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BIOCHEMICAL EFFECTS OF DUAZOMYCIN A IN THE MOUSE PLASMA CELL NEOPLASM 70429*

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Abstract—Sublines of the 70429 mouse plasma cell neoplasm exhibited cross-resistance between duazomycin A and the other glutamine antagonists, DON† and azaserine. Like these compounds, duazomycin A inhibited incorporation of formate into soluble purine nucleotides and into nucleic acids in growing tumor cells *in vivo*, and produced large accumulations of formylglycinamide ribotide in the soluble fraction of these cells. The utilization of hypoxanthine for purine nucleotide and nucleic acid synthesis was correspondingly increased. These findings are in agreement with a major site of duazomycin inhibition, as for the other two antagonists, at the glutamine-mediated amidination of formylglycinamide ribotide to formylglycinamidine ribotide. The accumulations of formylglycinamide ribotide were much reduced at higher levels of duazomycin, although purine nucleotide synthesis continued to be blocked; this would be consistent with an effective secondary inhibition earlier in the *de-novo* pathway of purine biosynthesis, in the glutamine-requiring synthesis of phosphoribosylamine. In this respect duazomycin resembled DON, but azaserine did not show this effect. Duazomycin was also an effective inhibitor of the conversion of uridine to cytidine nucleotides in these cells, although it was not so potent as DON in this respect. At the level tested duazomycin did not exert apparent inhibition of the synthesis of RNA guanylic acid from hypoxanthine, although such inhibition was evident with DON. Duazomycin was readily deacetylated, evidently to DON, by mammalian acylase I purified from hog kidney. In addition, acylase activity in mouse kidney deacetylated some duazomycin; acylase activity was weak in extracts of 70429 cells and had no detectable effect on duazomycin.

DUAZOMYCIN A (also called diazomycin A) was first described by Rao *et al.*[1] as an antibiotic and antitumor substance isolated from a culture broth of the actinomycete, *Streptomyces ambofaciens*. It was characterized as an aliphatic diazo compound related to O-diazoacetyl-L-serine (azaserine)[2] and to 6-diazo-5-oxo-L-norleucine (DON)†[3]; these antibiotics have likewise been isolated from *Streptomyces* cultures and found to possess antitumor activity. The chemical properties of duazomycin A have been well described by Rao *et al.*[1, 4]; the compound was found to be active against certain microorganisms[1, 5] and protozoa[6] and to possess activity against a number of

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† The abbreviations used are those accepted by the *Journal of Biological Chemistry*, with the following exceptions or additions: XMP, xanthosine-5′-phosphate; NAD, nicotinamide-adenine dinucleotide; DON, 6-diazo-5-oxo-L-norleucine; FGAR, formylglycinamide ribonucleotide; FGARiboside, formylglycinamide ribonucleoside.

neoplasms, both experimental and of human origin.[1,6–8] The present report is concerned with the further characterization of duazomycin A as a glutamine antagonist similar to azaserine and DON, and with the investigation of its biochemical effects, as such, in the mouse plasma cell neoplasm 70429.

MATERIALS AND METHODS

Chemicals

Sodium formate-^{14}C (3·6 mc/mmole), hypoxanthine-8-^{14}C (4·2 mc/mmole), and uridine-2-^{14}C (2 mc/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Crystalline azaserine and DON were gifts from Dr. John Dice, Parke, Davis and Co., Detroit, Mich., and from the Cancer Chemotherapy National Service Center, through the courtesy of Dr. R. B. Ross and Dr. Howard Bond. Duazomycin A (N-acetyl-6-diazo-5-oxo-L-norleucine) was furnished as a solution in sodium phosphate buffer; this was generously provided by Dr. Theodore Medrick of Charles Pfizer and Co., Brooklyn, N.Y., and by the Cancer Chemotherapy National Service Center. N-acetyl-L-glutamic acid was obtained from Sigma Chemical Co., St. Louis, Mo., and N-acetyl-L-norleucine from Cyclo Chemical Corp., Los Angeles, Calif. Purine and pyrimidine bases, nucleosides, and nucleotides, and other compounds used as chromatographic references, were for the most part obtained from California Corp. for Biochemical Research, Los Angeles, Calif., Pabst Labs., Milwaukee, Wis., or Sigma Chemical Co. Alkaline phosphatase was a chromatographically purified preparation from *Escherichia coli*, obtained from Worthington Biochemical Corp., Freehold, N.J., or a crude bovine intestinal preparation from Armour and Co., Chicago, Ill. Snake venom (*Crotalus atrox*) was purchased from Ross Allen's Reptile Institute, Silver Springs, Fla.

Procedure for total ascites cell count

The ascitic form of the plasma cell neoplasm 70429 was described and characterized by Potter and Law;[9] in our laboratory the cell lines of this tumor have been routinely carried in C3H mice. For total cell count, mice were killed by cervical dislocation or by carbon dioxide suffocation, and an essentially quantitative yield of ascites cells was obtained from each mouse by dipping the entire contents of the peritoneal cavity into three successive 25-ml portions of cold Locke's solution.[9] This combined cell yield was, for each mouse, then further diluted to 100 ml, and aliquots were taken for cell count. Such counts were routinely made 10 days after intraperitoneal inoculation of 1×10^6 ascites tumor cells. Each experimental group was made up of an equal number of male and female mice since the 70429 tumor produces a significantly higher cell count in female animals. The groups contained from six to ten mice, and the total cell count per mouse was averaged for each group. In each experiment cell count in the presence of inhibitor was then calculated as the percentage of the cell count for that particular tumor line in the absence of any inhibitor. Inhibitors were made up in phosphate buffer (0·1 to 0·2 M, pH 6·0 to 7·5*) and injected subcutaneously.

* Maximal stability for these diazo compounds is generally reported to be in the range of pH 6 to 8; DON and duazomycin may be somewhat more stable at pH 6·0 to 6·8. All three compounds are exceedingly labile to heat. Azaserine and DON are comparatively stable when stored in the solid state or in frozen solutions. Duazomycin A appears to be stable in relatively concentrated solution in phosphate buffer, pH 6·8, stored at 3°.

Chromatographic procedures

Two-dimensional chromatography. Aqueous phenol (72% v/v;[10] solvent 1) was used to develop sheets of Whatman 1 filter paper in the first dimension; chromatography in the second dimension was carried out in n-butanol:propionic acid:water (solvent 2, equal volumes of 93·8% aqueous n-butanol, v/v, and 44% aqueous propionic acid, v/v[10]). After this, chromatography papers were exposed to X-ray film (Eastman Kodak, single emulsion, blue sensitive) for 2 weeks. In this manner reproducible radioautographic patterns of the soluble metabolites were obtained from extracts of cells exposed to radioactive sodium formate, hypoxanthine, or uridine; similar radioautographic patterns have also been described in previous publications (e.g. Refs. 11-13).

One-dimensional chromatography. Analysis of nucleic acid hydrolysates, and of the soluble fraction of cells exposed to uridine-^{14}C (see below), was made by using descending chromatography on Whatman 3MM filter paper. Other one-dimensional chromatography was run either descending or ascending on Whatman 1 paper. The solvent systems used for the various aspects of this work were:

Solvent 3, *iso*butyric acid:ammonia, pH 4·6[14]
Solvent 4, *iso*propanol:water (7:3, v/v) in an ammonia atmosphere[15]
Solvent 5, *iso*butyric acid:0·5 N ammonia (10:6, v/v), pH 3·6[14]
Solvent 6, 0·1 M phosphate buffer, pH 6·8:saturated ammonium sulfate:n-propanol (100:60:2, v/v/v)[16]
Solvent 7, *iso*amyl alcohol:5% Na_2HPO_4 (1:2, v/v)[17]
Solvent 8, n-butanol:glacial acetic acid:water (8:2:2, v/v/v)[18]
Solvent 9, phenol:90% formic acid:water (34:1:17, w/v/v)[11]
Solvent 10, *iso*butyric acid:glacial acetic acid:water (100:1:50, v/v/v)[11]
Solvent 11, 95% ethanol:1 M ammonium acetate, pH 7·5 (75:30, v/v)[19]
Solvent 12, *iso*propanol:water (8:2, v/v)[1]

Administration of inhibitors and tracers and isolation of labeled ascites cells

Female C3H mice, 10 to 12 weeks old, were injected intraperitoneally with 1×10^6 ascites tumor cells. Ten days later groups of three tumor-bearing mice were used for tracer administration and exposure to inhibitor. Control groups received only tracer in the absence of any inhibitor; tracers were introduced in a single intraperitoneal injection in aqueous solution. Sodium formate-^{14}C was administered at a level of 50 μc per mouse, hypoxanthine-8-^{14}C, and uridine-2-^{14}C at a level of 5μc per mouse. The inhibitors—duazomycin A, azaserine, or DON—were injected intraperitoneally at specified dosages on a weight basis. Inhibitor was given 1 hr before the tracer administration, and cells were then harvested 1 hr after injection of the isotope. Azaserine and DON were dissolved immediately before use in 0·05 M phosphate buffer, pH 7·5. Duazomycin A was obtained as a stock solution in phosphate buffer, pH 6·8; it was further diluted immediately before injection with 0·05 M phosphate buffer, pH 7·5.

The animals were killed in groups of three by carbon dioxide suffocation, and the ascites tumor cells were removed with sterile Locke's solution. An aliquot of the total cell suspension was diluted for a quantitative cell count; total cell yields, per group of 3 mice, were usually 1 to 2×10^9 cells (approximately 1 to 2 ml of packed cells). The suspension of ascites cells was kept at 4° and then centrifuged at $250 \times g$. The packed cells were washed in 10 ml of cold physiological saline solution; they were then resuspended in 10 ml of distilled water, and this suspension was poured into 40 ml of

boiling ethanol and extracted for 5 min at the boiling point with constant stirring. The ethanol suspension was cooled and centrifuged at 6,000 × *g* to separate the aqueous alcohol-soluble supernatant from the solid material. These two portions were termed soluble fraction and cell residue respectively.

Analysis of the soluble fraction

This fraction from the ascites cells was concentrated under reduced pressure in a Rinco rotating evaporator,* and the concentrate was lyophilized. The lyophilized sample was redissolved in a volume of water proportional to the total number of cells extracted (1 ml water per 4 × 10^8 cells); any undissolved material was removed by centrifugation. Of the extracts thus obtained, 100-μl samples were subjected to two-dimensional chromatography as described above. Radioactive areas of the chromatograms were localized from the X-ray films (see Figs. 1–3), and R_f values of these areas were compared with those observed in previous work, as an aid to identification. These areas were then cut out as disks, and radioactivity was determined quantitatively in a toluene solution of organic scintillators in a Tri-Carb liquid scintillation spectrometer.†[20] Radioactive areas from duplicate chromatograms were eluted, lyophilized, and used for confirmation of identity by chromatography in other solvents with known reference compounds. (See, below, identification of formylglycinamide ribonucleotide, FGAR; cf. also Ref. 11.) The solvent systems used for such identification were solvents 2 and 5–11. Reference compounds were usually localized with an ultraviolet Mineralight or with ninhydrin spray. In the case of FGAR, additional confirmation was obtained by dephosphorylation of the ribonucleotide eluted from two-dimensional chromatograms and rechromatography of the resulting ribonucleoside. The dephosphorylation was accomplished by incubation with alkaline phosphatase; this was with *E. coli* alkaline phosphatase for 1 hr at 37°, pH 8, or with bovine intestinal phosphatase for 4 hr at 37° and neutral pH. In addition to FGAR and the corresponding riboside (Table 4), the soluble components identified in this fashion included adenine, adenosine, AMP, ADP, ATP, IMP, GMP, NAD, allantoin, glycine, alanine, serine, aspartic acid, glutamic acid, methionine, and lactic acid. By these techniques the total radioactivity in the soluble components could be summed, and the distribution of this activity in purine compounds, FGAR and its ribonucleoside, organic and amino acids, and other compounds could each be expressed as a percentage of this total activity.

Two-dimensional chromatographic analysis of the soluble fraction from cells exposed to uridine-^{14}C gave inadequate separation of uridine and cytidine nucleotides. To analyse the labeling of these compounds, samples (0·5 ml) of the soluble fraction were incubated with 1 mg crude crystalline *C. atrox* venom (final volume 1 ml); incubation was for 18 hr at 37° under toluene. Aliquots of the venom-treated samples, as well as untreated samples of the soluble fractions, were then spotted on Whatman 3MM paper for descending chromatography in solvent 3. R_f's of reference compounds were as follows: cytidine, 0·77; uracil, 0·68; uridine, 0·61; CMP, 0·56; CDP, 0·46; UMP, 0·40; CTP, 0·32; UDP, 0·28; and UTP, 0·17. Other experiments[21] had shown that UDP-glucose and UDP-N-acetylglucosamine travel between UDP and UTP in this solvent system. The papers were exposed to film as described above for localization

* A. S. Aloe Co., St. Louis, Mo.

† Packard Instrument Company, LaGrange, Ill.

of the radioactive areas. Snake venom completely hydrolysed radioactive nucleotides, and venom-treated samples showed radioactive spots only in the regions of uracil, uridine, and cytidine. Identity of these compounds was confirmed by eluting these radioactive areas, lyophilizing the eluates, and subjecting them to high-voltage electrophoresis on Whatman 3 MM paper in 0·05 M sodium tetraborate, pH 9, according to the procedure of Markham.[15] As previously described[21] this technique gave separation of uridine, cytidine, and uracil, in order of decreasing migration from the origin.

Isolation of the nucleic acid fraction and assay of specific activity

The cell residue from the hot alcohol extraction of ascites cells was washed with 80 % ethanol, resuspended in 10 ml of 10 % sodium chloride, and extracted at 95° for 30 min. This extraction was repeated twice with 5-ml portions of 10 % sodium chloride, and to the combined extracts was added an equal volume of ethanol. The solution was allowed to stand at ice temperature to precipitate sodium nucleate; the precipitate was then purified by redissolving it in hot water (1 to 2 ml) and reprecipitating with one volume of 0·3 N hydrochloric acid and two volumes of ethanol. After standing in ice for 60 min, the white precipitate was collected by centrifugation and dried with absolute ethanol and anhydrous ether.

A 1-mg sample of the nucleic acid thus isolated was dissolved in 10 ml water, and an aliquot of this diluted with 0·1 N sodium hydroxide for ultraviolet absorption measurements; λ_{max} for such preparations was 260 mμ, and the optical density at that wavelength was used to calculate the nucleic acid concentration. A nucleic acid solution having an optical density of 1·0 was considered to contain 40 mg/l, and this was used as a standard of purity from which to calculate the milligrams of nucleic acid in the solution read. The nucleic acid samples prepared as described above were 80 to 90 per cent pure; impurity was most likely sodium chloride. Additional aliquots, in triplicate, of the original 10 ml of aqueous solution were used for radioactivity determinations in gas-flow proportional counters. Specific activity of the nucleic acid samples was expressed in counts per second per milligram.

Analysis of the distribution of radioactivity in nucleic acid components

Samples of nucleic acid from experiments in which hypoxanthine-^{14}C or uridine-^{14}C were used as tracers were further analysed to determine the distribution of radioactivity in AMP and GMP, or UMP and CMP, respectively. Samples (2 mg) of isolated nucleic acid were incubated in 1 N potassium hydroxide for 18 hr at room temperature to hydrolyse the RNA, the alkaline hydrolysate was chilled and neutralized with 1 N perchloric acid, and the potassium perchlorate was removed by centrifugation. Samples of the supernatant (500 μl) were spotted as bands on Whatman 3MM paper for descending chromatography. For isolation of AMP and GMP from hypoxanthine-^{14}C, solvent 4 was used; for isolation of UMP and CMP labeled by uridine-^{14}C, the system used was solvent 3. Two clearly separated radioactive bands were localized in each case, by radioautography, and identified by comparison of the R_f's with those of reference nucleotides. Radioactivity was determined by counting the radioactive areas of the paper in a liquid scintillation spectrometer as described above. These areas were eluted with water; the eluates were then lyophilized, redissolved in water, and further identified by paper electrophoresis[15] with reference nucleotides on Whatman 3MM paper in 0·05 M ammonium formate buffer, pH 3·5. In this way identity and purity of these radioactive nucleic acid components were clearly established.

Deacetylation of duazomycin A by hog kidney acylase

Samples for deacetylation were incubated with a preparation of acylase I,* purified from hog kidney as described by Birnbaum *et al.*;[22] incubation was for 45 min at 37° in 0·1 M phosphate buffer, pH 7·2. The incubation samples were: (a) 11 μmoles duazomycin A + 6·25 mg enzyme; (b) 11 μmoles duazomycin A + 0·63 mg enzyme; (c) 3·75 μmoles neutralized (pH 5 to 7) N-acetyl-L-glutamic acid + 0·63 mg enzyme; and (d) appropriate controls of each substrate incubated in the absence of the enzyme. At the end of the incubation period the samples were divided for immediate chromatography in solvents 10, 11, and 12. Contents of the control tubes (d), were similarly chromatographed, and references of duazomycin, DON, and glutamic acid were also run simultaneously. The relevant R_f's in these solvent systems are: In solvent 10, duazomycin A, 0·68; DON, 0·55; glutamic acid, 0·31. In solvent 11, duazomycin A, 0·78; DON, 0·69; glutamic acid, 0·42. In solvent 12, duazomycin A, 0·45; DON, 0·30; glutamic acid, 0·07. The enzyme itself, in the absence of substrates, was likewise chromatographed. After development the chromatograms were localized by ultraviolet light and by ninhydrin spray. Both duazomycin and its deacetylated product, DON, could be localized under ultraviolet light, the solutions of duazomycin that were furnished us producing a purple, ultraviolet-absorbing spot with a yellow, fluorescent center; DON could also be detected by its reaction with ninhydrin, as could glutamic acid derived from acetylglutamate.

The rate of deacetylation of duazomycin by this enzyme was measured by the Van Slyke manometric ninhydrin method (cf. Ref. 22); these experiments were very kindly performed by Dr. Sanford Birnbaum, National Cancer Institute. Fifty μmoles of duazomycin A were incubated with 0·31 mg of the hog kidney acylase in 0·1 M phosphate buffer, pH 7·0 (total volume 3 ml); measurements of carbon dioxide were made at 10 and at 20 min. Another sample of 50 μmoles duazomycin that had been similarly incubated with 3·1 mg enzyme was completely deacetylated within the first 10 min and liberated a nearly stoichiometric amount of carbon dioxide based on the ultraviolet absorption of the original duazomycin. The behaviour of duazomycin was compared with that of N-acetyl-L-glutamic acid and N-acetyl-L-norleucine upon exposure to this same preparation of acylase. These tubes contained, respectively, 50 μmoles N-acetylglutamate + 0·16 mg enzyme, and 50 μmoles N-acetylnorleucine + 0·03 mg enzyme; the liberation of carbon dioxide was measured after 15 min.

Deacetylation of duazomycin A by extracts of mouse kidney and of 70429 *tumor cells*

Mouse kidney was homogenized by hand in two volumes of ice water in a chilled McShan–Erway glass homogenizer, and the homogenate was centrifuged at 1,500 × *g* for 20 min at 3°. The supernatant (0·08 ml/sample tube) was then incubated in 0·1 M phosphate buffer, pH 7·2, with a sample of 2 μmole duazomycin A, acetylglutamate, or acetylnorleucine. After 1 hr at 37°, the samples were deproteinized by heating in boiling water for 2 min; this procedure was shown in separate experiments to produce no deacetylation *per se*. Appropriate blank tubes containing the same amount of enzyme were incubated in the absence of substrate, and the substrate was then added to the tube after deproteinization. The deproteinized samples were subjected to

* This was generously donated by Dr. Sanford Birnbaum, National Cancer Institute, to whom we are also indebted for advice and help on the deacetylation experiments and for carrying out the studies on quantitative rates of deacetylation.

chromatography in solvent 11, with references of duazomcin, DON, glutamic acid, and norleucine (R_f for norleucine 0·75). Compounds were localized by ultraviolet light and ninhydrin spray.

In another experiment, 20-day-old 70429/S tumor cells were harvested and washed in Locke's solution, suspended in 7 volumes of 0·1 M phosphate buffer, pH 7·2, and disintegrated by high-frequency vibration with stainless steel beads;[23] the tissue suspension was centrifuged at 12,500 × *g* for 15 min at 3°. The supernatant (0·2 ml/sample tube) was incubated as before with samples of 2 μmole duazomycin A, acetylglutamate, or acetylnorleucine; enzyme-substrate blanks were again included. Chromatographic analysis was carried out in solvent 11 as in the previous experiment.

RESULTS

Cross-resistance between duazomycin A, azaserine, and DON

Duazomycin A had not been characterized at the time the present studies were begun, although certain chemical[1] as well as clinical[24] data had indicated a similarity to the glutamine antagonists, particularly DON. Our initial experiments were therefore undertaken to compare it with the known glutamine antagonists, azaserine and DON, by evaluating cross-resistance to duazomycin A in azaserine-resistant sublines of the mouse plasma cell neoplasm 70429. The first of these resistant lines, 70429/Az(la), was originally characterized by Potter and Law;[9] further characterization has been described more recently.[25] The other two resistant sublines, 70429/Az(A-I) and 70429/Az(A-II), have been developed in this laboratory; like the Az(la) line, each of these lines also arose in a single transplant generation. All these lines were selected for resistance to azaserine; all have also shown a high degree of cross-resistance to DON.

The results of such experiments are shown in Table 1, in which cell counts are reported for the parent-sensitive line of 70429 and for the three sublines selected for

TABLE 1. PER CENT SURVIVAL OF 70429 ASCITES TUMOR CELLS IN MICE GIVEN AZASERINE, DON, AND DUAZOMYCIN A*

Treatment	Tumor line			
	70429/S	70429/Az (la)	70429/Az (A–I)	70429/Az (A–II)
No inhibitor	100	100	100	100
Azaserine				
(5 mg/kg 6 days/week)	9	89	105	101
DON				
(1 mg/kg every third day)	9	113	79	55
Duazomycin A				
(16 mg/kg every fifth day)	3	54	60	53
(1 mg/kg every third day)	13	121	97	68
(0·3 mg/kg every third day)	76			

* Total cell count as per cent of that obtained in the absence of the inhibitor. Figures were based for each experiment on the average of individual cell counts on six to ten mice. In most cases the figure here was then the average of results from more than one experiment.

resistance to azaserine. Animals were injected intraperitoneally with 1 × 10^6 ascites cells of the respective tumor lines, and quantitative cells counts were made after 10 days. Inhibitors were administered intraperitoneally at dosage levels and schedules shown in Table 1.

For each experiment the average cell count on animals not exposed to inhibitor was taken as 100 per cent. It can be seen that, at the dosages indicated, azaserine and DON strongly inhibited cell proliferation of the sensitive neoplasm, whereas growth of the three resistant sublines was not appreciably inhibited. The highest level of duazomycin A reduced growth of the sensitive line to as low as 3 per cent of controls, whereas the resistant lines exhibited 54 per cent, 60 per cent, and 53 per cent growth respectively. Resistance to the lower level of duazomycin was even more complete. Thus, cross-resistance between azaserine, DON, and duazomycin was evident in all three resistant sublines, and from these initial findings it appeared likely that duazomycin A might be acting in a manner similar to the known glutamine antagonists azaserine and DON.

TABLE 2. EFFECT OF GLUTAMINE ANTAGONISTS ON FORMATE-^{14}C INCORPORATION INTO NUCLEIC ACIDS AND SOLUBLE PURINE NUCLEOTIDES IN 70429/S TUMOR CELLS *IN VIVO*

Treatment*	Activity in nucleic acids†	Activity in soluble purine nucleotides‡
No inhibitor	100	100
Duazomycin A (mg/kg)		
0·04	98	111
0·1	50	56
0·4	20	21
1·6	13	10
4·0	19	10
16·0	18	5
DON (mg/kg)		
0·005	42	
0·05	13	
Azaserine (mg/kg)		
1·25	10	

* Cells were exposed to inhibitor, at the dosage indicated, for 1 hr before administration of tracer. Isotope was administered in a single injection and cells were harvested 1 hr later and analysed.

† Specific activity (counts/sec/mg) as per cent of that observed in control cells in the absence of the inhibitor. Most figures were averaged from more than one experiment. Formate-^{14}C injected was 50 μc/mouse. Average specific activity of the nucleic acids from untreated controls (9 independent experiments) was 682 counts/sec/mg.

‡ Radioactivity in soluble purine nucleotides was expressed for both control and treated cells as per cent of total soluble radioactivity. The figures here are then:

$$\frac{\% \text{ activity in purine nucleotides in treated cells}}{\% \text{ activity in purine nucleotides in control cells}} \times 100.$$

Inhibition of biosynthesis de novo *of purines*

The mechanism of action of duazomycin was examined further in tracer studies of nucleic acid biosynthesis. ^{14}C-formate incorporation into nucleic acids was investigated in 70429 cells growing *in vivo*, and Table 2 shows the effect of a number of levels of duazomycin A. Similar results with low levels of DON and with a rather high dose of azaserine are also included for comparison. In each experiment specific activity of the nucleic acids from cells not exposed to inhibitor was taken as 100 per cent; values

in the presence of inhibitor then represent specific activity as percentage of that observed in the controls.

These figures for formate incorporation into nucleic acids represent almost exclusively uptake into the nucleic acid purines. As indicated in Table 2, a single injection of 0·4 mg/kg of duazomycin reduced this incorporation to 20 per cent of that in controls not exposed to inhibitor. Higher levels of duazomycin did not result in significantly greater inhibition of the incorporation, but lower levels produced correspondly less inhibition. A comparison of these levels with those in Table 1 shows that duazomycin A exhibited a real biochemical effect at doses appreciably below those necessary for marked inhibition of the tumor. Table 2 shows that azaserine produced equally marked inhibition of formate incorporation and that DON inhibited the incorporation at much lower levels; even 0·005 mg/kg of DON caused significant inhibition.

This marked inhibition of formate uptake into nucleic acids was a specific effect rather than one due to any general cessation of metabolism. Formate incorporation into soluble components of these cells was analyzed by two-dimensional paper chromatography and radioautography; it was apparent that, although labeling of such components as amino acids remained normal, incorporation of labeled formate into soluble purine nucleotides was inhibited by duazomycin A as well as by the other two glutamine antagonists. Incorporation into each of the radioactive areas was quantitatively determined; for purine nucleotide components this was then summed as a total figure that included uptake into compounds identified as AMP, IMP, GMP, ADP, ATP, and NAD. This sum of the activity in these components was then calculated as percentage of the total soluble radioctivity. This was done for both control and treated cells and, in experiments in which the total soluble activity was approximately the same in both (indicating comparable fixation of formate), the two figures were compared. Taking the figure for the control cells as 100 per cent, the figure for the treated cells could then be expressed as a percentage of this, and the reduction in incorporation into the purine nucleotides in the treated cells could be estimated. These figures are also shown in Table 2, and it can be seen that formate incorporation into the soluble purine nucleotides was inhibited by duazomycin in a manner paralleling that into the nucleic acids.

It is well known that glutamine antagonists exhibit such inhibition of purine biosynthesis by virtue of their inhibition of the synthesis *de novo* from small molecules. In Table 3 the effect of 0·6 mg/kg duazomycin on formate incorporation into soluble purine nucleotides and into nucleic acid purines is compared with its effect on the similar incorporation of hypoxanthine, as an example of a preformed purine precursor. These experiments were again carried out with 70429/S cells growing *in vivo*. Extent of isotope incorporation in the presence of duazomycin is given as percentage of that in the control populations not exposed to inhibitor, as described above.

Table 3 shows that duazomycin inhibited the incorporation of formate into both soluble and nucleic acid purines and that, concomitant with this inhibition of *de-novo* purine synthesis, there was increased utilization of the preformed purine, hypoxanthine. Formate labeling of soluble purine nucleotides was inhibited by this level of duazomycin to 6 per cent of the control value, whereas hypoxanthine utilization was increased to 204 per cent of that in controls. This effect on formate and hypoxanthine utilization

was also reflected in the pattern of incorporation of these tracers into the nucleic acid purines.

Accumulation of formylglycinamide ribotide and riboside

It has been shown by Buchanan and co-workers,[26–29] Tomisek *et al.*,[11,30] and LePage and associates[31,32] that inhibition of *de-novo* purine synthesis by glutamine antagonists occurs most dramatically at the site of amidination of formylglycinamide

TABLE 3. EFFECT OF DUAZOMYCIN A ON PATHWAYS OF PURINE SYNTHESIS IN 70429/S ASCITES TUMOR CELLS *IN VIVO*

Treatment	Activity in soluble purine nucleotides*		Activity in nucleic acids*	
	Formate-^{14}C	Hypoxanthine-8-^{14}C	Formate-^{14}C	Hypoxanthine-8-^{14}C
No inhibitor	100	100	100	100
Duazomycin† (6·4 mg/kg)	6	204	7	126

* Extent of isotope incorporation in the presence of duazomycin is given as per cent of that in the control populations not exposed to the inhibitor; these figures were calculated as in Table 2. Formate-^{14}C administered was 50 μc/mouse; hypoxanthine-^{14}C, 5 μc. Three separate experiments on this level of duazomycin were carried out with each tracer. Average specific activity of the nucleic acids from untreated controls was 595 counts/sec/mg for the three experiments with ^{14}C-formate and 487 counts/sec/mg for the three ^{14}C-hypoxanthine experiments.

† Cells were exposed to inhibitor and then to the respective tracers, as in Table 2.

ribotide. The radioautograms in Fig. 1 illustrate the effect of 0·4 mg/kg duazomycin on the incorporation of formate into soluble components in 70429/S cells *in vivo*. Purine nucleotides, as shown in the lower right-hand portion of the radioautogram, showed appreciably less labeling in the duazomycin-treated cells, with detectable incorporation appearing only in the nucleoside triphosphate area. The treated cells showed instead a concomitant accumulation of large amounts of radioactivity in the spot labeled FGAR, formylglycinamide ribotide. A similar pattern of FGAR accumulation, as purine nucleotide biosynthesis was suppressed, could be seen in comparable experiments on azaserine or DON inhibition of 70429/S cells. Such patterns are shown in Fig. 2 for exposure to 5 mg azaserine/kg and in Fig. 3 for 0·005 mg DON/kg. Thus, these effects of duazomycin A resembled those of the other two glutamine antagonists.

The compound that accumulates thus in the treated cells was identified as formylglycinamide ribotide by rechromatography in other solvent systems. The total spot labeled FGAR was eluted from chromatograms such as those represented in Figs. 1–3 and was found to migrate as a single entity in four other solvent systems. As shown in Table 4, in these four systems, as well as in the two solvents used for the original two-dimensional chromatography, the R_f of this material agreed with that of samples of FGAR kindly donated by Dr. Bruce Levenberg and Dr. Frank Henderson, and with R_f values reported in the literature by Tomisek *et al.*[11] Samples of FGAR from these laboratories have also been subjected to other and more complete identification.[11,26]

The spot labeled FGARiboside was likewise subjected to rechromatography. This spot also migrated as a single entity with R_f's as shown in Table 4; again these R_f's agreed well with literature figures.[11] As additional confirmation, FGAR eluted from two-dimensional chromatograms was dephosphorylated by incubation with alkaline

phosphatase, and the product of the incubation similarly rechromatographed. As can be seen in Table 4, this converted FGAR to a compound that traveled, in all solvent systems tested, like the formylglycinamide riboside eluted from the two-dimensional chromatograms.

TABLE 4. CHROMATOGRAPHIC CHARACTERIZATION OF FORMYLGLYCINAMIDE RIBOTIDE AND RIBOSIDE ACCUMULATED IN 70429 TUMOR CELLS DURING INHIBITION BY GLUTAMINE ANTAGONISTS

Compound	Chromatographic R_f*				
	Ethanol–ammonium acetate	Phenol–formic acid–water	*Iso*butyric acid–ammonia	*Iso*butyric acid–acetic acid–water	Two-dimensional chromatography†
FGAR					
Accumulated in duazomycin inhibition	0·18	0·33	0·22	0·10	0·22 : 0·09
Accumulated in azaserine inhibition	0·18	0·32	0·23	0·11	0·23 : 0·08
Accumulated in DON inhibition	0·18	0·31	0·24	0·10	0·25 : 0·08
Reference FGAR					
Sample 1‡	0·18	0·32	0·22	0·11	
Sample 2§	0·17		0·20	0·08	
Literature value¶		0·29		0·15	0·27 : 0·07
FGARiboside					
Accumulated in duazomycin inhibition	0·70			0·30	0·72 : 0·29
Accumulated in azaserine inhibition	0·70	0·80		0·36	0·75 : 0·29
Accumulated in DON inhibition	0·70			0·34	0·71 : 0·26
FGAR incubated with alkaline phosphatase	0·69	0·80		0·38	0·69 : 0·29
Literature value¶		0·79		0·38	0·69 : 0·29

* Localization by radioautography.
† Phenol–water followed by *n*-butanol–propionic acid–water.
‡ Gift from Dr. Bruce Levenberg.
§ Gift from Dr. Frank Henderson.
¶ Tomisek *et al.*[11]

Figure 4 summarizes the effect of various levels of duazomycin on the extent of formate incorporation into accumulated FGAR as well as into soluble purine nucleotides. The radioactivity in these components is expressed here simply as percentage of the total radioactivity in the soluble fraction. In the untreated control cells 40 per cent of this soluble radioactivity was present as purine nucleotides (hatched bars); less than 1 per cent was in FGAR. With increasing levels of duazomycin the activity in the purine nucleotides was depressed to a level that was less than 5 per cent of the total soluble activity. Concurrently, the labeling of FGAR (solid black bars) increased to a maximum value of 50 to 60 per cent of the total in the range of 0·16 to 0·6 mg/kg duazomycin. However, with higher levels of duazomycin, despite continued supression of nucleotide synthesis, FGAR accumulation then fell off again to 10 per cent or less of the total. Thus, these higher levels of inhibitor still blocked purine biosynthesis *de*

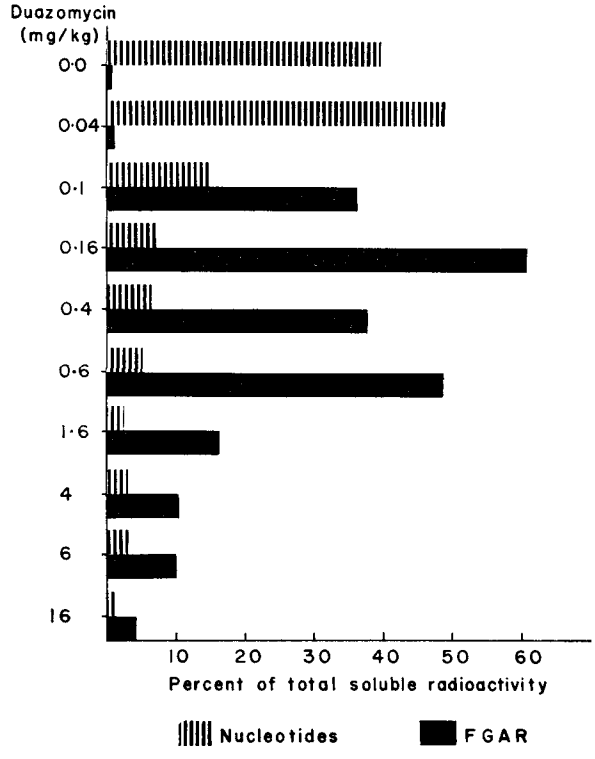

FIG. 4. Effect of various levels of duazomycin A on the extent of formate-^{14}C incorporation into soluble purine nucleotides and FGAR.

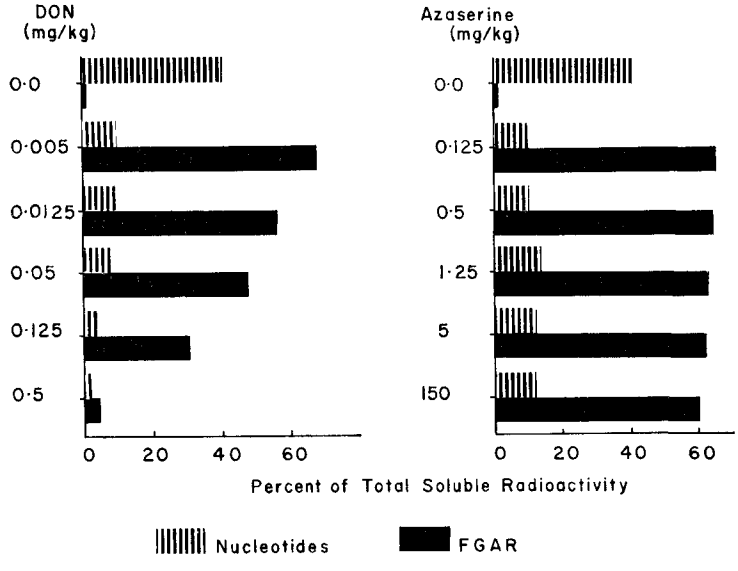

FIG. 5. Effect of various levels of DON and azaserine on the extent of formate-^{14}C incorporation into soluble purine nucleotides and FGAR.

novo but also prevented the accumulation of the FGAR intermediate in this pathway. This would be consistent with an inhibition of *de-novo* synthesis at a site prior to the formation of FGAR.

These results obtained with duazomycin can be compared with the effects of DON and azaserine, as shown in Fig. 5. Formate incorporation into soluble purine nucleotides (hatched bars, as in Fig. 4) was similarly inhibited by both of these antagonists. At low levels of DON, 0·005 mg/kg radioactivity, found in FGAR (black bars) accounted for as much as 70 per cent of the total activity in the soluble fraction. With increasing levels of DON, radioactive FGAR accumulation followed the pattern seen with duazomycin—i.e. it then decreased again to only 5 per cent of the total. Azaserine, however, unlike the other two inhibitors, did not inhibit the accumulation of FGAR even at doses as high as 150 mg/kg. From Figs. 4 and 5 it can be seen that DON was by far the most potent of the three inhibitors. DON produced accumulation of FGAR at concentrations much below effective levels of duazomycin and was also much more active than duazomycin in then inhibiting FGAR accumulation at higher levels.

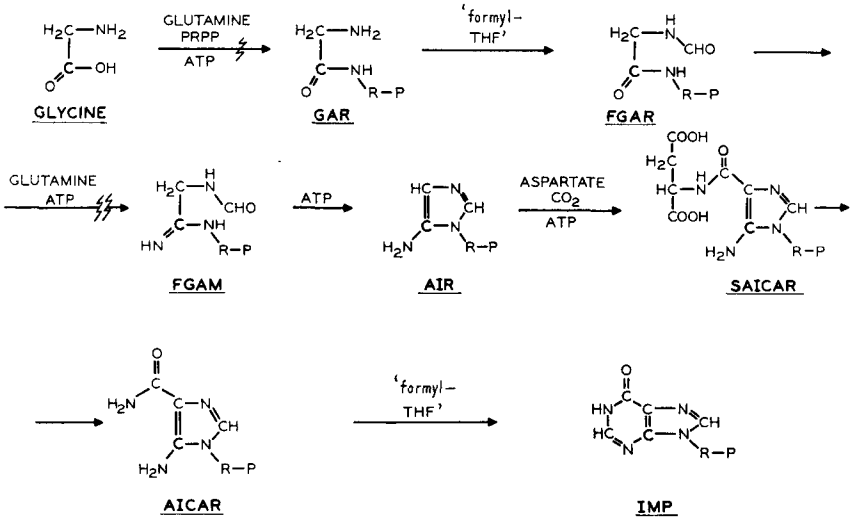

FIG. 6. Enzymatic reactions involved in the biosynthesis of inosinic acid *de novo*. The two reactions involving glutamine, and therefore vulnerable to inhibition by the glutamine antagonists, are indicated by the zig-zag lines; in the earlier of these two sites of action, glutamine is involved specifically in the synthesis of phosphoribosylamine from phosphoribosylpyrophosphate. and phosphoribosylamine then participates in the formation of glycinamide ribonucleotide. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; SAICAR, 5-succinylamino-4-imidazolecarboxamide ribonucleotide; AICAR, 5-amino-4-imidazolecarboxamide ribonucleotide; THF, tetrahydrofolic acid.

Effect of duazomycin A on other glutamine-requiring aminations

The effects of duazomycin described above are compatible with the known sites of action of the glutamine antagonists in the pathway for *de-novo* biosynthesis of purines, the amidination of formylglycinamide ribotide, and the synthesis of phosphoribosylamine (see Fig. 6). It therefore seemed of value to explore, in this same system, the

effect of duazomycin on other reactions involving nitrogen transfer from the amide group of glutamine, and likewise known to be inhibited by azaserine and/or DON. These include the amination of XMP to GMP[33] and that of uridine nucleotides to cytidine nucleotides.[34] Experiments were therefore set up to investigate the effect of duazomycin on these conversions in 70429 cells growing *in vivo*.

Although glutamine is required for the amination of xanthylic acid in the sequence of reactions, IMP → XMP → GMP, it is not involved in the conversion of IMP to AMP. In the tracer experiments with labeled hypoxanthine, the incorporation into nucleic acid AMP and GMP was therefore examined separately, and duazomycin A and DON were tested for their capacity to inhibit specifically the incorporation of hypoxanthine into nucleic acid GMP. Samples of the isolated nucleic acids were hydrolyzed to yield RNA nucleotides, and AMP and GMP were isolated, identified, and their radioactivity measured. At the rather high level tested (6 mg/kg), duazomycin did not significantly alter the distribution of radioactivity between these nucleotides from the ratio seen in uninhibited control cells. DON, however, even at a level of 0·5 mg/kg, markedly inhibited the incorporation into GMP.

The effect on the conversion of uridine nucleotides to cytidine nucleotides was likewise explored. In the experiments with uridine-^{14}C, the RNA from nucleic acid

TABLE 5. EFFECT OF DUAZOMYCIN A AND DON ON THE INCORPORATION OF URIDINE-2-^{14}C INTO RNA-UMP AND RNA-CMP IN 70429/S ASCITES TUMOR CELLS *IN VIVO*

Treatment	Distribution of radioactivity in RNA*	
	UMP	CMP
No inhibitor	66	34
Duazomycin A† (mg/kg)		
6·4	100	0
1·6	94	6
0·6	72	28
DON† (mg/kg)		
0·5	100	0

* Figures are the radioactivity found in RNA-UMP and RNA-CMP, respectively, as per cent of the total radioactivity in these two nucleotides. Uridine-2-^{14}C was administered at a level of 5 μc/mouse. Specific activity of the nucleic acids from untreated controls (average of two experiments) was 840 counts/sec/mg.

† Cells were exposed to inhibitor and then to tracer, as described in Table 2.

samples was similarly hydrolysed, UMP and CMP were isolated, and the incorporation of uridine into each of these two nucleotides was measured separately. Table 5 shows the effect of several levels of duazomycin on this incorporation into the two RNA pyrimidines; the effect of DON is also included for comparison. The figures are expressed as percentage of the total radioactivity found in these two nucleotides,

to show the distribution of radioactivity between the two. The highest level of duazomycin reduced uridine incorporation into nucleic acid CMP to zero, while incorporation into UMP continued. Lower levels of duazomycin also inhibited the incorporation into CMP but to a correspondingly smaller extent. The level of DON shown was approximately equimolar with the lowest level of duazomycin; DON was thus again more effective than duazomycin as an inhibitor since it completely blocked the uptake of uridine into nucleic acid CMP, whereas a comparable level of duazomycin allowed some conversion to take place.

It was desirable to examine this inhibition of CMP formation at the level of the soluble nucleotides as well, and this was done in experiments with 6 mg/kg duazomycin A and with 0·5 mg/kg DON. The soluble fractions of the cells were treated with snake venom to dephosphorylate the nucleotides, and analysis was made of the radioactivity in uridine and cytidine. The soluble fractions of cells from control animals showed radioactive spots corresponding to uracil, uridine, and cytidine. In the soluble fractions of cells from treated animals, only two such radioactive areas were detected, corresponding to uracil and uridine, and no radioactive cytidine was present. It can be reasonably concluded that at these levels the glutamine antagonists were effective inhibitors of the amination of uridine nucleotides.

Deacetylation of duazomycin A to DON

Duazomycin A thus appeared to have sites of action similar to those of the other glutamine antagonists, behaving more like DON than like azaserine, especially in the prevention of FGAR accumulation by high levels of inhibitor. While these studies were in progress, Rao[4] reported that duazomycin A had been characterized an N-acetyl-DON, and it appeared possible that the compound was being deacetylated *in vivo* to DON. A study was therefore made on the enzymatic deacetylation of duazomycin A.

Duazomycin was incubated, in several different experiments, with a sample of acylase I, purified from hog kidney;* this enzyme is known to hydrolyse the N-acyl bond in α-N-acylated-L-amino acids with aliphatic side chains.[35] In a preliminary experiment, deacetylation was estimated by chromatographic separation of the products of the incubation, with three different solvent systems. The deacetylation of duazomycin was compared with that of N-acetyl-L-glutamic acid. Duazomycin disappeared in the presence of the enzyme, as indicated by localization of the chromatograms with ultraviolet light, and an ultraviolet-absorbing, ninhydrin-positive compound appeared which migrated like DON in all three solvent systems. Similarly, acetylglutamate was readily deacetylated to a compound that traveled like glutamate in the three solvent systems used. Duazomycin thus appeared readily susceptible to the purified preparation of hog kidney acylase.

In a quantitative determination* of the rate of this deacetylation by hog kidney acylase, the effect on duazomycin was again compared with that on acetylglutamate and also on N-acetyl-L-norleucine. In comparative agreement with literature values,[22] acetylglutamate was deacetylated at a rate of 2,420 μmoles/hr per mg enzyme N, and acetylnorleucine at a rate of 11,280 μmoles/hr per mg N. The deacetylation of duazomycin A was comparable to that of acetylglutamate, the rate being 1,685 μmole/hr per mg enzyme N.

* See footnote p. 1340

The possibility of deacetylation of duazomycin *in vivo* in the mouse was explored as being more immediately relevant to the biochemical effects in the 70429 tumor cells. Acylase I has been found to be particularly active in kidney tissues,[35] but no data appeared to be available on its activity in mouse kidney. Accordingly, a crude extract of mouse kidney was incubated with samples of duazomycin A, acetylglutamate, or acetylnorleucine. As judged qualitatively from chromatography of the incubation products, the mouse kidney extract produced marked deacetylation of acetylnorleucine, definite but less extensive attack on acetylglutamate, and detectable but even less extensive breakdown of duazomycin A to DON, with some unreacted duazomycin left after the incubation. Acetylnorleucine would be expected to be the best of these three substrates for acylase I (see rate studies above).

Although hydrolytic activity for many peptides has been reported to be comparatively low in neoplastic tissue,[36,37] the deacetylation was also specifically explored in 70429/S cells by incubation of crude extracts with duazomycin A, acetylglutamate, or acetylnorleucine. From chromatography of the products of these incubations, there did appear to be definite deacetylation of acetylnorleucine and slight deacetylation of acetylglutamate by the tumor extracts. However, in the products of the duazomycin incubation, no DON could be detected by either ultraviolet light or ninhydrin, whereas unreacted duazomycin A could be clearly localized. Thus, although some acylase activity did appear to be present in this extract of 70429 cells, it had no detectable effect on duazomycin A under the conditions used.

DISCUSSION

The chemical properties of duazomycin A observed in these studies, i.e. ultraviolet absorption spectrum and behavior toward ninhydrin, resembled those reported by Rao *et al.*;[1,4] chromatographic R_f's have also been recorded here for solvent systems 10, 11 and 12. Antimicrobial properties of duazomycin A have been described for *E. coli* and for *Bacillus subtilis*;[1,5] these same species are susceptible to DON,[38,39] but Margison and Oleson[5] have reported differences in patterns of microbial resistance to these antagonists. Duazomycin has also been found to have activity against two protozoan species, *Ochromonas malhamensis* and *Crithidia fasciculata*;[6] similar activity was observed against these species for azaserine and DON. Antitumor activity of duazomycin A has previously been observed against sarcoma 180, adenocarcinoma 755, and leukemia L1210 in mice, as well as against some neoplasms of human origin.[1,7,8] In the present study, activity has also been observed against the mouse 70429 neoplasm, and in this case there was cross-resistance between duazomycin, azaserine, and DON. Duazomycin was also reported to be active against HeLa cells in culture,[6] although it has been stated in other work to be without marked activity against cells in culture.[40]

Glutamine analogs that contain the diazo moiety have been of considerable value in the elucidation of mechanisms for the biosynthesis of purine and pyrimidine nucleotides and have therefore been of significant biochemical interest.[41,42] It is well known that a major site of action of the antagonists, azaserine and DON, is in the pathway for *de-novo* biosynthesis of the purine ring. Early studies on azaserine indicated that this antimetabolite inhibited the incorporation of formate or glycine into nucleic acids

without inhibiting the uptake of preformed purine;[43,44] similar results have been observed with DON[39,45] and are reported here for duazomycin as well. Azaserine inhibition of *de-novo* purine biosynthesis was explored at the enzymatic level in pigeon liver preparations by Buchanan and his group,[28,29] and the most sensitive site in the pathway was shown to be the amidination of formylglycinamide ribotide to formylglycinamidine ribotide.[27] This site of inhibition was confirmed in mammalian[46] and bacterial[47] enzyme systems, and FGAR accumulation during azaserine inhibition was observed in cell-free preparations from pigeon liver[26] and in bacterial[11] and mammalian[31] cells. The accumulation of FGAR produced by duazomycin A in the present experiments with 70429 cells thus parallels that observed with the other glutamine antagonists (Figs. 1–3). DON has previously been found to be even more potent than azaserine in inhibiting FGAR amidination,[27,30,46,47] and similar results have been observed in the present study, based on the levels of inhibitor necessary to produce FGAR accumulation.

Inhibition by these antagonists of the earlier step in *de-novo* purine synthesis—the glutamine-requiring synthesis of phosphoribosylamine—has also been explored in several systems. These sites of inhibition in the *de-novo* pathway are shown in Fig. 6. In the present studies higher levels of both DON and duazomycin A were effective in preventing FGAR accumulation, indicating a block at the earlier step; however, azaserine produced no such effect even when administered at a level of 150 mg/kg. Azaserine has been clearly defined as an inhibitor of the synthesis of phosphoribosylamine *in vitro* in pigeon liver preparations,[27,48] although the conversion was much less sensitive to azaserine than was the amidination of FGAR.[27] DON was appreciably more potent than azaserine in inhibiting phosphoribosylamine synthesis in the pigeon liver preparations;[49–51] this difference has been quantitated with the purified enzyme from this source[51] and the differential between the two inhibitors shown to be even greater than for FGAR amidination.[50,51] The early step does not seem to have been explored at the enzymatic level in mammalian systems, and the insensitivity of the reaction to azaserine seen here in 70429 cells *in vivo* remains unexplained. Such insensitivity has also been indicated in bacterial cells,[52] and a difference between azaserine and DON in this respect has likewise been observed in earlier studies with mammalian tissues.[32] It seems unlikely that the conversion in these cells would prove to be completely insensitive to azaserine and yet sensitive to the other glutamine antagonists. It is possible that lower sensitivity to azaserine, combined with greater breakdown of this inhibitor,[53] could explain the difference observed here between azaserine and DON or duazomycin. It is also conceivable that even higher levels of azaserine might have achieved an effect; certainly exploration at the enzymatic level should prove enlightening.

An additional glutamine-requiring step in purine biosynthesis is the amination of xanthylic acid to guanylic acid,[33,54] and the enzyme catalysing this reaction, as purified from calf thymus, has likewise been shown to be sensitive to both azaserine and DON.[33] In other work the synthesis of GMP from labeled adenine or hypoxanthine was inhibited by very high levels of DON *in vivo* and by unspecified levels of DON in tumor tissue breis *in vitro*.[45] Synthesis of RNA-GMP was preferentially inhibited by azaserine in Ehrlich ascites tumor cells, especially *in vitro*.[55] In our experiments definite inhibition by DON of the incorporation of hypoxanthine into RNA-GMP

was evident in the 70429 cells, whereas uptake into AMP was not inhibited. Such inhibition of GMP synthesis was not apparent with duazomycin A at the level explored but might perhaps have been evident at higher levels.

A requirement for glutamine has also been established in pyrimidine nucleotide synthesis, for the amination of uridine nucleotides to cytidine nucleotides.[34,56,57] Inhibition of this conversion by DON has also been evident[34,58] and has been explored with enzymes from rat liver and Novikoff hepatoma[34] and from *E. coli*;[56,57] such inhibition was likewise strikingly apparent in 70429 cells here (Table 5). In this case strong inhibition by duazomycin was also evident; this serves to extend the metabolic sites sensitive to duazomycin to most of those already defined for the other glutamine antagonists.

The question remains open as to whether the molecule of duazomycin A is active *per se*, or acts after deacetylation to a molecule of DON. There seems to be no necessity for deacetylation of the compound before its blockage of amide nitrogen transfer from glutamine could occur. Buchanan (p.85)[29] has stated that when the amino group of azaserine was bound in peptide linkage there was no decrease in the effectiveness of such compounds as enzyme inhibitors as compared with azaserine; presumably this implies tests *in vitro* of such compounds in enzymatic experiments, although details of such experiments have not been published. Alazopeptin[59] is one of several diazo peptide compounds that have also been isolated from natural sources and found to have antibiotic and antitumor activity; this compound has been reported to be a peptide containing one molecule of α-alanine and two molecules of a diazoketoamino acid that appeared to be DON or an isomer thereof.[59] Alazopeptin has been found to inhibit an early step in *de-novo* purine synthesis, presumably formation of phosphoribosylamine.[49] Duazomycin B, which has biological activity similar to that of the compound studied in our work,[1] has also been reported to be "built up on a unit of DON."[4,60] Various other N-diazoacetyl derivatives of amino acids and peptides have also been reported to have antitumor activity.[61] The ratio between duazomycin and DON, in inhibitory activity, is apparently not necessarily constant in these experiments, as it might be expected to be if duazomycin were acting solely by conversion to DON. The extent of deacetylation might, of course, vary from one experiment to another. In some cases, notably the amidination of formylglycinamide ribotide (Figs. 4 and 5) and the amination of uridine nucleotides (Table 5), duazomycin was strikingly less effective than DON on an equimolar basis; it seems that deacetylation would have had to be very slight indeed to be consistent with such differences. Such deacetylation was indeed not even detectable in crude extracts of 70429 cells as prepared in these experiments although, of course, other preparations extracted in a different way, or from younger cells, might have produced more deacetylation of duazomycin; the compound was definitely susceptible to mammalian acylase. It appears that a conclusive answer to the question of the activity of the duazomycin molecule *per se* must await a comparison of this inhibitor with DON in purified enzyme systems.

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