Metabolism of Leukemic Cells in Culture; Azaserine Inhibition of J-128 (Osgood).* (28829)

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Since it has been demonstrated that azaserine interferes with several glutamine catalyzed enzyme reactions, *in vitro,* it has been concluded that the primary site of action involves inhibition of conversion of formylglycinamide-ribotide to formylglycinamidine-ribotide(1).

Formylglycinamide-ribotide + glutamine + ATP ⟶ formylglycinamidine-ribotide + glutamic acid.

Preformed purine or aminoimidazole carboxamide (AIC) reverse the inhibitory effect of azaserine on *E. coli* cells indicating that in these cells azaserine inhibits *de novo* purine synthesis(2). When cell free extracts of pigeon liver are incubated in the presence of azaserine, formylglycinamide-ribotide accumulates suggesting that the first glutamine catalyzed reaction of *de novo* purine synthesis shown below, is not markedly inhibited by azaserine(3). Pyrophosphorylribose-5-PO_4 + glutamine ⟶ ribosylamine-5-phosphate + glutamic acid.

Two mechanisms have been presented to describe the inhibitory action of azaserine. Baker(4) has suggested that azaserine reacts with pyridoxal phosphate yielding an inactive cofactor-azaserine complex while French(5) has recently reported that azaserine reacts with a sulfhydryl group of the enzyme which converts formylglycinamide-ribotide to formylglycinamidine-ribotide.

Although it would appear that azaserine inhibits growth of *E. coli* by interfering with *de novo* purine synthesis, our data indicate that this may not be the primary site of action in the case of a tissue culture cell line which was originally derived from a patient with chronic granulocytic leukemia (Osgood J-128).

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Materials. Hypoxanthine-8-C^{14} (4.10 millicuries/millimole), guanine-8-C^{14} (6.00 millicuries/millimole), and glycine (4.42 millicuries/millimole), were obtained from New England Nuclear Corp. Hypoxanthine, guanine and AIC were purchased from Mann Biochemical Corp. Bovine serum, minimum essential medium (MEM) and glutamine were obtained from Microbiological Associates. Dr. Dice, Parke-Davis Laboratories, generously provided the azaserine, and the initial culture of J-128 was kindly supplied by Dr. E. E. Osgood, Univ. of Oregon Med. School. Narrow mouth 8-oz Blake culture bottles were secured from Wheaton Co.

Methods. Monolayer cultures of J-128 were maintained on a medium consisting of 90% MEM, and 10% bovine serum. This medium was adjusted to pH 7.4 with sodium bicarbonate, made 2 mM with glutamine and enough penicillin was added to give the final concentration of 130 units per ml of medium. Twenty-five ml of this medium was added to each culture bottle. Stock cultures were subcultured every 4 days by releasing the cells with versene, diluting with fresh medium, and transferring to 2 fresh culture bottles. About 40% of the cells ruptured during this procedure and the number of cells attached to the glass after subculture was between 8 to 10 million. Cells cultured by this procedure for several months appeared uniform when observed with a low powered dissecting microscope and phase microscopy revealed the presence of only 1 to 2% giant cells. The cells contained from one to 5 nucleoli with over half the cells having 2 to 3 nucleoli. Phase microscopy revealed that each cell contained numerous round granules or mitochondria. The size of these particles varies greatly from cell to cell, and Wright stains of the cells did not reveal these bodies.

All experiments discussed below were carried out using pooled cultures to give the same number of cells in each culture bottle.

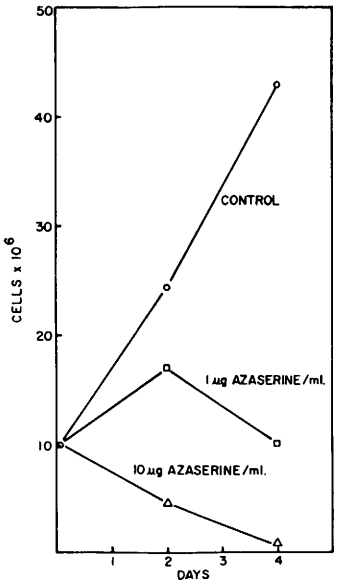

FIG. 1. Inhibition of growth of J-128 tissue culture cells by azaserine.

Compounds added to the cultures were made up in MEM and heat sterilized with the exception of azaserine, which was sterilized by passage through a 0.45 μ millipore filter. Growth of cultures was measured by disrupting the cells in citric acid and counting the nuclei in a hemocytometer. Adenine and guanine from total cell nucleic acid was prepared as described below: Cells were released with versene and washed twice with saline. Cells from 4 cultures were combined, and one ml of 5% ice cold perchloric acid then added. After standing for 15 minutes at 5°C the cells were collected by centrifugation, the supernatant discarded, and the pellet extracted a second time as described above. One-half ml of 5% perchloric acid was added to the cell pellet which was dispersed by gently tapping the tube. The tube was then placed in a boiling water bath for 15 minutes after which it was cooled to room temperature, centrifuged and 0.1 ml of the supernatant was applied to Whatman No. 1 chromatograph paper. Descending chromatography was carried out employing HCl-isopropanol-butanol-H_2O (1-5-4-20) as solvent. Adenine and guanine were located with a mineralite, the spots cut out and the bases eluted in 5 ml of 0.1 N HCl. The concentration of each purine solution was determined in a DU spectrophotometer and the radioactivity in each eluent measured with a Packard Tri-carb liquid scintillation counter.

Results. The inhibitory effect of azaserine on the growth of J-128 is demonstrated in Fig. 1. One μg of azaserine per ml of medium produces arrest of growth over a 4-day period. This experiment has been repeated numerous times with similar results. In some instances there was approximately 50% increase in the number of cells after 4 days. Ten μg of azaserine per ml of culture medium in all experiments caused cell death and an insignificant number of cells are found attached to the culture bottles on Day 4.

As may be observed from Table I, neither AIC, hypoxanthine, nor a combination of hypoxanthine and guanine reverse the inhibitory effect of azaserine. This is true whether azaserine is added at a concentration of 1 μg/ml medium or 10 μg/ml medium. In these experiments azaserine was added with the preformed purines or AIC on day zero, and after 4 days the cells attached to the glass were released, disrupted with citric acid, and the nuclei counted. The results are given as the number of cells present after 4 days divided by the number of cells which were

TABLE I. Failure to Reverse Azaserine Inhibition with AIC and Preformed Purines.

Culture No.	Additions to culture medium (μg/ml)	Cells, day 4 / Cells, day 0
1	None	4.4
2	Azaserine 1 μg	1.5
3	Azaserine 10 μg	.1
4	AIC 40 μg	5.1
5	AIC 40 μg +Azaserine 1 μg	1.6
6	AIC 40 μg +Azaserine 10 μg	.0
7	Hypoxanthine 40 μg	5.1
8	Hypoxanthine 40 μg +Azaserine 1 μg	.0
9	Hypoxanthine 20 μg +Guanine 20 μg	4.2
10	Hypoxanthine 20 μg +Guanine 20 μg +Azaserine 1 μg	1.5
11	Hypoxanthine 20 μg +Guanine 20 μg +Azaserine 10 μg	.1

TABLE II. Effect of AIC and Preformed Purines on Incorporation of Glycine-1-C^{14} into Nucleic Acid Adenine and Guanine of J-128.

Exp No.	Addition to media containing glycine-C^{14}*	cpm/μmole† Adenine	cpm/μmole† Guanine
		($\times 10^6$)	
1	None	.273	.277
2	"	.204	.243
3	"	.232	.200
4	Hypoxanthine 1 mg/culture	.004	.000
5	*Idem*	.008	.000
6	AIC 1 mg/culture	.060	.000
7	*Idem*	.012	.000
8	Guanine .2 mg/culture	.225	.177
9	" 1.0 "	.230	.110

* Glycine-1-C^{14} = 1 mg/culture, 0.730 $\times 10^6$ cpm/μmole.
† Avg of 4 experiments.

attached to the bottles on day zero. A value of 1.0 would indicate cell stasis while a value under one indicates a decrease in the number of cells from day zero. The amount of preformed purine or AIC added was at least in 10-fold excess of that required to meet the purine requirements of the cells for nucleic acid synthesis.

The ability of these cells to utilize preformed purine or AIC is illustrated in Table II. When preformed purines are not added to the medium the cells incorporated glycine-1-C^{14} into nucleic acid adenine and guanine. The added glycine-1-C^{14} had a specific activity of 0.73 CPM/μmole. When AIC or preformed purines were not added, nucleic acid adenine and guanine had a specific activity of approximately 0.2 CPM/μmole; approximately 30% that of the added glycine-1-C^{14}. When hypoxanthine was added to the culture medium along with glycine-1-C^{14} and the nucleic acid purines isolated after 4 days, insignificant amounts of radioactivity were found associated with the nucleic acid purine. The addition of AIC along with glycine-1-C^{14} yielded similar results, but a significant amount of radioactivity was found associated with radioactive nucleic-acid-adenine. Addition of guanine with glycine-1-C^{14} did not affect activity of nucleic acid adenine, but significantly lowered the specific activity of guanine isolated from nucleic acid.

Experiments were carried out adding radioactive hypoxanthine-8-C^{14} and guanine-8-C^{14} to culture medium and subsequently isolating nucleic acid guanine and adenine. The results obtained in these experiments were in agreement with those reported above.

Discussion. The failure of AIC or hypoxanthine to reverse azaserine inhibition of J-128 indicates that azaserine exerts a block other than preventing *de novo* purine synthesis, as is the case with *E. coli.* The data also suggest that azaserine does not exert its action by blocking conversion of xanthylic acid to guanylic acid, a reaction which has been shown to be inhibited only by high concentration of azaserine *in vitro*(6). However, the evidence in this instance is not as clear cut. Guanine is converted to guanylic acid, but whether it is converted directly or at a rate fast enough to meet cell requirements of guanylic acid is questionable.

Summary. Osgood's tissue culture J-128 may be cultured on a simple medium which does not contain preformed purine or AIC. When hypoxanthine or AIC are added to the culture medium the cells utilize these compounds as a source of nucleic acid, adenine and guanine. Addition of one μg of azaserine per ml of culture medium results in cell stasis and 10 μg of azaserine results in total cell death within 4 days. Hypoxanthine, AIC or guanine do not reverse the inhibitory action of azaserine. It would appear that azaserine causes cell death by some mechanism other than inhibition of *de novo* purine synthesis.

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