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EFFECT OF AZASERINE ON THE INCORPORATION OF ^{14}C-LABELED PURINES AND PYRIMIDINES INTO THE ACID-SOLUBLE AND NUCLEIC ACID FRACTIONS OF *TRYPANOSOMA EQUIPERDUM**

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Abstract—Azaserine decreased the incorporation of ^{14}C-labeled adenine, guanine, hypoxanthine, uracil, and orotic acid into the nucleic acid fraction and increased the incorporation of these compounds into the acid-soluble fraction of *Trypanosoma equiperdum* incubated *in vitro*. The amino acids L-glutamine, L-tryptophan, L-leucine, and L-tyrosine partially prevented the inhibition produced by azaserine on the incorporation of adenine-^{14}C into the nucleic acid fraction. Azaserine did not interfere with the incorporation of adenine-^{14}C into the acid-soluble adenine- and guanine-containing compounds but did inhibit the incorporation of uracil-^{14}C into acid-soluble cytosine-containing nucleotides and the UMP and CMP components of RNA. These results suggest that in *T. equiperdum* azaserine inhibits the conversion of UMP to CMP, and as a result of this inhibition there is a secondary inhibition of the biosynthesis of RNA. The extremely limited ability of *T. equiperdum* to anabolize tritiated cytosine or cytidine suggests that this protozoan species depends primarily upon the *de novo* pathway for the biosynthesis of CMP and indicates a possible site for exploitation with the appropriate antimetabolites.

THE finding by Jaffe[1] that azaserine markedly inhibited *in vivo* the growth of *Trypanosoma equiperdum* was intriguing, because it is generally accepted that the major mode of action of azaserine (in mammalian tissues) is to interfere with the *de novo* biosynthesis of purine nucleotides,[2–5] a metabolic pathway that has been shown to play only a minor role in *T. equiperdum*[6,7]. Since *T. equiperdum* possesses very active pathways for pyrimidine biosynthesis,[8] and since it has been demonstrated[9–11] that azaserine also inhibits the conversion of uridylic acid (UMP) to cytidylic acid (CMP), the possibility was investigated that the antitrypanosomal activity of this antibiotic might be due to its effect on certain pathways of pyrimidine metabolism.

Data presented in this paper indicates that the major mode of action of azaserine against *T. equiperdum* was to inhibit the enzyme-mediated conversion of UMP to CMP, and that, as a consequence of its causing a depletion of cytosine-containing nucleotides, azaserine caused a secondary inhibition of total ribonucleic acid (RNA) biosynthesis.

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MATERIALS AND METHODS

Adenine-8-^{14}C, guanine-8-^{14}C, hyposanthine-8-^{14}C, uracil 2-^{14}C, cytosine-2-^{14}C, and thymine-2-^{14}C were obtained from Calbiochem, Los Angeles, Calif.; cytosine-^{3}H and cytidine-^{3}H were obtained from New England Nuclear Corp., Boston, Mass. Azaserine (lot 205997 PL2), kindly supplied by Dr. L. M. Werbel (Research Laboratories, Parke, Davis & Co., Ann Arbor, Mich.), is a mixture containing 64% L-azaserine by weight, the main contaminant being the D-isomer. All concentrations of azaserine were calculated in terms of the L-isomer.

Trypanosoma equiperdum was maintained in Ha/ICR female albino mice by weekly intraperitoneal inoculations of blood from an infected mouse. When large amounts of trypanosomes were needed, Sprague-Dawley rats weighing about 500 g were inoculated i.p. with the blood of an infected mouse (1×10^6 trypanosomes/100 g body weight). The rats were sacrificed at about 40 hr, a time when the number of trypanosomes in the blood was about 1×10^9/ml. The blood was obtained after ether anesthesia by cardiac puncture with an 18-gauge needle attached to a 20-ml glass syringe containing 2 ml heparin (1 mg/ml). Eight-ml aliquots of blood were placed in 12-ml centrifige tubes at 0° and diluted with 4 ml modified Krebs phosphate buffer (pH 7·6, 0·116 M).[12] The tubes were centrifuged at about 1,700 *g* for 15 mins. The trypanosomes formed a thick white interphase between the supernatant-diluted plasma and packed red cells. The trypanosomal layer was carefully removed with a pipet, diluted with buffer to give a concentration of $10–70 \times 10^6$ trypanosomes/ml, and placed in unstoppered Erlenmeyer flasks on a Dubnoff metabolic shaking incubator at 27°. After preincubation of the trypanosomes in the presence of azaserine (0·25–1.0 μmole/ml) for 20–30 min, the radioactive compounds (0·1–10·0 μc) were added to the medium and the incubation contined for 40–60 min. At the end of the incubation period the contents of the flask were decanted and centrifuged at 0°, the supernatant removed, and the trypanosomal pellet washed twice with buffer.

The acid-soluble fraction was prepared by extracting the trypanosomal pellet with ice-cold 10% (w/v) trichloroacetic acid (TCA) three times. The acid-insoluble precipitate was suspended in 5% TCA and heated at 90° for 30 min to extract the nucleic acids components.[13] In the experiments where the isolation of purines, pyrimidines, RNA, and DNA was desired, 10% and 5% TCA was replaced by 0·4 N and 0·2 N perchloric acid respectively.

Adenine and guanine were isolated by a modification of the procedure used by LePage.[14] When adenine-8-^{14}C is used as a precursor, this procedure measures both metabolized and unmetabolized adenine-^{14}C. The acid-soluble fraction (0·2 N perchloric acid) was heated in a boiling-water bath for 60 min to convert the purine-containing compounds to their corresponding free purine bases. The sample was placed on Dowex 50–X8 (H^+form), the column was washed with 1 N HCl and H^2O, and the purines were eluted from the column with 6 N HCl, evaporated to dryness, and suspended in 0·5 N HCl. Adenine and guanine were separated on Whatman 3 MM. by the 65% (v/v) isopropanol–2 N HCl solvent system of Wyatt.[15]

Uridylic acid and cytidylic acid were isolated by the method of Rey and Fernandes.[16] Cold CMP (3·1 μmoles) was added to the acid-soluble fraction, which was then heated in a boiling-water bath for 40 min to convert the uracil- and cytosine-containing nucleotides to their monophosphate form. The pH of the solution was adjusted to 9 with NaOH, and the nucleotides were precipitated with $BaBr_2$ and 3

volumes of absolute ethanol at −10°. The precipitate was washed with cold ethanol and dried overnight in a desiccator. The dried sample was suspended in H_2O and the pH adjusted to 2 with HCl. The solution was placed on Dowex 50-X8 (H^+ form), and the UMP and CMP fractions were eluted with 0·05 N HCl. The fractions were concentrated and further purified by paper chromatography, as previously described for the purines.

In order to isolate RNA and DNA, the acid-insoluble fraction was extracted with 95% ethanol, and the residue suspended in 0·3 N KOH and incubated for 18 h at 37°. Concentrated perchloric acid (to give a final concentration of 0·2 N) was added to precipitate the DNA and protein, the supernatant contained the RNA fraction. The precipitate was heated in a boiling-water bath for 30 min to solubilize the DNA fraction, and the protein was removed by centrifugation. The u.v. absorption at 260 mμ (1-cm light path) of RNA and DNA fractions (in 0·1) N HCl) was measured and expressed as total optical density units (optical density × ml).

Cytidylic acid was isolated from the RNA fraction by the method of Cohn and Khym.[17] The RNA fraction was adjusted to pH 9 with KOH and centrifuged for 1 hr at 0° to remove $KClO_4$. The supernatant was placed on Dowex 1-X8 (Cl^-form) and the column washed with 0·01 N NH_4Cl; CMP was eluted from the column with 0·002 N HCl, and the other nucleotides (UMP fraction) were eluted simultaneously with 0·1 N HCl.

The purine and pyrimidine nucleotides were assayed for u.v. absorption at 260 and 280 mμ. All fractions (0·1 ml) were assayed for radioactivity by liquid scintillation counting with 10 ml of phosphor containing 25% ethanol and 3% 2,5-diphenyloxazole (PPO) in toluene. The results are expressed as the average of two or more experiments.

TABLE 1. EFFECT OF AZASERINE ON INCORPORATION OF ^{14}C-LABELED PURINES INTO ACID-SOLUBLE AND NUCLEIC ACID FRACTIONS OF *TRYPANOSOMA EQUIPERDUM* INCUBATED *IN VITRO**

Purine	Control		Azaserine	
	Acid-soluble	Nucleic acid	Acid-soluble	Nucleic acid
	(counts/min)			
Adenine-8-^{14}C	63,800	17,600	75,900	7,300
Guanine-8-^{14}C	23,400	11,700	29,100	5,200
Hypoxanthine-8-^{14}C	16,200	12,300	16,800	5,000

* Trypansomes 50 × 10^{16} were placed in 3 ml of modified Krebs phosphate buffer (pH 7·4, 0·116 M) and incubated for 30 min in presence of azaserine (0·2 μmole/ml), followed by 60-min incubation in presence of labeled purines. Adenine-8-^{14}C (0·1 μc, spec. act. 14·4 μc/μmole), guanine-8-^{14}C (0·28 μc, spec. act. 6·5 μc/μmole), hypoxanthine-8-^{14}C (0·1 μc, spec. act. 11·2 μc/μmole.) Temp. 27°.

RESULTS

Effect of azaserine on purine metabolism

Azaserine decreased the incorporation of adenine-8-^{14}C, guanine-8-^{14}C, and hypoxanthine-8-^{14}C into the nucleic acid fraction and correspondingly increased the incorporation of these ^{14}C-labeled purines into the acid-soluble fraction of *T. equiperdum* incubated *in vitro* (Table 1). The amount of inhibition produced by azaserine on the incorporation of each of these ^{14}C-labeled purines into the nucleic acid fraction was

about the same (ca. 60%). This effect of azaserine has been reported before in *T. cruzi*[18] and in mice bearing sarcoma 180.[19] Contrary to all these findings it has also been reported that azaserine stimulated the incorporation of adenine-^{14}C into the nucleic acid fraction of some mammalian tumor cells.[5, 20]

The presence of L-tryptophan, L-leucine, L-tyrosine, and L-glutamine in the incubation medium produced a partial reversal of the inhibition produced by azaserine on the incorporation of adenine-8-^{14}C into the nucleic acid fraction (Table 2). Of the

TABLE 2. EFFECT OF PRESENCE IN MEDIUM OF DIFFERENT AMINO ACIDS UPON ABILITY OF AZASERINE TO INHIBIT INCORPORATION OF ADENINE-8-^{14}C INTO NUCLEIC ACID FRACTION OF *TRYPANOSOMA EQUIPERDUM* INCUBATED *IN VITRO**

Azaserine (μmole/ml)	L-Amino acid (μmole/ml)	Nucleic acid (counts/min)
none	none	27,900
0·2	none	12,600
0·2	L-tryptophan (2)	14,900
0·2	L-leucine (2)	19,600
0·2	L-tyrosine (1)	20,100
0·2	L-glutamine (10)	25,300

* Trypanosomes (50 × 10^6) were placed in 3 ml of modified Krebs phosphate buffer (pH 7·4, 0·116 M) and incubated for 30 min in presence of azaserine and amino acids, followed by 60 min in presence of adenine-8-^{14}C (0·1 μc, spec. act. 14·4 μc/μmole.) Temp. 27°.

TABLE 3. EFFECT OF AZASERINE ON INCORPORATION OF ADENINE-8-^{14}C INTO ACID-SOLUBLE PURINES-^{14}C AND ON INCORPORATION OF URACIL-2-^{14}C INTO ACID-SOLUBLE PYRIMIDINE NUCLEOTIDES-^{14}C OF *TRYPANOSOMA EQUIPERDUM* INCUBATED *IN VITRO**

Compound isolated	Control		Azaserine	
	(counts/min)	(μmoles)	(counts/min)	(μmoles)
Adenine	912,000	1·40	1,100,000	1·50
Guanine	17,700	0·33	22,200	0·32
Uridine phosphate	2,770,000	0·60	3,610,000	0·65
Cytidine phosphate	8,200	1·9†	600	1·9†

* Trypanosomes (15 × 10^9) were placed in 200 ml of modified Krebs phosphate buffer (pH 7·4, 0·11 6 M) and incubated for 20 min in presence of azersarine (1·0 μmole/ml), followed by 40-min incubation in presence of adenine-8-^{14}C (8·75 μc, 3·24 μc/μmole) or in presence of uracil-2-^{14}C (10·0 μc 31·8 μc/μmole). Temp. 27°.

† Cold carrier CMP was added during the isolation procedure.

first three amino acids, L-tyrosine possessed the most potent reversing activity, L-leucine was intermediate, and L-tryptophan was least active. These same amino acids have been shown to reverse the inhibition produced by azaserine of the growth of *Escherichia coli*,[5] *Streptococcus faecalis*,[21] and *Kloeckera brevis*.[22]

Azaserine increased the incorporation of adenine-8-^{14}C into acid-soluble adenine-^{14}C and guanine-^{14}C-containing compounds, most of the radioactivity being present in the adenine fraction (Table 3).

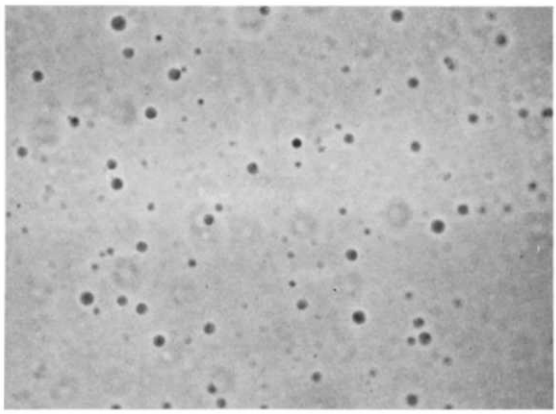

FIG. 4. Phase-contrast microphotograph of control mitochondria 10 min. after suspension in system given for Fig. 1. Magnification × 1850.

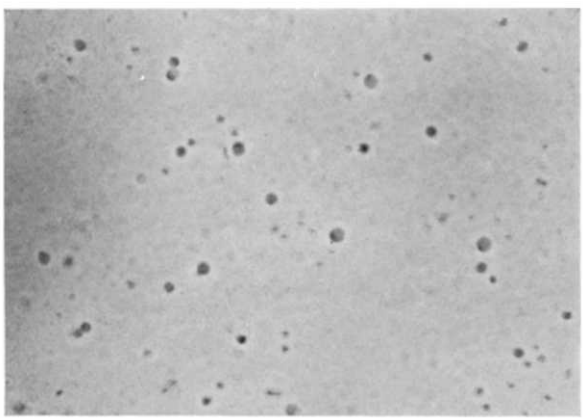

FIG. 5. Phase-contrast microphotograph of control mitochondria 20 min after suspension in system given for Fig. 1. Magnification × 1850.

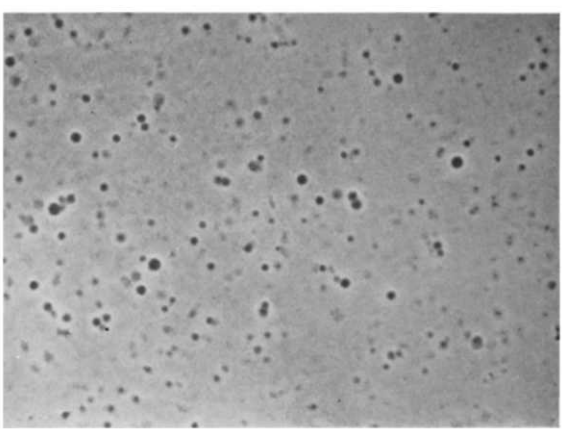

FIG. 6. Phase-contrast microphotograph of mitochondria 30 min. after suspension in system given for Fig. 1 plus 1 mM D-tubocurarine and 0·1 mM DL-Fe $(phen)_3^{++}$. Magnification × 1850.

facing page 242

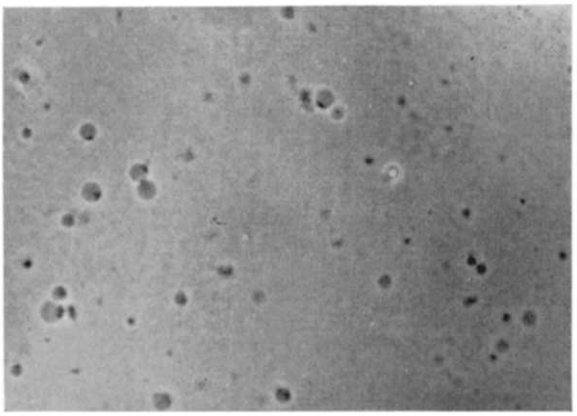

FIG. 7. Phase-contrast microphotograph of mitochondria 20 min. after suspension in system given for Fig. 1 plus 0·1 mM DL-Fe $(phen)_3^{++}$. Magnification × 1850.

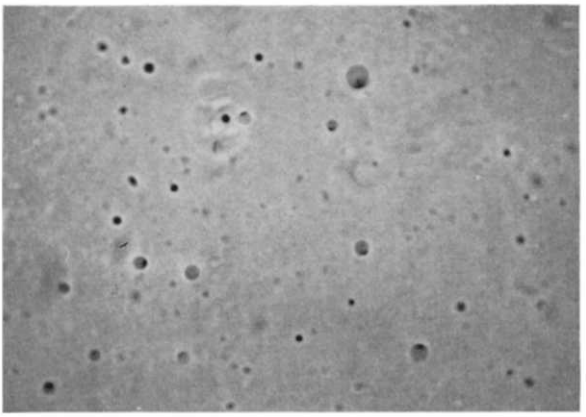

FIG. 8. Phase-contrast microphotograph of mitochondria 30 min after suspension in system given for Fig. 1 plus 0·2 mM L-Ru $(phen)_3^{++}$. Magnification × 1850.

Effect of azaserine on pyrimidine metabolism

Azaserine decreased the incorporation of uracil-2-^{14}C and orotic acid-2-^{14}C into the nucleic acid fraction and increased the incorporation of these ^{14}C-labeled pyrimidines into the acid-soluble fraction of *T. equiperdum* incubated *in vitro* (Table 4). Similar findings reported previously have shown that azaserine[9] and DON

TABLE 4. EFFECT OF AZASERINE ON INCORPORATION OF ^{14}C-LABELED PYRIMIDINES INTO ACID-SOLUBLE AND NUCLEIC ACID FRACTIONS OF *TRYPANOSOMA EQUIPERDUM* INCUBATED *IN VITRO**

Pyrimidine	Control		Azaserine	
	Acid-soluble	Nucleic acid	Acid-soluble	Nucleic acid
	(counts/minute)			
Uracil-2-^{14}C	24,800	14,700	27,000	4,400
Orotic acid-2-^{14}C	7,900	3,300	8,200	400
Cytosine-2-^{14}C	2,600	none	2,500	none
Thymine-2-^{14}C	2,800	none	2,500	none

* Trypanosomes (50×10^6) were placed in 3 ml of modified Krebs phosphate buffer (pH 7·4, 0·116 M) and incubated for 30 min in presence of azaserine (0·2 μmoles/ml), followed by 60-min incubation in presence of labeled pyrimidines, uracil-2-^{14}C (0·14 μc, sp act. 6·5 μc/μmole), orotic acid-2-^{14}C (0·28 μc, sp act. 4·2 μc/μmole), cystosine-2-^{14}C (0·14 μc, spec. act. 5·6 μc/μmole), thymine 2-^{14}C (0·14 μc/ spec. act. 14·8 μc/μmole). Temp. 27°.

(6-diazo-5-oxo-L-norleucine)[23] decreased the incorporation of orotic acid-6-^{14}C into the RNA fraction of mammalian tumor cells. At the amounts used there was no incorporation of cytosine-2-^{14}C or thymine-2-^{14}C into the nucleic acid fraction.

Azaserine increased the incorporation of uracil-2-^{14}C into acid-soluble uridine phosphate and decreased the incorporation of uracil-2-^{14}C into acid-soluble cytidine phosphate, most of the radioactivity being present in the uridine phosphate fraction (Table 3). In this experiment cold CMP was added as carrier during the isolation procedure; no detectable CMP (by u.v. measurements) could be isolated from the acid-soluble fraction of 15×10^9 trypanosomes (about 1·0 g wet weight) in the absence of CMP as carrier. Azaserine has been shown to inhibit the incorporation of orotic acid-6-^{14}C into acid-soluble CMP of Novikoff hepatoma.[9]

After the incorporation of uracil-2-^{14}C into the nucleic acid fraction, most of the radioactivity was recovered in the RNA fraction and comparatively little in the DNA fraction; azaserine decreased the incorporation of radioactivity derived from uracil-2-^{14}C into both of these fractions (Table 5). The decreased amount of radioactivity in the RNA fraction in the presence of azaserine was paralleled by a proportionate increase in radioactivity in the acid-soluble uridine phosphate fraction (Table 3). The total amount of RNA, but not DNA, as measured by optical density units at 260 mμ was decreased by azaserine.

Fractionation of the pyrimidine nucleotides of RNA revealed that the ratio of incorporation of radioactivity derived from uracil-2-^{14}C into UMP and CMP (UMP/CMP) was about 10 (Table 5). Azaserine decreased the incorporation of uracil-2-^{14}C into both the UMP and CMP fractions of RNA, the decrease in incorporation into the CMP fraction being considerably greater than that into the UMP fraction.

The uptake of cytosine-^{3}H or cytidine-^{3}H into the acid-soluble and nucleic acid fractions of *T. equiperdum* incubated *in vitro* was very low (Table 6); by comparison, the uptake of uracil-2-^{14}C and orotic acid-2-^{14}C into the acid-soluble and nucleic acid fractions was considerably greater (Table 4).

TABLE 5. EFFECT OF AZASERINE ON INCORPORATION OF URACIL-2-^{14}C INTO DNA, RNA AND URIDINE PHOSPHATE AND CYTIDINE PHOSPHATE FRACTIONS OF RNA OF *TRYPANOSOMA EQUIPERDUM* INCUBATED *IN VITRO**

Fraction	Control		Azaserine	
	(counts/min)	(O.D. units)	(counts/min)	(O.D. units)
DNA	4,600	11	2,200	11
RNA	1,510,000	145	528,000	136
Uridine phosphate (RNA)	1,630,000		575,000	
Cytidine phosphate (RNA)	128,000	22·9	900	22·5

* Trypanosomes (15×10^9) were placed in 200 ml of modified Krebs phosphate buffer (pH 7·4 0·116 M) and incubated for 20 min in presence of azasersine (1·0 μmole/ml), followed by 40-min incubation in presence of uracil-2-^{14}C (10·0 μc, 31·8 μc/μmole). Temp. 27°.

TABLE 6. INCORPORATION OF CYTOSINE-^{3}H AND CYTIDINE-^{3}H INTO ACID-SOLUBLE AND NUCLEIC ACID FRACTIONS OF *TRYPANOSOMA EQUIPERDUM* INCUBATED *IN VITRO**

Pyrimidine	Acid-soluble (counts/min)	Nucleic-acid (counts/min)
Cytosine-^{3}H	1,000	500
Cytidine-^{3}H	900	400

* Trypanosomes (75×10^6) were placed in 3 ml of modified Krebs phosphate buffer (ph 7·4, 0·116 M) and incubated in the presence of the labeled pyrimidines for 45 min. Cytosine-^{3}H (6·25 μc, spec. act. 1,130 μc/μmole), cytidine-H^3 (6·25 μc, 1,210 μc/μmole). Temp. 27°.

DISCUSSION

The minor importance of the *de novo* pathway for the biosynthesis of purine nucleotides in *T. equiperdum*,[6,7] a pathway that is markedly inhibited by azaserine in other systems,[2–5] suggested that biochemical studies of this trypanosome could possibly clarify the modes of action of azaserine and separate the distinct effects of this antimetabolite on purine metabolism from those on pyrimidine metabolism. The potent inhibition of the reproduction of *T. equiperdum* by azaserine[1] suggested that this antibiotic must be acting at a specific site other than the *de novo* purine biosynthetic pathway.

The partial reversal by L-glutamine, L-tryptophan, L-leucine, and L-tyrosine of the inhibition produced by azaserine on the incorporation of adenine-8-^{14}C into the nucleic acid fraction of *T. equiperdum* incubated *in vitro* (Table 2) could be due, as suggested by Jacquez,[24] to the competition of these compounds with azaserine for the active transport system of amino acids. Another possibility which should be considered in the case of L-glutamine is that this amino acid may also be acting at the enzymatic

site of inhibition of azaserine, since it has been found that L-glutamine can competitively reverse the inhibition produced by azaserine on certain glutamine-dependent enzymatic reactions.[2, 4, 10, 25]

Azaserine decreased the incorporation of ^{14}C-labeled purines and pyrimidines into RNA and also decreased the total amount of RNA (Tables 1, 4, 5). These findings suggested that one of the major effects of azaserine on *T. equiperdum* was to inhibit the biosynthesis of RNA.

Abrams and Bentley[25] found that azaserine inhibited the glutamine-mediated enzymatic conversion of XMP to GMP in calf thymus and Lowy and Williams[26] presented similar evidence in rabbit erythrocytes. The failure of azaserine to inhibit the incorporation of adenine-8-^{14}C into acid-soluble guanine-^{14}C-containing compounds suggested that azaserine does not block the conversion of XMP to GMP in *T. equiperdum* (Table 3). This finding excludes the possibility that inhibition at this site by azaserine explains the mechanism of inhibition of the biosynthesis of RNA.

In *T. equiperdum*, azaserine decreased the incorporation of uracil-2-^{14}C into acid-soluble CMP (Table 3) and into CMP of RNA (Table 5), evidence which strongly suggests that azaserine inhibits the conversion of UMP to CMP in this protozan. This interpretation of our findings is strengthened by the report of Kammen and Hurlbert[9] that azaserine inhibited the conversion of UMP to CMP in Novikoff hepatoma. Also supporting this interpretation are the reports that DON, an analog of azaserine, inhibited the conversion of UTP to CTP in cell-free extracts of Novikoff hepatoma[10] and *E. coli*.[11]

The inhibition of the conversion of UMP to CMP by azaserine in *T. equiperdum* may possibly explain the inhibition of the biosynthesis of RNA. The enzymatic synthesis of RNA in mammalian cells requires the presence as substrates of all four nucleotides (ATP, GTP, UTP, and CTP).[27, 28] The intracellular level of CMP was found to be much lower than AMP, GMP, and UMP in *T. equiperdum* (Table 3), a condition that was also found to exist in mammalian liver.[29] It is possible that azaserine, by inhibiting the conversion of UMP to CMP, decreased the intracellular level of CMP to such a point as to limit the rate of biosynthesis of RNA in *T. equiperdum*. This appears at this time to be the most likely mechanism by which azaserine inhibits the biosynthesis of RNA, but the data do not exclude the possibility that azaserine could inhibit the biosynthesis of RNA by other mechanisms such as direct inhibition of RNA polymerase.

The extremely limited uptake *in vitro* of cytosine-^{3}H and cytidine-^{3}H by T. *equiperdum* suggests that this organism very strongly depends upon the *de novo* pathway for the biosynthesis of CMP. On the other hand mammalian cells are able to utilize the preformed pathway for the synthesis of CMP[9]. The inhibition of the *de novo* synthesis of CMP with an antimetabolite such as azaserine may provide a site for exploitation in the chemotherapy of trypanosomiasis since mammalian cells can possibly be protected from the toxic effects of azaserine with preformed cytosine-containing compounds (also preformed purines since azaserine does inhibit *de novo* purine biosynthesis). The rapid deamination of cytidine by mammalian cells[30] could limit the protective effect of this compound and thus cytosine, because of its lower rate of deamination,[30] may be a more useful agent in this respect.

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