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# QUALITATIVE DIFFERENCES IN THE PYRIMIDINE METABOLISM OF *TRYPANOSOMA EQUIPERDUM* AND MAMMALS AS CHARACTERIZED BY 6-AZAURACIL AND 6-AZAURIDINE*

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**Abstract**—The protozoan organism, *Trypanosoma equiperdum*, converts 6-azauracil, an analog of uracil, directly to the 5′-ribonucleotide, 6-azauridylic acid, by condensation of the free base with 1-pyrophosphorylribose 5-phosphate. Azauridylic acid, in turn, can be further phosphorylated to the di- and triphosphate derivatives. These reactions proceed, presumably, via the normal pathway used by the organism for incorporation of preformed uracil. Although the ribonucleoside, 6-azauridine, can readily enter viable trypanosomes, the organism appears to lack any significant capacity to phosphorylate this ribose-containing analog to azauridylic acid. Since the latter is a potent inhibitor of orotidylic acid decarboxylase, the capacity of trypanosomes to convert azauracil, and not azauridine, to this derivative appears to account for the far greater potency of azauracil in inhibiting the *de novo*-synthesis of pyrimidines by *T. equiperdum* and, thus, in suppressing the growth of this organism in mice. The extensive capacity of trypanosomes to convert azauracil to azauridylic acid by direct condensation of the free base with 1-pyrophosphorylribose 5-phosphate, together with the limited capacity of mammalian cells to convert azauracil by a totally different reaction sequence to the same ribonucleotide (via the intermediate formation of azauridine), affords both a qualitative and a quantitative biochemical distinction between this protozoan and mammalian species.

IT WAS REPORTED recently by Jaffe[1] that 6-azauracil (AzU) markedly suppresses the rate of reproduction of *Trypanosoma equiperdum* and thus prolongs the survival time of mice infected with this parasitic organism. It was reported also that 6-azauridine (AzUR), the ribonucleoside of azauracil, at a dose that is capable of suppressing the growth of several transplantable murine neoplasms,[2] is ineffective in suppressing the reproduction of *T. equiperdum*.

In the present paper, data will be presented which indicate that both AzU and AzUR can readily enter viable trypanosomes and that only the former can be converted within the cell to a derivative which is a highly active competitive inhibitor of the *de novo*-synthesis of pyrimidine nucleotides. This derivative will be shown to be 6-azauridylic acid (AzUMP) and its formation from AzU will be shown to occur via condensation of the free base analog with 1-pyrophosphorylribose 5-phosphate

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(PRPP). Evidence will be presented which suggests that this condensation is accomplished in the same manner as is the conversion of preformed uracil to uridylic acid. In addition it will be shown that cell-free extracts of *T. equiperdum* have little, if any, phosphokinase activity for phosphorylating AzUR to the active form of inhibitor. These observations offer a reasonable explanation for the parasitostatic activity of AzU and for the ineffectiveness of AzUR in the treatment of trypanosomiasis in mice.

## MATERIALS AND METHODS

Orotic acid-$^{14}$COOH was obtained from the New England Nuclear Corp.; orotidylic acid-$^{14}$COOH was prepared by incubating orotic acid-$^{14}$COOH ($1 \times 10^{-4}$ M) with an ethanol extract of yeast[3] in the presence of azauridylic acid ($5 \times 10^{-5}$ M) and 1-pyrophosphorylribose 5-phosphate ($2 \times 10^{-4}$ M) in 0·05 M Tris-formate buffer (pH 7·0); this was followed by recovery of the labeled nucleotide by gradient elution from a Dowex 1-X4 (formate) column.[4]

Azauracil, azauridine, and azauridine mono-, di-, and triphosphates were generously supplied by the Cancer Chemotherapy National Service Center of the National Institutes of Health. Azauracil-2-$^{14}$C and azauracil-4,5-$^{14}$C were kindly prepared by Dr. Pauline Chang of this department.[5] Azauridine-2-$^{14}$C and azauridine-4,5-$^{14}$C were prepared biosynthetically from the corresponding labeled azauracil by the procedure of Škoda *et al.*[6] The 1-pyrophosphorylribose 5-phosphate was obtained from the Pabst Laboratories.

*T. equiperdum* was maintained in C57 × DBA/2 hybrid mice by weekly intraperitoneal inoculation of blood from an infected mouse into fresh recipients. In an attempt to develop an AzU-resistant organism, trypanosomes were transferred weekly through mice which were maintained on maximally tolerated levels of AzU. After 12 weeks of such passage there was no indication of a developing resistance of the parasite to AzU.

When large numbers of the organism were required for biochemical studies, Sprague–Dawley rats (500–600 g) were inoculated intraperitoneally with the blood of an infected mouse; approximately $1 \times 10^5$ trypanosomes were injected per 100 g of body weight. It was predetermined that this dose of trypanosomes would cause the death of the animals within 65–70 hr; at this time the number of parasites in the blood of these rats was above $2 \times 10^9$/ml. After 60–64 hr, when the blood concentration ranged between 1–2 $\times 10^9$/ml, the rats were anesthetized with ether, and blood was withdrawn by cardiac puncture. The blood was centrifuged at $850 \times g$ for 10 min; the trypanosomes formed a cream-coloured interphase between the supernatant plasma and packed erythrocytes. The clear plasma was withdrawn and discarded, and the parasitic organisms were aspirated and resuspended in a Krebs III buffer,[7] modified to include no $NaHCO_3$, but containing 23·1 $\mu$moles of sodium phosphate buffer and 84·7 $\mu$moles of NaCl per ml. This increase in the buffering capacity of the medium was essential to prevent destruction of the organisms by the large amounts of acid formed in the presence of glucose by concentrated suspensions of these protozoa. The suspension was again centrifuged at $850 \times g$ and the trypanosomes were resuspended in fresh buffer. This method yielded suspenstions of viable parasites that were contaminated with less than 1% erythrocytes. The trypanosomes prepared in this manner, in concentrations up to $2 \times 10^9$/ml, remained viable for at least 8 hr at 0 °C and for at least 1 hr at 37 °C.

Cell-free extracts of *T. equiperdum* were prepared by freezing and thawing suspensions of the viable organisms in a solid $CO_2$–alcohol bath. After this treatment less than 1% of the trypanosomes remained intact, as seen under the light microscope. Several cycles of freezing and thawing produced a progressive decrease in the enzymatic capacity of the preparation to decarboxylate orotic acid, and for this reason the trypanosomal suspensions were routinely frozen rapidly in the solid $CO_2$–alcohol mixture and then stored at −10 °C until needed, at which time they were thawed and used immediately. Injection of such preparations into mice did not result in a trypanosomal infection, indicating that these preparations were cell-free.

After the centrifugal fractionation of cell-free extracts, orotidylic decarboxylase activity was associated with a particulate fraction, and the activity could be sedimented by centrifugation at forces as low as 850 × *g*. Sonication and freezing and thawing of viable cells in water or in 0·25 M sucrose failed to solubilize this enzyme. Thus, in the experiments with cell-free preparations, nonfractionated extracts were used.

The effect of the various analogs on the conversion of orotic acid–$^{14}$COOH and of orotidylic acid–$^{14}$COOH to $^{14}CO_2$ and uridine nucleotides was determined with viable cells and with cell-free extracts. For this purpose 10-ml Erlenmeyer flasks were adapted to contain a center well into which was pipetted 0·2 ml of 2 N NaOH for the purpose of trapping the liberated $^{14}CO_2$. In the experiments on viable trypanosomes an aliquot containing 40–60 × $10^6$ trypanosomes was pipetted into the reaction flask; varying amounts of substrate and inhibitor and sufficient buffer (described above) were added to give a final volume of 1·4 ml. In the experiments on cell-free extracts of trypanosomes, an aliquot containing the equivalent of 120–180 × $10^6$ trypanosomes was added to the reaction flask which contained appropriate amounts of the substrate, inhibitor, and PRPP, as well as 0·50 ml of a mixture designed to generate PRPP. This mixture contained per 0·50 ml: $KH_2PO_4$ (5 μmoles), $MgCl_2$ (7·5 μmoles), ATP (0·5 μmoles), phosphoglyceric acid (10 μmoles), ribose-5-phosphate (3 μmoles), and sucrose (165 μmoles); the final pH of the mixture was 7·2. Sufficient buffer also was added to the reaction flasks to give a final volume of 1·4 ml.

The flasks were capped with rubber stoppers of the type used on serum bottles and shaken at 37 °C in a Dubnoff metabolic incubator. At the end of the incubation, 0·3 ml of 6 N $HClO_4$ was injected by hypodermic needle and syringe through the rubber stopper into the reaction mixture. After allowing another 10 minutes for complete absorption of $^{14}CO_2$, the stoppers were removed, and aliquots of the NaOH were withdrawn for assay of radioactivity in a Packard Tri-Carb liquid scintillation counter, as described previously.[8]

The distribution and metabolism of labeled AzU and AzUR by viable trypanosomes were assessed by incubation of suspensions of cells (1–4 × $10^8$) with the radioactive analog in stoppered Erlenmeyer flasks at 37 °C. At several intervals, aliquots of 5 ml were withdrawn from the incubation flask and centrifuged (at 0 °C) at 850 × *g* for 10 min in a 10-ml Kolmer graduated centrifuge tube. The ratio of the concentration of the analog per milliliter of packed cells to the concentration of analog per milliliter of suspending medium was determined as described by Pasternak *et al.*[9] This ratio is a measure of the transport of a given compound into a cell. The acid-soluble contents of the cell were extracted and fractionated on a Dowex 1-X4 (formate) column; the free bases and their nucleosides were eluted with 0·1 N formic acid and the nucleotides with 2 N ammonium formate at pH 5·0.

The nature of the phosphorylated derivatives of AzU in the acid-soluble fraction was determined by ion-exchange chromatography on Dowex 1-X4 (formate), by means of gradient elution with 1 N ammonium formate at pH 5·0.

## RESULTS

### *Effect of azauracil (AzU) and azauridine (AzUR) on viable trypanosomes*

Azauracil at a dose of 750 mg/kg/day, given intraperitoneally for 6 days, prolongs by 250 per cent the survival time of mice infected with *T. equiperdum*,[1] whereas azauridine at a dose of 120 mg/kg/day for 6 days has no effect. Although the latter dosage, on a molar basis, is only one-twelfth that of the former, the two doses result in equal toxicity in mice and are equally potent in inhibiting the growth of several murine neoplasms.[2] However, additional experiments now indicate that doses of AzUR up to 1500 mg/kg/day (i.e., equimolar with the effective dosage level of AzU) have no effect on the rate of development of a fatal trypanosomal infection in mice.

Since AzU and AzUR had been shown to inhibit the biosynthesis *de novo* of pyrimidine nucleotides in mammalian systems,[8] similar studies were undertaken with the trypanosomes. The inhibition by AzU and AzUR of the conversion of orotic acid–$^{14}$COOH to uridine nucleotides and $^{14}CO_2$ by viable trypanosomes is presented in Fig. 1; this figure shows that AzU at a concentration of 0·01 $\mu$moles per ml causes a

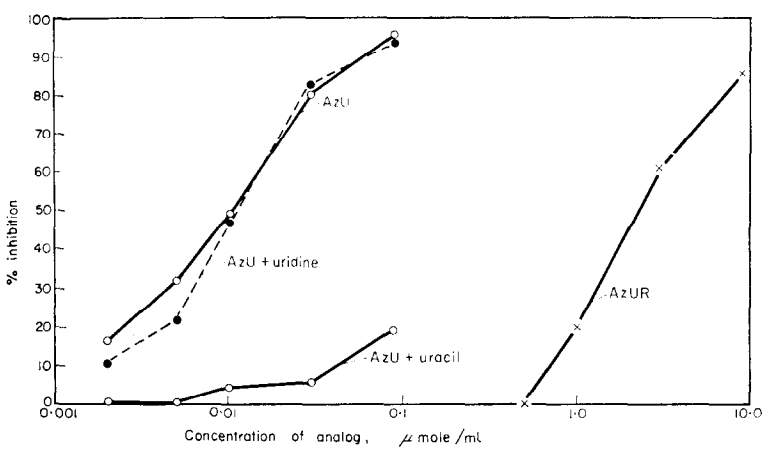


FIG. 1. Effect of azauracil and azauridine on the conversion of orotic acid — $^{14}$COOH to $^{14}CO_2$ and uridylic acid by viable *T. equiperdum*. Incubations were carried out *in vitro*, as described in the text. All flasks contained orotic acid — $^{14}$COOH: 0·018 $\mu$moles/ml, 85,700 cpm/ml. The percentage of inhibition for experimental flasks was calculated relative to control flasks to which the analog had not been added. The uracil and uridine were added, where indicated, at a concentration of 0·015 $\mu$moles per ml.

50 per cent inhibition of the *de novo*-formation of pyrimidine nucleotides, whereas the equi-inhibitory concentration of AzUR is 2·5 $\mu$moles per ml, or 250-fold higher. Thus, the greater potency of AzU as an inhibitor of the overall conversion of orotic acid to uridylic acid and $CO_2$ parallels its greater potency as a suppressor of trypanosomal reproduction. It should be pointed out that the concentrations of AzU, shown in Fig. 1 to be effective in inhibiting the formation of uridine nucleotides *in vitro*, are well within the blood levels attained in mice after the administration of effective doses of AzU.[10]

Fig. 1 shows that uracil, at a concentration of 0·015 μmoles per ml, reverses the inhibition caused by azauracil. This finding is consistent with the observation by Jaffe[1] that supplementation of the diet with uracil partially reversed the inhibitory effect of AzU on the reproduction of trypanosomes in mice. Fig. 1 also demonstrates that uridine at a concentration of 0·015 μmoles per ml has essentially no effect on the inhibition caused by azauracil. This reversal by uracil, coupled with the lack of effect of uridine, suggests that in the conversion of AzU to the active form of an inhibitor, the analog competes with uracil for a common metabolic pathway which does not involve the intermediate formation of the corresponding ribonucleoside.

The observed differences between azauracil and azauridine in their capacity to suppress the reproduction of trypanosomes *in vivo* and to inhibit the conversion of orotic acid to uridine nucleotide *in vitro* could be accounted for either by an inability of AzUR to enter the viable cell or by a relative inability of AzUR to be converted to a derivative which is the active inhibitor and to which AzU can be readily converted. Fig. 2 shows the results of an experiment designed to explore these possibilities. It

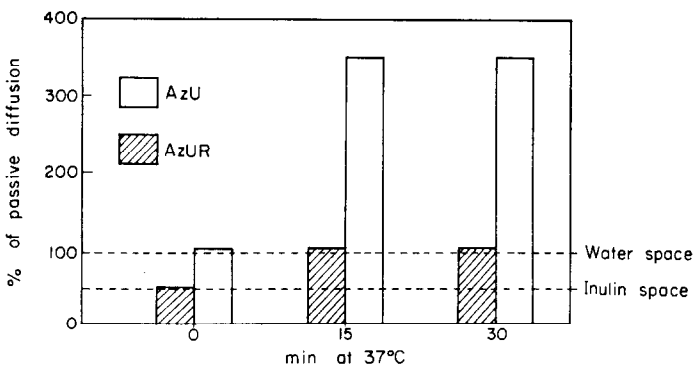


FIG. 2. Distribution of AzU and AzUR in *T. equiperdum*. Twenty ml of a suspension of viable trypanosomes was incubated with AzU-4,5-$^{14}$C (0·065 μmoles/ml, 4·25 × $10^5$ cpm/ml) or AzUR-4,5-$^{14}$C (0·065 μmoles/ ml, 5·65 × $10^5$ cpm/ml). Comparable incubations were performed with tritiated water (3·09 × $10^5$ cpm/ml) and with inulin-$^{14}$C (1·54 × $10^5$ cpm/ml). Further details concerning the determination of the transport of a given compound into the protozoa are presented in the text. The permeability of the trypanosomes to tritiated water was taken as a measure of passive diffusion, and the permeability to inulin-$^{14}$C was used as a measure of the extracellular space in the cell pack (based on the assumption that inulin does not penetrate intact trypanosomal membranes). The permeability of the trypanosomes to inulin, AzU, and AzUR was plotted as the per cent of passive diffusion.

can be seen that even at zero time (i.e., at 0 °C) AzU rapidly equilibrates across the trypansomal membrane, as is indicated by its ability to attain the same degree of entrance into the cells as that of tritiated water; AzUR at zero time is essentially not transported across the trypanosomal membrane, as is indicated by its ability to attain only the distribution seen with inulin. Within 15 min, however, AzUR also enters the trypanosomal cells, as can be seen by its distribution in the water space; at 30 min there was no further change in the intracellular accumulation of AzUR. On the other hand, AzU exhibited a marked uptake against a concentration gradient; this was evident at 15 min and was maintained at 30 min.

A possible mechanism for the apparent accumulation of AzU against a concentration gradient would be the conversion of AzU intracellularly to a nondiffusible derivative. Concomitant analysis of the acid-soluble contents of the trypanosomes by means of ion-exchange and paper chromatography indicates (Fig. 3) that incubations with

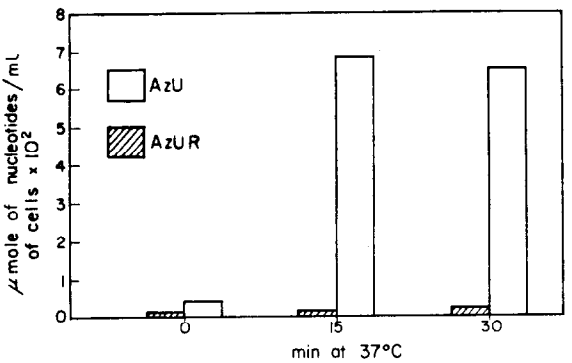


FIG. 3. Metabolism of AzU and AzUR in *T. equiperdum*. The acid-soluble contents of the trypanosomes from the experiment shown in Fig. 2 were fractionated on a Dowex 1-X4 (formate) column. The column was first eluted with three 10-ml portions of 0·1 N formic acid and then with three 10-ml portions of 2 N ammonium formate (pH 5·0). The micromoles of nucleotide were calculated from the counts per minute eluted from the column with the latter solvent.

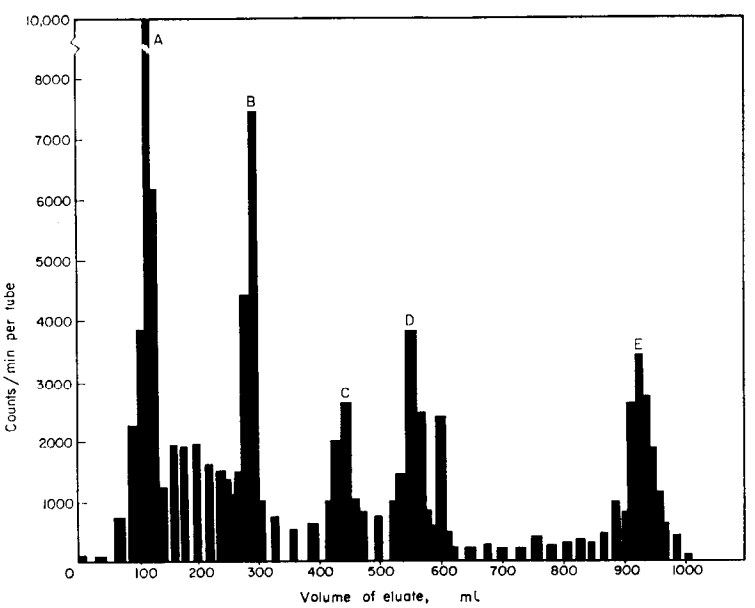


FIG. 4. Ion-exchange chromatogram of the cold acid-soluble extract obtained from a suspension of *T. equiperdum* (5·5 × $10^9$ organisms) which was incubated in a final volume of 50 ml of modified Krebs III buffer with azauracil-2-$^{14}$C (0·174 μmoles/ml, 348,000 cpm/ml). The acid-soluble extract contained a total of 117,000 cpm. Gradient elution was performed on a 0·78 × 20-cm column of Dowex 1-X4 (formate, 200–400 mesh) with 1 liter of water in the mixing chamber and 1 liter of 1 N ammonium formate (pH 5·0) in the upper chamber.

AzU results in the formation of large amounts of nucleotide derivatives, whereas incubation of the cells with AzUR does not. Since phosphorylated derivatives generally do not diffuse across biological membranes,[11] the intracellular formation of a nucleotide derivative of AzU is sufficient to account for the observed accumulation ofAzU against a concentration gradient. The inability of AzUR to yield nucleotide derivatives is consistent with the observed lack of concentration of AzUR within viable trypanosomes. Also, it may be pointed out that no evidence was found for the conversion of AzU to AzUR in these experiments.

Gradient elution of the acid-soluble fraction derived from viable trypanosomes which had been incubated with AzU-2-$^{14}$C yielded five peaks (Fig. 4). On the basis of gradient elution of the known compounds, these peaks have been identified as follows: A, AzU or AzUR; B, azauridine 5′-monophosphate; D, azauridine 5′-diphosphate; and E, azauridine 5′-triphosphate. Peak C, on the basis of its intermediate position between the mono- and diphosphate, could be an azauridine diphosphohexose derivative analogous to uridine diphosphoglucose.[12] In spite of the fact that AzU can be metabolically converted to the nucleoside triphosphate, no significant amounts of AzU-containing derivatives were incorporated into trypanosomal nucleic acids.

*Effect of azauracil and its derivatives on orotidylic decarboxylase in cell-free extracts of* T. equiperdum

As is indicated in Fig. 5, the inhibition by AzU of orotidylic acid decarboxylase in cell-free extracts requires the presence of pyrophosphorylribose 5-phosphate (PRPP). With increasing concentrations of PRPP the inhibitory capacity of AzU increases

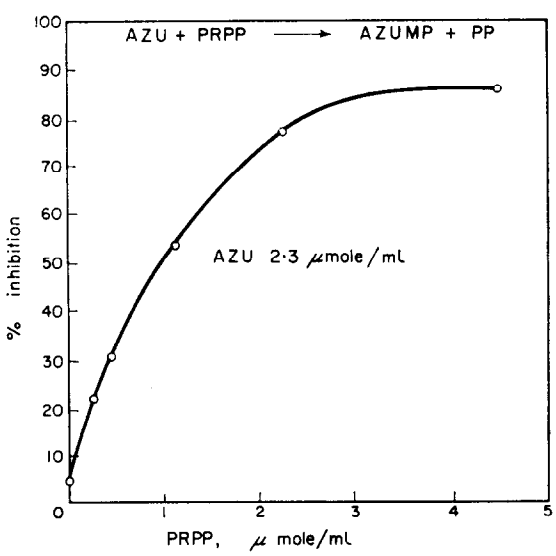


FIG. 5. Effect of PRPP on the inhibition of orotidylic acid decarboxylase by AzU in a cell-free extract of *T. equiperdum*. Each flask contained the following in a final volume of 1·6 ml: cell-free extract of *T. equiperdum* equivalent to 120 × $10^6$ trypanosomes; AzU 3·6 μmoles; PRPP-generating mixture (as described in the text), 0·5 ml; orotidylic acid-$^{14}$COOH, 0·001 μmoles, 6700 cpm; and varying amounts of PRPP, as indicated in the figure. All flasks were incubated at 37 °C for 30 min and enzymic activity was determined as indicated in the text. The percentage inhibition was calculated relative to control flasks to which AzU had not been added.

until a maximum is reached at about 1:1 molar ratio. Paper chromatography of cell-free extracts incubated with AzU and PRPP indicated that nucleotide derivatives of AzU were formed. These data suggest that AzU is converted to the active form of the inhibitor by a direct condensation with PRPP to form azauridylic acid and pyrophosphate, a reaction analogous to that shown by Canellakis[13] for the utilization of uracil by *Lactobacillus bulgaricus* 09X.

Table 1 indicates the levels of AzU and its derivatives that are required to produce a 50%-inhibition of orotidylic acid decarboxylase in cell-free extracts; of the compounds assayed, azauridylic acid is the most effective inhibitor of the enzyme. Kinetic analysis of all three inhibitors in the presence of PRPP indicated competitive antagonism. These data suggest that AzU and AzUR, as in other systems, act only after their conversion to AzUMP. However, the conversion of AzU to the nucleotide in trypanosomes is very much more extensive than is the phosphorylation of AzUR. In addition it should be pointed out that, in the determination of the concentration of AzUR required for 50%-inhibition of the decarboxylase activity (Table 1), high levels of PRPP were added to the reaction flasks. If AzUR were, indeed, being converted to AzUMP via phosphorylation, then elimination of PRPP from the reaction flasks should not decrease the amount of AzUMP formed and consequently should not reduce the suppression of orotidylic acid decarboxylase activity caused by AzUR.

TABLE 1. RELATIVE POTENCIES OF AZU, AZUR, AND AZUMP AS INHIBITORS OF OROTIDYLIC DECARBOXYLASE IN A CELL-FREE PREPARATION OF *T. equiperdum*

| Compound | Concentration required for 50%-inhibition of OMP decarboxylase* ($\mu$moles/ml) | Relative potencies (AzUR = 1) |
|---|---|---|
| AzUR | 19·3 | 1 |
| AzU | 0·68 | 28 |
| AzUMP | 0·001 | 19,300 |

* In the presence of optimal levels of PRPP and 0·001 $\mu$moles of orotidylic acid per ml.

However, the degree of inhibition was reduced from 50% to 9% when PRPP was eliminated from the assay mixture. These results suggest that the level of inhibition seen with AzUR may be attributed to a limited conversion of the nucleoside to AzU and subsequent condensation of the free base wtih PRPP to form AzUMP.

## DISCUSSION

Earlier investigations have indicated that AzU and AzUR suppress the cellular reproduction of several bacterial species as well as a number of experimental neoplasms in mice by inhibiting the *de novo*-synthesis of pyrimidine nucleotides.[8, 14] The inhibition is not accomplished by the analogs *per se*, but depends on their prior conversion to azauridylic acid (AzUMP),[8, 9, 15] a competitive inhibitor of orotidylic acid decarboxylase.[4] In general, the results presented in this paper for the protozoan

organism, *T. equiperdum*, are in agreement with this concept, but *T. equiperdum* exhibits certain interesting differences in the metabolism of azauracil and its derivatives. Thus, whereas bacterial[16] and mammalian cells[8] have been shown to convert AzUR directly to azauridylic acid by phosphorylation of the nucleoside, *T. equiperdum* exhibits little, if any, capacity to carry out this reaction. The lack of the phosphokinase for phosphorylating AzUR is manifest by the inability of AzUR to inhibit the reproduction of this organism *in vivo*.

With respect to AzU, mammalian cells[8] have only a limited capacity to convert this analogue to AzUMP; this low level of conversion proceeds via the intermediate formation of azauridine. Different species of bacteria have varying capacities to convert AzU to the ribonucleotide (the details of this conversion have not been described). *T. equiperdum*, however, can readily metabolize AzU to AzUMP, and it has been shown to do so by a direct conversion of AzU to the nucleotide level by condensation with PRPP, circumventing the intermediate formation of the ribonucleoside. This direct condensation probably involves the normal pathway for the conversion of preformed uracil to uridylic acid by the protozoa. The ability of trypanosomes to convert AzU to the nucleotide level is manifest in the profound inhibition of the reproduction of *T. equiperdum* in mice after their treatment with AzU.

Other points of difference were noted. Like bacterial,[17] but unlike mammalian cells,[8] *T. equiperdum* phosphorylates azauridylic acid to the di- and triphosphate derivatives. This difference may be important in the total understanding of the mechanism of action of AzU. For, although the pathways for the formation and degradation of nucleic acids in trypanosomes have not been defined, these fraudulent nucleotides may exert an inhibitory action at such sites. For example, inhibition *in vitro* of bacterial polynucleotide phosphorylase by azauridine diphosphate has been observed.[18] Finally, in spite of the ability to form adequate amounts of the di- and triphosphates, there was no incorporation of azauracil-containing derivatives into trypanosomal nucleic acids. This is in contrast to the results obtained with *Streptococcus faecalis*, in which the data suggest small, but significant incorporation.[17]

Thus it can be seen that *T. equiperdum*, occupying, as it does, a position in the biological spectrum between unicellular organisms of plant origin and multicellular organisms of animal origin, shares biochemical characteristics of both groups in terms of the form of the active inhibitor and possible site of action of AzU. In addition, this protozoan organism lacks a pathway which is found in both groups—the activation of AzUR by phosphorylation—and possesses a pathway for the activation of AzU not previously described for either biological group, but which is presumed to be the counterpart of the formation of uridylic acid from uracil and PRPP by *L. bulgaricus* 09X.[13] If the demonstrated difference in the utilization of preformed pyrimidines by mammalian cells and *T. equiperdum* should extend to other species of trypanosomes, then there would exist an exploitable biochemical difference for the development of potential chemotherapeutic agents against these parasitic organisms.

*Note added in proof.*—Recent studies carried out in conjunction with Dr. David Weinman of the Department of Microbiology at Yale University School of Medicine have shown that AzU, at the same dose level reported in this paper, also suppresses the reproduction of *Trypanosoma rhodesiense* in mice. Microscopic examination of this strain, which had been exposed to AzU, *in vivo*, revealed the presence of large numbers of multinucleate and anucleate forms. This has been interpreted as being due to delayed and/or irregular cytoplasmic divisions. The authors wish to express their appreciation to Dr. Weinman for the initial inoculum of *T. equiperdum* and for his interest in this project.

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