Azaserine Resistance in a Plasma-Cell Neoplasm without Change in Active Transport of the Inhibitor*

E. P. ANDERSON AND JOHN A. JACQUEZ

(National Cancer Institute, National Institutes of Health, Bethesda, Maryland; Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Graduate School of Medical Sciences, New York, New York)

SUMMARY

An investigation was made of azaserine entry into cells of a sensitive and resistant line of the plasma-cell neoplasm 70429. Transport experiments determined (a) the initial velocity of concentrative uptake in the first 1 or 2 minutes of exposure to the compound, (b) the total kinetics of uptake over 30-minute to 90-minute periods, and (c) the dependence of steady-state uptake on the extracellular concentration of the inhibitor.

Azaserine was rapidly taken up by these cells and concentrated intracellularly, even within 1-2 minutes, to a level tenfold that of the extracellular medium at low extracellular concentrations; this distribution gradient was less at higher extracellular concentrations. The uptake leveled off at 20-30 minutes and remained constant over longer time intervals. Older cells showed somewhat less active transport than did the 10-day-old cell harvests used routinely in these experiments.

The sensitive and resistant lines were found to be nearly identical in their capacity for active transport of azaserine; the kinetics and extent of concentrative uptake were very nearly the same in both lines over all the time periods examined. Over longer time intervals the resistant line was able to attain actually a slightly higher intracellular concentration, especially at higher extracellular concentrations of the inhibitor, but this may have been due to some damage of the sensitive cells by the inhibitor rather than to a real difference in transport capacity. The resistant line showed slightly less transport compared with the sensitive line only at very short time intervals (1-to 2-minute uptake) and this only at high concentrations of the inhibitor; even under these conditions uptake in the resistant line was 80-90 per cent of that in the sensitive line. It is concluded that a difference in transport capacity cannot explain the biological resistance of the 70429/Az(la) line nor account for the differences previously observed with intact cells in azaserine inhibition of purine biosynthesis.

The experiments also measured the intracellular breakdown of azaserine. Both the extent and kinetics of breakdown were nearly identical for the sensitive and resistant lines, and it is concluded that increased breakdown of azaserine in the resistant cells cannot account for the resistance.

O-Diazoacetyl-L-serine, azaserine, originally iso- to be an antagonist of glutamine and to block amide transfer from glutamine; it inhibits various glutamine-requiring aminations (2), specifically the biosyntheses of phosphoribosylamine (29) and especially of formylglycinamidine ribotide (23, 34) in de novo purine synthesis, of guanosine 5'-phosphate (1), of cytidine nucleotides (19), of anthranilic acid (31), and of diphosphopyridine nucleotide (26).

Received for publication July 14, 1961.

* This work was supported in part by grants T36 and T37 from the American Cancer Society and by CCNSC contract 1SA-45-ph-2445, National Cancer Institute, Public Health Service.

Available evidence indicates that resistance in
cell populations frequently arises by mutation, with a resistant population then being selected by the presence of the drug (21, 22). Considerable effort has been expended in attempts to correlate heritable resistance with specific alterations in biochemical characteristics, especially enzymatic activities, and recent progress along these lines has been most encouraging (2). Decreased entry of a drug into the resistant cells has often been cited as a possible mechanism of resistance; in the cases where entry has been shown to involve active transport linked to cell metabolism, this mechanism would fall into the category of a biochemical alteration in metabolism rather than simply a physical change in surface characteristics and can thus be more readily visualized as an enzymatic expression of a genetic change.

Azaserine is transported into cells and concentrated intracellularly by the amino acid transport system; in many cells the intracellular-extracellular concentration gradient attained is greater than that attained by "normal" amino acids (18). This transport has been studied in several cell systems (14, 15, 18, 24). The theoretical considerations in active transport of amino acids have recently been discussed in detail by Jacquez (16).

Previous studies in our laboratory on resistance to azaserine in the plasma-cell neoplasm 70429/Az-(1a) had provided evidence for an "intact cell factor" in the mechanism of this resistance. Azaserine inhibited purine biosynthesis in preparations of both the sensitive and resistant cells, but the difference between the two lines in the degree of this inhibition was greater in intact cell suspensions than it was in cell-free extracts (3). Since an obvious explanation for this could be decreased entry of azaserine into the resistant cells, compared with the sensitive cells, it appeared necessary to study specifically this entry, and toward this end the present studies were undertaken.

MATERIALS AND METHODS

Growth of cell lines and preparation of cell suspensions.—The ascitic form of the plasma-cell neoplasm 70429 was described and characterized by Potter and Law (25). This tumor is undifferentiated and has now been identified as of plasma-cell origin. The azaserine-resistant subline 70429/Az-(1a) and has, for our experiments, been routinely carried in the presence of the inhibitor up to the transplant generation used for experimental purposes. This line is approximately 78 per cent resistant (100 X 10-day cell count on azaserine/10-day cell count in the absence of azaserine) to a dose of 5 mg/kg of azaserine administered daily 6 days a week. Resistance in this subline has been maintained at approximately the same level for 110 additional transplant generations.

The cell lines were carried in C3H mice by periodic intraperitoneal injection of ascites cells. For experimental cell harvests mice were given injections of 1 X 10⁸ cells (by cell count) and killed on the 10th day of tumor growth. Ascites cells were collected from the abdominal cavity with a pipette and suspended in 5–10 volumes of fresh Krebs-Ringer bicarbonate containing 0.05 mg heparin/5 ml. This suspension was centrifuged at 800 × g in an International No. 2 centrifuge; the cells were washed in Krebs-Ringer bicarbonate without heparin and then resuspended in enough Krebs-Ringer bicarbonate to give a cytocrit of 0.1 or less for most experiments. Neither the sensitive nor the resistant line of the ascitic tumor is hemorrhagic, and no precautions were needed to separate the ascites cells from red blood cells.

Incubation of cells with azaserine and separation of cells.—The concentrative uptake of azaserine was examined as described previously by exposing measured amounts of cells to the inhibitor at 37° C. for specified periods of time and then determining intracellular and extracellular azaserine (14, 17). All procedures prior and subsequent to the actual incubations were carried out in the cold to prevent transfer between intracellular and extracellular phases; cooling to 2°–4° C. markedly slows down the active transport and diffusion across the cell membrane (14). Three ml. of ascites cell suspension in Krebs-Ringer bicarbonate, prepared as described above, was put into one arm of a Heinecke two-arm reaction vessel, and 3 ml. of Krebs-Ringer bicarbonate containing additives was put into the other arm. The vessels were then gassed with 5 per cent CO₂ in air and stoppered. They were equilibrated to 37° C., after which the contents of the two arms of the vessel were mixed and incubated at 37° C. for the specified periods of time. The vessels were then plunged into ice water and agitated to hasten temperature equilibration. Cells were separated from extracellular fluid at 3° C. Two ml. of the

1 Azaserine was a gift of the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Maryland, and of Dr. John Dice, Parke, Davis and Company, Detroit, Michigan.
incubation mixture was pipetted into a 100 × 13-mm. test tube and centrifuged at top speed in a Phillips-Drucker 708 centrifuge for 15 minutes. The supernatant was decanted, the tube drained for 15 minutes, and the inside of the tube swabbed dry with gauze down to within 1–2 cm. of the pellet. The pellet and a sample of the supernatant solution were then used for extraction and analysis as described below. Another 2-ml aliquot of the incubate was pipetted into a tared test tube and handled in the same way; the supernatant from this was discarded, and the wet and dry weights of the pellet were determined. The remainder of the suspension was used for a cytotic determination with Van Allen or Wintrobe hematocrit tubes, to determine the concentration of cells in each sample, and for a cell count with 1:4000 trypan blue vital stain in Krebs-Ringer phosphate, to determine the proportion of damaged cells. All procedures were carried out as soon as possible after incubation. The cytocrits for these experiments were in the range of 0.07–0.1 (pellet volumes, 0.14–0.2 ml.). From the trypan blue examinations, the damaged cells in these experiments were usually 2–8 per cent of the total cells counted.

**Extraction of the packed cell pellet and supernatant.**—For extraction of azaserine the pellet was thoroughly resuspended and mixed with 4.8 ml. of 79 per cent ethanol containing barium acetate for precipitation of phosphorylated compounds; this mixture consisted of 1 ml. of 5 per cent aqueous barium acetate per 0.2 ml. of pellet volume, added to 50 ml. of 79 per cent ethanol. When higher concentrations of barium acetate were used the extracts were often opalescent. After centrifugation at 230 × g in an International No. 2 centrifuge, the sediment was resuspended in 5 ml. of 0.005 per cent barium acetate containing comparable amounts of cellular material, and the combined extracts were used the extracts were often opalescent. After centrifugation at 230 × g in an International No. 2 centrifuge, the sediment was resuspended in 5 ml. of 0.005 per cent barium acetate in 75 per cent ethanol and again centrifuged. The combined extracts were diluted to an appropriate volume with 75 per cent ethanol for spectrophotometric estimation of azaserine.

For determination of extracellular azaserine 1 ml. of supernatant from the cell suspension was mixed with 4 ml. of 95 per cent ethanol containing barium acetate; this mixture was prepared by adding 0.25 ml. of 5 per cent barium acetate, for each 0.2 ml. of pellet volume, to 50 ml. of 95 per cent ethanol. After centrifugation the precipitate was likewise re-extracted by resuspension in 5 ml. of 0.005 per cent barium acetate in 75 per cent ethanol, and the combined extracts were diluted as before for spectrophotometry.

**Determination and calculation of azaserine content.**—Estimations of azaserine were made as described previously (14). Spectrophotometric read-

ings were made at five wave-lengths near the peak of the absorption spectrum, 242, 246, 250, 254, and 258 m/μ; the millimolar extinction coefficients used, for azaserine in 75 per cent ethanol at the wave-lengths, were 13.53, 16.86, 18.17, 16.25, and 10.99, respectively. In each transport experiment appropriate control "background" samples containing comparable amounts of cellular material, but no azaserine, were treated in the same fashion as the azaserine incubates; spectrophotometric readings at the above wave-lengths on aliquots of these samples gave the absorption due to cell background material. Exposure to azaserine did not, apparently, produce appreciable change in the relative absorbance of the background material—i.e., in its "absorption spectrum"; the background material could, therefore, for calculation purposes, be treated as a single absorbing component. Readings at the same wave-lengths on the cell samples exposed to azaserine gave the absorption due to both this background "compound" and to azaserine; from these readings azaserine concentrations could be calculated as described previously (14). The total error in this method of determination is the sum of the squares of the differences between the actual absorption and the absorption which can be accounted for by azaserine and by the background "compound"; this can be represented by:

$$\Sigma[(c_1 \epsilon_{1,1} + c_2 \epsilon_{2,1}) - A_i]^2,$$

where, for each wave-length, λ, A_i is the actual observed absorption, c_1 \epsilon_{1,1} is the absorbance due to azaserine (concentration, c_1, of azaserine times its extinction coefficient at that wave-length, \epsilon_{1,1}), and c_2 \epsilon_{2,1} is the absorbance due to the background "compound" (concentration, c_2, times its extinction coefficient, \epsilon_{2,1}). The best values for c_1 and c_2 are those which minimize this total error—i.e., those for which the derivative of this term with respect to both c_1 and c_2 equals zero. If this is done, and the resulting equations are solved simultaneously for c_1, the concentration of azaserine can be represented by the term:

$$\frac{[\Sigma(\epsilon_{1,1})^2][\Sigma(\epsilon_{1,1} A_i)] - [\Sigma(c_{1,1})^2][\Sigma(\epsilon_{1,1} A_i)]}{[\Sigma(\epsilon_{1,1})^2][\Sigma(\epsilon_{2,1})^2] - [\Sigma(c_{1,1})^2][\Sigma(c_{1,1} \epsilon_{2,1})]^2}.$$

These summations over all wave-lengths can be readily calculated from the known values for \epsilon_{1,1} for azaserine, the appropriate \epsilon_{2,1} values observed.

1 These are the averages from many determinations, all corrected to the same peak extinction coefficient to compensate for observed spontaneous breakdown of azaserine during storage at 3° C. These values may, therefore, vary slightly from the true values, but the relative values for the different wave-lengths are correct; this variation cannot appreciably affect the results of the present experiments.
in the background sample, and the absorbance
A observed in the corresponding sample containing
azaserine.

Determination of extracellular space in packed
pellets.—The extracellular fluid volume in the pel-
et of packed cells, as prepared under the condi-
tions of these experiments, was determined by
mixing the cells thoroughly with a solution of
sucrose, centrifuging according to the usual experi-
mental procedure, and measuring the amount of
sucrose retained in the packed pellets. Since sucrose
is not taken up or utilized by the cells, all that
remains in the pellet is held in the extracellular
space at the same concentration as in the extra-
cellular fluid; this space can therefore be calculated
from the determinations of the extracellular con-
centration of sucrose and the total sucrose in the
pellet.

Ascites-cell suspensions in Krebs-Ringer bico-
arbonate were prepared as described above to give
a high cytocrit. Increments of cell suspension were
then diluted with Krebs-Ringer bicarbonate to
a total volume of 5 ml. and mixed in penicillin
bottles with 1 ml. of 0.25 M sucrose. A 2-ml.
aliquot of each mixture was then used for high-
speed centrifugation of cell pellet and separation
of extracellular supernatant exactly as in theaza-
serine experiments. Another 2-ml. aliquot was
used for wet and dry weight determinations, and
additional samples for cytocrit and trypan blue
viability determinations. Final cytocrits of the
mixtures ranged from 0.05 to 0.3. Mixing, sam-
ples, and centrifugations were carried out at 3° C.

Sucrose was extracted from the pellet by re-
suspending the packed cells in 6 ml. of distilled
water, and adding 2 ml. of 0.5 N NaOH and
2 ml. of 10 per cent ZnSO₄·7 H₂O. The NaOH
was standardized to a phenolphthalein end-point
against the ZnSO₄ solution. The mixture was
thoroughly shaken to break up the gel, and the
precipitate was centrifuged off and washed once
with 10 ml. of distilled water. The combined
extract and wash were diluted to 25 ml. for sucrose
determination. For sucrose extraction from the
supernatant solution, 0.5 ml. of this solution was
diluted to 10 ml. with distilled water and extracted
with 0.5 ml. of 0.5 N NaOH and 0.5 ml. of 10 per
cent ZnSO₄·7 H₂O. The precipitate was cen-
trifuged off and washed as before, and the extract
plus wash was diluted to 100 ml. A 2-ml.
aliquot of each pellet and supernatant sample was used
for colorimetric sucrose determination with thiou-
rea-resorcinol by the method of Roe et al. (29).

The concentration of sucrose in the extracellular
fluid of the pellet (Cₑ) is the same as that deter-
mined in the supernatant solution. This concen-
tration (Cₑ) times the volume of the extracellular
fluid (Wₑ) equals the total sucrose retained in the
pellet (s). The value for s is also measured directly,
and Wₑ can, therefore, be calculated from s/Cₑ.

The extracellular space in the pellet was de-
termined for a range of pellet volumes covering
the experiments performed. A standard curve was
constructed by plotting the extracellular fluid vol-
ume of the pellets against the pellet volume; from
this the extracellular volume could be found for
any given pellet size in subsequent experiments
on azaserine uptake.

RESULTS

Experiments on azaserine transport were carried
out to determine: (a) the initial velocity of concen-
trative uptake in the first 1 or 2 minutes of ex-
pposure to the compound, (b) the total kinetics
of uptake over longer time periods, and (c) the
dependence of steady-state uptake on the extra-
cellular concentration of the inhibitor.

Initial velocity of uptake.—The uptake of various
concentrations of azaserine was examined in ex-
pirments in which the cells were exposed to
azaserine for 1 or 2 minutes. Ascites-cell suspensions in
Krebs-Ringer bicarbonate were prepared from both the sensitive and resistant
lines as described under “Methods,” and when possible the
experiments on the two lines were performed si-
multaneously in order to standardize the timing
of the exposures to the compound. In one experi-
ment equal aliquots of the cell suspensions were
exposed for 1 minute to a range of azaserine
concentrations between 0.00012 M and 0.0095 M.
In a second experiment aliquots of the cell suspen-
sions were exposed for 2 minutes to concentrations
between 0.0003 M and 0.019 M. Background sam-
ples for each cell line contained comparable ali-
quots of the cell suspension but no azaserine.
In the first experiment (1-minute uptake) the
cell suspensions were incubated at 37° C. for 30
minutes, centrifuged, and resuspended in fresh
Krebs-Ringer bicarbonate before the uptake exper-
iment. This was done to diminish the back-
ground absorbance by soluble small molecules.
A high background would introduce appreciable
error into the spectrophotometric measurements
when a low intracellular concentration of azaserine
is to be measured, as was the case in some of the
samples in these brief uptake experiments.

From the data accumulated on each sample, the
total water in the pellet, Wₚ, in grams, was
calculated from the difference between the wet
and dry weights of the pellet. The extracellular
water, Wₑ, in grams, was taken from the calibration
curve for extracellular sucrose space of the pellet,
the pellet volume being determined from the cytotocrit. The difference between these two was the grams of intracellular water, \( W_e \). The total millimoles of azaserine, \( A \), in the pellet was measured directly. Of this, the amount that was extracellular could be calculated as the product of the extracellular volume, \( W_e \), and the azaserine concentration, \( C_e \), in the extracellular fluid (directly determined as the concentration in the supernatant above the pellet). The total millimoles of intracellular azaserine was then \( A - W_e C_e \) and the intracellular figure would be this additional azaserine concentration factor. The extracellular concentration, \( \langle C_e \rangle \), as plotted, is the average of \( C_e \) and \( C_{e,0} \) for each sample; the values are multiplied by \( 10^4 \) to give the results in millmoles/kg water. The values for millmoles/kg and millmoles/l are not significantly different and were used interchangeably.

From Chart 1 it can be seen that the uptake of azaserine was very rapid and in 1 minute produced an intracellular concentration which was, for low concentrations of azaserine, several-fold

![Chart 1](chart1.png)

**Chart 1.** Concentrative uptake as a function of extracellular concentration of azaserine during early time stages of exposure. \( C_e \) and \( \langle C_e \rangle \) were calculated as described in the text.

1a: Uptake in 1 minute at 0.00012 M to 0.0085 M extracellular concentration of azaserine could be calculated from the equation:

\[
C_i = \frac{A - W_e C_e}{W_i}
\]

(14). This value times \( 10^4 \) was the intracellular concentration in millimoles per kg. water and is plotted against the extracellular concentration of azaserine to show the uptake. The results are shown in Charts 1a (1-minute uptake) and 1b (2-minute uptake). In the experiments the extracellular concentration changed somewhat, owing to the uptake of the compound by the cells, and the initial extracellular concentration, \( C_{e,0} \), was therefore estimated from the observed concentration, \( C_e \), plus a factor representing this additional loss of extracellular azaserine. If the total intracellular azaserine \( (A - W_e C_e) \) were distributed throughout the extracellular volume of the suspension (supernatant volume, estimated from the supernatant in the cytotocrit determination, plus extracellular space in the pellet, \( W_e \)), the resulting concentration of azaserine, \( C_i \), could be calculated from the equation:

\[
C_i = \frac{A - W_e C_e}{W_i}
\]

that of the extracellular medium. The \( C_i / \langle C_e \rangle \) ratio was 8–10 where \( \langle C_e \rangle \) was 0.3–0.4 mmole/kg; this concentration gradient was even greater than, for example, that previously found with leukemia L1210 cells. The intracellular concentration leveled off at higher extracellular concentrations. The uptake over a 2-minute period was not twice that observed in 1 minute, but the two experiments may not be directly comparable.

In both experiments the resistant line showed an initial velocity of azaserine uptake which was consistently slightly less than that of the sensitive line at concentrations greater than 0.5 mmole/kg water. At these concentrations uptake into the resistant cells was 80–90 per cent of that into the sensitive cells; at lower concentrations there was no appreciable difference between the two lines. The sensitive and resistant cell lines also did not differ appreciably in cytotocrit, dry weight, or total water determinations, nor in calculated cell volume.

In a third experiment somewhat older cells
of each line (14 days after transplant) were tested for 1-minute uptake over a series of low concentrations of azaserine ranging from 0.00006 M to 0.0017 M. In this experiment the concentrative uptake was somewhat below the 1-minute uptake for 10-day-old cells, indicating less active transport by these older cells; but the data showed the same pattern of slightly less uptake in the resistant line compared with the sensitive line. In this experiment this was evident even at the low concentrations.

**Kinetics of uptake over longer time periods.**—For a determination of the total kinetics of uptake, equal aliquots of the cell suspensions in Krebs-Ringer bicarbonate were exposed to a certain concentration of azaserine for varying periods of time. Background samples containing no azaserine were incubated for comparable periods of time. In one experiment the cells were suspended in 0.002 M azaserine for periods of time from 2 minutes to 32 minutes. In a second experiment cells were exposed to a much higher concentration, 0.011 M azaserine, for periods of time from 5 minutes to 90 minutes. Again the uptake experiments were usually performed on both lines simultaneously, so that the azaserine solution used was identical, as were the timing of the incubations. There is some argument in favor of the shorter time period, since physiological damage to the sensitive cell line might result from longer exposure.

In the samples collected at each time point, the extracellular azaserine, $C_e$, was determined directly, and the intracellular azaserine in the pellet, $C_i$, was calculated, as described above. Both of these values were plotted against time to show the total kinetics of uptake. The results are shown in Charts 2a (uptake from 0.002 M azaserine) and 2b (uptake from 0.011 M azaserine). Like Chart 1, these curves show the very rapid active transport of azaserine into the cell; within 5 minutes the intracellular concentration attained a level several-fold that in the extracellular fluid. The $C_i/C_e$ ratio was approximately 2 when $C_e$ was 0.011 M (Chart 2b), and even greater, approximately 9, at the lower extracellular concentration of 0.002 M (Chart 2a). The uptake leveled off at later time periods. The kinetics of uptake were somewhat different for the two concentrations of azaserine; this was examined in more detail in

![Chart 2](chart2.png)

**Chart 2.**—Kinetics of azaserine uptake and of azaserine breakdown during longer time periods of incubation. 2a: Uptake and breakdown over 30 minutes at 0.002 M extracellular azaserine. 2b: Uptake and breakdown over 90 minutes at 0.011 M extracellular azaserine. The lower portions of the figures show concentrative uptake as the change with time of both the intracellular concentration of azaserine, $C_i$, and the extracellular concentration, $C_e$; these quantities were determined as described in the text. The upper portions of the figures show the total recovery of azaserine at each time point and therefore indicate the kinetics and extent of azaserine destruction. The total disappearance of azaserine at the longest time period was, in each experiment, about 10 per cent of the amount of azaserine present initially.

- $\triangle$ = 70420/S, azaserine-sensitive.
- $\bullet$ = 70420/Az(1a), azaserine-resistant.
subsequent experiments on steady-state intracellular concentrations at different extracellular concentrations (see below).

In both experiments it can be seen that the resistant line was very close to the sensitive line in kinetics of uptake over all of the time period examined, but that the inhibitor attained a slightly higher final intracellular concentration in the resistant line, compared with the sensitive line. In both experiments this difference was a factor of only about 10 per cent of the total intracellular concentration.

A third experiment was done on 20-day-old cells exposed to 0.011 M azaserine for different time intervals over a 60-minute period. As indicated by the experiments on initial velocity, these older cells showed less active transport of the compound; this was apparent over the entire time course of the kinetics curve, and the final intracellular concentration attained was, for each line, about 75 per cent of that observed in 10-day-old cells. However, these older cells showed the same pattern of a final intracellular concentration which was somewhat higher in the resistant line than in the sensitive line.

The data accumulated on these samples also permitted calculation of the total azaserine recovered from both the pellet and supernatant. The total millimoles of azaserine, A, in the pellet was determined directly. The total millimoles in the supernatant could be calculated from the supernatant volume (estimated from the cytocrit) times the extracellular concentration, Ce. The sum of these two values gave the total recovery of azaserine for each time point and showed the kinetics of intracellular plus extracellular azaserine breakdown in each experiment. These curves are plotted in the upper portions of Charts 2a and 2b. The concentration of azaserine initially added was also determined. In this experiment the steadystate level of uptake could be calculated. These data showed a disappearance of about 10 per cent recovery could be calculated. These data showed a disappearance of about 10 per cent of the azaserine over the course of each experiment. However, in both experiments the extent of breakdown with the resistant cells was the same as that with the sensitive cells; the kinetics of breakdown followed approximately the same course for the two lines, and the final recovery was nearly identical in both lines. The breakdown of azaserine was also examined in the experiment on 20-day-old cells. The destruction in the incubations with these older cells was a little greater than with 10-day-old cells, but the kinetics of breakdown did not differ at all for either line from those observed in the experiments with younger cells.

**The steady-state.**—From the experiments described above it could be seen that both the initial velocity and the over-all time kinetics of uptake of azaserine depended somewhat on the external concentration of the compound. The dependence of total or maximal uptake upon this factor was studied further by determining the effect of extracellular concentrations on the uptake at the steady state—i.e., after the velocity of uptake had become almost constant. For this, cells were exposed for 90 minutes to concentrations of azaserine varying over a hundred-fold range from 0.0001 M to 0.01 M. In previous work cells of both of these lines remained viable and maintained normal respiration when suspended in Krebs-Ringer solutions for this period of time, and de novo purine biosynthesis continued during this time. There may still, of course, have been some other kind of physiological damage to the sensitive line by the inhibitor during this interval. However, transport reached a plateau level by 90 minutes (Chart 2b) with no indication of any fall-off in uptake during this period, and this time point would appear to be a valid one at which to measure the steady-state intracellular concentrations.

From the data accumulated, values for Ce and C, at 90 minutes were obtained, as before, and uptake, as measured by C, was plotted against C, with the results shown in Chart 3. The actual C/C ratios at the different extracellular concentrations are given in Table 1. It can be seen that at the steady state the intracellular concentration was ten- to twenty-fold that in the extracellular fluid at lower extracellular concentrations and that this distribution ratio decreased with increasing extracellular concentrations.

* E. P. Anderson, unpublished.
As in the experiments on kinetics of uptake, the resistant line was able to hold or maintain an intracellular concentration of azaserine that was slightly higher than that in the sensitive line; this was particularly apparent at extracellular concentrations greater than 0.004 M but was evident throughout most of the concentration range explored in this experiment. Even at the point of maximum difference, however, the two lines differed by only about 10–15 per cent.

**DISCUSSION**

Previous work has indicated that azaserine is actively transported into cells by the same process as amino acids (14). This uptake is dependent upon concentration, and, as for amino acids (13), it follows pseudo-monomolecular kinetics, complicated for longer time periods by the disappearance of azaserine (14). The uptake is highly concentrative, especially in rapidly growing tissues such as tumor cells (6, 15). In the case of amino acids and other nutrients, this may give such cells a selective advantage in the competition for growth (6). It could also be a factor in the sensitivity of tumor cells to an inhibitor (see, for example [15, 24]).

However, from the present data it would appear that a difference in capacity for concentrative uptake could not account for the difference in azaserine sensitivity in these two lines of the neoplasm 70429. The resistant line showed a slightly lower transport capacity compared with the sensitive line only at short time intervals (1- to 2-minute uptake) and this only at very high concentrations of the inhibitor (greater than 0.5 mmole/kg water, Chart 1). Even these differences were of small magnitude, uptake by the resistant line being 80–90 per cent of that in the sensitive line, and it seems unlikely that they could account for the very high level of biological resistance (78 per cent; see “Methods”). Biochemical effects of azaserine, such as inhibition of purine biosynthesis, can be exerted by very low concentrations (23); 0.001 mmole/1 azaserine markedly inhibited purine biosynthesis in cells of 70429/S (3). This striking activity can probably be explained by the rapid and essentially irreversible way in which azaserine can bind to a sensitive enzyme (23). Transport of levels this low could not be detected and specifically studied with these methods for measuring azaserine; however, both the sensitive and resistant lines evidently have the capacity to take up many times this concentration of azaserine, and it would seem very unlikely that transport could be a limiting factor in intracellular biochemical inhibition. In previous work an “intact cell effect” was observed in azaserine inhibition of purine biosynthesis in 70429 (3). Glycine-C\(^14\) incorporation into acid-soluble purines was, in cell-free extracts, 57 and 46 per cent inhibited by azaserine in 70429/S and 70429/Az(1a), respectively—i.e., the inhibition in the resistant line was approximately 80 per cent of that in 70429/S. However, with intact cell suspensions, inhibition of this metabolic pathway was, in the resistant line, only half that in the sensitive line (44 and 85 per cent inhibition, respectively). The difference between intact cells and extracts is significant, but, in view of the present data, the comparative resistance of the intact cells would not seem to be due to any cell impermeability. From Chart 1, biochemically inhibitory concentrations, and even much higher concentrations, entered cells of both lines readily and equally well.

In experiments over longer time periods, the resistant cells surpassed the sensitive ones in the final intracellular concentration of azaserine which they could maintain. This difference was greater for higher concentrations of the inhibitor (Chart 3); and it is possible that this may be owing not to a difference between the two lines in transport capacity but merely to some damage to the sensitive cells by the inhibitor. Similar results were obtained previously in comparable experiments with azaserine-sensitive and -resistant L1210 (18).
Cells of 70429/S do remain viable and maintain some metabolic activity in the presence of azaserine. But unrevealed metabolic damage could presumably reduce transport capacity, since active transport of azaserine is apparently linked to intermediary metabolism.

There was some breakdown of azaserine by these cells, as indicated by the slow decrease in recovery of intracellular plus extracellular azaserine in Chart 2. Reilly has found that azaserine is enzymatically broken down by certain other tumors and normal tissues (27); the initial reaction is apparently a deamination, after which the diazo keto acid is thought to break down further spontaneously (28). The same reaction may account for the disappearance of azaserine in these experiments. There was apparently no significant difference in the extent of azaserine destruction by the sensitive and resistant cell lines, and increased breakdown by the resistant cells cannot, therefore, account for the resistance. Comparable extents of breakdown have also been found with azaserine-sensitive and resistant sublines of L1210 leukemia (18). If the reaction studied by Reilly is indeed the mechanism by which azaserine is broken down by these cells, it could not in any case explain the observed cross-resistance of 70429/Az to 6-diazo-5-oxo-1-norleucine (3, 25), since the enzyme has no effect on this latter compound (28). The calculations do not show how much of the azaserine breakdown was intracellular, but the total breakdown was on the order of 0.2 mmoles/l over a 15-minute period, which is appreciable in comparison with biochemically active levels of the inhibitor. Apparently the cells can both take up and destroy many times the biochemically inhibitory concentration, which fact again emphasizes the effective action of azaserine by virtue of its rapid and irreversible attachment to a sensitive enzyme system. Data such as those acquired in these experiments shed no light, of course, on possible mechanisms of resistance at the enzymatic level, such as more rapid recovery of purine biosynthesis in resistant cells (30) or altered affinity of resistant cell enzymes for normal substrates or for the inhibitor.

Pine (24) has reported studies on azaserine uptake in a number of tumor lines including the sensitive and two azaserine-resistant sublines of 70429; one of these sublines is probably similar to the one studied here, since both were derived from the same original resistant line. Pine’s data indicated less uptake of the inhibitor by the resistant cells, especially at lower extracellular concentrations; uptake in the sensitive line was nearly twice that of the resistant cells at 0.1 mmole/kg, and it was concluded that this difference could account for the “intact cell effect” observed in this laboratory in the inhibition of purine biosynthesis (3). From the present studies this would seem unlikely; with time periods equivalent to those employed by Pine, our data indicated the steady-state uptake in the two lines to be essentially identical at these and at lower concentrations (C_i/C_s = 21.3 for 70429/S and 21.0 for 70429/Az-1a at C_i = 0.14 mmole/kg, Table 1). In both studies concentrative uptake by the sensitive line was about tenfold at 1-5 mmoles/kg extracellular azaserine (Table 1); but uptake seen here in the resistant line was, throughout the comparable concentration range, appreciably greater than that reported by Pine and was very close to that in the sensitive line. Several sources of error could have contributed to such differences. For one thing, residual extracellular water retained in the tubes after drainage and wiping is a larger percentage of the total extracellular water for small pellet volumes, and the ratio of extracellular water to total water in the pellet is constant only at pellet volumes greater than 0.1 ml. (18). At the low pellet volumes used by Pine (0.02–0.05 ml.), this ratio would vary widely with differences in pellet size, and even small errors in the determination of pellet volumes would introduce large errors in the estimation of the extracellular water, W_e, upon which the calculations depend. The pellet volumes used in our experiments were in the range of 0.14–0.2 ml. and should therefore be subject to less error from this source; also, reproducibility appeared to be relatively good in these experiments. Secondly, in Pine’s experiments the resistant cells were harvested at 21 days, whereas the sensitive cell harvest was only 11 days old. In our studies older cells showed less active transport, and this could have contributed to decreased uptake in the resistant cells studied by Pine. These two cell lines show similar rates of growth during the first 10 days after inoculation, and should be comparable at the same harvest age. Lastly, there are no data on the extent of azaserine breakdown in Pine’s experiments, and this may have been an additional variable under the conditions of her experiments.

In our experiments there was little apparent difference between the two sublines in any of the characteristics studied. The background absorbance of the control samples did indicate somewhat more ultraviolet-absorbing material in the sensitive cells, and a separate experiment was carried out to determine the absorption spectrum in 75 per cent ethanol of the background material in
both cell lines. From these data the sensitive cell line appeared to contain about 30 per cent more extractable material having a nucleotide-type absorption spectrum; this may indicate a difference between the two lines in pool size of intracellular soluble nucleotides. It remains to be seen whether this difference could have any relation to the pattern of resistance to azaserine.

ACKNOWLEDGMENTS

For interest in and support of these studies, the authors are grateful to Dr. L. W. Law and Dr. G. B. Mider, National Cancer Institute; and to Dr. C. C. Stock, Sloan-Kettering Institute for Cancer Research, whose generous provision of laboratory space and facilities made this collaborative work possible. We are indebted to Mr. Thomas Lin for his able technical assistance, and we wish to thank Dr. R. W. Brockman, Dr. L. A. Heppel, and Dr. E. S. Maxwell for valuable suggestions on the manuscript.

REFERENCES


Azaserine Resistance in a Plasma-Cell Neoplasm without Change in Active Transport of the Inhibitor

E. P. Anderson and John A. Jacquez


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/22/1_Part_1/27

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.