iBuS)$^{5'}$Ado uptake in normal CEF by 2'-deoxyglucose (—▲—▲—) using Lineweaver and Burk representation. Experiments were done at 37° (—●—●—) and at 4° (—○—○—). 2'-Deoxyglucose was added to the medium as described in Materials and Methods.

Table 3. Specificity of the (iBuS)$^{5'}$Ado uptake in normal CEF*

No.	Unlabelled compound 0.8 mM	^{14}C-(iBuS)$^{5'}$Ado uptake (10 min) 0.07 mM	Cell transformation (%)	
			%	(mM)
1.	Adenosine	80	100	1.0
2.	5'-*S*- methyl adenosine	34	5	0.5
3.	5'-*S*- propyl adenosine	51	17	0.5
4.	5'-*S*-allyl adenosine	39	1	0.5
5.	5'-*S*- methyl-thio-methyl adenosine	52	0	0.5
6.	5'-*S*- butyl-adenosine	34	14	0.5
7.	5'-*S*- (1 methyl propyl) adenosine	34	1	0.5
8.	5'-*S*- (2 methyl propyl) adenosine [=(iBuS)$^{5'}$Ado]	35	0	0.5
9.	5'-*S*-methyl allyl adenosine	45	0	0.5
10.	5'-*S*- (2 hydroxy 2 methyl propyl) adenosine	77	100	0.5
11.	5'-*S*- (2 hydroxy propyl) adenosine	81	13	0.5
12.	5'-*N*- (2 methyl propyl) adenosine	63	toxic	0.5
13.	5'-*S*- pyridyl adenosine	47	6	1.0
14.	5'-*S*- (DL) penicillamino adenosine	101	100	0.5
15.	*S*-adenosyl-(L) homocysteine (SAH)	89	82	1.0
16.	Sinefungin	106	0	0.5
17.	*S*-adenosyl-(L) methionine (SAM)	48		
18.	5'-*S*- (2 methyl propyl) uridine	81	100	1.0
19.	5'-*S*- (2 methyl propyl) cytidine	92	100	0.5
20.	5'-*S*- (2 methyl propyl) inosine [(iBuS)$^{5'}$Ino]	102	75	1.0
21.	5'-*S*- (2 methyl propyl) N^6 carboxymethyl adenosine	83	45	1.0
22.	5'-*S*- (1 methyl propyl) N^6 methyl adenosine	101	41	0.5
23.	5'-*S*- (2 methyl propyl)- 3 deaza adenosine	87	toxic	0.5
24.	5'-*S*- (2 methyl propyl)-8 methoxy ethoxy adenosine	105	100	0.5
25.	5'-*S*- (2 methyl-propyl)-arabinosyl adenine	108	0	0.5
26.	9-(*S*)-(2,3-dihydroxypropyl) adenine	92	toxic	0.5
27.	5'-*S*- (2 methyl propyl)-adenosine 2'3 phosphite	81	18	0.5

* Unlabelled compounds were added 15 min before the (iBuS)$^{5'}$Ado. Results are expressed in percentage (±7 per cent) of the initial velocity measured at 0.070 mM (^{14}C-iBuS)$^{5'}$Ado, and represent the mean value of two different experiments each in duplicate.

Deoxycoformycin [28] and erythro-9-(2-hydroxy-3-nonyl) adenine [29]. had no significant effect on (iBuS)$^{5'}$Ado uptake suggesting that deamination was not an important metabolic pathway of (iBuS)$^{5'}$Ado under conditions of the assay.

CONCLUSIONS

In conclusion a mixed transport system is involved in (iBuS)$^{5'}$Ado entry into chick embryo fibroblasts. We can distinguish a high affinity transport system for concentrations up to 0.1 mM and a low affinity system over 0.1 mM. The high affinity system is significantly inhibited by analogues with short side chains and by *S*-adenosyl-methionine. A non competitive inhibition is obtained with 5′-methyl-thioadenosine and with 2′-deoxyglucose and the system is sensitive to reagents blocking thiol groups. The low affinity system is not entirely due to simple diffusion since a competitive inhibition exists with 5′methyl-thioadenosine and with 2′deoxyglucose. Polyamines and reagents blocking thiol groups are also effective on this system.

The fact that 2′-deoxyglucose which is not structurally related to (iBuS)$^{5'}$Ado inhibits competitively its uptake is puzzling. However several compounds have been reported to inhibit competitively the uptake of structurally unrelated molecules [30–31]. As (iBuS)$^{5'}$Ado is not a natural compound, its uptake by the cell must be mediated by carriers already existing. It is thus interesting to compare the properties of (iBuS)$^{5'}$Ado uptake with those of structurally related natural compounds (adenosine, 5′methyl-thioadenosine, *S*-adenosyl-methionine). The uptake of (iBuS)$^{5'}$Ado has some common features with the uptake of either adenosine or SAM; in both cases two systems are involved in the uptake [32, 33]. As for adenosine, (iBuS)$^{5'}$Ado uptake is sensitive to SH blocking reagents [34] and to 2′-deoxyglucose [34]. As SAM (iBuS)$^{5'}$Ado is accumulated in the cell [35, 36], furthermore (iBuS)$^{5'}$Ado uptake is inhibited by SAM and not by adenosine, thus suggesting that it may enter by a similar way as SAM.

The possibility that the mixed transport system reflects the uptake of an (iBuS)$^{5'}$Ado metabolite is low: although (iBuS)$^{5'}$Ado can be deaminated by adenosine- and 5′-adenylic acid deaminases [37], or cleaved by methyl-thioadenosine phosphorylase [38, 39] and to a lesser degree by purine nucleoside phosphorylase (F. Lawrence, unpublished results), we observed that after 2 hr incubation of normal chick embryo fibroblasts with 1 mM labelled (iBuS)$^{5'}$Ado, 25 per cent of the radioactivity was found in (iBuS)$^{5'}$Ino and 10 per cent in 5′-isobutyl ribose and 5′-isobutyl ribose-1-phosphate. Thus during the 10 min incubation assay used for the uptake studies, much less than 35 per cent (iBuS)$^{5'}$Ado should be metabolized.

Although viral development induces changes in the permeability properties of the plasma membrane of the host cell [40] no significant differences in initial velocities have been noticed between normal and transformed cells.

It is interesting to compare the inhibition of (iBuS)$^{5'}$Ado uptake and inhibition of RSV-induced cell transformation: it appears that compounds which are good inhibitors of (iBuS)$^{5'}$Ado uptake (compounds 2, 4, 6–11) are good inhibitors of formation of foci, whereas compounds inactive on (iBuS)$^{5'}$Ado uptake (compounds 14, 15, 18–24) are also inactive on formation of foci, the exception being compounds 16, 25 and 26 [1, 8, 13, 14, 22, 41] thus uptake assay could be an easy and rapid pre-screening method to test inhibitors of cell transformation.

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