DL-α-Difluoromethylornithine (DFMO) causes polyamines of the AIDS-associated opportunistic pathogen Pneumocystis carinii to diminish 15 times more rapidly than mammalian host cells. The proposed mechanism was that, unlike mammalian cells, P. carinii is unable to regulate polyamine catabolism when synthesis is blocked. To test this, the responses of the polyamine catabolic enzymes spermidine/spermine acetyltransferase (SSAT) and polyamine oxidase (PAO) were determined using a new high-performance liquid chromatography assay to measure the products of these enzymes. The specific activities in untreated Pneumocystis carinii were 1.78 ± 0.5 pmol min⁻¹ mg protein⁻¹ for SSAT, similar to mammalian cells, and 6.42 ± 0.8 pmol min⁻¹ mg protein⁻¹ for PAO, 19% of that of mammalian cells. DFMO treatment for 12 h caused reductions of only 11 and 4% in SSAT and PAO, respectively, despite polyamine reductions of 94, 96, and 90% for putrescine, spermidine, and spermine, respectively. The P. carinii SSAT \( K_m \) value of 25 \( \mu \)M spermidine is 20% of that of mammalian cells, and the PAO \( K_m \) value of 14 nM \( \text{N}^1 \)-acetyl spermidine is 0.01% of that of mammalian cells. Acetylated polyamines continue to be lost from P. carinii even when exposed to DFMO. Collectively, these results support the hypothesis that P. carinii is unable to regulate polyamine catabolism.

Polyamines are small molecular weight, positively charged compounds that are ubiquitous in all living cells. The most important polyamines are putrescine, spermidine, and spermine. Although much of the evidence is indirect, it is clear that polyamines play many important roles in cell growth and differentiation (1–3). Consequently, inhibition of polyamine synthesis, as well as other means of manipulating polyamine concentrations, has been used as therapeutic approaches for the treatment of cancer and infectious diseases.

The most commonly used compound for manipulating polyamine metabolism is difluoromethylornithine (DFMO), \( \text{eflorornithine, Ornidyl®} \). DFMO is an enzyme-activated irreversible inhibitor of ornithine decarboxylase, an enzyme that catalyzes the first step in polyamine synthesis and is rate-limiting. Thus DFMO can completely block \( \text{de novo} \) polyamine synthesis. This drug is currently approved for treatment of human African sleeping sickness (4) and has been used as an investigational drug for cancer chemotherapy and for treatment of Pneumocystis carinii pneumonia (PCP), the most common opportunistic infection associated with AIDS. DFMO is effective against PCP, in an animal model (5) and in patients (6). Although clinical trials showed this drug to be less effective than drugs already approved, it was also less toxic (7). Animal studies, however, demonstrated that continuous infusion of DFMO greatly enhances the anti-PCP activity as compared with the intermittent dosage obtained by inclusion of the drug in the drinking water (8). The activity of DFMO against PCP demonstrates that polyamine metabolism is a valid therapeutic target for this disease. A better understanding of P. carinii polyamine metabolism will improve the ability to exploit anti-polyamine therapy PCP.

In most cells the metabolism of polyamines begins with parallel decarboxylation of ornithine and \( \text{S-adenosylmethionine} \) to produce putrescine and decarboxylated \( \text{S-adenosylmethionine} \), respectively. Spermidine synthase produces spermidine from putrescine via transfer of an aminopropyl group from decarboxylated \( \text{S-adenosylmethionine} \). Spermine synthase similarly produces spermine from spermidine by transfer of a second aminopropyl group. All four biosynthetic steps are highly exothermic and therefore essentially irreversible. However, catabolism results in back-conversion of spermine to spermidine and spermidine to putrescine. The initial and rate-limiting step in back conversion is the acetylation of spermine and spermidine by spermidine/spermine-\( \text{N}^3 \)-acetyltransferase (SSAT). SSAT is a cytosolic enzyme that utilizes acetyl-CoA to acetylate a terminal amino group of spermidine or spermine, thus converting them into \( \text{N}^3 \)-acetyl derivatives. The final step is removal of the acetylated aminopropyl group by polyamine oxidase (PAO), a \( \text{FAD-dependent enzyme which produces 3 \text{-acetoacidospropanal and either spermidine or putrescine from N-acylated spermine or spermidine, respectively. The catabolic intermediates (the acetylated polyamines) are less charged at physiologic pH than the parent compounds and, therefore, are easily lost from the cell. Consequently, SSAT activity can result in a net loss of polyamines from the cell as well as back-conversion to smaller polyamines. Because SSAT is critical for the maintenance of intracellular polyamine concentrations, it is usually highly regulated. SSAT can be induced by a number of factors, including environmental stress, exogenous polyamines, polyamine analogues, growth hormones, and various toxic agents (9). Tight control of this enzyme is reflected by the rapid turnover rate (\( t_{1/2} = 15 \text{ min} \)) observed in rats treated with thiocetamide to induce SSAT (10). Down-regulation of SSAT prevents polyamine depletion in mammalian cells when polyamine synthesis is blocked by DFMO (11). When P. carinii isolated from infected rat lungs are exposed to DFMO in vitro, intracellular polyamine concentrations decline much more rapidly than in other cell types. A 3-h exposure of P. carinii to DFMO in vitro reduced the putrescine,
and spermine concentrations by 88, 71, and 84%, respectively (12). In contrast, 3–4 days were required to deplete mammalian cells of putrescine and spermidine and, even then, spermine concentration was either not reduced or reduced by only 50% depending on the cell types (13–16). Since de novo polyamine synthesis in both P. carinii and mammalian cells is blocked by DFMO, these data led to the hypothesis that polyamine catabolism is not regulated in P. carinii, which would cause this pathogen to be particularly vulnerable to compounds that interfere with polyamine synthesis (8). The work reported here supports that hypothesis by demonstrating that SSAT and PAO activities are not reduced when cellular polyamine concentrations decrease due to inhibition of ornithine decarboxylase by DFMO. In addition, as internal polyamine concentrations decrease as a result of polyamine catabolism, excretion from the cell further reduces the polyamine pool.

EXPERIMENTAL PROCEDURES

Chemicals—Spermidine, putrescine, N2-acetyl spermidine, 1,7-diaminoheptane, and acetyl-CoA were purchased from Sigma; AccQ Fluor reagent kits were from Waters Corp. (Milford, MA); N1, N2-dihydrospermine was obtained from Tocris Cookson (St. Louis, MO); DFMO was from Ilex Oncology (San Antonio, TX).

Preparation of P. carinii Extracts for Enzymatic Analysis—P. carinii cells freshly isolated from rat lungs (12) were used for development of the N1-acetyl spermidine HPLC method. All other experiments were performed with cells grown in a newly developed continuous axenic culture system for P. carinii (17). Extracts were prepared by suspending P. carinii in NKPD buffer (2.68 mM KCl, 1.47 mM KH2PO4, 51.1 mM Na2HP04, 7.43 mM NaH2PO4, 62 mM NaCl, 1 mM EDTA, and 1.0 mM dithiothreitol), sonicating for 20 min at 40 watts and 70% duty cycle (Heat System, Ultrasonics Inc., Plainview, NY), clarifying by centrifugation at 30,000 × g for 30 min, and dialyzing the supernatant against two changes of 1000 ml of NKPD buffer. An aliquot of each extract was retained for protein assay (dotMetric assay, Geno Technology, Inc., St. Louis, MO), and the balance was used immediately for various experiments.

Polyamine Depletion Studies—The in vitro axenic culture of P. carinii was as described previously (17). Cultured P. carinii cells were centrifuged at 5000 × g for 10 min, suspended in RPMI 1640 tissue culture (Life Technologies, Inc.) with no serum, and divided into four aliquots. One aliquot was extracted for polyamine analysis immediately. DFMO (1 mM) was added to the other three aliquots, and they were incubated at 37 °C. One aliquot was extracted for polyamine analysis after a 3-h incubation with DFMO and another after a 12-h incubation. The fourth aliquot was incubated with DFMO for 3 h. The DFMO was removed by centrifuging the cells at 5000 × g for 10 min and resuspending them in fresh RPMI 1640; after an additional 1-h incubation at 37 °C to allow polyamine content to recover, this aliquot was extracted for polyamine analysis.

SSAT Assay—The assay for SSAT activity was adapted from Wallace and Evans (18). Each reaction had a final volume of 50 μl; 40 μl of the dialysate and 10 μl of NKPD buffer containing 3 mM spermidine and 250 μM acetyl-CoA. The mixture was incubated for the indicated time at 31 °C, and the reaction was stopped by heating in a boiling water bath for 2 min. Prior to precolumn derivatization, the sample was clarified by microfiltration for 10 min at 14,000 × g; 30 μl of the supernatant was used for derivatization and HPLC analysis.

PAO Assay—PAO assay was assayed in a total volume of 50 μl; 40 μl of the dialysate and 10 μl of 5 mM N1-acetyl spermidine. The mixture was incubated for the indicated time at 37 °C and the reaction stopped by heating in a boiling water bath. After microcentrifuging for 10 min at 14,000 × g, 30 μl of the supernatant was used for precolumn derivatization and HPLC.

Precolumn Derivatization of Samples—Precolumn derivatization was performed as described previously (19). An internal standard (5 μl of 5 μM 1,7-diaminoheptane) and 45 μl of borate buffer (0.1 μM sodium borate, 1 μM EDTA, pH 8.8) were added to 50 μl of either P. carinii extract or a mixture of standards (putrescine or N1-acetyl spermidine). After mixing, 20 μl of AccQ Fluor reagent was added.

Reversed Phase High-Performance Liquid Chromatography—A Waters HPLC system was used including a quaternary pump (model 625), a system controller (model 600E), an autosampler (model 715), and a fluorescence detector (model 470). Waters Millennium32 software (Version 3.05) was used to control the system and collect the data. A 5-μm silica particle C18, Microsorb-MV column (150 × 4.6 mm inner diameter) with a 100-Å pore size (Rainin Instrument Co., Inc., Woburn, MA) was used for SSAT assays, and a Zorbax CN column (250 × 4.6 mm inner diameter) (DuPont) was used for PAO assays. Fluorescence excitation was at 250 nm, and emission was collected at 395 nm for both assays. For SSAT assay the mobile phase gradient was as described previously for polyamine analysis (19). For PAO assay the mobile phase consisted of eluent A (140 mM sodium acetate and 17 mM triethanolamine, pH 5.05) and acetonitrile. Elution was at 1.0 ml min−1 and began with a linear gradient starting with 83% eluent A, 17% acetonitrile extending to 60% eluent A, 40% acetonitrile over 20 min. This was followed by a second linear gradient ending with 40% eluent A, 60% acetonitrile over the next 10 min. The mobile phase was held at 40% eluent A, 60% acetonitrile for 5 min before regenerating the starting conditions using linear gradient over 10 min. All analyses were performed at room temperature.

RESULTS

Most of the work reported here was done with cells grown in a new culture system for P. carinii (17). One critical experiment that had been done previously with cells isolated from infected animals was repeated with cultured cells for comparison and for validation of other results obtained with cultured cells; i.e. a study was made of the rate of polyamine depletion in cultured cells in which polyamine synthesis was blocked by DFMO. The results presented in Table I show that exposure to DFMO causes cultured cells to become rapidly depleted of polyamines in a manner similar to cells P. carinii isolated from infected animal lungs (12).

For measurement of SSAT activity in P. carinii, the previously reported polyamine analytical method (19) was adapted for detection of N1-acetyl spermidine, the key product of this enzyme. This method was calibrated and validated by demonstrating linearity (r > 0.99) and sensitivity in the subpicomole range using purchased authentic compounds. Fig. 1 presents two chromatograms of P. carinii lysate; chromatogram (A) was spiked with authentic N1-acetyl spermidine to identify the peak. SSAT activity in dialyzed cell lysates was measured by following the accumulation of N1-acetyl spermidine. To ensure that the enzyme was adequately stable during the incubation period, accumulation of product was measured over 30 min as shown in Fig. 2. Stability was judged to be good, since the plot is linear with time. The specific SSAT activity of cultured cells was 1.78 ± 0.5 pmol min−1 mg protein−1 (n = 3). At a constant acetyl-CoA concentration of 250 μM, a Km value of 25 μM for spermidine was calculated from the slope shown in Fig. 3.

PAO activity was measured by following the production of putrescine in a dialyzed cell lysate to which 125 μM N1-acetyl spermidine had been added. Fig. 4 presents two chromatograms of P. carinii lysate, with one chromatogram spiked with authentic putrescine to identify the peak. The stability of enzyme activity during 45 min of incubation is demonstrated by the linear rate of product accumulation with time as shown in Fig. 5. PAO specific activity was 6.42 ± 0.8 pmol min−1 mg protein−1 (n = 3). A Km value of 14 nM was calculated from the

| Exposure to 1 mM DFMO | Polyamine | Polyamine pmol μg protein−1 |
|----------------------|-----------|-----------------------------|
| 0 h                  | Putrescine| 698                          |
|                      | Spermidine| 582                          |
|                      | Spermine  | 432                          |
| 3 h                  | Putrescine| 84                           |
|                      | Spermidine| 87                           |
|                      | Spermine  | 76                           |
| 12 h                 | Putrescine| 42                           |
|                      | Spermidine| 23                           |
|                      | Spermine  | 43                           |
| 3.0 h plus 1-h recovery | Putrescine| 447                          |
|                      | Spermidine| 338                          |
|                      | Spermine  | 225                          |
Measurements were made of the response of SSAT and PAO activities to polyamine depletion. Cultured cells were incubated with 1 mM DFMO for 12 h to deplete polyamines (conditions as in Table I). Although SSAT and PAO activities were reduced by 11% (1.59 ± 0.4 pmol min⁻¹ mg protein⁻¹; n = 3), and 4% (6.14 ± 2.6 pmol min⁻¹ mg protein⁻¹; n = 3), respectively, compared with untreated cells, these changes are not considered significant.

N₁,N₁₂-Diethylspermine is a polyamine analogue that induces SSAT activity and causes depletion of polyamines in some cells (11). P. carinii cells were exposed to this compound to test the possibility that SSAT could be induced, even if it is not down-regulated by polyamine depletion. Cells were incubated with 100 μM N₁,N₁₂-diethylspermine for 24 h at 31 °C. At the end of the incubations (n = 3), SSAT activity was 1.86 ± 0.5 pmol min⁻¹ mg protein⁻¹, a 12% increase in activity compared with the control 1.66 ± 0.4 pmol min⁻¹ mg protein⁻¹. This increase is not considered significant.

Excretion of acetylated polyamines was studied by following the increase in N₁-acetylspermidine and N₁-acetylspermidine concentrations in the growth medium of a P. carinii culture. To enhance sensitivity, 2.5 ml of medium were deproteinized by boiling, clarified by centrifugation, lyophilized and redissolved.
in 100 μl of HPLC running buffer. N₁-Acetylspermine and N₁-acetylspermidine accumulated in the medium to concentrations of 124 ± 28 (n = 3) and 216 ± 37 (n = 3) pmol mg protein⁻¹ during the 48 h of the assay. To assure that the measured acetylated polyamines were exclusively from *P. carinii*, a control incubation was done without *P. carinii*. No acetylated polyamines were detected in the control. When polyamine synthesis was blocked by incubation of cells with 1 mM DFMO for 12 h, accumulation of N₁-acetylspermine and N₁-acetylspermidine was somewhat lower (21 and 34 pmol mg protein⁻¹, respectively) than for the controls without DFMO (30 and 48 pmol mg protein⁻¹, respectively).

**DISCUSSION**

The experiments reported here have the advantage of using cells grown in tissue culture so that they could be performed under conditions fully supporting *P. carinii* growth. After a 3-h exposure to DFMO, cells isolated from infected rat lungs were 88, 71, and 84% depleted of putrescine, spermidine, and spermine, respectively (12). After similar treatment, the cultured cells used here were 88, 85, and 82% depleted, respectively. The similarity of the responses indicates that cells isolated from animals and cells grown *in vitro* are equivalent in response to DFMO and likely most other aspects of polyamine metabolism.
For future work, however, cultured cells offer possibilities for experiments beyond what can be done with cells isolated from animals.

*P. carinii* has both of the conventional polyamine catalytic enzymes, SSAT and PAO. The specific activity of SSAT in *P. carinii*, 1.78 ± 0.5 pmol min⁻¹ mg protein⁻¹, is similar to that reported in mammalian cells, 1.27 pmol min⁻¹ mg protein⁻¹. However, the *Kₘ* for spermidine, 25 μM, is significantly lower than for mammalian cells, 130 μM (20). The specific activity of PAO in *P. carinii*, 6.42 ± 0.8 pmol min⁻¹ mg protein⁻¹, is lower than that reported in mammalian cells, 34 pmol min⁻¹ mg protein⁻¹. The *Kₘ* for N₁-acetylspermidine, 14.25 nM, is significantly lower than that reported for mammalian cells, 14 μM (21).

The unusual feature of *P. carinii* polyamine catabolism is the lack of modulation of SSAT and PAO activities when the cellular content of polyamines is sharply reduced by exposure to DFMO. In mammalian cells the effect of DFMO on polyamine
content is attenuated by down-regulation of SSAT which slows polyamine catabolism thus helping the cells to maintain the polyamines already synthesized. For example, exposure of mammalian CHO cells to 1 mM DFMO resulted in 60% reduction in SSAT activity by 24 h; putrescine and spermidine were substantially depleted after 3 days but even then spermine was only slightly reduced (22). This contrasts to the data presented here which show that a 3-h exposure of P. carinii to 1 mM DFMO causes only minimally reduced SSAT and PAO activities (11 and 4%, respectively), while causing severe reductions in the content of all three polyamines.

Even though P. carinii does not repress SSAT when polyamines decline, the possibility of induction was examined, since a drug-induced increase in SSAT activity could enhance the therapeutic activity of compounds that block ornithine decarboxylase. However, N\textsuperscript{3}, N\textsuperscript{12}-diethylsperrmine, which is known to induce SSAT in L1210 cells (11), was without effect in P. carinii. These results suggest that P. carinii SSAT is a nonregulated, constitutively expressed enzyme, a situation that has not been reported for any other cell type.

An expected consequence of polyamine acetylation is loss of acetylated polyamines from the cell due to the reduction in positive charge caused by the acetylation. Excretion of acetylated polyamines by P. carinii was examined by measuring the accumulation of acetylated polyamines in culture medium. On a picomole milligram protein\textsuperscript{-1} basis, the total acetylated polyamine accumulation in the medium over 48 h was 20% of the total normal polyamine content of the P. carinii cells. This excretion is similar to that reported in HT115 cells; 25% of the polyamine content was lost after 96 h (23). The addition of 1 mM DFMO for 12 h reduced excretion by 29%, compared with untreated controls; this reduction was most likely due to the declining gradient across the cell membrane caused by the inability to make new polyamines as acetylated polyamines were lost.

The data presented here provide biochemical confirmation for the hypothesis that the selective action of anti-polyamine therapy against P. carinii is based on inability of this fungus to compensate for inhibition of polyamine anabolism by down-regulating polyamine catabolism. These results also confirm the usefulness of cultured P. carinii for biochemical investigations.

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