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2-DEOXYCOFORMYCIN INHIBITION OF INTRACELLULAR PHOSPHORYLATION OF ADENOSINE IN NOVIKOFF RAT HEPATOMA CELLS

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Abstract—Treatment of cultured wild type and azaguanine-resistant Novikoff rat hepatoma cells with 0.1 to 100 μM 2-deoxycoformycin resulted in an inhibition of more than 50 per cent of the incorporation of 100 μM [8-^{3}H]adenosine into intracellular ATP and nucleic acids. In wild type cells, part of the effect resulted from an inhibition of adenosine deamination of deoxycoformycin, with a consequent decrease of incorporation of radioactivity from adenosine via inosine → hypoxanthine → IMP. This pathway, however, was blocked in azaguanine-resistant cells because of the lack of hypoxanthine/guanine phosphoribosyltransferase. The inhibition of adenosine incorporation by deoxycoformycin in these cells was not mediated at the level of adenosine transport or phosphorylation of AMP. We conclude, therefore, that the intracellular phosphorylation of adenosine is impaired in deoxycoformycin-treated cells. There was a close correlation between inhibition of adenosine deamination and adenosine incorporation, both with respect to effective concentrations of deoxycoformycin and to irreversibility of the inhibition. In addition, intracellular concentrations of adenosine above 1–5 μM were found to strongly inhibit the phosphorylation of adenosine *in situ*, reflecting substrate inhibition of adenosine kinase. The results indicate that the inhibition of adenosine phosphorylation in deoxycoformycin-treated cells was caused by the accumulation of free adenosine in these cells due to adenosine deaminase inhibition.

2-Deoxycoformycin (dCF) is well recognized for its potent inhibition of adenosine deaminase (EC 3.5.4.4) [1–6], but other potential toxic effects of this adenosine analog have received less consideration. For example, dCF was found to inhibit the incorporation of radioactivity from 200 μM extracellular radiolabeled adenosine into the nucelotide pool in Ehrlich ascites cells [2]. We have observed a similar inhibition of adenosine incorporation in P388 mouse leukemia cells [7]. In Ehrlich ascites cells, the conversion of extracellular adenine to intracellular ATP was only slightly affected by dCF, and the inhibition of adenosine incorporation was readily reversed by removal of dCF from the medium [2]. The authors concluded that the effect of dCF on adenosine incorporation was not due to an inhibition of adenosine kinase (EC 2.7.1.20), and they attributed it rather to an inhibition of adenosine entry into the cell [2]. Our adenosine transport† studies in P388 cells contradicted the latter conclusion [7], but the mechanism of the inhibition remained unexplained. In this context it should be considered that, because of the rapid deamination of adenosine in mammalian cells [6, 8], the radioactivity in base-labeled adenosine is incorporated into ATP both via direct phosphorylation by adenosine kinase and via inosine → hypoxanthine → IMP. The ratio of radioactivity incorporated from adenosine via the two pathways decreases with an increase in adenosine concentration because the Michaelis–Menten constant for adenosine of adenosine deaminase is about one order of magnitude higher than that of adenosine kinase (see Ref. 7). Our studies with P388 cells suggested that the conversion of adenosine to ATP was inhibited to a greater extent by dCF than could be accounted for by the inhibition of adenosine deamination and its consequent incorporation via the second pathway [7]. This conclusion has been substantiated in the present study, in which we compared the effect of dCF on adenosine transport and metabolism in wild type and azaguanine-resistant Novikoff rat hepatoma cells. In the latter cells, the flow of adenosine to ATP via hypoxanthine is blocked due to lack of hypoxanthine/guanine phosphoribosyl-transferase (EC 2.4.2.8). The results also confirm the negligible effect of dCF on adenosine transport and indicate that it is the phosphorylation of adenosine that is inhibited in dCF-treated cells. The inhibition of adenosine kinase is caused by the free adenosine accumulating in cells in which adenosine deamination is inhibited (substrate inhibition).

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† "Transport" denotes solely the transfer of unmodified substrate across the cell membrane as mediated by a saturable, selective carrier. "Uptake" denotes the total intracellular accumulation of radioactivity from exogenous labeled substrate regardless of metabolic conversions. "Incorporation" is used interchangeably with uptake, but generally refers to the transfer of radioactivity from labeled substrate to a specified intracellular material.

MATERIALS AND METHODS

Cell culture. Novikoff rat hepatoma cells (subline N1S1-67) and an azaguanine-resistant subline

thereof [9, 10] were propagated in suspension culture in Swim's medium 67 and enumerated in a Coulter counter as described previously [11]. Cells to be used in experiments were harvested from exponential phase cultures by centrifugation at 400 *g* for 1–2 min and suspended in basal medium 42B (BM42B) [12]. Where indicated, cells were depleted of ATP by preincubation in glucose-free BM42B containing 5 mM KCN and 5 mM iodoacetate at 37° for 10 min [13].

Measurement of adenosine, adenine and hypoxanthine uptake. Suspensions of cells were supplemented with dCF and [8-^{3}H]adenosine, [8-^{3}H]adenine or [G-^{3}H]hypoxanthine as indicated in the appropriate experiments. At specified times during incubation at 25°, duplicate 0.5 ml samples of cell suspension were layered over 200 μl of an oil mixture (density = 1.034 g/cm^3, see Ref. 14) in 1.5 ml microcentrifuge tubes, and the cells were separated from the medium by centrifugation through the oil at 12,000 *g* in an Eppendorf microcentrifuge. From selected samples, 0.4 ml of the culture fluid was removed immediately after centrifugation, mixed with 0.1 ml of 1 N trichloroacetic acid, and then chromatographed on Whatman 3MM paper with a solvent composed of 79 ml of saturated ammonium sulfate, 19 ml of 0.05 M phosphate buffer (pH 6.0), and 2 ml of isopropanol (solvent 9) for the separation of adenosine, inosine, and hypoxanthine as described previously [7, 12]. Otherwise, the culture fluid was removed when convenient. Residual radioactivity from the culture fluid was removed from the upper portion of the centrifuge tube by layering 1 ml of H_2O over the oil and aspirating it along with most of the oil. The pelleted cells were resuspended in 0.2 ml of 0.5 N trichloroacetic acid, heated at 70° for 30 min, and analyzed *in toto* for radioactivity by liquid scintillation counting [12]. The values were corrected for substrate trapped in the extracellular space of the pellet as estimated by use of [carboxyl-^{14}C]inulin [14]. The extracellular space represented between 12 and 15 per cent of the total H_2O space in the cell pellets. The intracellular H_2O space was taken as the total H_2O space estimated by the use of [^{3}H]H_2O minus the inulin space [14]. The corrected values are referred to as radioactivity in total cell material. Additional duplicate 0.5 ml samples of suspension were analyzed for radioactivity in acid-insoluble material (nucleic acids) as described previously [12].

In experiments where it was desired to quench metabolism rapidly for the purpose of fractionating the acid-soluble pool of labeled cells, the cells were centrifuged through an oil layer of 350 μl directly into 100 μl of an extracting solution composed of 0.5 N trichloroacetic acid and 10% (w/v) sucrose (density = 1.04 g/ml). After centrifugation, the tubes were immediately placed in ice and the medium was aspirated. The tubes were then filled with an H_2O rinse that was aspirated along with most of the oil. Each acid extract was extracted twice with ether to remove residual oil and most of the acid. ATP, GTP, AMP, IMP, adenosine, inosine, and hypoxanthine in acid extracts were separated by ascending chromatography on Whatman 3MM paper with solvent 9, solvent 28 (30 ml of 1 M ammonium acetate, pH 5, and 70 ml of 95% ethanol), and solvent 22 (66 ml of isobutyric acid, 1 ml of concentrated NH_4OH and 33 ml of H_2O) as described previously [7, 12].

*Measurements of zero-*trans* and equilibrium exchange transport of adenosine.* Adenosine zero-*trans* influx and equilibrium exchange transport in azaguanine-resistant Novikoff cells, which were ATP-depleted and pretreated with 10 μM dCF for 5 min [7], were measured by a rapid kinetic technique described previously [7, 14, 16]. To influx data pooled for seven to eight adenosine concentrations was fitted by the method of least squares, the following zero-*trans* integrated rate equation which describes the accumulation of substrate to transmembrane equilibrium by a completely symmetrical system [14, 16, 17]:

$$S_{2,t} = S_1 \left[1 - \exp\left(-\frac{Vt + (1 + S_1/K)\, S_{2,t}}{K + 2S_1 + S_1^2/K}\right)\right] \quad (1)$$

where $S_{2,t}$ = concentration of substrate inside the cell at time t ($S_{2,o} = 0$); S_1 = exogenous substrate concentration; K = Michaelis–Menten constant and V = maximum velocity.

Equilibrium exchange data pooled for seven adenosine concentrations were analyzed in a similar manner, except that we applied the following integrated rate equation, which describes the time course of intracellular accumulation of radioactively labeled substrate to transmembrane equilibrium under conditions where the absolute intra- and extracellular substrate concentrations are equal [14, 16, 17]:

$$N_{2,t} = N_{2,\infty}\left[1 - \exp\left(-\frac{V^{ee}\, t}{K^{ee} + S}\right)\right] \quad (2)$$

where $N_{2,t}$ = intracellular concentration of radioactivity at time t; $N_{2,\infty}$ = intracellular concentration of radioactivity at $t = \infty$, which is equal to N_1, the concentration of radioactivity per equivalent volume of medium, and K^{ee} and V^{ee} are the apparent Michaelis–Menten parameters for equilibrium exchange.

Measurement of adenosine kinase activity in cell-free extracts. Cell-free extracts were prepared from azaguanine-resistant Novikoff cells and were assayed for the phosphorylation of adenosine and tricyclic nucleoside at 37° as described previously [7]. The final reactions contained 2.5 mM $MgCl_2$, 100 mM Tris–HCl (pH 7.4), 200 μg bovine serum albumin/ml, 10 mM ATP and 125 μM [8-^{3}H]adenosine (100 μCi/μmole), 170 μM 1,4,5,6,8-pentaazaacenaphthalene, 3-amino-1,5-dihydro-5-methyl-[5-^{14}C]-1-β-D-ribofuranosyl ([^{14}C]tricyclic nucleoside; NSC-154020), or concentrations of [8-^{3}H]adenosine indicated in the appropriate experiments. The substrates and reaction products were separated by chromatography with solvent 28.

Materials. [8-^{3}H]Adenosine and [8-^{3}H]adenine were purchased from Schwarz/Mann (Orangeburg,

* As defined by Eilam and Stein [15], zero-*trans* designates the transport of a substrate from one side of the membrane to the other side, where its concentration is zero. "Equilibrium exchange" designates the unidirectional flux of radioactively labeled substrate from one side of the membrane to the other side, where substrate is held at equal concentration. We designated arbitrarily the outside and inside faces of the membrane as 1 and 2 respectively.

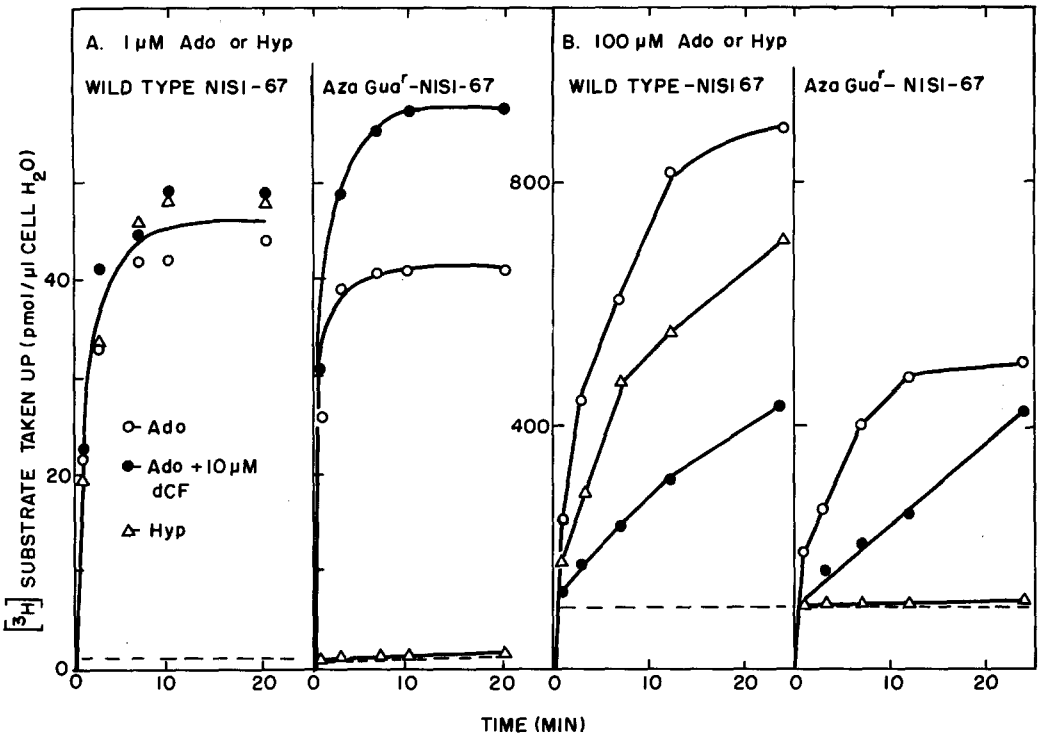

Fig. 1. Effect of dCF on adenosine uptake by wild type and azaguanine-resistant (AzaGua^r) Novikoff rat hepatoma cells. Samples of suspensions of about 1.7×10^7 cells of either cell line/ml of BM42B were supplemented where indicated with 10 μM dCF and incubated at 37° for 15 min. After equilibration at 24°, the suspensions were supplemented with either (A) 1 μM [^{3}H]adenosine (600 cpm/pmole) or [^{3}H]hypoxanthine (570 cpm/pmole) or (B) 100 μM [^{3}H]adenosine (7 cpm/pmole) or [^{3}H]hypoxanthine (6 cpm/pmole). At various times of incubation at 24°, the cells from duplicate 0.5 ml samples of each suspension were collected by centrifugation through oil and analyzed for radioactivity. Average radioactivity/cell pellet was corrected for substrate trapped in extracellular H_2O of the cell pellet (1.3 and 1.5 μl/pellet for wild type and azaguanine-resistant cells respectively) and converted to pmoles/μl cell H_2O on the basis of an intracellular H_2O space of 10.6 and 8.8 μl/pellet respectively. The broken lines indicate the intracellular concentration of substrate-equivalents equal to the extracellular substrate concentration at zero time. Parallel duplicate samples of cells were analyzed for radioactivity in acid-insoluble material. Acid extracts were prepared from samples of the same cells after 1, 12 and 25 min of incubation and chromatographed as described in Materials and Methods. Representative data on the composition of the acid-soluble pools and culture fluids after 25 min of incubation with 100 μM [^{3}H]adenosine are presented in Table 1.

NY), and [G-^{3}H]hypoxanthine was from Amersham/Searle (Arlington Heights, IL). Labeled and unlabeled tricyclic nucleoside was supplied by the National Cancer Institute under Contract N01-CM-43788. Other chemicals were reagent grade from standard suppliers.

RESULTS AND DISCUSSION

Figure 1 illustrates the time courses of uptake of [^{3}H]adenosine and [^{3}H]hypoxanthine by wild type and azaguanine-resistant Novikoff cells and the effects of dCF on adenosine uptake. dCF had markedly different effects on adenosine uptake depending on whether the extracellular adenosine was present at 1 μM (Fig. 1A) or 100 μM (Fig. 1B). At 1 μM [^{3}H]adenosine, dCF had no significant effect on overall uptake of radioactivity by wild type cells. At this concentration an intracellular steady-state concentration of adenosine is attained (below 0.1 μM; see Ref. 7) that is insufficient to saturate adenosine kinase (K_m = 0.6 to 6 μM; see Ref. 6). Thus, inhibition by dCF of the adenosine incorporation via inosine → hypoxanthine → IMP seemed to be compensated for by increased direct phosphorylation of imported adenosine to AMP, so that the overall incorporation of radioactivity into the cells was unaltered. The incorporation of radioactivity had almost ceased by 8 min of incubation because the medium had become practically depleted of labeled substrate. The capacity of the cells to take up hypoxanthine was about the same as for adenosine at the equivalent concentration of 1 μM (Fig. 1A), and chromatographic analysis showed that over 85 per cent of the radioactivity in the acid soluble pools of cells exposed to either [^{3}H]adenosine or [^{3}H]hypoxanthine was associated with ATP (see also Refs. 7, 10 and 12).

Analogous reasoning explains the stimulation by dCF of uptake of 1 μM adenosine that was observed in azaguanine-resistant cells (Fig. 1A). These cells possess less than 1 per cent of the hypoxanthine/guanine phosphoribosyltransferase activity of wild type cells [9, 10]. When exposed to hypoxanthine they rapidly accumulate free hypoxanthine to a concentration equivalent to that in the

Table 1. Composition of culture fluid and acid-soluble pools of wild type and azaguanine-resistant (AzaGua^r) Novikoff cells labeled for 25 min with [^{3}H]adenosine or [^{3}H]hypoxanthine*

Substrate†	Cells	dCF‡	Culture fluid (μM)				Acid-soluble pool§ (μM)						
			Ado	Ino	Hyp	Total	GTP	ATP	IMP	AMP	Ado	Ino	Hyp
Adenosine	Wild type	−	2	35	36	63	131	650	<15	<20	<5	45	33
		+	83	0‖	0	83	0‖	220	0	<30	110	0	0
	AzaGua^r	−	1	28	32	61	<10	420	<10	<20	<5	63	80
		+	84	0	0	84	0	220	0	<30	122	0	0
Hypoxanthine	Wild type	−	<1	3	67	70	108	470	57	<7	<7	21	112
	AzaGua^r	−	<1	3	93	96	<5	<5	<5	<5	<5	<5	110

* Details of the experiment are described in the legend to Fig. 1. The values were estimated from chromatographic analyses of the culture fluid with solvent 9 and of the acid-extracts with solvents 9, 22 and 28, as described in Materials and Methods.

† [^{3}H]Adenosine (100 μM) or [^{3}H]hypoxanthine (100 μM) was added at 0 time.

‡ Cells were preincubated with (+) or without (−) 10 μM dCF at 37° for 15 min.

§ Pools are expressed not as chemical concentrations, but as equivalents of isotopic substrate incorporated into the designated compound. The values are corrected for substrate trapped from the culture fluid (12 and 13 pmoles/μl cell H_2O for wild type and AzaGua^r cells respectively) on the basis of the experimentally determined composition of the culture fluid.

‖ Below detectable levels; since preincubation with 10 μM dCF completely inhibited adenosine deamination, concentration is assumed to be zero.

medium, but fail to phosphoribosylate it to a significant extent [10] [see Fig. 1 (A and B) and Table 1]. Thus, adenosine is converted to ATP in these cells only via direct phosphorylation to AMP, and the degradation products resulting from adenosine deamination are released into the medium [7]. An inhibition of deamination of adenosine by dCF makes more adenosine available for direct phosphorylation by adenosine kinase and thus stimulates overall uptake.

In contrast to the results with 1 μM adenosine, dCF strongly inhibited the uptake of adenosine when present at a concentration of 100 μM (Fig. 1B). At 100 μM adenosine and in the absence of dCF wild type cells established within seconds (at 25°) intracellular steady-state concentrations of adenosine and hypoxanthine that saturate adenosine kinase and hypoxanthine/guanine phosphoribosyltransferase, respectively, and the conversion of adenosine to nucleotides via both pathways occurs at a maximum rate (see Refs 7 and 10). This condition is attained because the Michaelis–Menten constants and maximum velocities for adenosine influx (see below) and for adenosine deaminase and purine nucleoside phosphorylase exceed those for the intracellular conversion of adenosine and hypoxanthine to nucleotides by one to two orders of magnitude [7, 10]. Addition of 10 μM dCF inhibited the uptake of 100 μM adenosine not only in wild type Novikoff cells, but also in the azaguanine-resistant mutant (Fig. 1B). Thus, the reduction of adenosine uptake in dCF-treated cells was not solely due to inhibition of incorporation of radioactivity into nucleotides via inosine → hypoxanthine → IMP resulting from the blockage of adenosine deaminase by dCF. The following analyses and experiments were designed to elucidate the mechanism of this inhibition.

First, let us consider the nature and concentrations of labeled components present in cells from the experiment depicted in Fig. 1B and in their culture fluid (Table 1). Inosine and hypoxanthine made up each about one-half of the radioactivity remaining in the medium of either wild type or azaguanine-resistant cells after 25 min of incubation with 100 μM [^{3}H]adenosine in the absence of dCF, whereas little labeled adenosine was left. Most of the radioactivity in the acid-soluble pool was associated with ATP, but the presence of label also in GTP in wild type cells confirms the incorporation of radioactivity into nucleotides via inosine → hypoxanthine → IMP in these cells. This pathway was blocked in the hypoxanthine/guanine phosphoribosyltransferase-deficient cells, which accounts for the lower overall uptake of adenosine in azaguanine-resistant cells (Fig. 1B; compare left and right frames), the lower level of radioactivity in ATP, and the lack of labeled GTP in the acid-soluble pool of these cells (Table 1). Some radioactivity was recovered in AMP and IMP in both types of cells, but because of the relatively small proportion of total radioactivity in these fractions and some overlap in chromatograms, the amounts could not be quantitated accurately. Labeled inosine and hypoxanthine were present intracellularly at concentrations approximately equal to those in the medium, but little if any adenosine was present.

In contrast, when the cells were treated with 10 μM dCF, about 84 per cent of the adenosine was still left in the medium after 25 min of incubation, and the intracellular steady-state concentration of adenosine was about equal to that in the medium. The chromatographic analyses also clearly demonstrated that the dCF treatment strongly inhibited the labeling of ATP in wild type and azaguanine-resistant cells. In fact, in the presence of dCF, the uptake of adenosine (Fig. 1B) and the labeling of intracellular components (Table 1) were about the same in both types of cells. This marked decrease in labeled ATP formation in dCF-treated cells explains why the incorporation of labeled adenosine into nucleic acids was

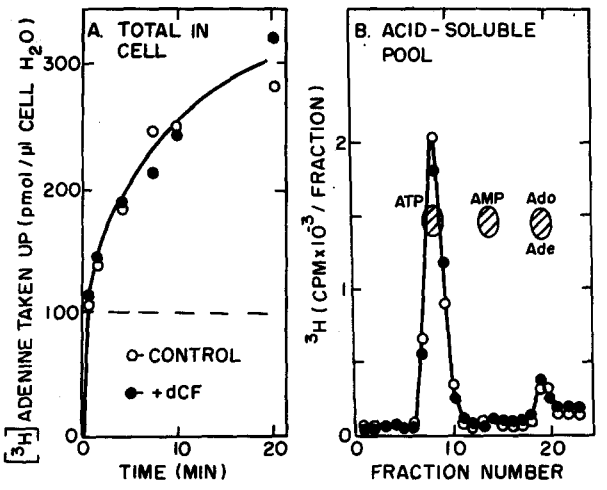

Fig. 2. Lack of effect of dCF on the uptake of adenine. Panel A: Samples of a suspension of about 2.5×10^7 azaguanine-resistant Novikoff cells were supplemented with 100 μM [^{3}H]adenine (4 cpm/pmole). Where indicated, the cells were preincubated with 10 μM dCF for 15 min. At various times of incubation at 25°, the cells from duplicate 0.5 ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. Radioactivity/cell pellet was corrected for substrate trapped in extracellular space (4.2 μl/pellet). Average radioactivity/sample was converted to pmoles/μl cell H_2O on the basis of an intracellular H_2O space of 16.6 μl/sample. Panel B: Acid extracts were prepared from additional samples of the same cell suspensions after 20 min of incubation and chromatographed with solvent 28.

diminished by dCF to an extent similar to that of its uptake into the acid-soluble pool (data not shown).

The absence of any unusual accumulation of labeled AMP in dCF-treated wild type or azaguanine-resistant cells (Table 1) indicates that the inhibition of adenosine uptake by dCF was not due to an inhibition of the conversion of AMP to ATP. This conclusion is supported by the data in Fig. 2A, which show that the long-term uptake of 100 μM adenine by azaguanine-resistant Novikoff cells was not inhibited by dCF. dCF also had no effect on the distribution of radioactivity in the acid-soluble pool of adenine-labeled cells (Fig. 2B).

In a previous study [7], we have shown that, in ATP-depleted P388 cells in which adenosine phosphorylation was blocked, dCF, even at a concentration of 1 mM, had no significant effect on the zero-*trans* influx of adenosine. The zero-*trans* influx of adenosine into ATP-depleted, azaguanine-resistant Novikoff cells was similarly unaffected by dCF (Fig. 3). Thus, the effect of dCF on long-term adenosine uptake was also not mediated at the transport step. The nucleoside carrier of Novikoff cells has been shown to be functionally completely symmetrical with thymidine and uridine as substrate [16, 17]. Our present study of adenosine transport in dCF-treated, ATP-depleted, azaguanine-resistant cells is in agreement with this conclusion. Results from a zero-*trans* influx experiment with seven adenosine concentrations (10–640 μM) yielded the best fitting Michaelis–Menten constant (K) and maximum velocity (V) for a completely symmetrical carrier of 103 ± 8 μM and 17.2 ± 0.5 pmoles/μl cell H_2O · sec respectively (correlation coefficient $r_{y,\hat{y}} = 0.9819$). Results from an equilibrium exchange experiment with the same adenosine concentrations yielded similar apparent kinetic parameters: $K^{ee} = 136 \pm 27$ μM and $V^{ee} =$ $= 11.1 \pm 1.0$ pmoles/μl cell H_2O · sec ($r_{y,\hat{y}} = 0.9556$). These values are comparable to those obtained for adenosine transport in P388 mouse leukemia cells [7]. Thus, the results indicate that adenosine influx at 100 μM adenosine in the medium exceeded the long-term rate of adenosine uptake in either dCF-treated or untreated Novikoff cells (Fig. 1B) 10- to 100-fold and was not rate-limiting. In fact, the rapid initial phase of uptake of [^{3}H]adenosine during the first minute of incubation (Fig. 1) reflects the rapid influx of adenosine, whereas the long-term accumulation of radioactivity reflects the formation of nucleotides. This fact was established previously for the uptake of adenosine [7] and other nucleosides and nucleobases [16, 17].

By a process of elimination we come thus to the conclusion that it is the adenosine kinase reaction that is inhibited in dCF-treated cells. We have considered three possible mechanisms for this inhibition. First, adenosine phosphorylation could be inhibited due to a depression of the ATP level in dCF-treated cells. We have not further investigated this possibility, but consider it unlikely since the ATP concentration in Novikoff cells is 3–4 mM [18] and the ATP concentration would have to be reduced by at least 90 per cent (values between 25 and 400 μM have been reported for the K_m of adenosine kinase for ATP [6] to reduce *in situ* adenosine phosphorylation by 50 per cent. The inhibition of adenosine uptake by dCF seems too rapid to be a consequence of such ATP pool reduction.

Second, the adenosine kinase reaction could be directly inhibited by dCF or by a phosphorylated form of dCF. We have repeatedly found that the presence of dCF reduced the phosphorylation of 170 μM tricyclic nucleoside (an adenosine analog that is a substrate for adenosine kinase, but not for adenosine deaminase [19]) in a cell-free extract from Novikoff cells (Table 2). The phosphorylation of 100 μM adenosine was similarly inhibited, but the results were less reproducible (data not shown). In

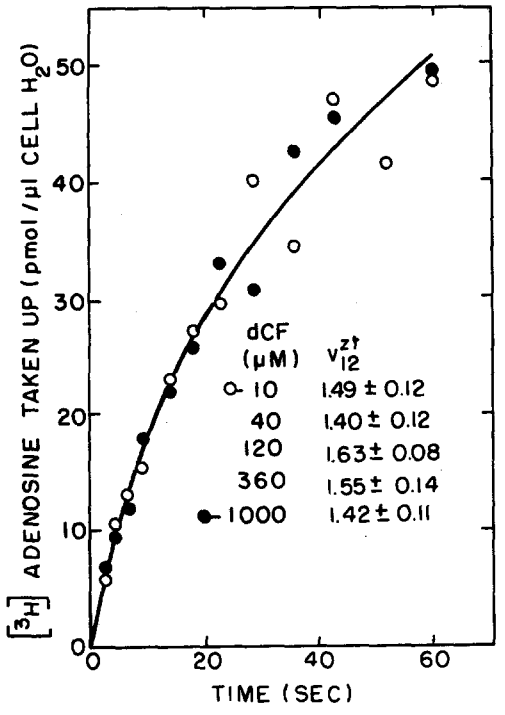

Fig. 3. Lack of effect of dCF on the zero-*trans* influx of adenosine in azaguanine-resistant Novikoff cells in which adenosine metabolism was blocked by treatment with dCF and depletion of ATP. The cells were suspended to about 4×10^7 cells/ml in glucose-free BM42B containing 5 mM KCN and 5 mM iodoacetate, and the suspension was incubated at 37° for 10 min. Samples of the suspension were then supplemented with dCF as indicated, equilibrated at 25°, and then assayed for zero-*trans* influx of 120 μM [^{3}H]adenosine (4 cpm/pmole) by the rapid kinetic technique as summarized in Materials and Methods. All $S_{2,t}$ values were calculated as pmoles/μl cell H_2O on the basis of experimentally determined intracellular H_2O space and corrected for substrate trapped non-specifically [14, 16]. Equation 1 was fit to each individual time course of adenosine accumulation with K fixed at 120 μM (see text) to yield estimates of V. The listed initial zero-*trans* velocities ($v_{12}^{zt} \pm$ S.E. of the estimate) were calculated by substituting K and the estimated values of V and $S_1 = 120$ μM into the zero-*trans* rate equation at $S_2 = 0$: $v_{12}^{zt} = V/(K/S_1 + 1)$. Only the time points of accumulation of adenosine in the presence of 10 and 1000 μM dCF are shown.

Table 2. Effect of dCF on adenosine kinase activity in cell-free extracts of Novikoff cells*

dCF (μM)	Nucleotides formed (% of total substrate)			
	Expt. 1		Expt. 2	
	5 min	30 min	2 min	10 min
0	25.0	49.1	16.3	57.0
10	24.1	48.2		
100	21.7	43.1		
500	21.4	36.7	11.9	30.1

* The phosphorylation of 170 μM [^{14}C]tricyclic nucleoside was measured as described in Materials and Methods.

both cases, however, an inhibition was observed only at dCF concentrations far in excess of those maximally inhibiting adenosine deaminase (see below), and this effect, therefore, seems insufficient to account for the inhibition of adenosine phosphorylation in whole cells. Furthermore, dCF had no effect on the phosphorylation of adenosine by a crude cell-free extract from P388 cells, even though adenosine uptake by P388 cells was inhibited by dCF to an extent similar to that in Novikoff cells [7]. These experiments also cannot distinguish between an inhibition by dCF itself or by a phosphorylated form of dCF, since a phosphorylation of dCF, if it occurs, would be expected to occur in the adenosine kinase reaction mixture containing crude cell-free extract. A significant, although slow, phosphorylation of dCF—probably by deoxyadenosine kinase—has been observed recently in L1210 cells [20], whereas the phosphorylation of adenosine by highly purified rabbit liver adenosine kinase was little affected by dCF [21].

We suggest, therefore, a third mechanism—that the inhibition of the *in situ* phosphorylation of adenosine in dCF-treated cells is most likely caused primarily by the free adenosine accumulating in these cells as a consequence of the inhibition of adenosine deaminase. Substrate inhibition of adenosine kinase has been demonstrated by several investigators [22–25]. For example, partially purified adenosine kinase from sarcoma 180 cells was inhibited about 50 per cent by 100 μM adenosine [22]. We observed a similar substrate inhibition of adenosine phosphorylation by cell-free extracts from Novikoff cells (Fig. 4). More important, however, is the demonstration that substrate inhibition also operated *in situ* (Fig. 5). The design of the experiment was based on the fact, explained already, that the long-term uptake of radioactivity from radiolabeled nucleosides above equilibrium levels reflects the accumulation of nucleotides and that, in the absence of deamination, the phosphorylation of adenosine is the rate-limiting step in the conversion of extracellular adenosine to intracellular ATP. Representative time courses of uptake of adenosine at various concentrations between 0.1 and 160 μM by dCF-treated, azaguanine-resistant Novikoff cells are presented in Fig. 5A. The data clearly demonstrate a decrease in adenosine uptake at higher adenosine concentrations. Complete inhibition of adenosine deamination in these cultures and formation of labeled nucleotides were confirmed by chromatographic analysis of the culture fluid and of acid extracts of these cells respectively. As summarized in Fig. 5B, after 4 min of incubation with 0.1 to 10 μM [^{3}H]adenosine, over 97 per cent of the intracellular radioactivity was in nucleotides (mostly ATP), and intracellular free adenosine was below measurable levels. At higher adenosine concentrations, on the other hand, adenosine kinase became saturated, phosphorylation was relatively slow compared to influx, and free adenosine accumulated close to equilibrium level.

Rates of intracellular phosphorylation were estimated from the linear phases of uptake, which developed after the initial rapid influx of adenosine (Fig. 5A), and are plotted in Fig. 5B as a function of the adenosine concentration. The rate of intra-

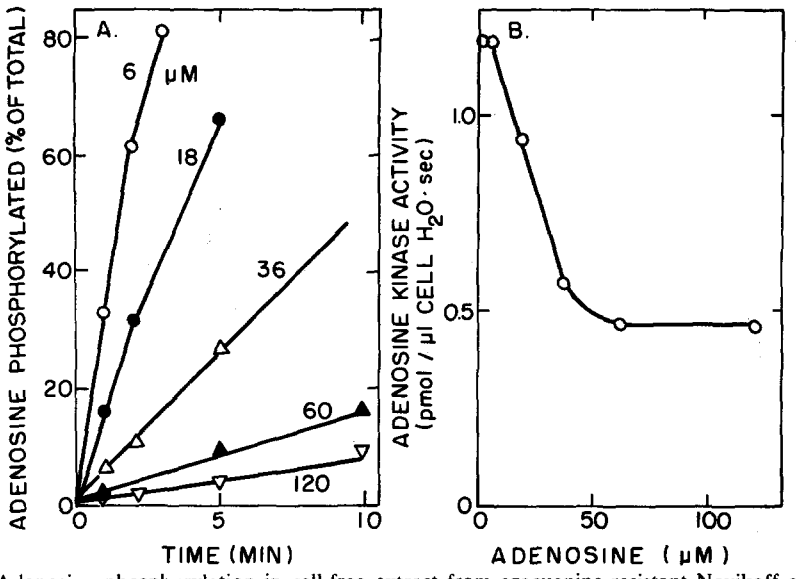

Fig. 4. Adenosine phosphorylation in cell-free extract from azaguanine-resistant Novikoff cells as a function of adenosine concentration. The phosphorylation of 3–120 μM [^{3}H]adenosine (16 μCi/ml, irrespective of concentration) was measured at 37° as described in Materials and Methods. The final reaction mixture contained cell extract equivalent to 26 or 52 μl of cell water/ml (0.4 and 0.8 mg protein/ml) with 3 and 6, or 18–120 μM adenosine respectively.

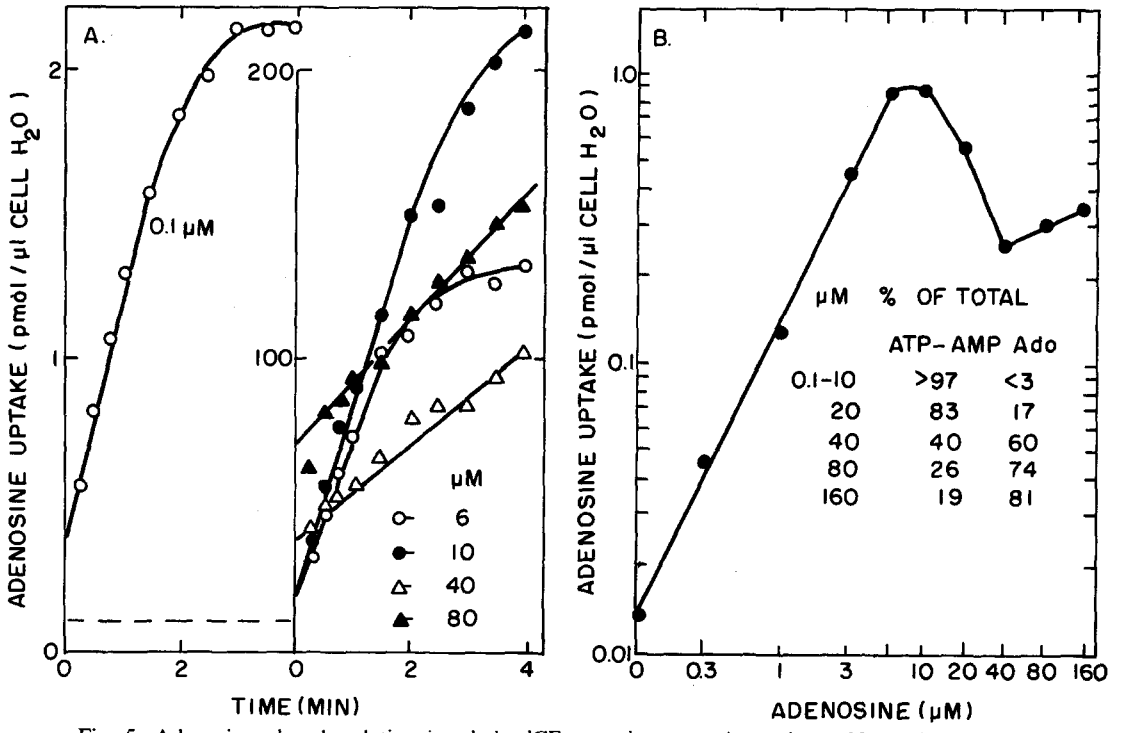

Fig. 5. Adenosine phosphorylation in whole dCF-treated, azaguanine-resistant Novikoff cells as a function of adenosine concentration. A suspension of 2.2×10^7 cells/ml was incubated with 5 μM dCF at 25° for 5 min. Then (0 time) samples of the suspension were supplemented with the indicated concentrations of [^{3}H]adenosine (430 cpm/ml, irrespective of concentration) and, at various times of incubation at 25°, the cells from 0.5 ml aliquots were collected by centrifugation through oil and analyzed for radioactivity. Radioactivity per cell pellet was corrected for that attributable to extracellular water space (3.5 μl) and converted to pmoles/μl cell water on the basis of an intracellular water space of 19.1 μl. Rates of intracellular phosphorylation (panel B) were estimated from the linear portions of the uptake curves between 0.5 and 4 min of incubation (panel A). The broken line in A signifies the intracellular concentration of adenosine equivalents equal to the initial adenosine concentration in the medium. The acid-soluble pools were extracted from replicate samples of cells after 4 min of incubation, and the acid extracts and culture fluid were analyzed chromatographically as described in Materials and Methods. The distribution of radioactivity among adenine nucleotides (ATP, ADP and AMP) and adenosine (uncorrected for adenosine trapped in extracellular space) are summarized in panel B.

cellular phosphorylation was directly proportional to the extracellular adenosine concentration between 0.05 and about 3 μM, but it was markedly reduced at higher concentrations. A maximum reduction of about 70 per cent occurred at 40 μM adenosine. The inhibition in whole cells (Fig. 5B) was similar to that observed in cell-free extracts (Fig. 4B), but it was unusual for simple substrate inhibition in that only about 70 per cent of phosphorylation was inhibitable by adenosine. One possible explanation compatible with the data is that a second high K_m phosphorylation mechanism that is not subject to substrate inhibition is operating in the cells.

Substrate inhibition of adenosine kinase explains why the inhibition of adenosine uptake by dCF was apparent only when the uptake of high concentrations of adenosine was measured. In the absence of dCF, deamination of adenosine was so rapid that its intracellular steady-state concentration was maintained at a level that allowed phosphorylation to occur unimpaired even when the extracellular adenosine concentration was 100 μM or higher (Table 1). When deamination was inhibited, on the other hand, adenosine equilibrated across the cell membrane (Table 1) and inhibited phosphorylation when its concentration was above 5–10 μM (Fig. 5).

The view that the inhibition of adenosine uptake by dCF primarily reflects substrate inhibition of adenosine kinase by the adenosine accumulating in dCF-treated cells was further supported by the existence of a close correlation between the inhibition of adenosine deaminase and of adenosine uptake by dCF. Comparison of the data in Fig. 6 (A and B) shows that the uptake of radioactivity from 100 μM [^{3}H]adenosine by whole cells was inhibited by dCF in a concentration-dependent manner similar to that of the deamination of adenosine by these cells, measured as the accumulation of inosine plus hypoxanthine in the culture fluid. Both processes were maximally inhibited by 0.08 to 0.4 μM dCF. The only difference between the inhibition of the two processes was that adenosine deamination was inhibited close to 100 per cent, whereas adenosine uptake was maximally inhibited 70–75 per cent. Increasing the concentration of dCF to 10 or 100 μM had no additional effect (data not shown, see Fig. 1B). This difference, however, is consistent with the postulated mechanism of inhibition of adenosine uptake. This degree of inhibition is all that could be expected by the steady-state concentration of 80–100 μM adenosine present in cells in which adenosine deamination has been completely inhibited by dCF. Increasing the concentration of dCF could not have any additional effect.

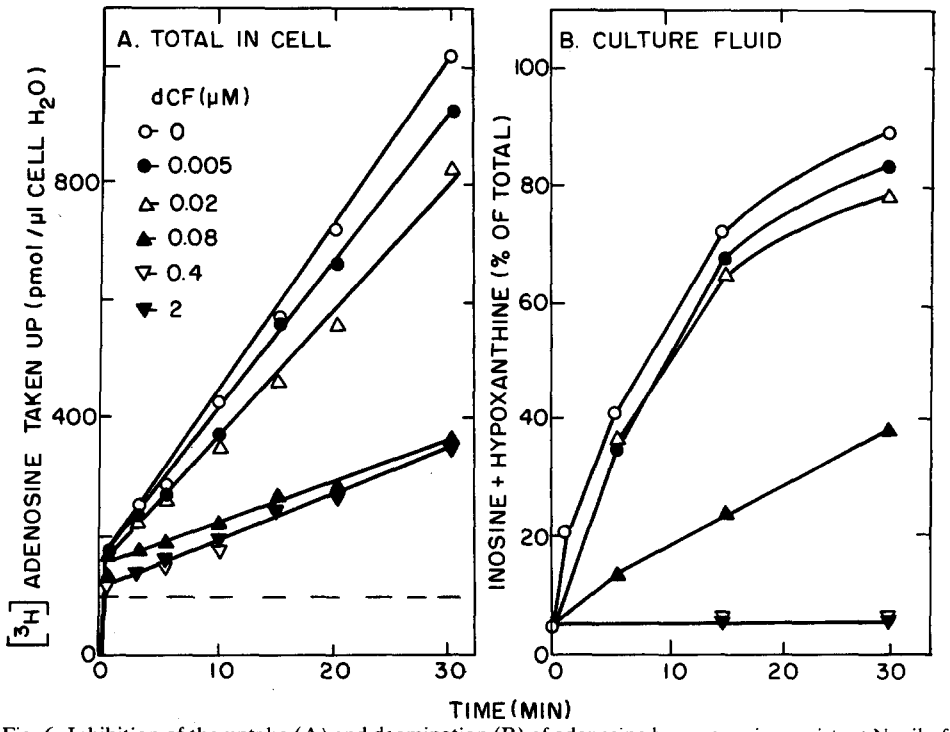

Fig. 6. Inhibition of the uptake (A) and deamination (B) of adenosine by azaguanine-resistant Novikoff cells as a function of dCF concentration. Samples of a suspension of about 1×10^7 cells/ml of BM42B were supplemented with the indicated concentrations of dCF. After 10 min of incubation at 25° (0 time), [^{3}H]adenosine (5.7 cpm/pmole) was added to each suspension to a final concentration of 100 μM. After various times of further incubation at 25°, the cells from duplicate 0.5 ml samples of suspension were collected by centrifugation through oil. Samples of the cell-free supernatant fraction were chromatographed. The percent values of total radioactivity associated with inosine plus hypoxanthine are summarized in panel B. The cell pellets were analyzed for radioactivity which was corrected for substrate trapped in extracellular space (2.4 μl/pellet). Average radioactivity/sample was converted to pmoles/μl cell H_2O on the basis of an intracellular H_2O space of 10.8 μl/sample. The broken line in panel A signifies the intracellular concentration of adenosine equivalents equal to the initial adenosine concentration in the medium.

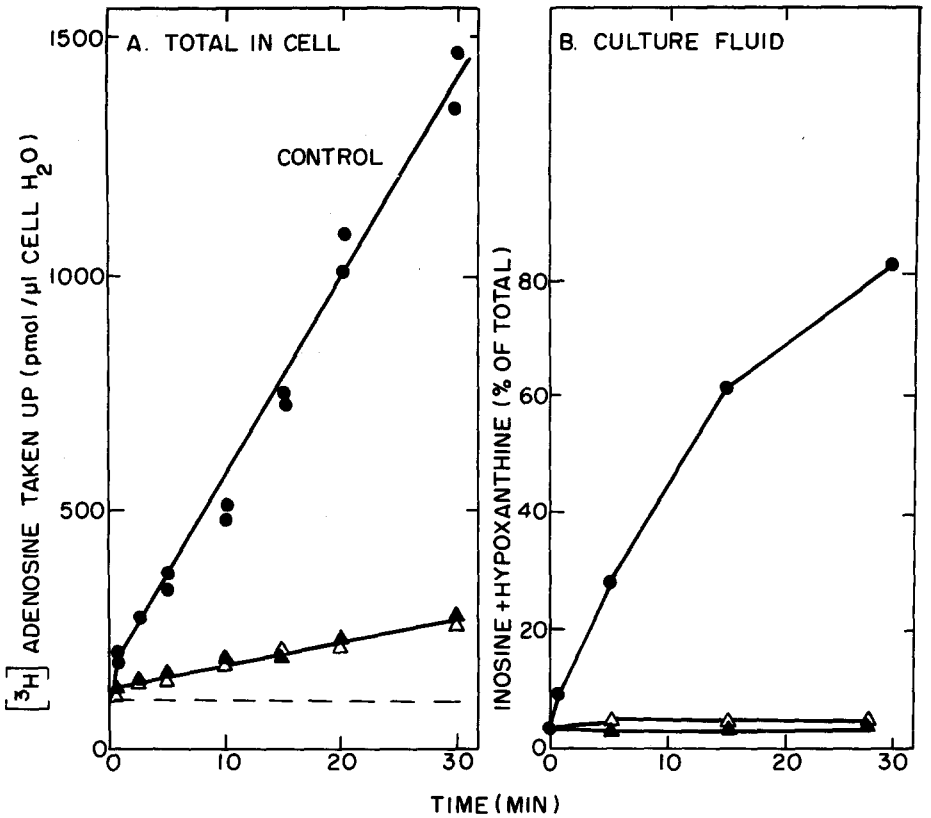

Fig. 7. Lack of reversibility of the inhibition by 1 µM dCF of the uptake (A) and deamination (B) of adenosine by azaguanine-resistant Novikoff cells. One sample of a suspension of 2×10^6 cells/ml of BM42B was supplemented with 1 µM dCF. After 10 min of incubation at 37°, the cells of the dCF-treated suspension and of an untreated control suspension were collected by centrifugation, washed once by resuspension to 4×10^6 cells/ml of BM42B, and recentrifuged. The cells were then resuspended to 8×10^6 cells/ml in BM42B, and duplicate samples of the untreated (●—●) and dCF-pretreated suspensions were supplemented with 100 µM [^{3}H]adenosine (3 cpm/pmole). One sample of the dCF-pretreated suspension also received 1 µM dCF (▲—▲), while the other sample remained without dCF (△—△). At various times of incubation at 25°, the cells from duplicate 0.5 ml samples of suspension were collected by centrifugation through oil. Samples of cell-free culture fluid were chromatographed. The percentages of total radioactivity in inosine plus hypoxanthine are summarized in panel B. The collected cell pellets were analyzed for radioactivity which was corrected for substrate trapped in extracellular space (1.1 µl/pellet). Average radioactivity/sample was converted to pmoles/µl cell H_2O on the basis of an intracellular H_2O space of 7.7 µl/sample. The broken line in panel A signifies the intracellular concentration of adenosine equivalents equal to the initial concentration of adenosine in the medium.

In addition, the data in Fig. 7 demonstrate that neither the inhibition of adenosine uptake nor the inhibition of adenosine deamination caused by a 10-min incubation with 1 µM dCF was significantly reversed by removal of dCF from the medium. This apparent irreversibility of the inhibition of adenosine uptake contrasts with the results reported for Ehrlich ascites cells [2], but it is consistent with the view that the inhibition of adenosine phosphorylation in dCF-treated cells was a consequence of the inhibition of adenosine deaminase. The inhibition of adenosine deaminase was difficult to reverse, probably because of the tight binding of dCF to the enzyme [1].

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