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RESISTANCE TO 4-AMINOPYRAZOLO(3,4-*d*)PYRIMIDINE*

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Abstract—From H. Ep. #2 cells in culture, a subline was selected for high resistance to 4-aminopyrazolo(3,4-*d*)pyrimidine (APP). Upon culture in the absence of APP, the selected line reverted partially to sensitivity. The APP-resistant cells were also highly resistant to APP-ribonucleoside and 6-methylthiopurine ribonucleoside, but showed a much smaller degree of resistance to 2-fluoroadenine, 2-fluoroadenosine, tubercidin and certain other analogs of purines and purine nucleosides. There was generally good correlation between concentrations of purine and nucleoside analogs required to produce inhibition of *de novo* synthesis of purines and those required to produce growth inhibition. Metabolic and other studies showed that: (1) sensitive and resistant cells were equally permeable to APP; (2) extracts of sensitive and resistant cells had about the same activities of AMP and IMP-pyrophosphorylases and some capacity to convert APP to its nucleotide; (3) extracts of resistant cells had adenosine kinase activity, but it was less than in extracts of sensitive cells; (4) intact resistant cells grown in the presence of APP-^{14}C or 6-methylthiopurine-^{35}S ribonucleoside contained little or no radioactive nucleotides, whereas they metabolized 2-fluoroadenosine-^{3}H to phosphate derivatives to about the same extent as did sensitive cells; (5) extracts of resistant cells were more active than extracts of sensitive cells in dephosphorylating nucleotides (5′-AMP, 5′-IMP, APP-ribonucleotide, and 6-methylthiopurine ribonucleotide); (6) when treated with azaserine, sensitive cells incorporated formate-^{14}C predominantly into formylglycinamide ribonucleotide, whereas in resistant cells the corresponding ribonucleoside was the principal product; (7) resistant cells were more active than sensitive cells in metabolizing adenine (inosine and hypoxanthine were the major soluble products). Resistance to APP thus appears to be associated with incapacity of the cells to accumulate APP-ribonucleotides and this incapacity may be related to the higher rate of degradation of APP-ribonucleotides in resistant cells.

Sensitive cells grown in the presence of APP-^{14}C contained, in addition to APP-ribonucleotides, small amounts of APP-ribonucleoside and its deamination product, 4-hydroxypyrazolo(3,4-*d*)pyrimidine ribonucleoside. APP-ribonucleoside was a substrate for calf intestinal adenosine deaminase; the K_m value was $2{\cdot}5 \times 10^{-4}$ M and the V_{max} was 15 μmoles/min/mg protein.

A WIDESPREAD mechanism of resistance to purine analogs is a loss of capacity to convert the analog to the nucleotide; this loss may or may not be associated with a loss of capacity to form a nucleotide from the corresponding natural purine.[1] This mechanism of resistance has been demonstrated for analogs of hypoxanthine and

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guanine[1] and also for several analogs of adenine, such as 8-azaadenine,[2] 2,6-diaminopurine[3-7] and 2-fluoroadenine.[8, 9] Of the cytotoxic analogs of adenine, APP* has been of particular interest because of its antitumor activity.[10] Despite a number of studies of the metabolism of APP and of its metabolic effects,[11-20] the site or sites of action responsible for its toxicity and antitumor activity have not been defined. Studies in resistant cells have provided useful information on the metabolism and action of other purine analogs; but, except for the isolation of a resistant *Neurospora*,[21] resistance to APP has not been studied. We report here the isolation and some metabolic characteristics of a line of H. Ep. #2 cells selected for resistance to APP.

EXPERIMENTAL

Compounds. APP and 6-MeMP-ribonucleoside were obtained from the Cancer Chemotherapy National Service Center through the courtesy of Dr. Harry B. Wood. Tubercidin (7-deazaadenosine) was a gift of Dr. C. G. Smith of the Upjohn Company, Kalamazoo, Mich., and 4-aminoimidazo(4,5-*d*)pyridazine was a gift of Dr. J. A. Carbon, Abbott Laboratories, Chicago, Ill. 8-Azaadenosine, synthesized by Dr. Leon Goodman, Stanford Research Institute, was obtained from the Cancer Chemotherapy National Service Center. 1-β-D-Ribofuranosyl-4-aminopyrazolo(3,4-*d*)pyrimidine (APP-ribonucleoside),[22] 2-fluoroadenine,[23] 2-fluoroadenosine[24] and 6-MeMP-^{35}S-ribonucleoside[25] were synthesized in our laboratories by Dr. J. A. Montgomery and his associates. APP-6-^{14}C was prepared as described earlier.[17] Small amounts of the ribonucleoside monophosphates of APP-6-^{14}C and 6-MeMP-^{35}S, which were used in certain experiments, were isolated by paper chromatography from extracts of H. Ep. #2 cells (parent line) grown in the presence of APP-6-^{14}C or 6-MeMP-^{35}S-ribonucleoside. 2-Fluoroadenosine was tritiated (Wilzbach method) by New England Nuclear Corp., Boston, Mass., and was purified in our laboratories by recrystallization and by chromatography on paper. The following compounds and preparations were obtained from the suppliers indicated: inulin-^{14}C, inulin-^{3}H, $^{3}H_2O$, adenine-8-^{14}C, hypoxanthine-8-^{14}C and sodium formate-^{14}C, New England Nuclear Corp.; AMP-8-^{14}C and IMP-8-^{14}C, Schwarz BioResearch, Orangeburg, N. Y.; crude snake venom (*Crotalus atrox*), Ross Allen's Reptile Institute, Silver Springs, Fla.; calf intestinal adenosine deaminase, Sigma Chemical Co., St. Louis, Mo.

Isolation and propagation of APP-resistant cells. Human epidermoid carcinoma cells of the line (H. Ep. #2) established in culture by Moore *et al.*[26] were grown either on glass or in suspension culture under conditions described previously.[27] For selection of the resistant line, cells were subjected to serial passage in the presence of levels of APP that were increased gradually from 0·3 to 80 μg/ml. From cells that had been grown in suspension in the presence of 80 μg/ml, clones were selected by plating in the presence of 40, 80, 120 and 160 μg/ml. The selected clones were allowed to grow until they formed a confluent sheet and were then transferred for many generations in the presence of 160 μg APP/ml. From these cells, colonies were again selected by plating in the presence of 320 μg APP/ml. (For reference, the resistant line is designated H. Ep. #2/APP and the parent line H. Ep. #2/S.) In the presence of APP at concentrations of 160 μg/ml or greater, the growth of H. Ep. #2/APP cells was relatively

* Abbreviations: APP, 4-aminopyrazolo(3,4-*d*)pyrimidine; HPP, 4-hydroxypyrazolo(3,4-*d*)pyrimidine; 6-MP, 6-mercaptopurine; 6-MeMP, 6-methylthiopurine; FGA, formylglycinamide; FGAR, formylglycinamide ribonucleotide.

slow in comparison with that of the parent line; and cells were, therefore, routinely carried in the presence of APP at 80 μg/ml. After the resistant line had been carried for many generations in the presence of APP, resistant cells were cultured in the absence of drug and checked for sensitivity to APP after 3, after 11, and after 20 generations on normal medium.

Response of sensitive and resistant cells to inhibitors. The responses of parent and resistant cells to analogs of purines and nucleosides were measured by their capacity for colony formation.[9]

Chromatography and autoradiography. Paper chromatography was used for the isolation of metabolic products and for their identification. The solvents used had the following compositions:

A. equal volumes of 93·8% *n*-butanol and 44% aqueous propionic acid;
B. 2,2,3,3-tetrafluoro-1-propanol, H_2O, 90% formic acid (25:15:0·5) (v/v);[28]
C. 0·1 M sodium phosphate (pH 6·8), solid $(NH_4)_2SO_4$, *n*-propanol (100:60:2) (v/w/v);[29]
D. isobutyric acid, conc. NH_4OH, H_2O (57:4:39) (v/v);
E. 70% aqueous isopropanol in an ammonia atmosphere;
F. methanol, conc. HCl, H_2O (50:17:33) (v/v);
G. *n*-butanol saturated with NH_4OH;
H. for two-dimensional chromatography: phenol, H_2O (72:28) (v/v) in the first direction and solvent A in the second.

Radioactive areas of chromatograms were located by autoradiography, cut out, and assayed for ^{14}C, ^{35}S or ^{3}H in a Packard liquid scintillation spectrometer.[30]

Enzyme assays. Assays for pyrophosphorylase activities for AMP, IMP and APP-ribonucleotide were performed by procedures described in detail elsewhere.[2, 17] For the assay of adenosine kinase activity, 6-MeMP-^{35}S-ribonucleoside was used. This compound is a good substrate for adenosine kinase and can be used to measure the activity of the enzyme in crude supernatants in which adenosine cannot be used because of the high activity of adenosine deaminase.[25, 31] Degradation of nucleotides by cell-free extracts was determined by procedures described in Fig. 3.

Evaluation of APP-ribonucleoside as a substrate for bovine adenosine deaminase was accomplished spectrophotometrically as described in Fig. 4. The molar extinction coefficient for APP-ribonucleoside at 275 mμ was 11,400 $M^{-1}cm^{-1}$. The ribonucleoside of HPP was not available for assay, but the extinction coefficient of this compound was calculated from the optical density remaining after deamination of a solution of APP-ribonucleoside had ceased. The value was 3270 $M^{-1}cm^{-1}$ at 275 mμ. These extinction coefficients are about the same as those reported for the deoxyribonucleosides of APP and HPP.[32]

Protein was determined by the method of Lowry *et al.*[33]

Inhibition of an early step of synthesis of purines de novo. For measurement of the effect of purines and derivatives on *de novo* synthesis of purines, the procedures of LePage and Jones[34] and Henderson,[35] as modified in our laboratory,[36] were used. In this procedure azaserine is used to produce a buildup of FGAR-^{14}C in cells grown in the presence of sodium formate-^{14}C; the capacity of a compound to reduce the amount of FGAR accumulating is a measure of its capacity to inhibit a step of *de novo* synthesis prior to the formation of FGAR.

Metabolism of purines by intact cells. Studies of the intermediary metabolism of labeled adenine, APP, 2-fluoroadenosine and 6-MeMP-ribonucleoside and of their incorporation into polynucleotides by sensitive and resistant cells were accomplished by previously described methods.[17, 30]

Permeability studies. Permeability of sensitive and resistant cells was determined by comparison of the distribution of inulin-^{3}H, inulin-^{14}C, $^{3}H_2O$ and APP-6-^{14}C in suspensions of sensitive and resistant cells. The procedure used was essentially that of Bieber and Sartorelli.[37]

RESULTS

Response of APP-resistant cells to inhibitors. The high degree of resistance of H. Ep.#2/APP cells to APP is shown by the facts that: (1) they grew well insuspension culture in the presence of APP at concentrations up to 160-fold greater than that resulting in the destruction of cells of the parent line (Fig. 1); and (2) colony form-

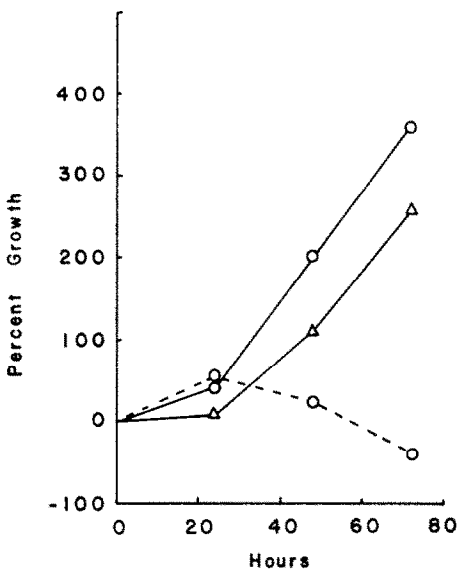

FIG. 1. Growth of H. Ep. #2/S and H. Ep. #2/APP cells. ○——○, H. Ep. #2/S cells on normal medium; ○- - - -○, H. Ep. #2/S cells on medium containing APP (3·7 μM); △——△, H. Ep. #2/APP cells on medium containing APP (590 μM). Cells were grown in suspension culture and APP, when present, was added at the time the cells were placed in the medium. Cells were stained with vital stain and counted in a hemocytometer. The ordinate represents per cent growth; values < 0 indicate partial destruction of cell inoculum.

ation was not decreased by concentrations of APP up to 740 μM, whereas a concentration of 3 μM inhibited the formation of colonies by cells of the parent line by 50 per cent or more (Table 1). When the cells were cultured in the absence of APP, some degree of sensitivity to APP was regained slowly; however, after 20 doubling times in the absence of drug, there was still a considerable degree of resistance (Fig. 2). Resistance to APP conferred resistance to certain other purine analogs, but the magnitudes of the resistance varied (Table 1). For example, there was high resistance to APP-ribonucleoside and 6-MeMP-ribonucleoside, much less resistance to 2-fluoroadenine,

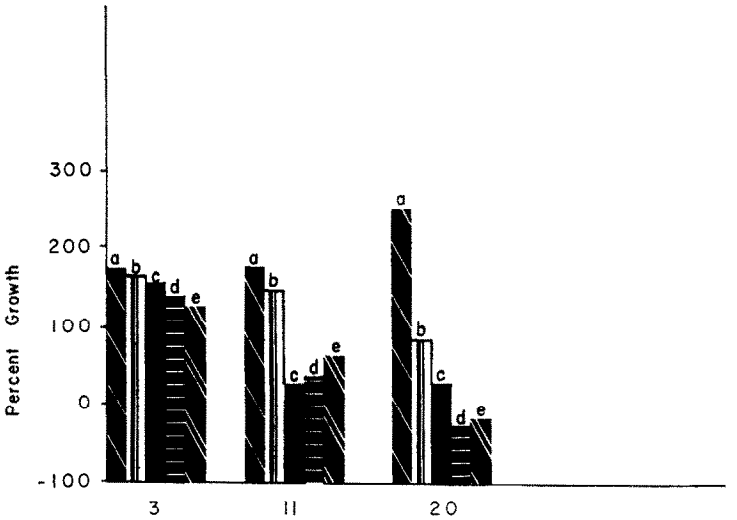

FIG. 2. Effects of growth in absence of drug on resistance in H. Ep.#2/APP cells. Cells were grown in suspension culture for 3, 11 or 20 doubling times in the absence of APP and then assayed for sensitivity to various concentrations of APP (see Fig. 1 for other details). The ordinates represent per cent growth as determined by cell count 72 hr after inoculation in the specified medium. a, no APP; b, APP, 7·4 μM; c, APP, 37 μM; d, APP, 74 μM; e, APP, 296 μM.

TABLE 1. RESPONSE OF H. EP. #2/S AND H. EP. #2/APP CELLS TO INHIBITORS

Inhibitor*	Inhibitor concn (μM) for H. Ep. #2/S cells†	Ratio of inhibitory concn: H. Ep. #2/APP/ H. Ep. #2/S
APP	3	> 250
APP-ribonucleoside	0·2	>2000
6-MeMP-ribonucleoside	1	> 330
2-Fluoroadenine	0·03	10–30
2-Fluoroadenosine	0·02	10–40
Tubercidin (7-deazaadenosine)	0·002	2–10
6-Methylpurine	4	*ca.* 1
6-Methylpurine ribonucleoside	0·003	1–3
4-Aminoimidazo (4,5-*d*) pyridazine	4	*ca.* 20
6-Mercaptopurine	0·6	*ca.* 5
6-Thioguanine	0·06	3–10

* One hundred cells were placed in 4 oz prescription bottles containing, for control cultures, 10 ml medium, and for treated cultures, 10 ml medium in which the inhibitors were present at the desired concentrations. After the bottles had been incubated at 37° for 7–10 days, the colonies were stained and counted. The inhibitory concentrations given for the parent cell line have been reported earlier.[9]

† Concentrations inhibiting the formation of colonies by 50 per cent or more. For many of the compounds, there was considerable variation in the concentrations required for inhibition of the resistant cells; these variations are indicated either by range or by *ca.* before the value.

2-fluoroadenosine, 6-mercaptopurine and 6-thioguanine, and little or no resistance to 6-methylpurine, 6-methylpurine ribonucleoside and tubercidin.

Nucleotide pyrophosphorylase and nucleoside kinase activities. The resistant cells retained capacity to convert adenine, hypoxanthine and APP to their nucleotides

(Table 2). In some experiments the AMP pyrophosphorylase activity of resistant cells was below that of the parent cell line; however, this was not consistently so and the differences between sensitive and resistant cells were not greater than the variations observed in either cell line in experiments performed with batches of cells grown independently. The activities of IMP pyrophosphorylase of resistant cells were within

TABLE 2. ACTIVITIES OF PURINE NUCLEOTIDE PYROPHOSPHORYLASES AND PURINE NUCLEOSIDE KINASE IN CELL-FREE EXTRACTS FROM H. EP. #2/S AND H. EP. #2/APP CELLS*

Substrate	Nucleotide formation (n-moles/mg protein/hr)	
	H. Ep. #2/S	H. Ep. #2/APP
Adenine-8-^{14}C	414–606 (4)	312–519 (4)
Hypoxanthine-8-^{14}C	519–839 (2)	519–609 (2)
APP-6-^{14}C	20–67 (4)	10–26 (4)
6-Methylthiopurine-^{35}S ribonucleoside	139–251 (2)	79–127 (2)

* For assays of nucleotide pyrophosphorylases, the reaction mixture contained the ^{14}C-labeled purine base (0·25 μmole), the magnesium salt of PRPP (2 μmoles), crude enzyme (100,000 *g* supernatant) fraction or acetone powder (0·20 mg protein) and Tris buffer (50 μmoles) in a final volume of 0·5 ml, pH 7·5. Incubation was for 1 hr at 37°; the reaction was stopped by addition of EDTA and immersion of the reaction vessel in a boiling water bath. For assay of nucleoside kinase activity, the reaction mixture contained 6-MeMP-^{35}S-ribonucleoside (1 μmole), ATP (2·5 μmoles), $MgCl_2$ (0·25 μmole), potassium phosphate buffer (50 μmoles) and crude enzyme (25,000 *g* supernatant) fraction (5·5 mg protein) in a final volume of 1·0 ml, pH 7·0. Incubation was for 30 min at 25°; the reaction was stopped by immersion of the vessel in boiling water. After the removal of denatured protein, portions of each reaction mixture were subjected to paper chromatography in solvent E (for pyrophosphorylase assays) or A (for kinase assays). The number of assays is given in parentheses.

the range of those found in the parent cells. As has been noted earlier,[17] the extent of conversion of APP to nucleotide is small relative to that of adenine to AMP, and the very low conversions in both sensitive and resistant cell lines make doubtful the significance of the observed differences between the two cell lines. Cell-free supernatants from sensitive and resistant cells both had capacity to phosphorylate 6-MeMP-^{35}S-ribonucleoside; however, the activity of resistant cells was less than that of sensitive cells. Earlier studies[25, 31, 38] have shown that adenosine kinase catalyzes the phosphorylation of the ribonucleosides of 6-MeMP, APP, 2-fluoroadenine, purine, 6-methylpurine and a number of other bases.

Inhibition of accumulation of FGAR and FGA-ribonucleoside. Treatment with azaserine caused an accumulation of FGAR and FGA-ribonucleoside in both sensitive and resistant cells (Table 3). The cell lines differed in the proportions of nucleotide and nucleoside that accumulated. Although the relative amounts of these two compounds varied to some extent from one experiment to another, in all experiments the major product was FGAR in the sensitive cells and FGA-ribonucleoside in the resistant cells. The individual experiments shown in Table 3 are a random selection from a large number of experiments. The resistant cells incorporated much less formate into soluble metabolites than did sensitive cells.

TABLE 3. ACCUMULATION OF FGAR, FGA-RIBONUCLEOSIDE AND OTHER COMPOUNDS IN AZASERINE-TREATED CELLS*

Cell line	FGAR (cpm)	FGA-ribonucleoside (cpm)	Polyphosphates (cpm)	Ratios of cpm: FGAR/FGA-ribonucleoside	Ratios of cpm: Chromatogram/medium
H. Ep.#2/S					
Expt. 1	82,800	10,100	31,000	8·2	$2·26 \times 10^{-3}$
Expt. 2	63,600	19,100	29,900	3·3	$2·21 \times 10^{-3}$
Expt. 3	67,000	10,600	30,800	6·3	$2·16 \times 10^{-3}$
H. Ep.#2/APP					
Expt. 1	8900	19,600	4300	0·46	$0·59 \times 10^{-3}$
Expt. 2	9900	22,800	4000	0·43	$0·66 \times 10^{-3}$
Expt. 3	15,600	28,200	5000	0·56	$0·90 \times 10^{-3}$

* To cells in suspension culture (4×10^7 cells in 100 ml medium) azaserine was added to a final concentration of 10 μg/ml followed 30 min later by the purine or nucleoside analog and 30 min thereafter by 25 μc sodium formate-^{14}C. To control flasks only azaserine and formate were added. Cells were harvested 2 hr after addition of formate and extracted with 80% aqueous ethanol. The water-soluble portion of the extract was subjected to chromatography in solvent H. These data represent individual experiments randomly selected from those on which the summary data of Table 4 are based. The 'polyphosphates' have been shown to consist largely of polyphosphates of FGA-ribonucleoside.[30] The principal spot on the chromatograms, other than those shown in the table, was serine; the ^{14}C content of serine varied widely among experiments, but has been included in the summation of total ^{14}C for the last column, which shows the relative uptake of formate by the two cell lines.

Several purines and nucleosides, known to inhibit accumulation of FGAR and FGA-ribonucleoside in H. Ep. #2/S cells,[36] were evaluated for capacity to inhibit accumulation of these compounds in H. Ep. #2/APP cells (Table 4). Adenine,

TABLE 4. CONCENTRATIONS OF INHIBITORS REQUIRED FOR 50 PER CENT INHIBITION OF *DE NOVO* PURINE SYNTHESIS*

Inhibitor	Inhibiting concn (μM): H. Ep.#2/S	Inhibiting concn (μM): H. Ep.#2/APP
Adenine	4	4
APP	>74	
APP-ribonucleoside	7	>296
2-Fluoroadenine	4	4–7
2-Fluoroadenosine	4	< 15
8-Azaadenosine	4	4–7
Tubercidin	4	< 7
4-Aminoimidazo(4,5-*d*)pyridazine	15	> 94

* The concentrations given are those required to inhibit by 50 per cent the accumulation of FGAR and FGA-ribonucleoside in azaserine-treated cells (see Table 3 for details). Some of the values given for sensitive cells are taken from an earlier publication.[36]

2-fluoroadenine, 2-fluoroadenosine, 8-azaadenosine and tubercidin inhibited accumulation of FGAR and FGA-ribonucleoside at concentrations about the same as those that had been found earlier to inhibit accumulation in sensitive cells. APP-ribonucleoside and 4-aminoimidazo(4,5-*d*)pyridazine, at the relatively high concentrations used, did not inhibit accumulation of these derivatives in resistant cells. In the sensitive cell line, APP-ribonucleoside was an effective inhibitor, whereas APP was not.

Degradation of nucleotides by cell-free extracts. The fact that FGA-ribonucleoside rather than FGAR was the predominant compound accumulating in H. Ep.#2/APP cells treated with azaserine suggested that the resistant cells might differ from the sensitive cells in the activity of a phosphatase acting on nucleotides. Nucleotide-degrading capacity was evaluated by incubating crude cell-free supernatants with labeled nucleotides and analyzing the mixture chromatographically at various times thereafter (Fig. 3). The resistant cells were much more active than the sensitive cells in

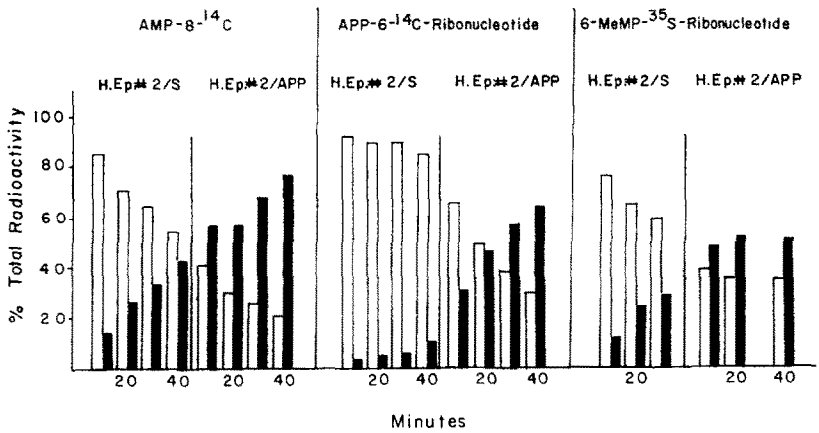

FIG. 3. Degradation of nucleotides by cell-free supernatants from H. Ep. #2/S and H. Ep. #2/APP cells. □, Nucleotide used as substrate plus the corresponding di- and triphosphates (if found); ■, degradation products (nucleosides and free bases). When AMP-^{14}C was substrate, the degradation products were inosine and hypoxanthine; when APP-^{14}C nucleotide was the substrate, the degradation products were APP-ribonucleoside and HPP-ribonucleoside; when ^{35}S-6-MeMP-ribonucleotide was the substrate, the principal degradation product was 6-MeMP-ribonucleoside. Small amounts of radioactivity present in unidentified compounds were not included among either the anabolic or catabolic products. Each incubation mixture contained crude 25,000 *g* supernatant (4·4 mg protein) and the labeled nucleotide in a final volume of 0·6 ml of 0·02 M Tris buffer, pH 7·5. After incubation at 37° for 10, 20, 30 or 40 min, the reaction was stopped by immersion of the vessel in boiling water; protein was removed by centrifugation and the radioactive compounds present in the supernatant were isolated and assayed by the procedure described in Table 6.

degrading three nucleotides: 5′-AMP and the ribonucleotides of 6-MeMP and APP. At all incubation times the preparations from sensitive cells had more undegraded nucleotides than degradation products, whereas after 20 min the preparations from resistant cells contained more degradation products than nucleotides. The differences between the cell lines in rates of degradation were most marked when APP-ribonucleotide was the substrate. Within 10 min the resistant cell preparations had degraded about 60 per cent of the AMP, 30 per cent of the APP-ribonucleotide and 50 per cent of the 6-MeMP-ribonucleotide, whereas the sensitive cell preparations had degraded less than 15 per cent of each of these nucleotides. Similar but more limited experiments, which for the sake of brevity are not shown, were also accomplished with IMP-8-^{14}C and adenosine-8-^{14}C. The results with IMP were similar to those with the other nucleotides. When adenosine was the substrate, no marked differences were noted between sensitive and resistant cells in the extent of formation of inosine and hypoxan-

thine. The results indicate an increased nucleotidase activity of resistant cells with no marked change in adenosine deaminase or inosine phosphorylase activities.

Since these cell lines do not possess significant xanthine oxidase activity, the principal degradation products were nucleosides and free bases (see Fig. 3). Whereas the principal products derived from AMP were inosine and hypoxanthine, the major products from APP-ribonucleotide were the ribonucleosides of APP and of HPP. Both of these nucleosides have been previously identified as metabolites of APP in *Cordyceps militaris*[19] and APP-ribonucleoside is a metabolite in mammalian cells.[12] The principal degradation product of 6-MeMP-ribonucleotide was the corresponding ribonucleoside; this finding is in accord with earlier observations that this nucleoside is not a substrate for phosphorylases in mammalian cells.[25, 31, 39]

Metabolism of adenine in intact cells. Resistant cells took up approximately three times more adenine than did sensitive cells and metabolized it differently (Table 5).

TABLE 5. METABOLISM OF ADENINE-8-^{14}C BY H. EP. #2/S AND H. EP. #2/APP CELLS*

	Concentrations (μM) of adenine in medium					
A. Soluble fraction	7·4	15	30	7·4	15	30
	Distribution of ^{14}C among metabolites (% of total in cell extract)					
Metabolite	H. Ep. #2/S			H. Ep. #2/APP		
AMP	31	29	23	5	3	3
ADP	14	15	18	6	6	5
ATP	4	7	13	2	2	3
NAD	22	25	24	15	15	10
Adenine	1	1	1	1	1	1
Inosine	3	2	2	15	11	12
Hypoxanthine	15	9	8	47	55	59
Other metabolites	10	12	10	9	8	9
Relative uptake†	0·08	0·17	0·41	0·25	0·53	1·4
B. Polynucleotides	Relative specific activities‡					
	H. Ep. #2/S			H. Ep. #2/APP		
DNA-adenine	4·7	8·2	14	4·5	7·9	9·7
guanine	0·57	2·1	5·4	1·4	3·0	5·4
RNA-adenine	4·7	8·7	15	5·8	9·8	14
guanine	0·73	2·4	6·5	1·9	4·0	7·5

* Cells were grown in suspension culture and harvested 24 hr after addition of adenine-8-^{14}C. Each flask contained 10 μc adenine diluted with nonradioactive adenine to give the concentrations indicated. After harvesting, the cells were washed free of medium with 0·9% NaCl and extracted with hot 80% ethanol. The water-soluble portion of the extract was subjected to paper chromatography in solvent H. From the residue remaining after extraction with ethanol, the DNA and RNA purines were separated and hydrolyzed to the free bases, which were separated by paper chromatography.[17,30,40]

† Relative uptake = total counts in soluble fraction ÷ specific activity of administered adenine.

‡ Relative specific activity = sp. act. of isolated purine ÷ sp. act. of administered adenine.

In the soluble fraction of the sensitive cells, 71–78 per cent of the ^{14}C was present as nucleotides (AMP, ADP, ATP, NAD), whereas in the resistant cells 62–71 per cent was present as hypoxanthine and inosine. The relative specific activities of adenine in RNA and DNA did not differ greatly between the cell lines, but the relative specific activity of guanine was generally higher in the resistant cells. This latter difference

may be due in part to the fact that the resistant cells took up more adenine than the sensitive cells; the data of Table 5 show that the extent of conversion of adenine to nucleic acid guanine is dependent on the concentration of adenine in the medium.

Metabolism of APP, 2-fluoroadenosine and 6-MeMP-ribonucleoside. H. Ep. #2/S cells incorporated APP and converted it in significant amounts to nucleotides, which were the predominant intracellular products; small amounts of APP and the ribonucleosides of APP and HPP were also present (see Tables 6 and 7). H. Ep. #2/APP cells grown

TABLE 6. METABOLISM OF 4-AMINOPYRAZOLO(3,4-*d*)PYRIMIDINE-6-^{14}C, 6-METHYLTHIOPURINE-^{35}S-RIBONUCLEOSIDE AND 2-FLUOROADENOSINE-^{3}H BY H. EP. #2/S AND H. EP. #2/APP CELLS*

Compounds isolated	Counts/min/10^6 cells	
	H. Ep. #2/S	H. Ep. #2/APP
APP-^{14}C as precursor		
APP-ribonucleoside monophosphate	327	3
APP-ribonucleoside triphosphate	786	3
APP-ribonucleoside	25	4
APP	40	7
HPP-ribonucleoside	45	7
Unidentified metabolite	25	2
6-MeMP-^{35}S-ribonucleoside as precursor		
6-Methylthiopurine-^{35}S ribonucleoside monophosphate	18,200	448
2-Fluoroadenosine-^{3}H as precursor		
Mono-, di-, and triphosphates of 2-fluoroadenosine-^{3}H	16,230	15,780

* To suspension cultures of H. Ep. #2/S or H. Ep. #2/APP cells (3–6 · 10^5 cells/ml) was added APP-6-^{14}C (50 μmoles, 0·028 μc/ml medium), 6-MeMP-^{35}S-ribonucleoside (32 n-moles, 0·11 μc/ml medium) or 2-fluoroadenosine-^{3}H (1·7 n-moles, 0·02 μc/ml medium). Four hr thereafter, the cells were collected by centrifugation and alcohol extracts were prepared as described in Table 5. The water-soluble portion of the extract was subjected to chromatography either in solvent H (for metabolites of APP and 2-fluoroadenosine) or in solvent A (for metabolites of 6-MeMP-ribonucleoside).

6-MeMP-ribonucleotide was characterized as described earlier.[25] Phosphates of 2-fluoroadenosine were characterized (1) by their migration like the corresponding adenine nucleotides upon paper electrophoresis in borate buffer, pH 9·2; and (2) by their conversion to 2-fluoroadenosine upon treatment with crude snake venom. See Table 7 for characterization of metabolites of APP.

under the same conditions contained very little radioactivity. This low uptake conceivably could be the result of the fact that the resistant cells had been cultured in the presence of APP up to the time of the experiment and that, therefore, some of the cells in the culture may have contained APP which diluted the radioactive substrate. That this probably is not a contributing factor is shown by the similar very low uptake of 6-MeMP-^{35}S-ribonucleoside by the resistant cells; it is to be noted that this is one of the compounds to which H. Ep. #2/APP cells are highly cross-resistant. H. Ep. #2/APP cells were as active as sensitive cells in forming nucleotides from 2-fluoroadenosine, a compound to which they had a much lower degree of resistance.

The polynucleotides were also isolated from H. Ep. #2/S cells grown for 24 hr in the presence of APP-^{14}C. The crude sodium nucleate fraction was separated into DNA and RNA fractions; each of these fractions was hydrolyzed by acid and the silver salts of

the purines were isolated.[40] The purines were then released from the silver salts by acid hydrolysis and carrier APP was added to the hydrolysate. Adenine, guanine and APP were isolated by paper chromatography in solvent G. The sodium nucleates had a specific activity of 0·11 nc/mg, most of which was found in the APP fraction from RNA. (Small amounts of ^{14}C were also present in the adenine and guanine fractions from both DNA and RNA.) The amount of ^{14}C associated with polynucleotides was too small to permit isolation and characterization of APP-nucleotide from an RNA

TABLE 7. IDENTIFICATION OF METABOLITES OF 4-AMINOPYRAZOLO(3,4-*d*)PYRIMIDINE*

Compound	R_f values in solvent					
	A	B	C	D	E	F
Known compounds						
AMP	0·17	0·48	0·29	0·62	0·53	
ATP	0·06	0·27	0·37	0·50	0·49	
APP	0·72	0·78	0·15	0·88	0·73	0·67
APP-R†	0·56	0·74	0·22	0·80	0·72	0·82
HPP-R	0·44	0·65		0·64	0·66	0·79
Metabolic products‡						
APP-R monophosphate§	0·19	0·50	0·29	0·63	0·56	
APP-R triphosphate§	0·06	0·28	0·41	0·54	0·53	
APP-R	0·57	0·76	0·20	0·79	0·72	0·75
HPP-R	0·44	0·58		0·64	0·64	0·75
Venom-treated APP-R phosphates‖	0·57					
Acid-hydrolyzed APP-R phosphates¶	0·69					

* Chromatographic separations were made on Whatman No. 1 or Schleicher–Schüll white paper. Known compounds were detected by illumination of the paper with ultraviolet light and unknown radioactive compounds by autoradiography. The 'metabolic products' were eluted from chromatograms obtained in the experiments described in Table 6 and Fig. 3.

† Abbreviation: R = ribonucleoside.

‡ The identities indicated for the first 4 compounds are those suspected from their migration upon two-dimensional chromatography (see Table 6) and confirmed by their migration in the solvents listed here.

§ These nucleotides migrated similarly to the corresponding adenine nucleotides upon paper electrophoresis at 1250–1500 V for 60–90 min in 0·05 M formate buffer, pH 3·6, or in 0·05 M borate buffer, pH 9·2.

‖ The ^{14}C appearing in the nucleotide area of a two-dimensional chromatogram was eluted and treated with crude snake venom for 4 hr at 37°. The reaction was stopped by heating in boiling water and the supernatant was subjected to chromatography in the solvent indicated.

¶ The ^{14}C appearing in the nucleotide area of a two-dimensional chromatogram was eluted and incubated with HCl-saturated methanol for 24 hr at room temperature, after which the resulting hydrolysate was subjected to chromatography in the solvent indicated.

hydrolysate and thus it cannot be said that these experiments show true incorporation. Even if all the ^{14}C present represented true incorporation, the amount incorporated is very small; only one of each 2000 adenine residues in RNA would have been replaced. Evidence for a small amount of incorporation of APP-^{14}C into polynucleotides *in vivo* has been reported for tumors and normal tissues.[12, 17]

Deamination of APP-ribonucleoside. The isolation of HPP-ribonucleoside as a metabolite of APP suggested that APP-ribonucleoside was a substrate for adenosine deaminase. This nucleoside was found to be acted upon by bovine intestinal adenosine deaminase (Fig. 4); the K_m was $2{\cdot}5 \times 10^{-4}$ M and the V_{max} was 15 μmoles/min/mg protein. The K_m value was of the same order of magnitude as that reported for APP-

deoxyribonucleoside.[32] The K_m value and V_{max} for adenosine, determined with the same enzyme preparation, were $3{\cdot}3 \times 10^{-5}$ M and 233 μmoles/min/mg protein.

Permeability studies. Suspensions of sensitive and resistant cells, when incubated with inulin-^{14}C, $^{3}H_2O$ or APP-^{14}C, showed similar patterns in distribution of radioactivity; this result indicates that resistance is not associated with a difference in

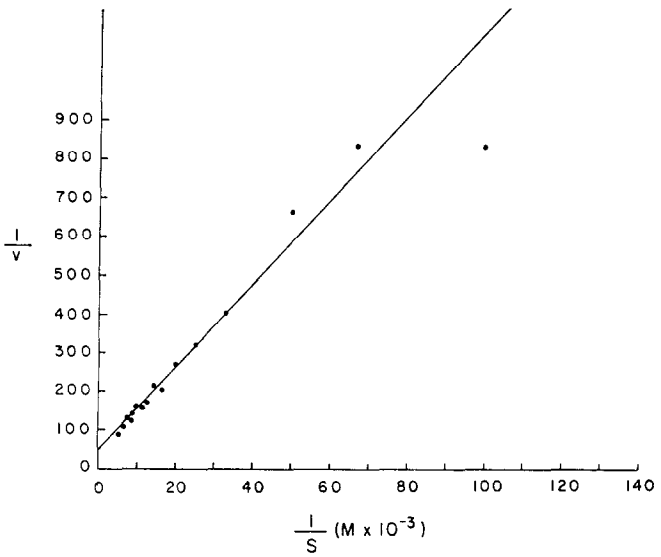

FIG. 4. Lineweaver–Burk plot of the deamination of APP-ribonucleoside by adenosine deaminase. The reaction mixtures, containing 1·67 μg of Sigma calf intestinal adenosine deaminase and APP-ribonucleoside (concentrations varying from 10 to 275 μM) per 1·0 ml of 0·05 M phosphate buffer, pH 7·5, were incubated at 25°. The rate of deamination was followed by determination of change in optical density at 275 mμ.

permeability to APP. The data show that APP-^{14}C and $^{3}H_2O$ occupied about the same volumes in the cell suspensions. In fact, the dilution of radioactivity from APP-^{14}C resulting from the addition of cells to the incubation medium was consistently slightly greater than the dilution of $^{3}H_2O$; this difference may result from some fixation of APP by the cells (Fig. 5).

DISCUSSION

Resistance to APP in the selected cell line was associated with a failure to accumulate APP-nucleotides. The essentially complete absence of APP-nucleotides in resistant cells cannot be accounted for by impermeability of the resistant cells to APP or by loss of nucleotide pyrophosphorylase activity. Similarly, loss of nucleoside kinase activity does not account for the high degree of resistance of H. Ep. #2/APP cells to APP-ribonucleoside and 6-MeMP-ribonucleoside, since there was adequate, though somewhat lower, kinase activity in resistant cells. The patterns of metabolism of inhibitors correlate well with the responses of the cells to the inhibitors; thus, cells of the resistant line were highly resistant to APP and 6-MeMP-ribonucleoside and formed little or no nucleotides from these compounds, whereas these cells had a much smaller degree of resistance to 2-fluoroadenosine which they metabolized extensively to phosphate derivatives (Table 6).

The failure of resistant cells to accumulate nucleotides derived from some analogs but not from others probably is related to the rate at which the analog is converted to the nucleotide relative to the rate that the nucleotide, once formed, is degraded.

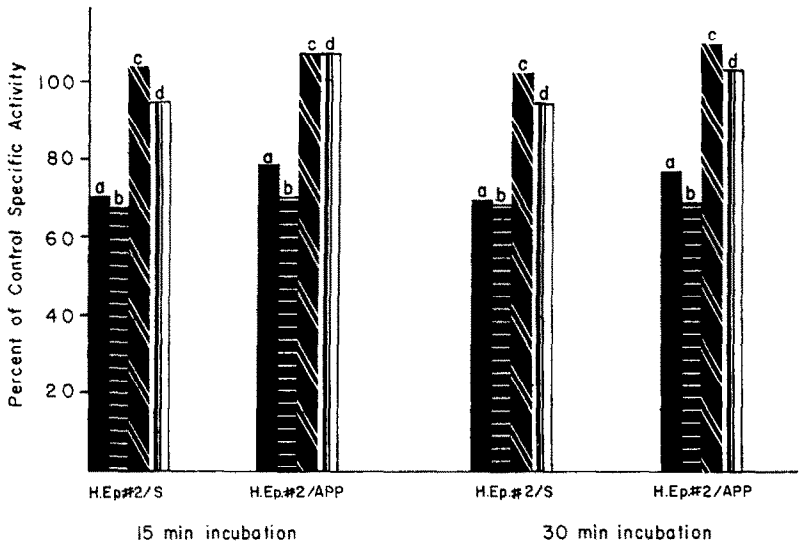

FIG. 5. Permeability of H. Ep. #2/S and H. Ep. #2/APP cells to APP. Cells were suspended in 0·9% aqueous saline solution at a concentration of about 2·5 × 10^8 cells (1 g wet wt.) per ml. One ml of this suspension was added to incubation tubes containing either APP-^{14}C + ^3H_2O, APP-^{14}C + inulin-^{3}H, or ^3H_2O + inulin-^{14}C with sufficient saline to make a total volume of 1·50 ml. Similar mixtures containing 1·0 ml of 0·9% saline solution instead of the cell suspension served as controls. After 15 or 30 min of incubation with shaking, the cells were separated by centrifugation and the specific activities of the supernatants with respect to both ^{3}H and ^{14}C were determined in a Packard Tri-Carb liquid scintillation spectrometer. The ordinates are the specific activities of the incubation mixtures containing cells as percentages of the specific activities of those of the control mixtures. a, ^3H_2O; b, APP-6-^{14}C; c, inulin-^{14}C; d, inulin-^{3}H.

Experimental observations do, in fact, indicates that the resistant cells, as compared with sensitive cells, phosphorylate nucleosides more slowly (Table 2) and degrade nucleotides more rapidly (Fig. 3). The differences between sensitive and resistant cells in nucleotidase activity were much greater than the differences in adenosine kinase activity; hence, increased degradative capacity may be the major factor in the observed resistance patterns. For example, the much more rapid degradation of ribonucleotides of APP and 6-MeMP by resistant cells could well account for the resistance of these cells to APP, APP-ribonucleoside and 6-MeMP-ribonucleoside and for their failure to accumulate labeled nucleotides when they were grown in the presence of labeled APP or 6-MeMP-ribonucleoside. Increased capacity for degradation of nucleotides could also account for the observations on the metabolism of adenine in resistant cells; the rapid degradation of AMP as it is formed would tend to increase the uptake of adenine from the medium with the net result of an increased turnover of the AMP pool and an accumulation of degradation products.

The fact that the resistant cells were not highly resistant to a number of analogs can be explained in terms of increased degradative capacity only if some analog nucleotides are degraded more rapidly by resistant cells than by sensitive cells while others are

degraded by the cell lines at about the same rates. The data obtained, although they do not provide complete evidence on this point, do indicate that nucleotides may be degraded at different rates (Fig. 3). Since the resistant cells had only a small degree of resistance to 2-fluoroadenine and 2-fluoroadenosine, it would be expected that 2-fluoro-AMP would be degraded by sensitive and resistant cells at about the same rate. Unfortunately, 2-fluoro-AMP was not available for a direct study of its degradation; however, the approximately equal accumulation in sensitive and resistant cells of nucleotides derived from 2-fluoroadenosine (Table 6) would suggest that degradation of these nucleotides, if it does occur, proceeds at about the same rate in both cell lines.

Evidence that degradative capacity is not the sole factor in resistance is provided by the data on the metabolism of APP in intact cells. Resistant cells grown in the presence of APP-^{14}C contained surprisingly small quantities of degradation products (Table 6). By analogy with the results with adenine (Table 5), extensive intracellular accumulation of APP-ribonucleoside, HPP-ribonucleoside, and HPP might have been expected.

The H. Ep. #2/APP cell line differs from other cell lines selected in our laboratory for resistance to purine analogs in at least two respects: (1) resistance was not entirely stable when the cells were cultured in the absence of drug; and (2) loss of nucleotide pyrophosphorylase activity did not appear to be the basis for resistance. It is of interest that, although H. Ep. #2 cells lacking AMP pyrophosphorylase activity are highly resistant to APP,[9] APP itself was ineffective as an agent for selection of cells lacking AMP pyrophosphorylase. H. Ep. #2/APP cells have been carried in culture in the presence of APP for as long as 2 years with retention of capacity for conversion of both adenine and APP to nucleotides. In some respects, H. Ep. #2/APP cells may be compared with a 6-MP-resistant line of Ehrlich cells studied by Paterson *et al.*[41–43] This cell line had enzymic capacity to convert 6-MP to the nucleotide, but the nucleotide did not accumulate in intact cells. In contrast to the findings with H. Ep. #2/APP cells, this 6-MP-resistant Ehrlich line had a decreased nucleotidase activity.[43] The H. Ep. #2/APP cells may also be compared with 6-thioguanine-resistant cell lines studied by Bieber and Sartorelli,[37] Sartorelli *et al.*[44] and Ellis and LePage,[45] and a line of Ehrlich cells resistant to 6-MP and 6-MeMP-ribonucleoside studied by Henderson *et al.*;[46] in these lines resistance was associated with an increased degradative capacity. With respect to the metabolism of APP, H. Ep. #2/APP cells are also comparable with certain 'naturally' resistant tumors which, relative to sensitive tumors, either formed smaller amounts of APP nucleotides or degraded them more rapidly.[12]

The effectiveness of selected compounds as feedback inhibitors in sensitive and resistant cells (Table 4) correlates moderately well with their effectiveness as growth inhibitors. Compounds, such as APP-ribonucleoside and 4-aminoimidazo(4,5-*d*)-pyridazine, to which the cells were highly resistant, did not produce feedback inhibition; whereas compounds to which the cells showed no resistance, or a low degree of resistance, produced feedback inhibition at about the same concentrations as those effectively inhibiting in H. Ep. #2/S cells. The fact that the relatively low (2- to 40-fold) degrees of resistance to growth inhibition by 2-fluoroadenine, 2-fluoroadenosine and tubercidin were not reflected in resistance to feedback inhibition may be a result of the difference in duration of the two types of experiment. The feedback inhibition experiments measure the effects of the inhibitor over a period of only a few hours, whereas growth inhibition studies are the results of the exposure of cells to inhibitors for several days. In regard to feedback inhibition, it is noteworthy that in H. Ep. #2/S

cells APP-ribonucleoside was a much more effective inhibitor than was APP and was much more toxic.[9] Since it appears that the nucleotide or a nucleotide derivative is the active inhibitor, this result indicates that intracellular APP-nucleotide accumulates much more quickly with APP-ribonucleoside as precursor than with APP as precursor, and is in accord with the observations that APP-ribonucleoside is an excellent substrate for adenosine kinase,[38] whereas APP is a poor substrate for AMP pyrophosphorylase.[17] (See also Table 2.)

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