Journal of Cellular Biochemistry 46:54–59 (1991)

# $Na^+$-Dependent, Active Nucleoside Transport in S49 Mouse Lymphoma Cells and Loss in AE-1 Mutant Deficient in Facilitated Nucleoside Transport

**Peter G.W. Plagemann**

Department of Microbiology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455

**Abstract** S49 murine lymphoma cells were examined for expression of various nucleoside transport systems using a non-metabolized nucleoside, formycin B, as substrate. Nitrobenzylthioinosine (NBTI)-sensitive, facilitated transport was the primary nucleoside transport system of the cells. The cells also expressed very low levels of NBTI-resistant, facilitated nucleoside transport as well as of $Na^+$-dependent, concentrative formycin B transport. Concentrative transport was specific for uridine and purine nucleosides, just as the concentrative nucleoside transporters of other mouse and rat cells. A nucleoside transport mutant of S49 cells, AE-1, lacked both the NBTI-sensitive, facilitated and $Na^+$-dependent, concentrative formycin B transport activity, but $Na^+$-dependent, concentrative transport of α-aminoisobutyrate was not affected.

**Key words:** $Na^+$/nucleoside cotransport, AE-1 nucleoside transport mutant, S49 mouse lymphoma cells

With a few exceptions, all mammalian cells express facilitated nucleoside transport with broad nucleoside substrate specificity [1,2]. Two forms of facilitated transport can be distinguished on the basis of sensitivity to inhibition by nitrobenzylthioinosine (NBTI). One form is strongly inhibited by nanomolar concentrations of NBTI (designated NBTI-sensitive), resulting from the binding of NBTI to high affinity binding sites on the plasma membrane (Kd $\leq$ 1 nM). The other form is not associated with such binding sites and is inhibited only by micromolar concentrations of NBTI (designated NBTI-resistant). A second basic type of nucleoside transport, $Na^+$-dependent, concentrative transport, is prevalent in epithelial cells of the kidney and intestine and brush border membranes thereof [3–7]. At least two forms of concentrative nucleoside transport have been distinguished on the basis of substrate specificity. One form transports uridine and purine nucleosides; the other form transports preferentially various pyrimidine nucleosides [5]. Both concentrative transporters are highly resistant to inhibition by NBTI and various other inhibitors of facilitated nucleoside transport [4–7].

Recently, it has been shown that all types of mouse cells examined so far exhibit $Na^+$-dependent, concentrative nucleoside transport, but at different levels relative to the facilitated nucleoside transport activity in these cells [8–13]. In mouse macrophages and B and T lymphocytes, concentrative transport represents a major nucleoside transport component [10–12]. It is specific for uridine and purine nucleosides [10, 12,13], just as one of the concentrative nucleoside transporters of brush border membranes. Its Km for formycin B transport is about two orders of magnitude lower than that for the facilitated transport of formycin B [10,12]. In contrast to these cells, in different mouse cell lines, derived variously from T and B lymphocytes (P388, L1210, LK35.2), macrophages (RAW 309Cr.1), and fibroblasts (L929), $Na^+$-dependent, concentrative nucleoside transport represents only a minor transport component [11–13]. In fact, in most of these cells it is only detectable by the $Na^+$-dependent concentrative accumulation of substrate in the presence of inhibitors of facilitated nucleoside transport, such as NBTI or dipyridamole [11,12]. The concentrative substrate accumulation results from the inhibition of the efflux via facilitated transport of the nucleoside that is actively transported into the cells [11–13]. In contrast to these mouse cell lines, no $Na^+$-dependent, concentrative nucleoside transport has been de-

Received August 13, 1990; accepted December 12, 1990.

tected in human HeLa cells, three human T cell lines, Novikoff rat hepatoma cells, or a line of *Mus dunni* cells [11,12]. Facilitated nucleoside transport is the only nucleoside transport system of these cells. It is also the major nucleoside transport system of the various mouse cell lines, consisting to varying proportions of NBTI-resistant and sensitive transport [2]. The presence of three transport systems in all of these mouse cell lines complicates the isolation of nucleoside transport-deficient mutants from these cells. However, special selective conditions have been developed to isolate variants that lack one or the other of the nucleoside transport systems [13–15].

The only mouse cell line from which single step mutants have been isolated that are practically devoid of nucleoside transport activity are S49 mouse lymphoma cells [16,17]. This isolation has been possible probably because NBTI-sensitive nucleoside transport accounts for at least 99% of the transport activity of these cells [18]. A S49 mutant lacking NBTI-sensitive transport (AE-1) has been isolated from MNNG-mutagenized S49 cells by a single-step procedure in medium containing a toxic concentration of adenosine plus an inhibitor of adenosine deamination [16]. The AE-1 cells express only very low levels of NBTI-resistant facilitated nucleoside transport that is insufficient to allow growth of these cells when made nucleoside-dependent by treatment with methotrexate [16,19]. In the present study we show that S49 cells, like all other mouse cells that have been investigated, express low levels of $Na^+$-dependent, concentrative nucleoside transport and that this activity has been lost in the AE-1 mutant. One interpretation of these findings is that in various mouse cell lines $Na^+$-dependent, concentrative transport and NBTI-sensitive facilitated transport share a genetically controlled component.

## MATERIALS AND METHODS

### Cell Culture

S49 and AE-1 cells were originally provided by Dr. B. Ullman [16] and propagated in suspension culture as described previously [18]. Cells were harvested from late exponential phase cultures and suspended to (2–5) $\times$ $10^7$ cells/ml of RPMI, if not indicated otherwise. For measuring nucleoside or amino acid transport in $Na^+$-free medium, the harvested cells were first washed once in 145 mM choline chloride containing 5 mM Tris-HCl (pH 7.4; Tris-choline chloride) and then were suspended in the same or 145 mM KCl or NaCl containing 5 mM Tris-HCl (pH 7.4; Tris-KCl and Tris-NaCl, respectively). Cultures were routinely ascertained to be free of mycoplasma contamination using an adenosine phosphorylase assay with 2′-deoxyadenosine as substrate [20].

### Formycin B and α-Aminoisobutyrate Transport Measurements

Samples of cell suspension were supplemented, where indicated, with NBTI, dipyridamole or gramicidin. After 5–10 min of incubation, time courses of uptake of formycin B were measured under zero-*trans* conditions by rapid kinetic techniques using a dual syringe apparatus (12 time points/time course) or by manual sampling for longer time points as described previously [10,11,21]. The procedure involves separating the cells from the medium by rapid centrifugation through an oil layer and analyzing the pelleted cells for radioactivity. Radioactivity/cell pellet was corrected for that attributable to extracellular space in the cell pellet as estimated with [$^{14}$C]inulin [22]. Intracellular $H_2O$ space was measured with $^3H_2O$ [22]. Where indicated, unlabeled nucleosides were added simultaneously with [$^3$H]formycin B.

For estimating the kinetic parameters of facilitated formycin B transport, uptake was measured at 6 concentrations ranging from 40–1280 μM. The concentration of [$^3$H]formycin was kept constant in all samples, while the specific radioactivity was altered by addition of unlabeled formycin B. An appropriate integrated rate equation based on the simple carrier model was fitted to the time courses of uptake assuming directional symmetry and the Michaelis-Menten parameters extracted by least-squares regression [1,2,21]. The uptake of [$^{14}$C] α-aminoisobutyrate was measured as described for formycin B uptake.

### Analysis of Metabolism of Formycin B

After 60 or 90 min of incubation with [$^3$H]formycin B, cells were collected by centrifuguation through an oil layer into a solution composed of sucrose and 0.5 M trichloroacetic acid [22]. The acid extract was further processed and analyzed by ascending paper chromatography [9] using a solvent composed of 30 ml 1 M

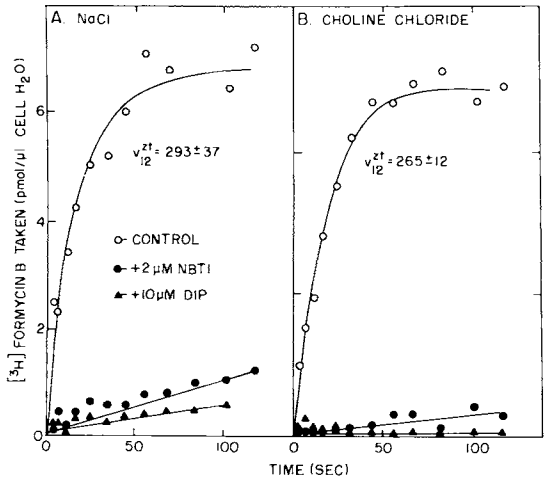


**Fig. 1.** Effects of NBTI and dipyridamole on the transmembrane equilibration of formycin B in S49 cells in Tris-NaCl **(A)** or Tris-choline chloride **(B)**. S49 cells were washed once in Tris-choline chloride and then suspended to $2 \times 10^7$ cells/ml in Tris-NaCl or Tris-choline chloride. Samples of each suspension were supplemented as indicated with 2 µM NBTI or 10 µM dipyridamole and then the uptake of 5 µM [$^3$H]formycin B (22 cpm/pmol) was measured by rapid kinetic techniques at 25°C. An integrated rate equation was fitted to the uptake time courses with Km fixed at 200 µM and the rate of zero-*trans* entry ($v_{12}^{zt}$ in fmole/µl cell water × s) was calculated as the slope to each uptake curve at t = 0 [2,19].

ammonium acetate (pH 5) and 70 ml 95% ethanol (solvent 28).

### Determination of $Na^+$ Content of Cells

Replicate samples of $2.6 \times 10^7$ cells were collected by centrifugation, washed once in Tris-choline chloride and then analyzed for $Na^+$ concentration by flame photometry (conducted by R.H. Ophaug, School of Dentristy, University of Minnesota).

### Materials

[$^3$H]Formycin B was purchased from Moravek Biochemicals (Brea, CA), [1–$^{14}$C] α-amino isobutyrate from ICN (Irvine, CA) and unlabeled nucleosides and gramicidin from Sigma (St. Louis, MO).

## RESULTS AND DISCUSSION

First, we determined by rapid kinetic techniques the uptake of 5 µM formycin B by S49 cells in NaCl and choline chloride based media (*Fig. 1*). Formycin B, a C-analog of inosine, was used as substrate because: 1) it is only poorly phosphorylated in mammalian cells and not metabolized in any other way, so that its transmembrane equilibration can be measured unimpeded by intracellular metabolic conversions [4,10,21]; and 2) it is a good substrate for both the facilitated nucleoside transporters and the $Na^+$-dependent, active nucleoside transporter of various mammalian cells [4,10,12,13,21]. The Km for formycin transport at 25°C for the two types of transporters are 200–300 µM and about 5 µM, respectively [10,12,21]. The kinetic parameters for facilitated formycin B transport at 25°C by S49 cells determined in the present study were Km = 175 ± 15 µM, and Vmax = 22.3 ± 0.7 pmol/µl cell water × s.

The transmembrane equilibration of formycin B in S49 cells was about the same whether the cells were suspended in Tris-NaCl or washed and suspended in Tris-choline chloride (Fig. 1A,B). Furthermore, in agreement with earlier studies in which uridine was used as substrate [2], formycin B transport in the NaCl medium was inhibited about 97% by 2 µM NBTI (Fig. 1A), confirming that NBTI-sensitive facilitated nucleoside transport was the primary transport system of these cells. Formycin B transport in the NaCl medium was inhibited about 98% by 10 µM dipyridamole (Fig. 1A). However, formycin B transport was even more inhibited by NBTI and dipyridamole when the cells were suspended in Tris-choline chloride (99% and 99.5%, respectively; Fig. 1B). This finding suggested the potential presence of a low level of $Na^+$-dependent, NBTI/dipyridamole-resistant nucleoside transport in these cells.

The operation of such a system in S49 cells is confirmed by the finding that upon long-term incubation in presence of dipyridamole, formycin B accumulated concentratively in these cells (Fig. 2A). In untreated cells, formycin B had equilibrated across the plasma membrane at 1 min of incubation at 37°C, the first time point taken, in agreement with the data in Figure 1A. Formycin B accumulated slightly against a concentration gradient, but this finding is typical for all purine nucleosides in all cells that have been investigated and occurs independent of the presence of an active transporter [23,24]. It seems to reflect binding of these nucleosides to some intracellular component(s) and has not been observed with pyrimidine nucleosides [24]. The presence of 10 µM dipyridamole caused an inhibition of the initial influx of formycin B, but upon continued incubation formycin B eventually accumulated to thrice the extracellular concentration (Fig. 2A). This finding has been con-

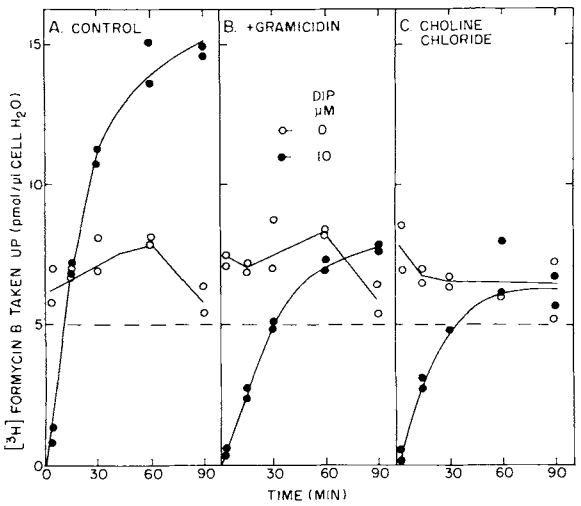


Fig. 2. Effect of dipyridamole on the long-term uptake of formycin B by control (A), gramicidin-treated (B) and $Na^+$-depleted (C) S49 cells. S49 cells were suspended to $2 \times 10^7$ cells/ml in RPMI which contained, in B, 50 μg gramicidin/ml and the suspensions were incubated at 37°C for 15 min. In C, the cells were washed in Tris-choline chloride and then suspended in the same. One half of each suspension was supplemented as indicated with 10 μM dipyridamole and then the uptake of 5 μM [$^3$H]formycin B (42 cpm/pmol) was measured at 37°C. The broken lines indicate the intracellular concentration of formycin B equal to that in the medium.

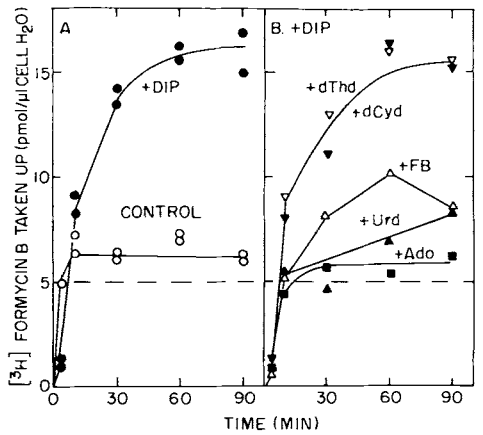


Fig. 3. Effects of nucleosides on the concentrative accumulation of formycin B by dipyridamole-treated S49 cells. A suspension of $3.8 \times 10^7$ S49 cells/ml of RPMI was supplemented with 10 μM dipyridamole (DIP, ●—●), except for a small portion which remained untreated (○—○). Then the uptake of 5 μM [$^3$H]formycin B (46 cpm/pmol) was measured at 37°C. Where indicated, 100 μM unlabeled formycin B (FB, △—△), uridine (Urd, ▲—▲), adenosine (Ado, ■—■), thymidine (dThd, ▽—▽) or deoxycytidine (dCyd, ▼—▼) was added to dipyridamole containing suspensions in B simultaneously with the [$^3$H]formycin B. All points represent averages of duplicate samples of cell suspension. The results in A are for two independent cell suspensions. The broken lines indicate the intracellular concentration of formycin B equal to that in the medium.

sistently observed in repeated experiments (Fig. 3 and data not shown). The concentrative accumulation of formycin B in dipyridamole-treated S49 cells was inhibited by treatment of the cells with gramicidin, a $Na^+/K^+$ ionophore (Fig. 2B), and was not observed when the cells were suspended in Tris-choline chloride (Fig. 2C). These results are comparable to those observed with all other mouse cell lines that have been investigated [11,12].

We have examined the substrate specificity of the $Na^+$-dependent, concentrative nucleoside transporter of S49 cells by determining the effects of various unlabeled nucleosides, all at a concentration of 100 μM, on the concentrative uptake of 5 μM [$^3$H]formycin B by dipyridamole-treated S49 cells. Under the conditions of the experiment, [$^3$H]formycin B uptake was strongly inhibited by unlabeled formycin B, uridine, and adenosine, whereas thymidine and deoxycytidine were without effect (Fig. 3B). The effectiveness of inhibition of uptake was adenosine > uridine > formycin B, which was the same as that reported for $Na^+$-dependent, concentrative formycin B transport in rat and mouse macrophages and mouse lymphocytes [10–12]. In the presence of 100 μM adenosine, formycin B accumulated in dipyridamole-treated S49 cells to only about the same level (Fig. 3B) as observed in cells not treated with dipyridamole (Fig. 3A). The results indicate that the concentrative nucleoside transporter of S49 cells exhibits the same substrate specificity as those observed in other mouse and rat cells.

Compared with wild-type S49 cells, the influx of 5 μM formycin B in the transport mutant AE-1 was very slow (3 fmol/μl cell water × s; see Fig. 4C). Transmembrane equilibration of formycin B in these cells was minimally affected by 10 μM dipyridamole or treatment with gramicidin (Fig. 4A) and was about the same in media containing $Na^+$ or $K^+$ (Fig. 4B). Dipyridamole clearly did not cause a concentrative accumulation of formycin B as in wild-type S49 cells. Thus, there was no evidence that the residual formycin B influx in these cells involves $Na^+$-dependent, concentrative transport. This conclusion is supported by the finding that the *residual* influx of formycin B in these cells was inhibited by thymidine (Fig. 4C), which is not a substrate for the $Na^+$-dependent, concentrative transporter commonly found in mouse and rat cells (Fig. 3B) [10–12]. Inhibition of formycin B influx by both thymidine and uridine and by di-

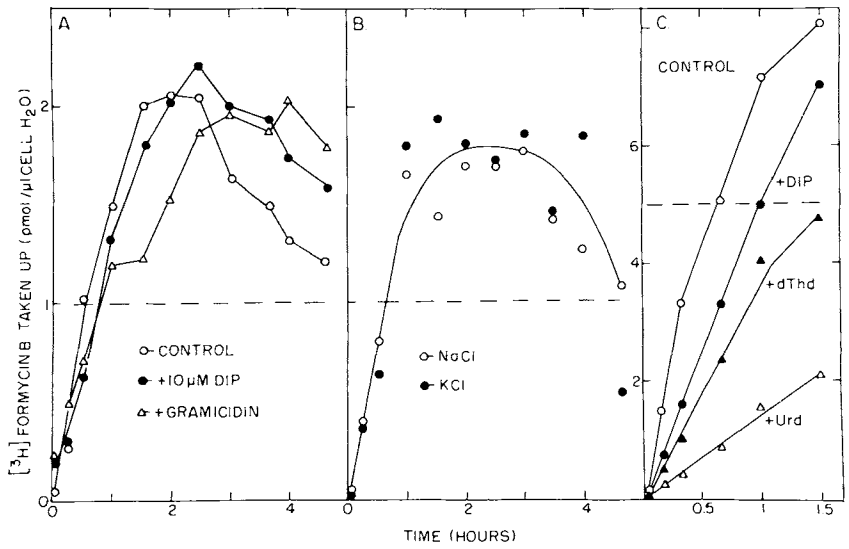


**Fig. 4.** Formycin B uptake by AE-1 cells under various experimental conditions. **A:** Samples of a suspension of 3.2 × $10^7$ cells/ml of RPMI were supplemented as indicated with 10 μM dipyridamole or 100 μg gramicidin/ml or **B:** the cells were washed once in Tris-choline chloride and then suspended in the same. Then the uptake of 1 μM [$^3$H]formycin B (280 cpm/pmole) was measured at 37°C. **C:** The uptake of 5 μM [$^3$H]formycin B (46 cpm/pmole) was measured at 37°C in samples of a suspension of 2.5 × $10^7$ cells/ml of RPMI. Where indicated, 20 μM dipyridamole or 1 mM uridine or thymidine were added simultaneously with the [$^3$H]formycin B. The broken lines indicate the intracellular concentration of formycin B equal to that in the medium.

pyridamole (Fig. 4C) is in agreement with our previous results, which led to the conclusion that the residual nucleoside transport in the AE-1 mutant reflects NBTI-resistant, facilitated transport in addition to non-mediated permeation [18].

In contrast to the loss of $Na^+$-dependent, concentrative nucleoside transport in AE-1 cells, the $Na^+$-dependent transport of α-aminoisobuytrate [25] was about the same in AE-1 and wild type S49 cells (Fig. 5). Moreover, the $Na^+$ content of both types of cells was about the same, 66 ± 6 and 64 ± 2 mM, respectively. These results indicate that the lack of $Na^+$-dependent, concentrative nucleoside transport in AE-1 cells was not simply due to disturbance of the transmembrane $Na^+$ gradient in these cells.

The simultaneous loss by AE-1 cells of both NBTI-sensitive, facilitated and $Na^+$-dependent, concentrative nucleoside transport is unexpected and difficult to explain at present, since the mutant has been isolated from the parent S49 cells by a single-step selection procedure [16]. One could argue that the loss of the $Na^+$-dependent, concentrative transporter was a secondary event that occurred during the continuous cultivation of these cells for at least 1 year, which was interspersed by two periods of storage in liquid $N_2$. However, the parent S49 cells were cultured and stored in the same manner without loss of either system and all mouse cell lines cultured in our laboratory under the same conditions have retained both facilitated and

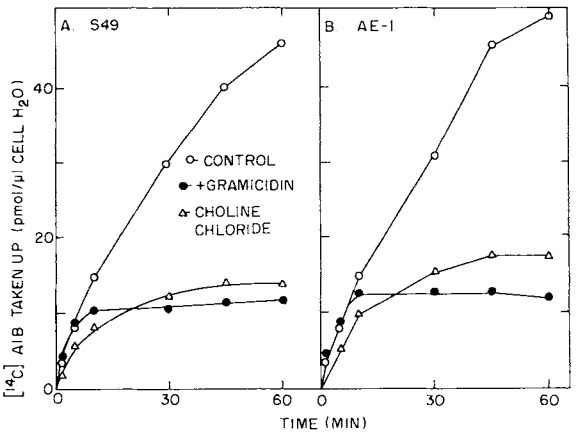


**Fig. 5.** Comparison of uptake of α-aminoisobutyrate by S49 and AE-1 cells. Samples of suspensions of 4 × $10^7$ S49 or AE-1 cells/ml of balanced salt solution were supplemented, where indicated, with 100 μg gramicidin/ml and incubated for 10 min at 37°C. Other samples of cells were washed once in Tris-choline chloride and suspended to the above density in the same. Then the uptake of 8 μM [$^{14}$C] α-aminoisobutyrate (AIB) was measured as described for formycin B uptake. All points represent averages of duplicate samples of cell suspension.

concentrative transport systems [11]. Since no information is available on the frequency of loss of either NBTI-sensitive, facilitated nucleoside transport or $Na^+$-dependent, concentrative nucleoside transport in mutagenized S49 cells, another related possibility is that the AE-1 cells represent a double mutant that was selected under the single-step selection procedure. On the other hand, an alternate possibility is that the two transport systems share a genetic component. Resolution of this question will probably be supplied by amino acid sequence determinations of these transporters.

## ACKNOWLEDGMENTS

I thank Dr. R.H. Ophaug for conducting the $Na^+$ measurements, Laurie Erickson for excellent technical assistance, and Colleen O'Neill for typing the manuscript. This work was supported by USPHS research grant GM 24468.

## REFERENCES

1. Plagemann PGW, Wohlhueter RM: Curr Top Membr Transp 14:225, 1980.
2. Plagemann PGW, Wohlhueter RM: Biochim Biophys Acta 947:405, 1988.
3. LeHir M, Dubach UC: Pflügers Arch 404:238, 1985.
4. Jacobs ES, Paterson ARP: Biochem Biophys Res Comm 14:1028, 1986.
5. Vijayalakshmi D, Belt J: J Biol Chem 263:19419, 1988.
6. Lee CW, Cheeseman CJ, Jarvis SM: Biochim Biophys Acta 942:139, 1988.
7. Jarvis SM: Biochim Biophys Acta 979:132, 1989.
8. Darnowski JW, Holdridge C, Handschuhmacher RE: Cancer Res 47:2614, 1987.
9. Plagemann PGW, Woffendin C: Biochim Biophys Acta 981:315, 1989.
10. Plagemann PGW, Aran JM, Woffendin C: Biochim Biophys Acta 1022:93, 1990.
11. Plagemann PGW, Aran JM: Biochim Biophys Acta, 1025:32, 1990.
12. Plagemann PGW, Aran JM: Biochim Biophys Acta, 1028:289, 1990.
13. Crawford CR, Ng CYC, Noel LD, Belt JA: J Biol Chem 265:9732, 1990.
14. Belt JA, Noel LD: J Biol Chem 263:13819, 1988.
15. Dagnino L: PhD Thesis, Dep Pharmac, Univ Alberta, 1988.
16. Cohen A, Ullman B, Martin DW Jr: J Biol Chem 254: 112, 1979.
17. Aranow B, Toll D, Patrick J, McCartan K, Ullman B: J Biol Chem 261:14467, 1986.
18. Plagemann PGW, Woffendin C: Mol Cell Biol 7:160, 1987.
19. Plagemann PGW, Wohlhueter RM: Biochim Biophys Acta 855:25, 1986.
20. Plagemann PGW, Woffendin C: J Cell Biochem 43:161, 1990.
21. Plagemann PGW, Woffendin C: Biochim Biophys Acta 1010:7, 1989.
22. Wohlhueter RM, Plagemann PGW: Methods Enzymol 173:714, 1989.
23. Plagemann PGW, Wohlhueter RM: Cancer Res 45: 6418, 1985.
24. Plagemann PGW, Woffendin C: Mol Pharmacol 36:185, 1989.
25. Lever JE: Proc Natl Acad Sci USA 73:2614, 1976.