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Characterization of Na^+-dependent, active nucleoside transport in rat and mouse peritoneal macrophages, a mouse macrophage cell line and normal rat kidney cells

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Peritoneal rat macrophages expressed solely an Na^+-dependent, concentrative nucleoside transporter, which possesses a single Na^+-binding site and transports purine nucleosides and uridine but not thymidine or deoxycytidine. The Michaelis-Menten constants for formycin B and Na^+ were about 6 μm and 14 mM, respectively, and the estimated Na^+:formycin B stoichiometry was 1:1. Rat macrophages accumulated 5 μM formycin B to a steady-state level exceeding that in the medium by about 500-fold during 60 min of incubation at 37°C. Concentrative formycin B transport was resistant to inhibition by nitrobenzylthioinosine, lidoflazine, dilazep and nifedipine, but was slightly inhibited by high concentrations of dipyridamole (> 10 μM) and probenicid (> 100 μM). Mouse peritoneal macrophages and lines of mouse macrophages and normal rat kidney cells expressed Na^+-dependent, active nucleoside transport but in addition significant Na^+-independent, facilitated nucleoside transport. Facilitated nucleoside transport in these cells was sensitive to inhibition by nitrobenzylthioinosine, dilazep and dipyridamole. The presence of these inhibitors greatly enhanced the concentrative accumulation of formycin B by these cells by inhibiting the efflux via the facilitated transporter of the formycin B actively transported into the cells. Whereas rat macrophages lacked high-affinity nitrobenzylthioinosine-binding sites, mouse macrophages and normal rat kidney cells possessed about 10000 such sites/cell. Rat and mouse erythrocytes, rat lymphocytes, and lines of Novikoff rat hepatoma cells, Chinese hamster ovary cells, *Mus dunni* cells and embryonic monkey kidney cells expressed only facilitated nucleoside transport.

Introduction

Two principle types of nucleoside transport have been recognized in mammalian cells [1–3]: (1) facilitated, non-concentrative transport with broad substrate specificity, which has been found in almost all types of cell investigated, and (2) Na^+-dependent, concentrative nucleoside transport, which has been detected in epithelial cells or brush-border membranes of rat, mouse and rabbit kidney and intestine [4–9], as well as in rabbit choroid plexus [10,11] and mouse spleen cells [12–14]. Facilitated transport exists in two forms; one form is strongly inhibited by nanomolar concentrations of nitrobenzylthioinosine (NBTI; designated NBTI-sensitive), whereas the other form is only inhibited by micromolar concentrations of NBTI (designated NBTI-resistant). Both forms are about equally sensitive to most other nucleoside transport inhibitors such as dipyridamole [2,15]. In contrast, concentrative nucleoside transport seems generally to be resistant to inhibition by NBTI and dipyridamole [4–14].

The presence of concentrative nucleoside transporters in epithelial cells of the kidney and intestine probably serves an important function in nutrient adsorption as suggested by their localization in brush-border membranes, but the potential physiological function of concentrative nucleoside transport in choroid plexus and mouse lymphocytes is unclear. In addition, recent studies have shown that all of five mouse cell lines, but not human HeLa cells, three human T lymphocyte cell lines or pig spleen lymphocytes, exhibit low levels of concentrative formycin B transport, in addition to high levels of facilitated transport [16]. The latter was detectable only in the mouse cell lines by the concentrative accumulation of formycin B during long-term incubation in the presence of dipyridamole [16]. Dipyridamole exerts this effect by inhibiting the rapid efflux via the facilitated carrier of the formycin B taken up via the dipyridamole-resistant, active carrier. In ad-

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dition, preliminary experiments showed that active nucleoside transport represents a major transport component in mouse macrophages [16] just as it does in mouse lymphocytes [13,14]. In the present study we have investigated in greater detail the characteristics of the concentrative nucleoside transport systems of mouse and rat macrophages and a number of additional cell lines from various species.

We have used formycin B as a substrate in a recent [14] and the present study, since it has been shown to be an efficient substrate for both the Na^+-dependent, concentrative [6,7,14] and facilitated [17] nucleoside transporters of mammalian cells, but to be only very poorly phosphorylated by these cells and resistant to enzymatic phosphorolysis [6,17]. Thus, it is an ideal substrate for measuring concentrative and facilitated nucleoside transport unimpeded by metabolic reactions.

Experimental procedures

Cells. Adult Swiss mice and rats were obtained from Biolabs (St. Paul, MN) and were injected with 2–3 or 10–20 ml, respectively, of thioglycollate medium. 2–4 days later peritoneal macrophages were harvested from these animals and cultured in 24-well tissue culture plates (2 cm^2/well) with RPMI plus 10% (v/v) fetal bovine serum and 10% (v/v) L-cell-conditioned medium as described elsewhere [18]. Except where indicated otherwise, the cultures were used for transport assays after 1–2 days of incubation with one medium change.

Spleens and blood were obtained from the thioglycollate medium-injected rats or from groups of 40–60 Swiss mice used in other studies [19]. Single cell suspensions of spleen cells free of erythrocytes were prepared in phosphate buffered saline (pH 7.4) containing 5 mM glucose (PBS-glucose) as described previously [13,14]. Erythrocytes were washed three times in cold saline containing 5 mM Tris-HCl (pH 7.4) (Tris-NaCl).

Normal rat kidney (NRK) cells and RAW 309 Cr.1 cells (an Abelson leukemia virus-transformed mouse macrophage line) were obtained from the American Type Culture Collection and lines of *Mus dunni* cells [20], of MA-104 embryonic monkey kidney cells [21] and human CEM T lymphocytes from Drs. J. Coffin, P. Jarling, and B. Chesebro, respectively. All these cell lines were routinely propagated in tissue culture T-flasks with RPMI plus 5% (v/v) fetal bovine serum and 5% (v/v) newborn calf serum [19]. For transport or NBTI binding studies CEM cells were propagated in 2 liter roller bottles, whereas all the other cell lines cited above were seeded into 24-well culture plates and the cultures used when confluent.

Novikoff rat hepatoma (N1S1-67) and Chinese hamster ovary (CHO) cells were propagated in suspension culture on a gyrotory shaker as described previously [22]. All cell lines were routinely examined for mycoplasma contamination by assaying cells and culture fluid for adenosine phosphorylase activity [23]. All cells used in the present study were free of mycoplasma.

Formycin B transport measurements. Cells were suspended in basal medium 42B (BM42B; Ref. 24), RPMI or PBS-glucose or overlayed with RPMI when assayed in 24-well plates and they were pretreated with various inhibitors, as indicated in appropriate experiments. Or, the cells were first washed with isotonic choline chloride containing 5 mM Tris-HCl (pH 7.4) (Tris-choline chloride) and then suspended in or overlayed with different media. Where indicated, cells were depleted of ATP by incubation in glucose-free medium containing 5 mM KCN and 5 mM iodoacetate at 37 °C for 10–20 min [25]. Time-courses of uptake of [^{3}H]formycin B were measured under zero-*trans* conditions at 25 °C or 37 °C. Formycin B uptake by suspensions of cells was measured using a dual syringe apparatus (12 time points/time-course) or manual sampling for longer time points as described previously [1,2,26,27]. The procedure involves separating the cells from the medium by rapid centrifugation through an oil layer and analyzing the cell pellet for radioactivity. Radioactivity/cell pellet was corrected for that attributable to extracellular space as estimated with [^{14}C]inulin [26]. Intracellular H_2O space was measured with ^3H_2O [26]. Cells in suspensions were enumerated in a Coulter counter.

Formycin B uptake by adhering cells propagated in 24-well tissue culture plates was measured by adding, at timed intervals, [^{3}H]formycin to the desired concentration to 6 or 11 wells in a plate. At the completion of the incubation, the medium was dumped out and the wells were rinsed rapidly three times (within 15 s) with ice-cold balanced salt solution (BSS). For kinetic analyses, the concentration of [^{3}H]formycin B was kept constant in all samples of an experiment, while the specific radioactivity was altered by addition of unlabeled formycin B. Cell densities were estimated by counting the cells in several fields of an inverted microscope and converting this number to cells/well on the basis of the areas of the microscopic field and the well [18].

Equilibrium binding of NBTI. The equilibrium binding of [^{3}H]NBTI to cells in suspension was determined as described previously [27]. An equation corresponding to a single, saturable binding site plus a non-specific binding component was fitted to the data. NBTI binding to adhering cells was measured in 24-well plates. Cultures of cells in duplicate wells were overlayed with RPMI (0.5 ml/well) containing [^{3}H]NBTI to final concentrations of 0.025 to 21 nM. After 20 min of incubation the culture fluid was analyzed for radioactivity. The wells were rinsed three times with ice-old BSS and the rinsed cells analyzed for radioactivity. Measured radioactivity was converted to concentrations of free ligand (Lf) and bound ligand (Lb), respectively, and the data were analyzed by the method of Scatchard [28].

Materials. ^{3}H-labeled nucleosides, hypoxanthine and NBTI were purchased from Moravek Biochemicals (Brea, CA) and diluted to the desired specific radioactivity with unlabeled substrate. Unlabeled nucleosides, ouabain and gramicidin (from *Bacillus brevis*), were obtained from Sigma (St. Louis, MO) and unlabeled NBTI from Calbiochem (San Diego, CA). Dipyridamole (Persantin) was a gift from Geigy Pharmaceuticals (Yonkers, NY).

Results

Na^+-dependent active formycin B transport by rat and mouse peritoneal macrophages

The results in Fig. 1A show that rat macrophages overlayed with isotonic Tris-NaCl rapidly accumulated 5 μM formycin B to concentrations greatly exceeding those in the medium. In fact, after 5 min of incubation at 25 °C, the intracellular concentration of formycin B exceeded that in the medium about 50 fold (Fig. 1A) and after 60 min of incubation at 37 °C about 500 fold (Fig. 1C). Chromatographic analysis of acid extracts of the cells indicated that the formycin B accumulated concentratively in an unmodified form; < 10% of the radioactivity present in cells incubated with 5 μM [^{3}H]formycin B for 90 min at 37 °C was associated with nucleotides and > 90% co-migrated with authentic formycin B. The concentrative uptake of formycin B was Na^+-dependent (Fig. 1A). A Michaelis-Menten fit of initial rates of uptake at various concentrations of Na^+ (v_{Na}) yielded a Michaelis-Menten constant for Na^+ (K_{Na}) of about 14 mM. This value is similar to those reported for Na^+-dependent, concentrative nucleoside transport in mouse lymphocytes [13,14] and brush-border membrane vesicles [8,9]. We have also analyzed the data in the Eadie Scatchard plot of the Hill equation [28]:

$$v_{Na}/[Na^+]^n = v_{Na}/K_{Na} + V_{max}/K_{Na}$$

where n = Hill coefficient. The linearity of the plot $v_{Na}/[Na^+]$ vs. v_{Na} (Fig. 1B) indicates $n = 1$. Combined the results indicate independent binding sites for Na^+ and nucleosides and a minimum Na^+ : nucleoside substrate stoichiometry of 1 : 1.

The Na^+-dependence of formycin B uptake by rat macrophages was further proven by the complete inhibition of the concentrative accumulation of formycin B by pretreatment of the cells with the ionophore gramicidin (50 μg/ml: Fig. 1C), which abolishes Na^+ and K^+ gradients across the plasma membrane [29]. Furthermore, no concentrative uptake of 5 μM for-

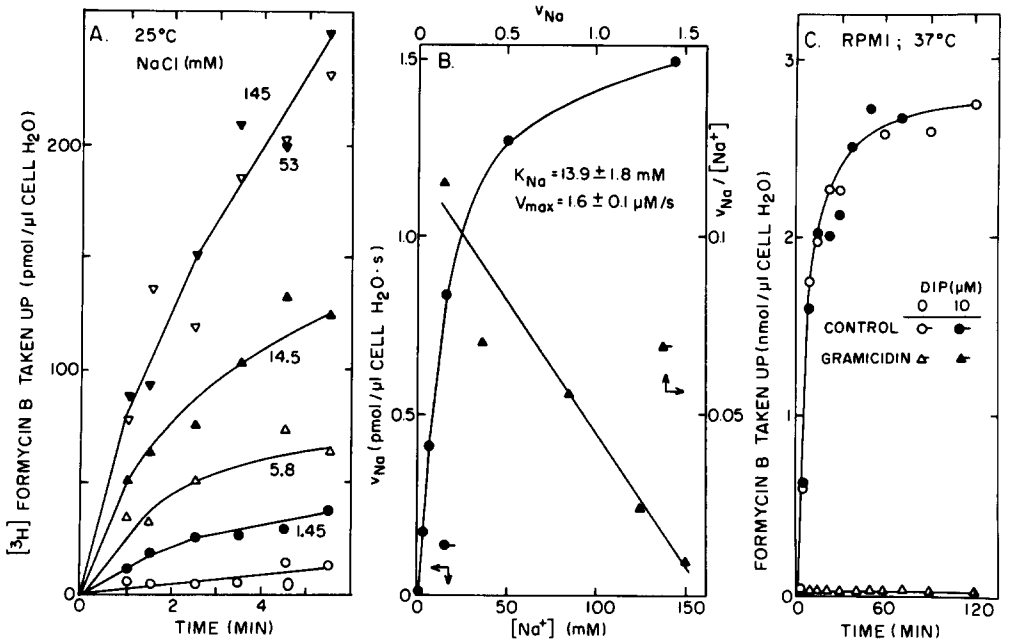

Fig. 1. Na^+-dependency of concentrative uptake of formycin B by rat macrophages. (A) Cultures of rat macrophages in 24-well plates ($3.3 \cdot 10^4$ cells/well) were rinsed once with Tris-choline chloride and then overlayed (0.5 ml/well) with mixtures of Tris-choline chloride and Tris-NaCl to the indicated final concentrations of NaCl. Then the uptake of 5 μM [^{3}H]formycin B (80 cpm/pmol) was measured at 25 °C as described under Experimental procedures. Cell-associated radioactivity/well was converted to pmol/μl cell water on the basis of the specific radioactivity of formycin B, the cell density and a cell water volume of 1.5 μl/10^6 cells. (B) Initial velocities of uptake (v_{Na}) are plotted as a function of the Na^+ concentration (●———●) and v_{Na}/Na^+ (▲———▲; see text). K_{Na} was calculated by fitting the Michaelis-Menten equation to the v_{Na} values. (C) Cultures of rat macrophages ($6.6 \cdot 10^4$ cells/well) were overlayed with RPMI (0.5 ml/well), which contained where indicated 50 μg gramicidin/ml, and incubated at 37 °C for 15 min. Then one half of the wells in each group was supplemented with 10 μM dipyridamole and the uptake of 5 μM [^{3}H]formycin B (106 cpm/pmol) was measured at 37 °C as in (A) except over a longer time period.

mycin B was detectable whether Tris-choline chloride washed rat macrophage cultures were overlayed with Tris-choline chloride (Fig. 1A), with Tris-KCl or with Tris-LiCl (all at 0.145 M; data not shown).

The finding that little if any formycin B uptake was observed in rat macrophages in the absence of Na^+ (Fig. 1A) or after pretreatment with gramicidin (Fig. 1C) indicates that Na^+-dependent, concentrative nucleoside transport is the predominant nucleoside transport system in rat macrophages. This is also indicated by the finding that the presence of 10 μM dipyridamole did not enhance the concentrative accumulation of 5 μM formycin B by rat macrophages (Fig. 1C). This finding contrasts sharply with results obtained with mouse macrophages and lymphocytes and other cell types that exhibit both Na^+-dependent, concentrative and facilitated nucleoside transport (Refs. 14,16 and see later Figs. 5A, 9 and 10).

Earlier studies had indicated that Na^+-dependent, concentrative nucleoside transport systems are highly resistant to inhibition by NBTI as well as other inhibitors of facilitated nucleoside transport [4–14]. Our studies with mouse spleen cells indicated that Na^+-dependent formycin B transport, though completely resistant to 2.5 μM NBTI, might be slightly inhibited by dipyridamole [14], but in cells exhibiting considerable facilitated as well as concentrative transport, inhibitor effects on the two transport systems are difficult to distinguish unequivocally. The finding that formycin B influx in rat macrophages is primarily via Na^+-dependent, concentrative transport made it possible to assess the effects of potential inhibitors on active formycin B transport more directly. The results showed that, although concentrative formycin B transport was not significantly affected by 5 μM NBTI, 50 μM dilazep, 10 μM lidoflazine, and 10 μM nifedipine, inhibitors of facilitated nucleoside transport [2], it was slightly inhibited in a concentrative-dependent manner by dipyridamole (data not shown; see later Fig. 6; $IC_{50} \geq 10$ μM), but less effectively than facilitated nucleoside transport in other rat cells ($IC_{50} = 0.3–1$ μM; Refs. 2, 15). Probenicid, which inhibits the active exit transport of cyclic AMP [1] had relatively little effect on the concentrative transport of 5 μM formycin B. The maximum inhibition by 100–250 μM probenicid was 35% (data not shown). The failure of NBTI to inhibit formycin B uptake correlated with a lack of detectable high-affinity NBTI-binding sites on rat macrophages (Fig. 2); only low-level, non-saturable binding was observed in two independent experiments, just as in Novikoff rat hepatoma cells that lack high affinity NBTI-binding sites (Ref. 2; see Fig. 2). The failure to detect high affinity NBTI binding to rat macrophages was not due to the low cell density of the macrophage cultures in 24-well plates, since high affinity binding could readily be detected in similar cultures of mouse

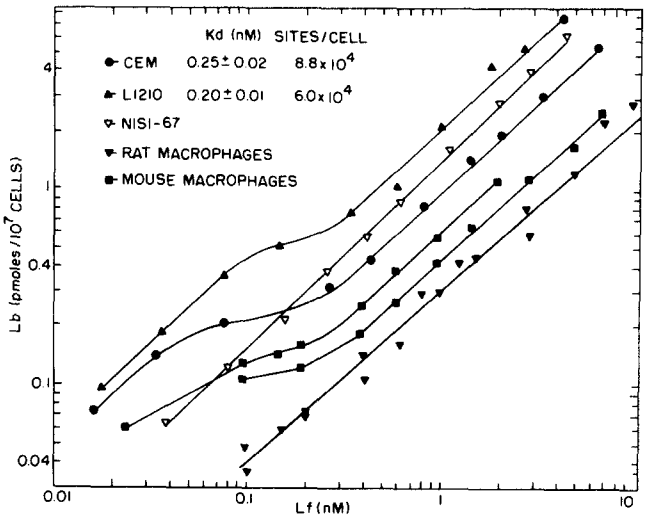

Fig. 2. Amounts of [^{3}H]NBTI bound (Lb) at equilibrium by the indicated types of cells as a function of the concentration of free NBTI (Lf). The equilibrium binding of [^{3}H]NBTI at concentrations ranging from 0.1 to 21 nM or 0.025 to 2 nM was determined as described under Experimental procedures. The values for rat and mouse macrophages are from two independent experiments. The cell densities were as follows: $8.5 \cdot 10^6$ CEM cells/ml; $2.5 \cdot 10^7$ L1210 cells/ml, $1.2 \cdot 10^7$ N1S1-67 cells/ml, $1.3 \cdot 10^5$ rat macrophages/well, and $2.5 \cdot 10^5$ mouse macrophages/well.

macrophages and NRK cells (Fig. 2 and data not shown). However, the low cell density of the adhering cell cultures precluded accurate kinetic analyses of NBTI binding by the latter cells. Nevertheless, the NBTI binding curves were similar to those obtained in companion experiments for mouse L1210 and human CEM leukemia cells (Fig. 2).

The concentrative influx of 5 μM [^{3}H]formycin B by rat macrophages was reduced about 85% by the presence of 100 μM unlabeled formycin B, indicating saturability of active formycin B transport in these cells at a relatively low concentration of formycin B. It was also reduced about 70% by 100 μM tubercidin, 92% by 100 μM uridine and 99% by 100 μM inosine or guanosine, but not significantly affected by 100 μM thymidine or deoxycytidine (data not shown). The substrate specificity of the concentrative transporter of rat macrophages was further explored by measuring the uptake of various ^{3}H-labeled nucleosides and of hypoxanthine (all at 2 μM) by these cells (Fig. 3). The macrophages concentratively accumulated formycin B, uridine, guanosine, deoxyadenosine and inosine but not thymidine, deoxycytidine or hypoxanthine. Thus, the substrate specificity of the active transporter of rat macrophages resembles that of one of the concentrative transporters of mouse intestinal epithelial cells [7]. Chromatographic analysis of acid extracts of rat macrophages that had been incubated with 2 μM [^{3}H]uridine, [^{3}H]guanosine or [^{3}H]inosine showed that less than 20% of the intracellular radioactivity was associated with

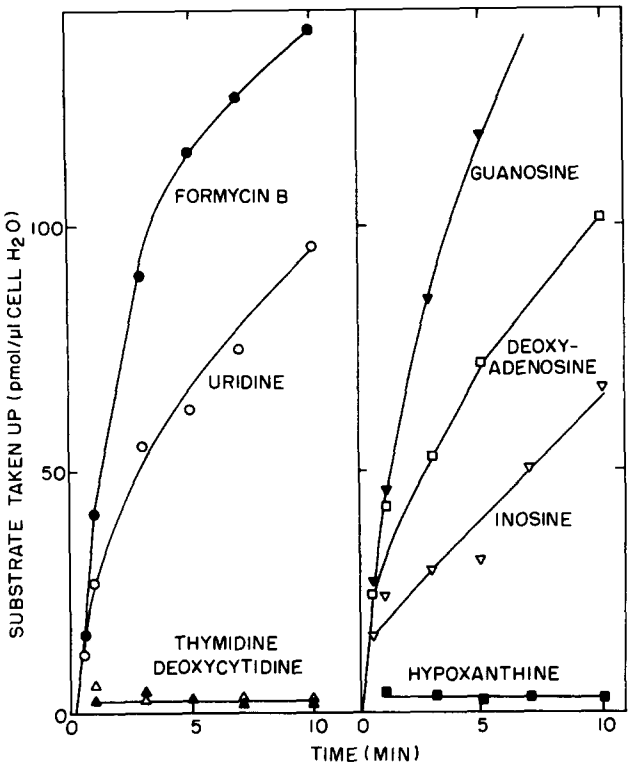

Fig. 3. Concentrative uptake of various nucleosides by rat macrophages. Cultures of rat macrophages in 24-well plates were overlayed with RPMI (0.5 ml/well) and then the uptake of 2 μM [^{3}H]formycin B, [5-^{3}H]uridine, [*methyl*-^{3}H]thymidine, [5-^{3}H]deoxycytidine, [2-^{3}H]inosine, [2-^{3}H]deoxyadenosine, [8-^{3}H]guanosine or [2-^{3}H]hypoxanthine (150–400 cpm/pmol) was measured at 25 °C as described in the legend to Fig. 1A.

nucleotides or nucleobasis (data not shown). Thus, these nucleosides were concentratively accumulated by the cells in an unmodified form. Furthermore, other experiments showed that the concentrative uptake of guanosine and uridine was completely inhibited in gramicidin-treated cells (data not shown).

Results from a few comparable studies with mouse macrophages are illustrated in Fig. 4. As in rat macrophages, the concentrative uptake of 5 μM formycin B by mouse macrophages was reduced by treatment with gramicidin or the absence of Na^+ (Fig. 4C). Thus, at a concentration of 5 μM formycin B entry was primarily via concentrative transport. Furthermore, formycin B uptake was similarly affected by various nucleosides (all at 100 μM; Fig. 4B) as that in rat macrophages. On the other hand, various inhibitors of facilitated nucleoside transport all enhanced the concentrative accumulation of 5 μM formycin B (Fig. 4A), because mouse macrophages exhibit both facilitated and concentrative transport and the inhibitors preferentially inhibited the efflux of the formycin B entering the cells via the concentrative transport system. As discussed already, the presence of a NBTI-sensitive facilitated nucleoside transporter in mouse macrophages was confirmed by demonstrating the presence of high affinity NBTI-binding sites on the cells (Fig. 2).

The initial rates of uptake of formycin B were measured as a function of the formycin B concentration in rat macrophages in the presence of an excess of Na^+ (Fig. 5A); the concentration of [^{3}H]formycin B was kept constant while the absolute formycin B concentration was adjusted by addition of unlabeled formycin B. A Michaelis-Menten plot of the initial velocities (v_0) yielded a K_m for formycin B of 3.3 μM (Fig. 5B), but somewhat higher values were obtained in two additional independent experiments in which [^{3}H]formycin B uptake was measured over lower concentration ranges (K_m = 10.6 and 6.4 μM; Fig. 5B). The results show that the affinity of formycin B for the concentrative nucleoside transporter is about 50-times higher than for the facilitated transporter ($K_m \approx 300$ μM; Refs. 16, 17).

Earlier studies have indicated that the facilitated transport of nucleosides by cultured cells changes little, if at all, with the growth stage of the cells, which contrasts with the activities of some enzymes which do change with the growth stage of the cells [1,2,30]. Concentrative nucleoside transport may fall in the latter category. The results in Fig. 6 indicate that the Na^+-dependent, concentrative transport of formycin B by rat macrophages decreased close to 70% during 4 days in culture. This decrease was not associated with a significant loss of cells or any obvious morphological changes of the cells. Whether the decrease was due to loss of concentrative transporter per se or mediated through changes in Na^+-gradients or other indirect factors cannot be decided at present.

When rat and mouse macrophages were loaded with [^{3}H]formycin B, then rinsed thrice with cold BSS and overlayed with 1.5 ml RPMI/well, the [^{3}H]formycin B was rapidly lost from both types of macrophage (Fig. 7). The $t_{1/2}$ at 25 °C was 0.5–1 min. The extracellular fluid space exceeded the intracellular space about 15 000-times and thus effectively acted as a sink for the formycin B released from the cells. Efflux of formycin B from rat macrophages was accelerated by the presence of 100 μM uridine and formycin B in the extracellular fluid (the *trans*-side) but not by 100 μM thymidine (Fig. 7C) which is not a substrate for the concentrative nucleoside transporter. Efflux was also little, if at all, affected by 10 μM dipyridamole or 50 μM dilazep (Fig. 7C).

Concentrative and facilitated nucleoside transport in NRK and RAW 309 Cr. 1

NRK (normal rat kidney) and RAW 309 Cr. 1 (mouse macrophage line) cells exhibited both Na^+-dependent, concentrative and facilitated nucleoside transport. This conclusion is indicated by the following findings illustrated for RAW 309 Cr.1 cells. (1) The cells concentratively accumulated 5 μM formycin B in the pres-

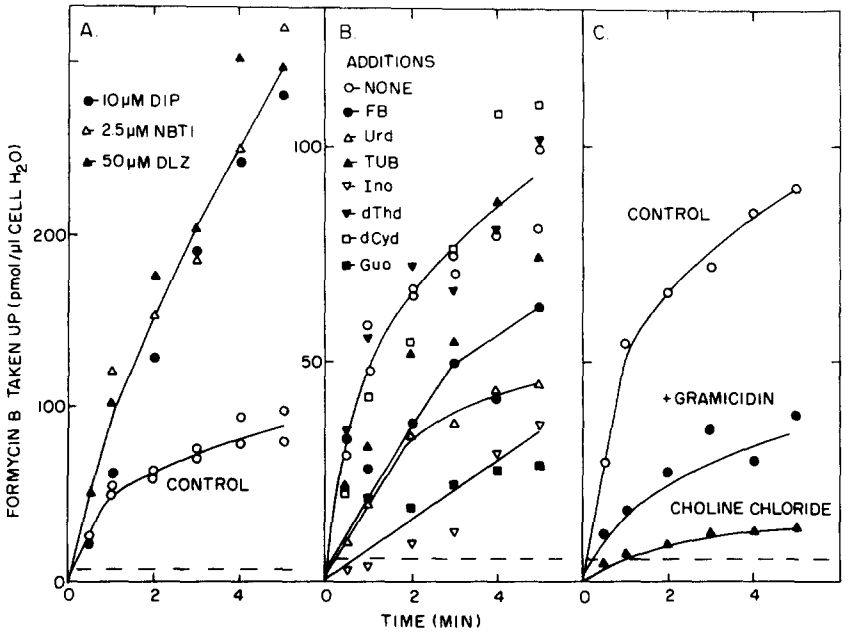

Fig. 4. Na^+-dependent, concentrative uptake of formycin B by mouse macrophages. Cultures of mouse macrophages in 24-well plates ($1.7 \cdot 10^5$ cells/well) were overlayed, if not indicated otherwise, with 0.5 ml RPMI/well and then the uptake of 5 μM [^{3}H]formycin B (120 cpm/pmol) was measured at 25 °C as described in the legend to Fig. 1A. (A) Where indicated, the medium was supplemented with 10 μM dipyridamole (DIP), 2.5 μM NBTI or 50 μM dilazep (DLZ) before formycin B uptake was measured. (B) As indicated, 100 μM unlabeled formycin B (FB), uridine (Urd), tubercidin (TUB), inosine (Ino), thymidine (dThd), deoxycytidine (dCyd) or guanosine (Guo) all at a concentration of 100 μM were added simultaneously with [^{3}H]formycin B. (C) Where indicated, the medium was supplemented with 50 μg gramicidin/ml and the cells incubated at 37 °C for 15 min before [^{3}H]formycin B uptake was measured, or the wells were rinsed with 1.5 ml Tris-choline chloride, overlayed with 0.5 ml of the same and [^{3}H]formycin B uptake measured. The broken lines indicate the intracellular concentration of [^{3}H]formycin B equal to that in the medium.

ence of excess Na^+ (Fig. 8A). NRK and RAW 309 Cr.1 cells accumulated formycin B to 8- and 5-times the extracellular concentration, respectively. (2) The concentrative accumulation of formycin B was abolished by the omission of Na^+ from the medium (Fig. 8B), and pretreatment of the cells with gramicidin (Fig. 8D) or

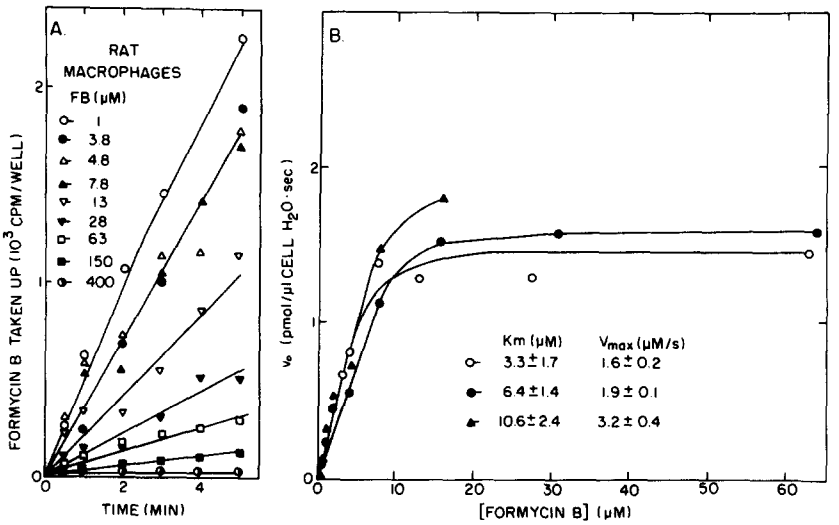

Fig. 5. Kinetic analyses of Na^+-dependent transport of formycin B by rat macrophages. (A) Cultures of rat macrophages in 24-well plates ($6.6 \cdot 10^4$ cells/well) were overlayed with RPMI (0.5 ml/well) and then the uptake of the indicated concentrations of [^{3}H]formycin B (340 cpm/μl, irrespective of absolute concentration) was measured as described in the legend to Fig. 1A. Initial velocities of uptake (v_0) were estimated from the initial linear portions of the uptake curves and are plotted in (B) along with data from two additional independent experiments as a function of the formycin B concentration. In the latter two experiments, the formycin B concentration ranges were 0.25–16 μM and 0.5–64 μM.

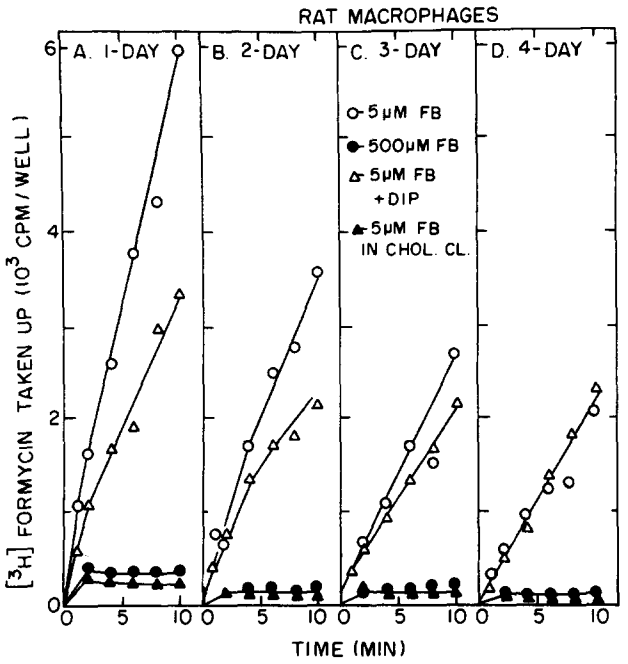

Fig. 6. Formycin B uptake by rat macrophages as a function of time in culture. Replicate cultures of rat macrophages in 24-well plates ($1.3 \cdot 10^5$ cells/well,) were incubated in a CO_2 incubator at 37 °C for the indicated periods of time. Then the cells in six wells of a plate were rinsed with Tris-choline chloride (Chol. Cl.) and overlayed with 0.5 ml of the same. The other wells of the plate received 0.5 ml RPMI and six of the latter wells were supplemented with 10 μM dipyridamole (DIP). Then, as indicated, the uptake of 5 or 500 μM [^{3}H]formycin B (FB; 510 cpm/μl, irrespective of concentration) was measured at 25 °C as described in the legend to Fig. 1A.

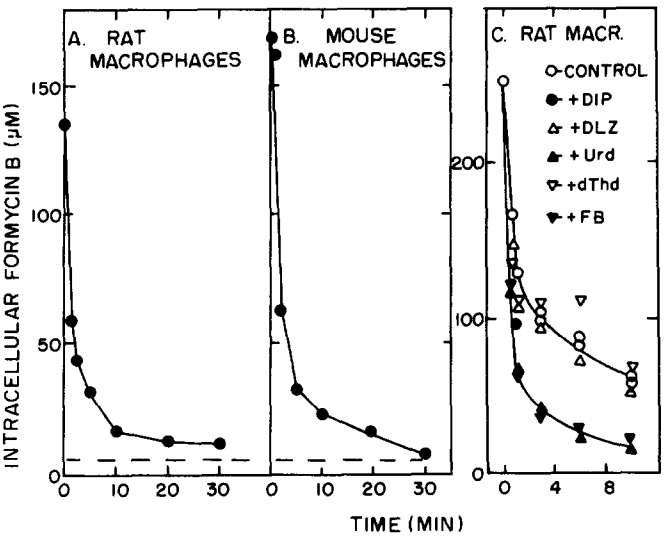

Fig. 7. Release of formycin B from preloaded, cultured rat and mouse macrophages. One-day cultures of rat and mouse macrophages ($1.3 \cdot 10^5$ and $3 \cdot 10^5$ cells/well, respectively) were incubated with 5 μM [^{3}H]formycin B (105 cpm/pmol) at 37 °C for 1 h and then rinsed thrice with cold BSS. The wells received 1.5 ml of RPMI (at 25 °C), containing as indicated in (C), 10 μM dipyridamole (DIP), 50 μM dilazep (DLZ), or 100 μM unlabeled uridine (Urd), thymidine (dThd), or formycin B (FB). At various times thereafter, the culture fluid was removed and the cells analyzed for radioactivity.

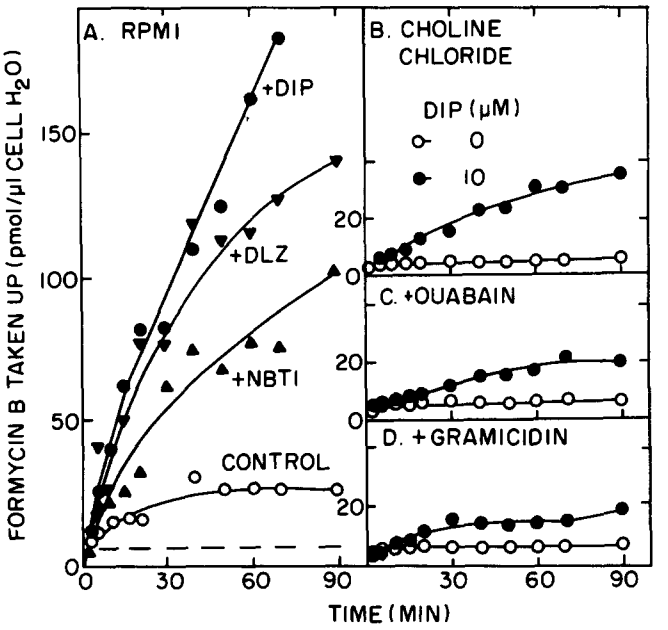

Fig. 8. Long-term formycin B uptake in the presence and absence of inhibitors of facilitated transport by RAW 309 Cr.1 cells in RPMI (A)- or choline chloride (B)- and by ouabain-(C)- and gramicidin (D)-treated cells. RAW 309 Cr.1 cells were propagated in 24-well plates to confluency ($1 \cdot 10^6$ cells/well). If not indicated otherwise, the cells were overlayed with 0.5 ml RPMI/well containing, where indicated, 2.5 mM ouabain or 50 μg gramicidin/ml and the plates were incubated at 37 °C for 15 min. In B the wells were rinsed with 1 ml Tris-choline chloride and the cells were overlayed with 0.5 ml the same. Then, as indicated, the medium was supplemented with 10 μM dipyridamole (DIP), 2.5 μM NBTI or 50 μM dilazep (DLZ) and the uptake of 5 μM [^{3}H]formycin B (96 cpm/pmol) was measured at 37 °C. The broken line (A) indicates the intracellular concentration of formycin B equal to that in the medium.

ouabain (Fig. 8C), whereas it was considerably stimulated by the presence of inhibitors of facilitated nucleoside transport (Fig. 8A). These results are similar to those previously reported for mouse B and T lymphocytes [14], mouse macrophages and various lines of mouse cells (L929, P388, L1210, and LK35.2; Ref. 16).

Lack of concentrative nucleoside transport in rat and mouse erythrocytes, rat lymphocytes and various cell lines of different species origin.

Fig. 9A shows that the initial rate of uptake of 5 μM formycin B by rat erythrocytes was slow and not reduced by omission of Na^+ or pretreatment of the cells with gramicidin. On the other hand, it was strongly inhibited by 10 μM dipyridamole under all three conditions. Steady-state levels of intracellular formycin B were attained only after about 20 min of incubation at 25 °C (Fig. 9B). These were slightly higher than those of the medium, but this slight concentrative accumulation of formycin B was not affected by treatment of the cells with gramicidin (Fig. 9B) and has also been observed for many different purine nucleosides, but not pyrimidine nucleosides, in a variety of cell types [14,16,31]. It

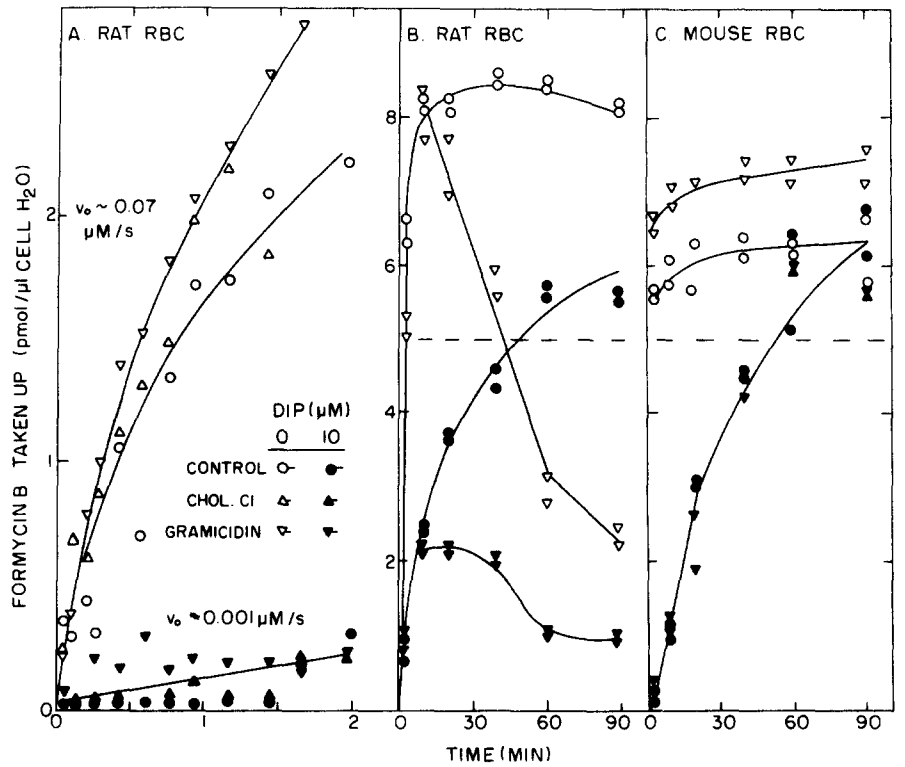

Fig. 9. Short-term (A) and long-term (B and C) uptake of formycin B by rat (A and B) and mouse (C) erythrocytes in Tris-NaCl and Tris-choline chloride and effect of gramicidin. Rat red blood cells (RBC) were washed once in Tris-choline chloride and suspended to $5 \cdot 10^8$ cells/ml of the same (△———△, ▲———▲). Or the cells were suspended in Tris-NaCl (○———○, ●———●) containing where indicated 50 μg (A and C) or 100 μg (B) gramicidin/ml (▽———▽, ▼———▼) and the latter suspensions were incubated at 37 °C for 15 min and then equilibrated at 25 °C. One-half of each suspension was supplemented with 10 μM dipyridamole (DIP) and then the uptake of 5 μM [^{3}H]formycin B (126 cpm/pmol) was measured at 25 °C as described under Experimental procedures. The initial velocities of uptake (v_0) were estimated in A from the initial linear phases of the uptake curves. The uptake of formycin B by mouse erythrocytes (C) was measured in the same manner, except that the cell density was $1.8 \cdot 10^9$ cells/ml. The broken lines indicate the intracellular concentrations of formycin B equal to that in the medium.

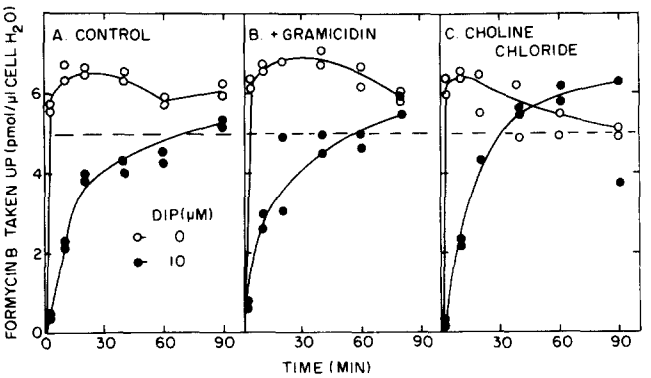

Fig. 10. Formycin B uptake in the presence and absence of dipyridamole by Novikoff rat hepatoma cells (N1S1-67) in media containing (A and B) or lacking (C) Na^+ and effect of gramicidin (B). N1S1-67 cells were washed in Tris-choline chloride and suspended to $1.7 \cdot 10^7$ cells/ml of the same (C). Or the cells were suspended to the same cell density in BM42B (A) containing as indicated 50 μg gramicidin/ml (B). The latter suspensions were incubated at 37 °C for 20 min and then equilibrated at 25 °C. One-half of each suspension was supplemented with 10 μM dipyridamole (DIP) and then the uptake of 5 μM [^{3}H]formycin B (46 cpm/pmol) was measured in all suspensions as described under Experimental procedures. The broken lines indicate the intracellular concentration of formycin B equal to that in the medium.

seems to reflect the binding of purine nucleosides to some intracellular component(s). The loss of formycin B from gramicidin-treated rat erythrocytes at 10 min of incubation was due to progressive lysis of cells (Fig. 9B). Results comparable to those presented for rat erythrocytes were obtained with mouse erythrocytes (Fig. 9C) and rat lymphocytes (data not shown). The slightly higher accumulation of formycin B in gramicidin-treated mouse red cells than control cells (Fig. 9C) reflected an increased volume of the cells due to disruption of the Na^+-gradient across the membrane [14], which was not corrected for in the present experiments.

Results similar to those in Fig. 9 were also obtained with suspensions of N1S1–67 rat hepatoma N1S1–67 cells (Fig. 10) and CHO cells (data not shown) as well as with monolayer cultures of lines of *M. dunni* cells and MA-104 monkey kidney cells (data not shown). In all instances, the uptake of formycin B was the same in media containing and lacking Na^+ and whether or not the cells were pretreated with gramicidin, whereas uptake was similarly inhibited under all conditions by 10 μM dipyridamole. Thus, in all these cells no significant Na^+-dependent, concentrative nucleoside transport was detectable.

Discussion

Rat macrophages represent the only type of cultured cells so far identified that expresses solely an Na^+-dependent, concentrative nucleoside transporter. In contrast, mouse macrophages and many other cell types have been found to express both active and facilitated nucleoside transport, but in different proportions. In some types of cell, such as mouse macrophages, lymphocytes [13,14] and intestinal epithelial cells [7], and lines of mouse macrophages (RAW 309 Cr.1), rat intestinal epithelial cells (EEC-6; Ref. 6), and rat kidney cells (NRK), concentrative nucleoside transport represents a major component of the total cellular nucleoside transport capacity. These cells concentratively accumulate nucleosides in an Na^+-dependent manner, which, however, is enhanced by inhibition of facilitated diffusion as a consequence of preventing efflux of the nucleoside actively transported into the cells. In other types of cell, on the other hand, concentrative transport represents only a minor transport component. In these cells, active nucleoside transport was only detected by the finding that inhibition of facilitated transport eventually leads to the concentrative accumulation of formycin B [16]. It is of interest that all types of mouse cell derived from *M. musculus* that have been analyzed so far express both concentrative and facilitated nucleoside transport. These include mouse macrophages, lymphocytes and intestinal epithelial cells as well as cell lines derived from macrophages, connective tissue, lymphomas and B-lymphocytes (RAW 309 Cr.1, L929, P388, L1210 and LK 35.2). It was, therefore, a surprise that a fibroblast cell line derived from *M. dunni* lacked detectable levels of concentrative nucleoside transport; it expressed only facilitated transport. These properties, however, are shared by many other cell lines derived from other species, including those from the rat (Novikoff hepatoma cells), Chinese hamster (CHO) and humans (HeLa, and three T-lymphocyte lines) as well as rat and pig lymphocytes [16] and rat and mouse erythrocytes. Thus clear species as well as cell type-specific differences in the expression of active nucleoside transport are apparent.

The joint presence in many cells that express concentrative nucleoside transport of similar or higher levels of facilitated transport has presented problems in the accurate characterization of the former [14]. The finding that rat macrophages express only concentrative nucleoside transport has solved this problem, though the limited number of macrophages that can be harvested from a rat presents another technical difficulty. Nevertheless, our studies have clearly demonstrated the strict Na^+-dependence of the transporter and allowed the determination of its kinetic properties and substrate specificity. The concentrative transporters of rat macrophages and mouse lymphocytes efficiently transport purine nucleosides and uridine, whereas thymidine and deoxycytidine are not transported. Thus they resemble one of the transporters identified in mouse intestinal epithelial cells [7]. The other transporter found in these cells that transports thymidine and deoxycytidine seems absent and so is the K^+-dependent active nucleoside transporter reported to be present in renal brush-border membrane vesicles [8].

No information is presently available as to the physiological significance of the Na^+-dependent, concentrative nucleoside transporter present in many cells other than brush borders of epithelial cells of the kidney and intestine. Also unclear is whether the expression of the concentrative nucleoside transporter is subject to some regulatory control as a function of the growth stage, cell cycle or state of differentiation of body cells, or is affected in a positive or negative manner as a consequence of cell transformation. Such controls do not seem to regulate the expression of the facilitated nucleoside transporter [1,2]. In this respect further information is required on the distribution of the concentrative transporter among different cell types in the body. Our experiments have shown that concentrative nucleoside transport of rat macrophages decreases with time in culture but it is unclear whether this decrease reflects loss of transporter per se or a regulation of its function.

It stands to reason that the presence of an active nucleoside transporter may enhance the capacity of cells to salvage nucleosides from the circulation, since it allows the accumulation of higher intracellular concentrations of the substrate. This possibility still needs to be explored. Nevertheless, if this assumption is correct, it could partly explain differences in sensitivity of certain cells to various cytotoxic natural nucleosides or nucleoside analogs which perhaps could be explored in chemotherapy. For example, because of the expression of both concentrative and facilitated nucleoside transport, dipyridamole enhances the salvage and cytotoxicity of 2′-deoxyadenosine in L1210 cells if deamination is blocked by treatment with deoxycoformycin [23,32].

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