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Guanine Uptake and Metabolism in *Neurospora crassa*

CLINT W. MAGILL,[1]* RICHARD L. SABINA,[1] THEODORE L. GARBER,[1] AND JANE M. MAGILL[2]

Genetics Section, Department of Plant Sciences,[1] and Department of Biochemistry and Biophysics,[2] Texas A&M University, College Station, Texas 77843

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Guanine is transported into germinated conidia of *Neurospora crassa* by the general purine base transport system. Guanine uptake is inhibited by adenine and hypoxanthine but not xanthine. Guanine phosphoribosyltransferase (GPRTase) activity was demonstrated in cell extracts of wild-type germinated conidia. The K_m for guanine ranged from 29 to 69 μM in GPRTase assays; the K_i for hypoxanthine was between 50 and 75 μM. The kinetics of guanine transport differ considerably from the kinetics of GPRTase, strongly suggesting that the rate-limiting step in guanine accumulation in conidia is not that catalyzed by GPRTase. Efflux of guanine or its metabolites appears to have little importance in the regulation of pools of guanine or guanine nucleotides since very small amounts of ^{14}C label were excreted from wild-type conidia preloaded with [8-^{14}C]guanine. In contrast, excretion of purine bases, hypoxanthine, xanthine, and uric acid appears to be a mechanism for regulation of adenine nucleotide pools (Sabina et al., Mol. Gen. Genet. **173**:31–38, 1979). No label from exogenous [8-^{14}C]guanine was ever found in any adenine nucleotides, nucleosides, or the base, adenine, upon high-performance liquid chromatography analysis of acid extracts from germinated conidia of wild-type or *xdh-1* strains. The ^{14}C label from exogenous [8-^{14}C]guanine was found in GMP, GDP, GTP, and the GDP sugars as well as in XMP. Xanthine and uric acid were also labeled in wild-type extracts. Similar results were obtained with *xdh-1* extracts except that uric acid was not present. The labeled xanthine and XMP strongly suggest the presence of guanase and xanthine phosphoribosyltransferase in germinated conidia.

In comparison with other nucleic acid bases, relatively little work has been reported on guanine uptake and utilization. This may be due in part to the limited solubility of guanine and its analogs which make transport and metabolic studies more difficult.

The initial step in guanine uptake is its transport across the cell membrane. Previous work (9) suggested that guanine is taken up by the general purine base transport system in *Neurospora*. Whether purine bases are phosphorylated during uptake, or accumulated by facilitated diffusion, the conversion by phosphoribosyltransferases (PRTases) to the impermeable phosphoribosylated form provides an obvious means of concentrating bases inside the cell. In many organisms, the enzyme which catalyzes the formation of GMP from guanine also catalyzes IMP formation from hypoxanthine and is termed hypoxanthine-guanine PRTase (HGPRtase). In a few bacterial species (4, 6), separate hypoxanthine PRTase (HPRTase) and guanine PRTase (GPRTase) enzymes have been demonstrated.

Guanine may be deaminated to yield xanthine by the enzyme guanase. Burridge et al. (1) reported that, in *Saccharomyces cerevisiae*, guanine was extensively deaminated to xanthine. Xanthine may then be degraded to uric acid by xanthine dehydrogenase and eventually excreted, or xanthine may be converted to XMP by a PRTase. In at least one organism (2), HGPRTase catalyzes XMP formation from xanthine. XMP is then converted to GMP by GMP synthetase.

In some bacteria, purine auxotrophs are capable of utilizing guanine as the sole purine supplement, whereas others, like *Neurospora*, can use only adenine or hypoxanthine to satisfy the purine requirement of purine auxotrophs (5). It has been assumed that organisms incapable of satisfying their purine requirement with guanine lack the enzyme GMP reductase which catalyzes the conversion of GMP to IMP, the usual precursor for adenine nucleotide synthesis. To test this assumption, several investigators have incubated cells with radioactively labeled guanine and then analyzed the nucleotide pools using thin-layer or paper chromatography to separate adenine, guanine, and hypoxanthine compounds. In *S. cerevisiae*, Burridge et al. (1) found a small amount of label from [^{14}C]guanine

in adenine nucleotides as did Zoref et al. using human cells (18). Greer et al. (5) showed label in adenine and hypoxanthine incubation of *Neurospora* conidia with [8-^{14}C]guanosine and subsequent depurinization of nucleic acids. However, neither organism has sufficient GMP reductase activity to allow growth of purine auxotrophs from exogenous guanine or guanosine.

In this report, we examined the transport of exogenous guanine, the initial steps in its metabolism, and its fate in cellular nucleotides.

MATERIALS AND METHODS

Chemicals. The ^{14}C-labeled purine bases and Omnifluor were purchased from New England Nuclear Corp. Unlabeled purine bases and 5-phosphoribosyl-1-pyrophosphate were obtained from Sigma Chemical Co. Glass fiber filters were obtained from Reeve-Angel, Division of Whatman Inc. The DEAE-cellulose circles (DE 81) also were obtained from Whatman Inc. The membrane filters used to collect conidia (pore size 0.45 μm) were purchased from Millipore Corp.

Neurospora strains. Wild-type strains, 74A, and the mutant strains, *ad-8* and *xdh1*, were obtained from the Fungal Genetics Shock Center, Humboldt College, Arcata, Calif. (Table 1).

Transport assay. Conidia from 5- to 9-day cultures were harvested in Vogel minimal media (17) (pH 6.0). The conidial suspension was filtered through sterile cheese-cloth and incubated with stirring at 30°C for 5 h. The suspension was centrifuged, and the conidia were suspended in minimal medium at 4×10^6 conidia per ml (about 250 μg/ml [dry weight]). Initial velocity of guanine transport was determined using 2- or 4-min incubation times. (The rate of [8-^{14}C]-guanine uptake was found to be linear for 12 min at 100 μM guanine under transport conditions.) A 2.0-ml volume of conidial suspension (4×10^6 per ml) was added to tubes containing [8-^{14}C]guanine to initiate transport. Uptake was terminated by rapid filtration of tube contents through glass fiber filters. The filters with conidial residue were washed immediately with cold Vogel medium and then dried overnight. The dried filters with conidial residue were added to 5 ml of Omnifluor scintillation fluid for counting. This and all other determinations of radioactivity were made using a Beckman 250 liquid scintillation spectrometer.

Incorporation into PCA precipitates. To determine the rate of incorporation of [8-^{14}C]guanine into nucleic acids, 2.0-ml volumes of conidial suspensions were added to 2 ml of cold 2 N perchloric acid (PCA), incubated in ice for 20 min, and filtered through glass fiber filters. The filters containing the PCA precipitate were dried, and radioactivity was determined as before.

Efflux assays. Germinated conidia were incubated in 10 μM [8-^{14}C]guanine, (50 μCi/μmol) for 20 min, caught on a membrane filter (Millipore Corp.), washed with cold minimal medium, and then suspended at 4×10^6 per ml in Vogel minimal medium or minimal medium plus 0.25 mM guanine. Portions (2.0 ml) of this conidial suspension were filtered, and the filtrate obtained was analyzed for purines by high-performance liquid chromatography (HPLC). The filter with the conidial residue was dried and added to 5 ml of Omnifluor, and the radioactivity was counted.

TABLE 1. *N. crassa* strains used

Strain designation	Enzyme lacking	Reference
74A	None	
ad-8	Adenylosuccinate synthetase (EC 6.3.4.4)	3
xdh-1	Xanthine dehydrogenase (EC 1.2.3.2)	12

A volume (200 μl) of the filtrate from the efflux assays as chromatographed by HPLC on a Waters C_{18} μBondapak column. Peaks were identified by retention time compared to a mixed standard and also spectrophotometrically by their ratio of absorbance at 280/254 nm. Quantitation was achieved by integration of peak areas using a chromatography control module data processor from Laboratory Data Control.

Enzyme assays. Conidia from 5- to 9-day cultures were harvested in Vogel minimal media and incubated with stirring for 5 h at 30°C. The germinated conidia were caught on a membrane filter (Millipore Corp.), suspended in 50 mM Tris buffer containing 10 mM dithiothreitol, and homogenized in a Ten Broeck homogenizer, using a motor-driven pestle. Centrifugation of this crude extract at 20,000 × *g* for 20 min (4°C) yielded a clear supernatant. HPRTase and GPRTase activities were determined using a modification of the method of Sabina et al. (15) in which [8-^{14}C]hypoxanthine or [8-^{14}C]guanine was incubated with 10 μl of the supernatant, 1.0 mM dithiothreitol, 1.0 mM 5-phosphoribosyl-1-pyrophosphate, 50 mM Tris-hydrochloride buffer (pH 7.5), 5.0 mM $MgCl_2$, and 1.0 mM ADP in a total volume of 100 μl. The reaction was terminated by adding 50 μl of 300 mM EDTA. A volume (50 μl) of the reaction mixture was added to DEAE circles which were washed with 10 ml of 1 mM ammonium formate, followed by 10 ml of distilled water. The circles were dried, and the radioactivity was measured as before in Omnifluor.

Acid extraction of nucleotides, nucleosides, and bases from germinated conidia. The extraction procedure is a modification of the method of Khym (8). Samples of germinated conidia were collected on Metricel membrane filters (0.45-μm pore size, 47-mm diameter) and washed with distilled water. The conidial residue was scraped from the filter and placed in freshly prepared, ice-cold 6% trichloroacetic acid (6.4×10^8 conidia in 1.0 ml of 6% trichloroacetic acid), and kept in ice for 30 min. After centrifugation at 1,200 × *g* for 3 min, the supernatant was removed and added to 1.0 ml of freshly prepared 0.5 N tri-*N*-octyl amine in Freon. The mixture was blended in a Vortex mixer and then centrifuged at 1,200 × *g* for 2 min. The aqueous upper layer containing bases, nucleosides, and nucleotides was removed and stored in a glass vial at −20°C.

Analysis of extracts by HPLC. Before injection onto the HPLC column, all samples were filtered through a membrane filter (Millipore Corp., type HA, 0.45-μm pore size, 1.3-cm diameter). Standards containing 0.1 mM nucleotides or 0.1 mM nucleosides and bases were added to conidial extracts before injection onto the HPLC column (10-μl standard per 100-μl extract).

Nucleotide separation was achieved on a Whatman Partisil-10 SAX column (25 cm by 4.6 mm) using the methods of Rose and Brockman (13). Nucleosides and bases were separated using a Waters C_{18} μBondapak column (14). A Laboratory Data Control HPLC equipped with two Constametric III pumps, two UV Spectromonitors, and a chromatography control module was used. Peaks were identified by retention time compared to a mixed standard and also by their ratios of absorbance at 280/254 nm. Quantitation of peaks was achieved by integration of peak areas using the Chromatography control module data processor. The radioactivity was determined by adding each fraction (0.5 ml) to 7.5 ml of dioxane-based scintillation fluid (300 g of naphthalene and 15 g of Omnifluor per 3 liters of scintillation-grade dioxane) and counted. The elution profile of the radioactivity was then compared to the chromatogram for the sample.

RESULTS

The kinetics of uptake of [8-^{14}C]guanine by wild-type germinated conidia is shown in Fig. 1. An intermediary plateau is evident in the Michaelis-Menten plot (V versus [S]), and a curved line is obtained in the double-reciprocal plot. These results are similar to those obtained for adenine and hypoxanthine transport in this organism (9, 11) and indicate that: (i) more than one transport system may be binding guanine, or (ii) the transport system has more than two binding sites which exhibit negative cooperativity for binding at lower concentrations and positive cooperativity at higher concentrations (16). Guanine uptake in wild-type cells was inhibited by adenine and hypoxanthine (Fig. 1) and stimulated by NH_4^+.

In germinated conidia from the *ad-8* strain, guanine transport capacity was decreased considerably from that of wild type (Fig. 1). Initial velocities of transport were compared as nanomoles of [8-^{14}C]guanine accumulated per 8×10^6 cells, germinated under identical conditions. The kinetics of uptake of guanine by *ad-8* cells were similar to that of wild type, and guanine uptake was inhibited by adenine and hypoxanthine in a manner similar to wild-type cells.

Initial velocity studies of GPRTase from cells extracts of 74A are shown in Fig. 2. A straight line was obtained in Linweaver-Burk plots, and the range of K_m values was between 30 and 69 μM. Dixon plots using unlabeled hypoxanthine as an inhibitor of [8-^{14}C]guanine yielded K_i values of 50 to 75 μM. Initial velocities for HPRTase were determined in identical wild-type cell extracts. A range of K_m values of 25 to 50 μM was obtained for hypoxanthine. In similar extracts from 74A, xanthine PRTase activity was detected using [8-^{14}C]xanthine in an assay similar to that for GPRTase, described in Materials and Methods. Unlabeled xanthine also inhibited conversion of [8-^{14}C]hypoxanthine to IMP.

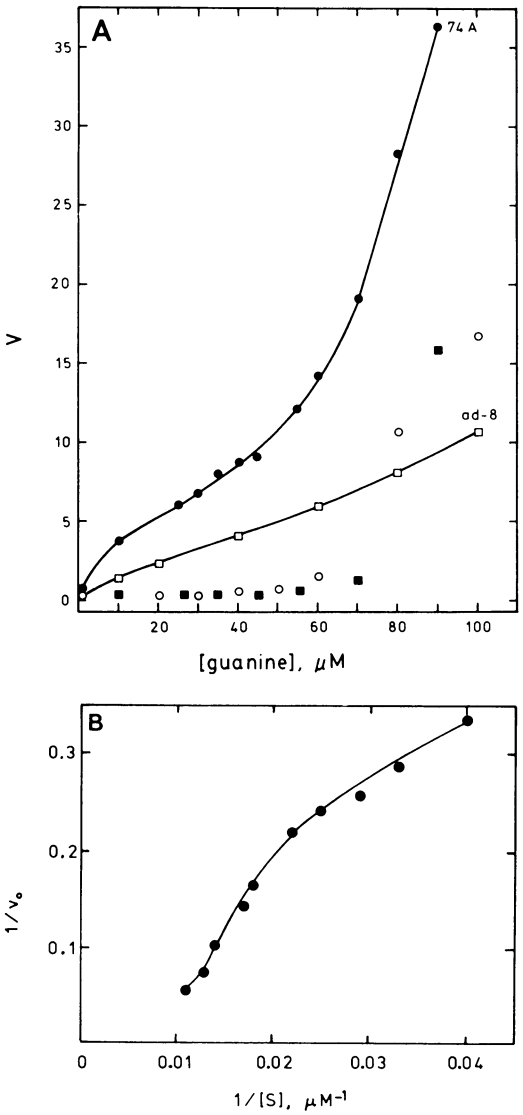

FIG. 1. (A) Initial velocities of [8-^{14}C]guanine transport at varying concentrations of guanine by germinated conidia of *N. crassa* strains, 74A (wild type) (●) and *ad-8* (□). Inhibition of guanine transport by 1.0 mM adenine (○) or 1.0 mM hypoxanthine (■) in germinated conidia of 74A strain. Initial velocity, V, is expressed as nanomoles of guanine accumulated per 8×10^6 conidia in 2.0-min incubation time. (B) Lineweaver-Burk plot of the data from (A) for the wild-type strain (74A) in the absence of inhibitors.

The efflux of purines derived from exogenous [8-^{14}C]guanine was studied in wild type and the *xdh-1* strains. The results (Fig. 3) indicate that very little ^{14}C label is lost from cells preloaded for 20 min with [8-^{14}C]guanine and then placed in minimal medium plus unlabeled guanine. In

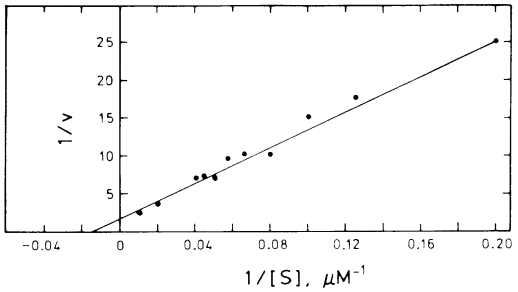

FIG. 2. Lineweaver-Burk plot of GPRTase activity in extracts of wild-type (74A) germinated conidia. The K_m for guanine is 66 μM.

the *xdh-1* strain where xanthine dehydrogenase activity is lacking (12), any guanine or xanthine excreted should come from transported guanine or degradation of guanine or xanthine nucleotides (see Fig. 6). The presence of NH_4^+ in the medium represses uricase (12) and thus should maximize pools of xanthine and guanine in wild-type cells. Under these conditions, the *xdh-1* or wild-type cells, preloaded with [8-^{14}C]-guanine, effected very little ^{14}C label. However, very small amounts of xanthine were effluxed as determined by HPLC analysis of the efflux medium from *xdh-1* or wild-type cells.

The incorporation of exogenous [8-^{14}C]guanine into nucleic acids was measured by adding germinated conidia to cold 1 N PCA, and the radioactivity of the dried precipitates was determined. After 20 min of incubation in [8-^{14}C]guanine, approximately 60% of the counts found in whole cells were in the PCA precipitates. Cells which were incubated with [8-^{14}C]guanine for 20 min and then washed and placed in minimal media showed an increase in incorporation of guanine into nucleic acids (Fig. 3) up to 40 min after removal of [^{14}C]guanine from the medium. The total amount of [8-^{14}C]guanine incorporated into nucleic acids in cells of the *ad-8* strain was much less than in wild-type or *xdh-1* cells as may be expected since guanine transport is significantly reduced in the *ad-8* strain.

The incorporation of exogenous [8-^{14}C]guanine into pools of cellular nucleotides was determined by HPLC analysis of acid extracts of germinated conidia from 74A (wild type) and *xdh-1* strains incubated with [8-^{14}C]guanine for 25 min. Figure 4 shows the distribution of radioactivity in the eluted nucleotides from wild-type conidial extracts. Significant amounts of radioactivity were found in compounds, presumably nucleosides and bases which do not bind to the anion-exchange column and thus are eluted in the first 2 min. The ^{14}C label was found in guanine nucleotides (GMP, GDP, GTP) as well as GDP hexoses. One peak of radioactivity eluted with XMP at approximately 5.5 min. IMP had a retention time of 6 min, eluting between XMP and GMP. In some experiments, a small

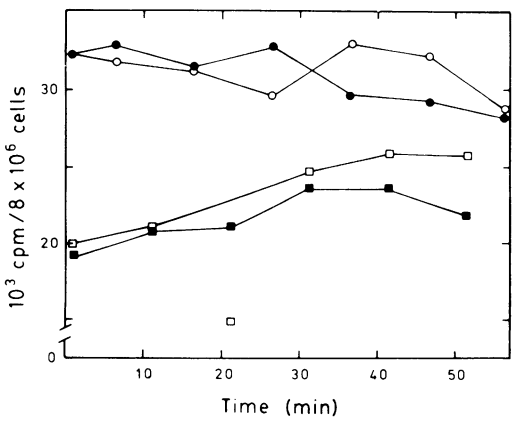

FIG. 3. Efflux of ^{14}C label from germinated wild-type conidia preloaded with [8-^{14}C]guanine (51 μCi/μmol) washed and suspended in minimal medium (○) or minimal medium plus 0.25 mM guanine (unlabeled) (●). PCA precipitates from duplicate samples of conidia in minimal medium (□) or minimal medium plus 0.25 mM guanine (■).

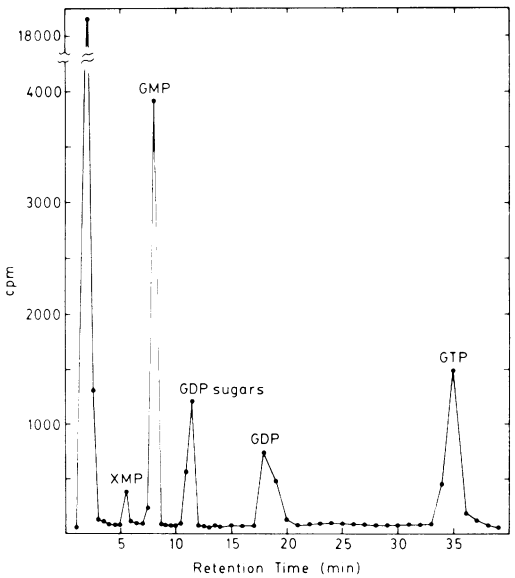

FIG. 4. Distribution of label in nucleotide pools from wild-type germinated conidia incubated with [8-^{14}C]guanine for 20 min. Nucleotides were separated by HPLC using a Whatman Partisil 10-SAX column with a linear gradient, 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7), over 40 min with a flow rate of 2.0 ml/min.

amount of ^{14}C label eluted at 6 min, but always appeared to be trailing the XMP peak. No label found in any adenine nucleotides, adenine nucleotide sugars, NAD^+, or $NADP^+$. The same results were obtained with the *xdh-1* conidial extracts.

To determine the distribution of ^{14}C label from exogenous [8-^{14}C]guanine among purine bases and nucleosides, identical samples of wild-type or *xdh-1* conidial extracts were injected onto a Waters C_{18} μBondapak column, and separation was effected by eluting isocratically with 50 mM $NH_4H_2PO_4$ (pH 6.0). The results using wild-type conidial extracts are shown in Fig. 5. The large number of counts between 1.5 and 3.0 min represents labeled nucleotides which bind poorly to the reverse phase column under these conditions. No label was ever found in adenine which eluted after 24 min or in adenosine which eluted at 18 min. The ^{14}C label appeared in uric acid and xanthine. Although it is difficult to separate xanthine and hypoxanthine by this method, when a linear gradient of 50 mM $NH_4H_2PO_4$ (pH 2.8), and 60/40 methanol/H_2O over 20 min is used at a flow rate of 1.5 ml/min, hypoxanthine elutes with uric acid more than 1 min before xanthine. In the *xdh-1* strain, no uric acid is formed due to the lack of xanthine dehydrogenase activity. In conidial extracts of *xdh-1*, no label was ever found in the hypoxanthine peak.

DISCUSSION

Purine bases are transported into *Neurospora* conidia by three different transport systems, the general purine base system (9) which binds adenine and hypoxanthine, the adenine-specific system (9, 11), and the system which binds xanthine and uric acid (12). In wild-type cells of *Neurospora*, guanine appears to be transported chiefly by the general purine base transport system. Evidence for this lies in the fact that

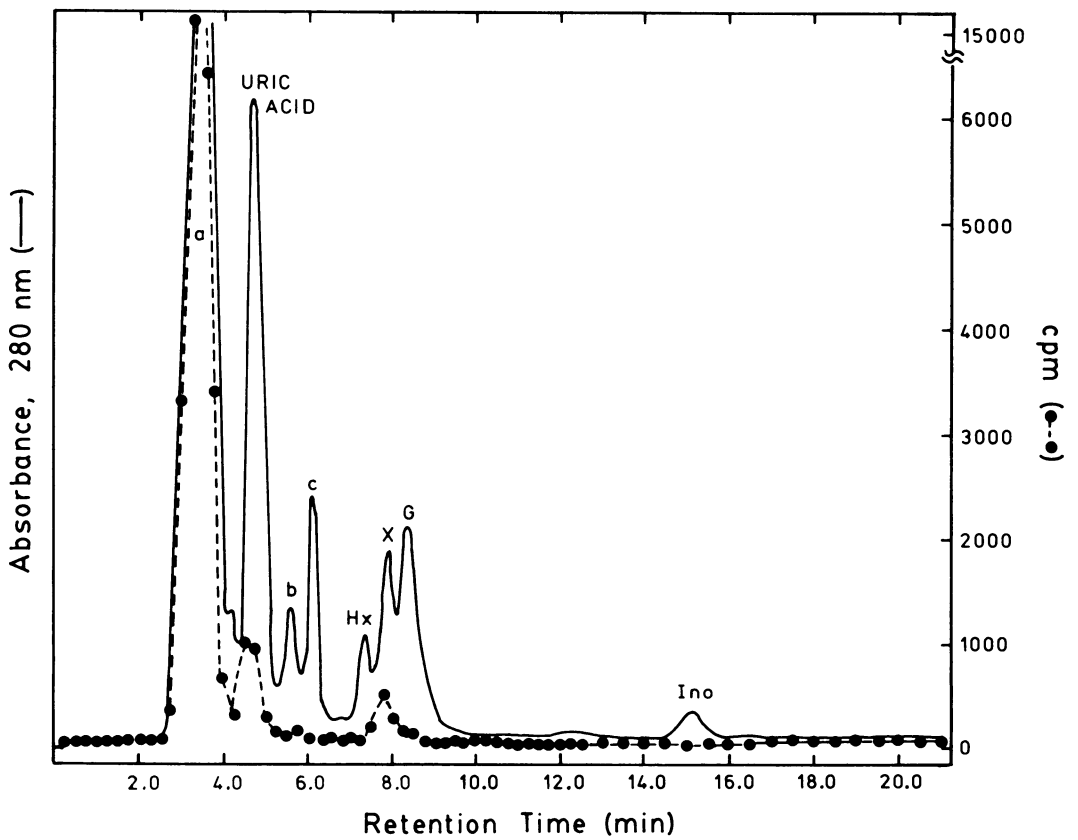

FIG. 5. Distribution of label in nucleosides and bases from wild-type germinated conidia incubated in [8-^{14}C]guanine for 20 min. Compounds were separated on a C_{18} μBondapak column, isocratically with 50 mM $NH_4H_2PO_4$ (pH 6.0), at a flow rate of 2.0 ml/min. Peak a contained nucleotides; peak b was ADP; and peak c was AMP.

both adenine and hypoxanthine strongly inhibit guanine uptake by wild-type cells. The intermediary plateau in the plot for initial velocity of guanine transport versus guanine concentration is also found in adenine and hypoxanthine transport studies (9, 11). Also, guanine transport capacity is severely reduced in the *ad-8* strains, and previous work (9) has shown that the activity of the general purine base transport system is markedly reduced in this strain. No evidence was found for a separate guanine-specific transport system.

The kinetics of guanine uptake differ markedly from the kinetics of GPRTase, indicating that the rate-limiting step in guanine accumulation in the cell is probably not conversion to GMP. These results suggest that the transport process may be the rate-limiting step in guanine uptake. Also suggested by these results is that group translocation is not the mode of transport by which purine bases are brought into the cell since phosphoribosylation of the purine base appears to be separate from transport. However, the results presented in this paper do not completely rule out the possibility that a separate GPRTase may be membrane bound and may participate in a group translocation mode of guanine transport.

The formation of purine nucleoside monophosphates from purine bases occurs as a result of PRTase activities in the cell. In some bacteria (4, 6), there appear to be three separate enzymes, one (adenine PRTase) specific for adenine, another (HPRTase) which binds only hypoxanthine, and a third (GPRTase) specific for guanine or xanthine. In *Saccharomyces* (1) and mammalian cells, hypoxanthine and guanine are converted to IMP and GMP, respectively, by the same enzyme HGPRTase (2). The similarity in K_m and K_i values for hypoxanthine and guanine suggests that *Neurospora* contains HGPRTase, similar to the yeasts. Since xanthine is converted to XMP in crude extracts of wild-type *Neurospora* and unlabeled xanthine inhibits GMP formation from [8-^{14}C]guanine in these same crude extracts, it would appear that HGPRTase in *Neurospora* catalyzes the phosphoribosylation of xanthine as well. The labeling of XMP from [8-^{14}C]guanine most likely results from the initial conversion of guanine to xanthine by guanase, and subsequent formation of XMP (Fig. 6).

The efflux of purine bases, hypoxanthine, xanthine, and uric acid appears to be an important factor in regulating adenine nucleotide pools in *Neurospora* (14). However, labeled compounds do not appear to be excreted from conidia loaded with [^{14}C]guanine even when unlabeled guanine is added to the efflux medium. These observations suggest that guanine and guanine nucleotide pools are not regulated by the efflux of purine bases as adenine and adenine nucleotide pools appear to be.

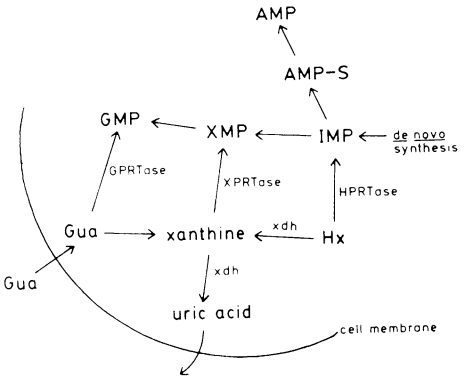

FIG. 6. Metabolic pathways followed by exogenous guanine in germinated conidia of *N. crassa*.

Adenine nucleotides were never found to be labeled when exogenous [8-^{14}C]guanine was incubated with wild-type or *xdh-1* cells. The labeling of adenine nucleotides from [^{14}C]guanine is possible only if the enzyme GMP reductase is present to catalyze the formation of IMP from GMP. Since purine auxotrophs of *Neurospora* cannot grow when guanine or guanosine is the sole purine supplement (5), it has been assumed that no GMP reductase is present in *Neurospora*. However, Greer et al. (5) showed that, in *Neurospora* conidia, a small portion of the label from ^{3}H- or ^{14}C-labeled guanosine was found in adenine and hypoxanthine after conidia were incubated in 0.4 M PCA for 1 h at 100°C to depurinate nucleic acids. Since the label in adenine and hypoxanthine in the study by Greer et al. (5) may have come from cellular pools of these bases, we determined the distribution of label in nucleosides and bases in our extracts. However, we found no label in adenine, adenosine, or inosine. Some label was found in xanthine and uric acid in wild-type strains, but labeled hypoxanthine could not be demonstrated in wild type or the *xdh-1* strain. It would appear from our studies that, in these strains of *Neurospora*, only uric acid, xanthine, and nucleotide derivatives of xanthine and guanine can be formed from exogenous guanine.

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