

Purine Base Transport in *Neurospora crassa*

JANE M. MAGILL* AND CLINT W. MAGILL

Department of Biochemistry and Biophysics* and Department of Plant Sciences, Texas A & M University,
College Station, Texas 77843

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Observations presented in this paper point to the presence of dual transport mechanisms for the base adenine in *Neurospora crassa*. Competition for transport, as well as growth inhibition studies using an *ad-1* auxotroph, show that the purine bases adenine, guanine, and hypoxanthine share at least one transport mechanism which is insensitive to adenosine, cytosine, and a variety of other purine base analogues. On the other hand, uptake of adenine by an *ad-8* mutant strain unable to transport [8-¹⁴C]hypoxanthine at any concentration was not inhibited by guanine or hypoxanthine. This observation demonstrates the existence of an adenine-specific transport system which was also found to be insensitive to inhibition by other purine base analogues, adenosine or cytosine. Recombination analysis of *ad-8* by wild-type crosses showed that the inability to transport [8-¹⁴C]hypoxanthine was a consequence of the *ad-8* lesion or a closely linked mutation. Saturation plots of each system gave intermediary plateaus and nonlinear reciprocal plots which, based on comparison with pure enzyme kinetic analysis, suggest that either each system consists of two or more uptake systems, at least one of which exhibits cooperativity, or that each system is a single uptake mechanism which possesses more than two binding sites where the relative affinity for the purine base first decreases and then increases as the sites are filled.

In view of the potential roles of base analogues as mutagens and antitumor agents, the relative scarcity of information on transport of bases is surprising. The very existence of adenine auxotrophs in *Neurospora* insures that adenine can permeate the cellular membranes of this organism. Information from other organisms reveals that a variety of mechanisms with varying specificities are used for purine uptake. Adenine uptake in vesicles of *Escherichia coli* is directly coupled to its conversion to adenosine 5'-monophosphate (AMP) (6) so that the specificity of uptake is that of the conversion enzyme, adenine phosphoribosyl transferase. A similar situation was postulated for purine uptake in *Bacillus subtilis* (1). However, this is not the case universally. In *Salmonella typhimurium*, 8-azaguanine-resistant cells gave lowered rates of guanine and hypoxanthine uptake without a concomitant loss of phosphoribosyl transferase activity (14), and a 4-aminopurine-resistant mutant strain of *Saccharomyces cerevisiae* actually had elevated levels of the phosphoribosyl transferases (9). Competition patterns for uptake in yeast provide evidence for separate systems capable of transporting: (i) adenine and hypoxanthine as well as the pyrimidine base cytosine (9, 10), (ii) guanine and hypoxan-

thine (9), and (iii) adenine, guanine, hypoxanthine, and cytosine after preincubation in glucose (11). Resistance to 8-azaguanine and 2-thiouric acid in *Aspergillus* was postulated to involve faulty hypoxanthine-adenine-guanine and xanthine-uric acid permeases, respectively, though no direct measurements of purine uptake were made (3).

In each of the previously mentioned studies where kinetic parameters were measured, no deviations from typical Michaelis-Menten saturation kinetics were apparent, although rather narrow substrate ranges were employed in some cases. By contrast, double-reciprocal plots of adenine transport in rabbit leukocytes gave curved lines, concave downward, suggesting the presence of at least two different saturable adenine transport systems with K_m values of 7 μ M and 100 mM. Adenine uptake by the low K_m system was inhibited by hypoxanthine, xanthine, purine, and guanine, whereas the high K_m system was specific for adenine (5).

In this study we have examined the kinetics of adenine and hypoxanthine uptake in germinated conidia of *Neurospora crassa* in two different adenine auxotrophs and in wild-type strains. In addition, we have screened other purine bases to determine their effect on the

uptake of these compounds and their effects on the growth of the adenine auxotrophs.

MATERIALS AND METHODS

Chemicals. All nonradioactive chemicals were purchased from Sigma Chemical Co. [$8\text{-}^{14}\text{C}$]adenine, [$8\text{-}^{14}\text{C}$]hypoxanthine, and Omnifluor were obtained from New England Nuclear. Glass-fiber filters (934H) were obtained from Reeve-Angel Corp.

Neurospora strains. The adenine-requiring strain, *ad-8*, was obtained from V. W. Woodward. Strain *ad-1* (allele no. Y234M419), 74A, and 73a (wild type) were obtained from the Fungal Genetics Stock Center, Arcata, Calif. The purine-requiring strains were maintained on Vogel minimal medium (15) containing 20 mg of adenine per 100 ml. Crosses between the *ad-8* and wild-type strains were made on cornmeal agar supplemented with adenine (20 mg/100 ml). The *ad-8* strain lacks adenylosuccinate synthetase activity (4). The *ad-1* strain appears to lack aminoimidazolecarboxamide ribonucleotide formyltransferase or inosine 5'-monophosphate (IMP) cyclohydrolase activities or both (2).

Preparation of conidia for transport studies. Conidia from 5- to 7-day cultures were harvested in Vogel minimal medium containing 20 mg of adenine per 100 ml. The conidial suspension was filtered through sterile cheesecloth and incubated with stirring at 30 C for 5 h. A portion of the conidial suspension was centrifuged, and the conidial pellet was resuspended in Vogel minimal medium to give a final concentration of 4×10^6 conidia/ml. After 2 h of incubation in Vogel minimal medium (with stirring, at 30 C) to deplete any existing pools of adenine, [$8\text{-}^{14}\text{C}$]hypoxanthine or [$8\text{-}^{14}\text{C}$]adenine uptake was measured.

Time course studies. To initiate uptake, [$8\text{-}^{14}\text{C}$]adenine or [$8\text{-}^{14}\text{C}$]hypoxanthine was added to flasks containing a conidial suspension (4×10^6 conidia/ml) and at various time intervals 2.0-ml samples were removed, collected on a glass-fiber filter, and washed with 10 ml of ice-cold Vogel minimal medium. The filters with conidial residue were dried overnight, added to 10 ml of Omnifluor liquid scintillation fluid (New England Nuclear), and counted in a Beckman liquid scintillation counter.

Kinetic studies. After conidial preparation as outlined above, 2.0 ml of conidial suspension (4×10^6 /ml) was added to tubes containing [$8\text{-}^{14}\text{C}$]adenine or hypoxanthine to make a total volume of 4.0 ml. After an incubation time of 2 or 5 min the tube contents were filtered through a glass-fiber filter, and the conidial residue was washed, dried, and counted as above.

Growth rate determinations. The growth rates of the *Neurospora* strain, *ad-1*, were measured in growth tubes (12) containing Vogel minimal medium supplemented with adenine or hypoxanthine. Various other purine bases and their analogues were also added at concentrations 10 times higher than that of adenine or hypoxanthine. Growth was measured (in millimeters) at 12-h intervals, and growth rates were determined from the linear part of the growth curve (12 to 84 h). Growth rates of the *ad-8* strain were measured as above and are reported in a previous paper (8).

RESULTS

The accumulation of [$8\text{-}^{14}\text{C}$]hypoxanthine by *ad-1* and wild type or of [$8\text{-}^{14}\text{C}$]adenine by *ad-1*, *ad-8*, and the wild-type strains was linear with time of incubation for at least 15 min at substrate concentrations of 0.01 mM (Fig. 1), 0.10 mM, 0.40 mM, and 0.60 mM. In the presence of 1.0 mM 2,4-dinitrophenol (Fig. 1) or 1.0 mM sodium azide, very little [$8\text{-}^{14}\text{C}$]hypoxanthine was taken up by conidia of the *ad-1* or wild-type strains, suggesting that hypoxanthine is actively transported into these cells. Similar inhibition of [$8\text{-}^{14}\text{C}$]adenine uptake by 2,4-dinitrophenol and sodium azide was found in these same strains and *ad-8*.

The initial rate of uptake of [$8\text{-}^{14}\text{C}$]adenine and [$8\text{-}^{14}\text{C}$]hypoxanthine into germinated conidia of the *ad-1* strain was tested over a wide range of concentrations, and the saturation curves were similar for both compounds (Fig. 2). For neither base were simple Michaelis-Menten saturation curves obtained. Instead, in both cases a plateau was observed around the substrate concentration of 0.2 mM, above which concentration initial velocity of uptake increased again (Fig. 2). Double-reciprocal plots of the same data are shown in Fig. 3 and can be interpreted as indicative of at least two substrate affinities. The data for low substrate concentrations give apparent affinity constants of 7 μM for adenine and 10 μM for hypoxanthine, and high concentrations gave apparent affinities in the range of 0.1 to 1.0 mM. It should be emphasized that these values are only given as approximations, for it is obvious that the saturation curves cannot be fully described by the two parameters necessary for describing a rectangular hyperbole of the Michaelis-Menten type and from which the term K_m is derived.

The kinetics of [$8\text{-}^{14}\text{C}$]adenine transport by germinated conidia of the *ad-1* strain (Fig. 2b) appear to be almost identical to the kinetics of [$8\text{-}^{14}\text{C}$]hypoxanthine uptake (Fig. 2a), suggesting both compounds might be transported by a common mechanism. In competition studies, adenine inhibited [$8\text{-}^{14}\text{C}$]hypoxanthine uptake (Fig. 3a) to a greater degree than hypoxanthine inhibited [$8\text{-}^{14}\text{C}$]adenine uptake (Fig. 3b). Guanine appeared to inhibit hypoxanthine transport (Fig. 3a) more than adenine transport (Fig. 3b). Xanthine, 8-azaadenine, cytosine, allopurinol, and 4-aminopyrazolo-(3,4-d)-pyrimidine failed to inhibit uptake of the [$8\text{-}^{14}\text{C}$]hypoxanthine or [$8\text{-}^{14}\text{C}$]adenine in the *ad-1* and wild-type strains. In each instance, the concentration of the competing compound was 0.25 mM.

Adenine uptake in the *ad-8* strain gave a

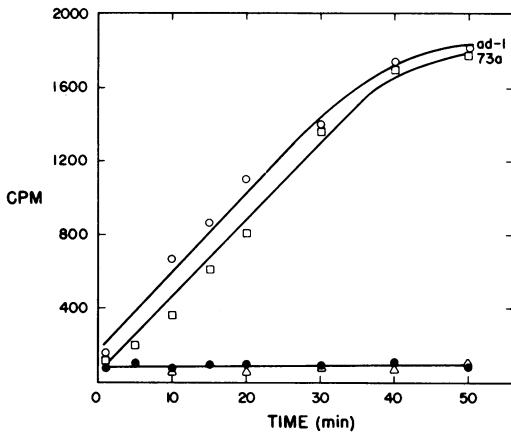


FIG. 1. Uptake of $[8-^{14}\text{C}]$ hypoxanthine (0.01 mM , specific radioactivity $0.1\ \mu\text{Ci}/\mu\text{mol}$) by germinated conidia of *N. crassa* strains *ad-1* (O), wild type (73a) (□), and *ad-8* (Δ). Also the uptake of $[8-^{14}\text{C}]$ hypoxanthine by *ad-1* (●) was measured in the presence of 1.0 mM 2,4-dinitrophenol.

saturation curve nearly identical to those found in *ad-1* and wild-type strains except that the amount of adenine accumulated per unit time was considerably lower for the same sample size (Fig. 4). Again an intermediary plateau occurred in the vicinity of 0.2 mM adenine. Because of the unusual nature of this curve many repetitions of this experiment using 2-, 5-, and 10-min fixed-time assays have been performed. In each case a plateau was observed between 0.1 and 0.3 mM adenine. The apparent affinity constant for lower substrate concentrations in this case is $30\ \mu\text{M}$. Also in contrast to the situation in *ad-1* and wild type, hypoxanthine and guanine had no inhibitory effect on the uptake of adenine into germinated *ad-8* conidia. Other compounds which were tested but had no effect on adenine uptake in the *ad-8* strain were: cytosine, xanthine, 8-azaadenine, adenosine, deoxyadenosine, inosine, and uridine. Thus the *ad-8* strain seems to have only a very specific mechanism for adenine uptake.

The *ad-8* mutation eliminates adenylosuccinate synthetase activity, the first of two enzymes required for the conversion of IMP to AMP (4). Therefore, even if it is converted to IMP by the addition of phosphoribosyl pyrophosphate (PRPP), hypoxanthine cannot satisfy the purine requirement of this strain. For this reason, the *ad-8* strain seemed to offer an opportunity to examine hypoxanthine uptake separated from its utilization. However, in contrast to the *ad-1* and wild-type strains, *ad-8* conidia failed to transport $[8-^{14}\text{C}]$ hypoxanthine at any concentration between 0.01 mM and 0.40 mM . To determine whether this inabil-

ity to transport $[8-^{14}\text{C}]$ hypoxanthine was due to the presence of another mutation, progeny of a cross between *ad-8* and wild-type strain 73a were tested for the ability to transport $[8-^{14}\text{C}]$ hypoxanthine. None of the nine adenine auxotrophic progeny tested took up any hypoxanthine at 0.4 mM concentration, whereas all 17 of the non-adenine-requiring progeny transported hypoxanthine at rates similar to the 73a parent. There was 61% germination from this *ad-8* by 73a cross.

The growth rates of the *ad-1* and *ad-8* strains were measured using Vogel minimal medium with 0.1 mM concentrations of adenine or hypoxanthine in the presence and absence of other purines. A 10-fold excess of guanine inhibited, slightly, *ad-1* growth on 0.1 mM adenine but severely inhibited *ad-1* growth when 0.1 mM hypoxanthine was used to satisfy the purine requirement (Table 1). Xanthine, allopurinol, and 4-aminopyrazolo-(3,4-d)-pyrimidine failed to significantly inhibit growth of the

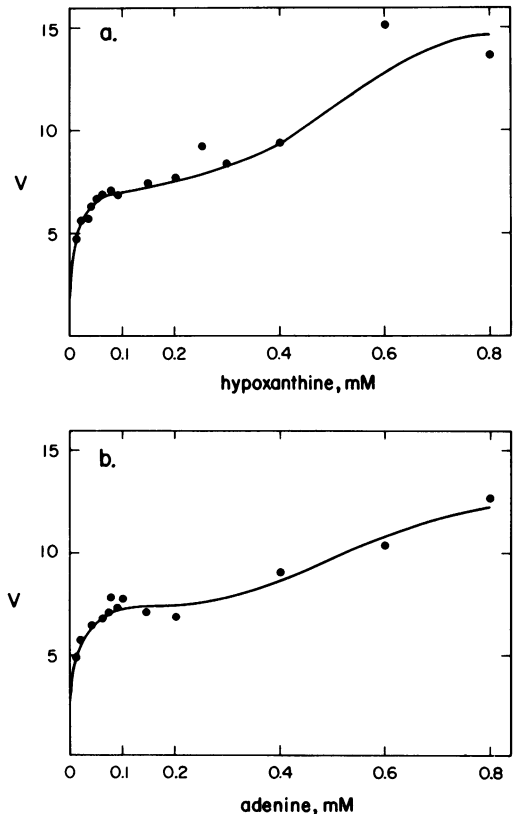


FIG. 2. Initial velocities of transport of (a) $[8-^{14}\text{C}]$ hypoxanthine and (b) $[8-^{14}\text{C}]$ adenine by germinated conidia of the *ad-1* strain. Velocity (V) is expressed as nanomoles of purine base accumulated in 5 min by 8×10^6 conidia.

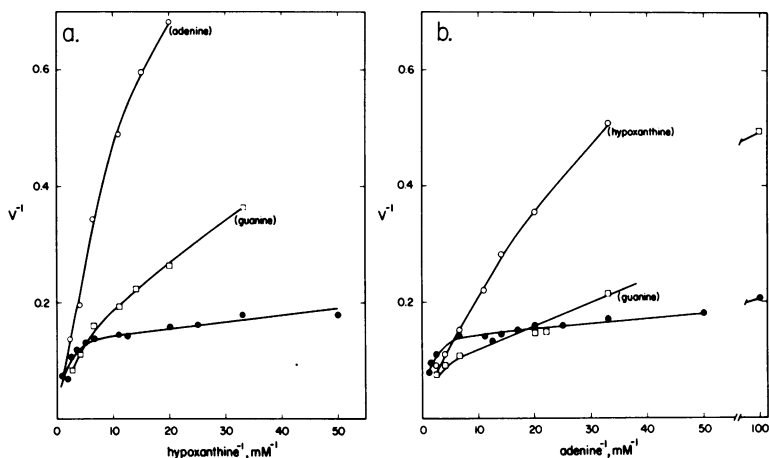


FIG. 3. Lineweaver-Burk plots showing (a) inhibition of $[8-^{14}\text{C}]$ hypoxanthine uptake by 0.25 mM adenine (O) or 0.25 mM guanine (□); and (b) inhibition of $[8-^{14}\text{C}]$ adenine uptake by 0.25 mM hypoxanthine (O) or 0.25 mM guanine (□). Velocity (V) is expressed as in the legend to Fig. 2.

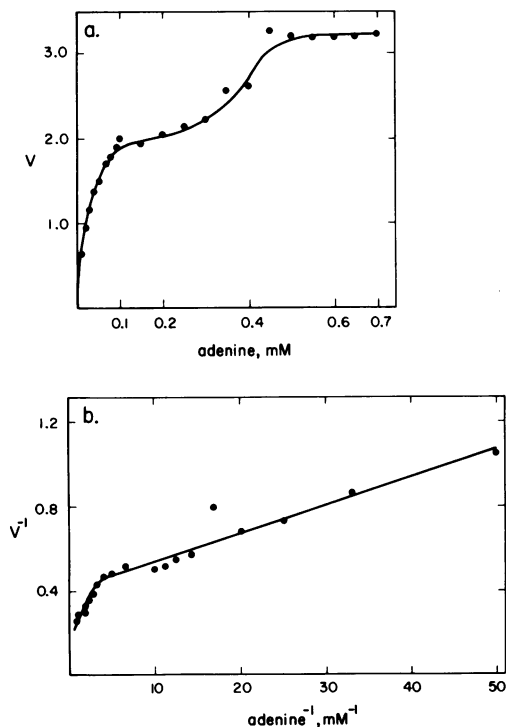


FIG. 4. The uptake of $[8-^{14}\text{C}]$ adenine by germinated conidia of the *ad-8* strain. (a) Initial velocities of transport at varying $[8-^{14}\text{C}]$ adenine concentrations; (b) Lineweaver-Burk plots of the data shown in (a). Velocity (V) is expressed as nanomoles of adenine accumulated in 5 min by 8×10^8 conidia.

ad-1 strain with either 0.1 mM adenine or 0.1 mM hypoxanthine as the purine supplement. The *ad-8* strain grew at a slower rate than the *ad-1* strain on 0.1 mM adenine alone (8), and no

significant growth inhibition was seen with a 10-fold excess of hypoxanthine, guanine, xanthine, allopurinol, or 4-aminopyrazolo-(3,4-d)-pyrimidine.

DISCUSSION

Evidence for multiple purine base transport systems has been obtained in yeast and rabbit leukocytes. Several lines of evidence point to the presence of at least two adenine transport systems in *Neurospora*. In the *ad-1* and wild-type strains, hypoxanthine and guanine compete for uptake with adenine, though they do not totally prevent adenine uptake even at low adenine concentrations. On the other hand, adenine is much more effective in inhibiting hypoxanthine uptake at low concentrations in this strain. Whereas these results are compatible with the presence of a shared transport system (or systems) with greater affinity for adenine than hypoxanthine, they are also compatible with a model requiring the presence of at least one shared mechanism along with an adenine-specific transport mechanism. The existence of an adenine-specific carrier is revealed in the *ad-8* strain, in which hypoxanthine

TABLE 1. Growth rates (mm/h) of *ad-1* strain on Vogel minimal medium supplemented with hypoxanthine or adenine

Inhibitor (1.0 mM)	Purine supplement (0.1 mM)	
	Hypoxanthine	Adenine
None	2.04	2.26
Guanine	NG ^a	1.87

^a NG, No growth.

neither is transported nor competes with adenine for uptake. In this *ad-8* strain the rate of adenine uptake is considerably lower than in the *ad-1* and wild-type strains, as would be expected if only a single adenine-specific system functions in the former, whereas both an adenine-specific system and a system shared with hypoxanthine and guanine function in the latter. The transport data are not sufficient to exclude the possibility that a second mechanism for hypoxanthine transport may exist in *ad-1* or wild-type as well.

The growth rates of the *ad-1* auxotroph on hypoxanthine and adenine in the presence and absence of other purines reflects the transport data presented above and eliminates the possibility of a hypoxanthine-specific transport mechanism. Guanine (which can not satisfy the *ad-1* purine requirement) inhibits *ad-1* growth only slightly when 0.1 mM adenine is used but completely prevents growth when 0.1 mM hypoxanthine is used. Guanine had no inhibitory effect on *ad-8* growth when adenine was the purine supplement (8). This further supports the hypothesis that there is another mechanism for adenine uptake which is separate from the hypoxanthine transport system and which is not inhibited by guanine or hypoxanthine.

In several species of bacteria and yeast the uptake of adenine appears to follow simple Michaelis-Menten kinetics, characterized by straight lines in double-reciprocal (Lineweaver-Burk) plots. In rabbit leukocytes (5) double-reciprocal plots resulted in a curved line, concave downward, and Hawkins and Berlin (5) interpreted these results as demonstrating two separate adenine transport systems, both showing Michaelis-Menten kinetics, one with a low K_m the other with a high K_m . As in the case of rabbit leukocytes, double-reciprocal plots of adenine (and hypoxanthine) uptake in *Neurospora* gave curved lines, concave downward. However, in *Neurospora* the response does not appear to be the result of effects of two simple Michaelis-Menten transport systems with different affinities or to negative cooperativity alone, which also gives curved double-reciprocal plots (13). This conclusion is based on the consistent appearance of a plateau in the saturation plots for adenine. The additive model for velocity versus substrate plots in which each component obeys Michaelis-Menten kinetics (or has negative cooperativity) gives a smooth hyperbolic curve with no plateau. Teipel and Koshland (13) have shown that enzyme saturation curves with an intermediary plateau, such as that shown in Fig. 2 and 4, can be produced by a single multiple-subunit enzyme

possessing more than two substrate binding sites if the relative affinity for the substrate first decreases then increases as the enzyme is saturated. Though these models are based on predictions from pure enzyme systems, each reflects a need or role for protein interactions which certainly could play an important role in a complicated process such as transport. An alternate mechanism for producing an intermediary plateau is the presence of two separate systems acting on the same substrate, one exhibiting Michaelis-Menten kinetics or negative cooperativity and the other showing positive cooperativity.

Intermediary plateaus were found when measuring adenine uptake in conidia from strains *ad-8*, *ad-1*, and 73a. If such plateaus do represent interacting binding sites, the fact that *ad-8* has only a specific transport mechanism for adenine makes it obvious that this mechanism possesses more than two interacting substrate binding sites on one or more carriers. The additive effects of this mechanism functioning simultaneously with transport by a less specific mechanism in *ad-1* or wild type would be expected to lead to plateaus in the saturation plots of adenine uptake, even if the less specific system obeys simple Michaelis-Menten kinetics.

Saturation plots for hypoxanthine uptake in the *ad-1* strain should provide a specific measure for the kinetic properties of the shared purine base transport system, assuming the absence of other hypoxanthine uptake mechanisms. However, the saturation curves (Fig. 2a) for the shared system displayed a small intermediary plateau and definitely gave non-linear double-reciprocal plots similar to those found for the adenine-specific transport mechanism (Fig. 4). Thus, both of the purine base transport systems which could be examined displayed similar, though unusual, kinetic properties. The similarities in function and kinetics suggest that the systems may have evolved from a single genetic precursor.

The *ad-8* strain reportedly lacks adenylosuccinate synthetase activity (4), making it unlikely that the *ad-8* mutation directly alters a component of the general purine-base uptake mechanism. It is possible that the strain contains a second mutation which eliminates hypoxanthine transport activity, but if so the mutant locus must be closely linked to *ad-8*, as no recombinants were found among 26 progeny. An alternate and more attractive hypothesis is that uptake is in some manner coupled to utilization. The *ad-8* strain should accumulate IMP from the de novo purine synthesis pathway, or

hypoxanthine, as a result of IMP breakdown, as has been suggested by Giles et al. (4). IMP is the product of the condensation of hypoxanthine and PRPP catalyzed by purine phosphoribosyl transferase (EC 2.4.2.8). Thus, if a shared purine phosphoribosyl transferase is involved directly or even as a second step in uptake, it could be inhibited by simple mass action or a more complicated regulatory mechanism due to high internal concentrations of IMP or hypoxanthine in the *ad-8* strain.

The data presented here show that purine uptake in *Neurospora* differs in several aspects from that in other organisms. The presence of an adenine-specific and a shared purine transport mechanism is similar to the situation in rabbit leukocytes, though in *Neurospora* the uptake mechanisms do not show Michaelis-Menten kinetics. Yeast also has multiple transport systems with affinity for adenine, but in each case the pyrimidine, cytosine competes for transport, which is not the case in the *Neurospora* strains examined here. In *E. coli*, adenosine is converted extracellularly to adenine which is then transported by group translocation through the addition of PRPP to give AMP (6). If the same situation for adenosine utilization existed in *Neurospora*, adenosine would be expected to inhibit the uptake of adenine, which does not occur.

In many organisms selection for base-analogue resistant mutants has been useful in determining if phosphoribosyl transferases are directly involved in transport. Mutants giving resistance to 8-azaguanine and 8-azaadenine have been reported in *Neurospora*, but were found only by using strains isolated for sensitivity to 5-methyltryptophan and were found concomitantly to be sensitive to azaadenine. Azaadenine did not compete for adenine uptake in the sensitive strains (7), nor did they or other base analogues inhibit transport of adenine or hypoxanthine in this study. This strongly suggests that these compounds are excluded from transport into normal *Neurospora* by the specificity of the various purine base uptake systems.

ACKNOWLEDGMENTS

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