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Metabolic Properties of an Azaguanine-Resistant Variant of Chinese Hamster Ovary Cells (aza^{r}ts) With Normal Levels of Hypoxanthine-Guanine Phosphoribosyltransferase Activity

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Aza^{r}ts Chinese hamster ovary cells were 20 to 50 times more resistant to 8-azaguanine and 50 to 10 times more resistant to both 6-thioguanine and 6-mercaptopurine than wild-type cells. Resistance correlated with a failure of aza^{r}ts cells to incorporate 8-azaguanine into the nucleotide pool and into nucleic acids. The uptake of hypoxanthine and guanine, on the other hand, was about the same in both types of cells and the hypoxanthine-guanine phosphoribosyltransferase of the aza^{r}ts cells as measured in cell lysates was unaltered both in concentration and kinetic properties with hypoxanthine as well as 8-azaguanine as substrate. Plasma membrane permeability to 8-azaguanine and the regulation of intracellular pH were also not altered in aza^{r}ts cells and there was no significant degradation of 8-azaguanine or azaguanine nucleotides. We conclude therefore that in aza^{r}ts cells the phosphoribosylation of 8-azaguanine per se is specifically blocked but that this effect is abolished upon cell lysis.

Key words: CHO cells, azaguanine-resistant, hypoxanthine, phosphoribosyltransferase, hypoxanthine transport

Variants of cultured mammalian cells resistant to 8-azaguanine (AzaGua) can be isolated with relatively high frequency (10^{-6}–10^{-7}) [1,2]. Most of the variants have been found to be resistant to AzaGua because of a decreased ability to phosphoribosylate it, caused by a deficiency in hypoxanthine(Hyp)-guanine(Gua) phosphoribosyltransferase (HPRT). However, some AzaGua-resistant variants have been isolated that possess normal levels of HPRT activity, and some types of cells exhibit natural resistance to AzaGua, but the basis of resistance of these cells to the analog is incompletely understood [1] and can vary with the cell type or isolate. Several alternate mechanisms have been postulated to account for AzaGua resistance of cells that possess HPRT (see below). Among them a defect in purine transport* reported by Harris and Whitmore [3] and suggested by earlier work [summarized in 1] was of

**Transport* denotes solely the transfer of unmodified substrate across the cell membrane as mediated by a saturable, selective carrier. *Uptake* denotes the total intracellular accumulation of radioactivity from exogenous labeled substrate regardless of metabolic conversions.

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particular interest to our research [4,5]. Harris and Whitmore [3] isolated a highly AzaGua-resistant variant of Chinese hamster ovary (CHO) cells (designated aza^{r}ts) that exhibited a temperature-dependent difference in the rate of uptake* of AzaGua and kinetics of Hyp uptake. Aza^{r}ts cells propagated and analyzed at 38.5°C took up [^{14}C]AzaGua at a rate of only about 20% that observed for aza^{r}ts cells propagated and analyzed at 34°C or for wild-type CHO cells propagated and analyzed at either temperature. The K_m for Hyp uptake was 7 μM for wild-type cells as well as for aza^{r}ts cells grown and assayed at 34°C. However, the K_m for Hyp uptake was 18 μM when the aza^{r}ts cells were grown and assayed at 38.5°C, whereas V_{max} was about the same in all cases. The authors concluded that the AzaGua resistance of the aza^{r}ts mutant might be attributable to a decreased affinity of the purine base transporter for its substrates, including AzaGua. This conclusion seemed to find support in the results of Prasad et al [6]. These investigators confirmed the temperature-dependent difference in the kinetics of Hyp uptake by 34°C-grown and 38.5°-grown aza^{r}ts cells and reported that membrane vesicles derived from these cells exhibited a similar difference in the K_m for Hyp uptake.

On the basis of various lines of indirect evidence, we have previously questioned the validity of the conclusion that the AzaGua resistance of the aza^{r}ts variant can be accounted for by a decreased affinity of the Hyp transporter for its substrates [5]. First, it seemed questionable that the observed two to three-fold difference in K_m for Hyp uptake without change in V_{max} could account for the approximately 30-fold higher resistance to AzaGua of the aza^{r}ts cells. Second, it has become apparent that the saturation of Hyp uptake measured over periods of 5 to 30 min reflects the saturation of the in situ phosphoribosylation of Hyp rather than that of its transport into the cell [4,5]. Third, we failed to find any evidence that AzaGua is a substrate for the Hyp transporter. On the contrary, all evidence supports the view that AzaGua entry into cells is mainly nonmediated [7]. However, because of the generally poor understanding of the basis of the AzaGua resistance of variants with normal HPRT activity, and because the availability of Hyp transport mutants would be of great utility in dissecting the number and substrate specificities of the transport systems for nucleosides and nucleobases [5], we have reanalyzed the properties of the aza^{r}ts variant.

MATERIALS AND METHODS

Cell Culture

CHO (Pro$^-$) cells, here referred to as wild-type, were originally obtained from Dr. L. Siminovitch and the aza^{r}ts variant from Dr. G.F. Whitmore. Both strains were propagated in suspension culture in Eagle's minimal essential medium (MEM) for spinner culture supplemented with 4% (v/v) bovine serum, 4% (v/v) horse serum, and 2% (v/v) newborn calf serum and 0.53 mg Pluronic F-68/ml [8] as well as in monolayer culture in regular Eagle's MEM supplemented with sera as indicated above. Cells were enumerated with a Coulter counter and viability was assessed by staining with trypan blue. Cultures were ascertained to be free of mycoplasma [9].

Measurement of Uptake of Radiolabeled Substances

Cells were harvested by centrifugation from late exponential-phase cultures and suspended to about 1×10^7 cells per ml of basal medium 42B (BM42B [10]) adjusted

to pH 7.0–7.2 if not indicated otherwise. Suspensions were supplemented with radiolabeled substrates as indicated in appropriate experiments. At various times of incubation the cells from duplicate 0.5-ml samples were collected by centrifugation through an oil mixture [11] and analyzed for radioactivity. All uptake values were corrected for substrate trapped in extracellular space (between 10% and 15% of total radioactivity) in pellets and converted to pmol/μl cell water on the basis of an experimentally determined cell water space. Intracellular and extracellular water spaces in pellets were estimated by the use of ^3H_2O and [^{14}C]inulin [11]. For the analysis of labeled components in the culture fluid and in the acid-soluble pool of cells, labeled cells were centrifuged through oil directly into an underlying acid solution [11]. Samples of the culture fluid and the cell extracts were analyzed by ascending paper chromatography with solvents composed of 79 ml saturated ammonium sulfate, 19 ml of 0.05 M phosphate buffer (pH 6.0), and 2 ml isopropanol (solvent 9) or 30 ml 1 M ammonium acetate, (pH 5.0) and 70 ml 95% ethanol (solvent 28) or by high-performance liquid chromatography (HPLC) using a Whatman Partisil ODS-10 reversed-phase column and a solvent composed of 10 mM NH_4Cl, 10 mM $NH_4H_2PO_4$, and 6% (v/v) methanol (pH 4.9). Samples of suspension were analyzed for radioactivity in acid-insoluble material as described previously [7].

Hyp Transport

The kinetics of Hyp transport were determined as described previously [4,12]. Time courses of transmembrane equilibration of six to eight concentrations of [^{3}H]Hyp (40–5120 μM, constant concentration of ^{3}H, irrespective of absolute concentration of Hyp) were determined under zero-*trans* conditions by rapid kinetic techniques with suspensions of 1–2 $\times$ 10^7 cells/ml of BM42B. The appropriate integrated rate equation was fitted to these time courses pooled for all the concentrations assayed assuming directional symmetry of the carrier and equality of movement of empty and Hyp-loaded carrier as previously demonstrated for Novikoff cells [12]. The fit yields best-fitting parameters for K, the Michaelis-Menten constant, and V, the maximum velocity.

Assay of Phosphoribosylation and Metabolism of AzaGua and Hyp by Cell Extracts

Cells were collected by centrifugation and suspended in a solution composed of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM triethanolamine. The suspensions were treated twice with a Branson sonifier (model 185) for 10 sec at 0°C and the lysate was used directly where indicated or centrifuged at 100,000g for 30 min.

For the HPRT assay, the supernatant fraction was used directly or was heated at 70°C for 10 min and then clarified by centrifugation in an Eppendorf microfuge for 5 min. Samples of the supernatant fluid were assayed for AzaGua phosphoribosylation similarly as described by Kong and Parks [13]. The reaction mixture contained 100 mM sodium cacodylate, pH 6.2, 2 mM $MgCl_2$, 2 mM P-Rib-PP, unlabeled AzaGua (varied between 1 and 80 μM), and cell extract equivalent to about 10 μl cell water in a total volume of 1 ml. The rate of formation of AzaGMP was estimated from the increase in absorbance at 260 nm at room temperature (molar absorbance 6,100). In another assay the reaction mixture contained 80 mM 3-(N-morpholino)propanesulfonate, phosphate or Tris-HCl at various pH values, 5 mM $MgCl_2$,

50 μM [^{14}C]AzaGua, 1 mM P-Rib-PP, 0.2 mg bovine serum albumin, and appropriate amounts of cell extract. At various times of incubation at 37°C, samples of reaction mixture were heated in a boiling water bath for 1 min and then clarified by centrifugation. AzaGua and AzaGua monophosphate in the supernate were separated by chromatography in solvent 9. For measuring Hyp phosphoribosylation, the final reaction mixture contained 50 mM sodium cacodylate, pH 6.2, 1 mM $MgCl_2$, 250 μM P-Rib-PP, [^{14}C]Hyp (1–80 μM), and cell extract equivalent to 1 μl cell water in a total volume of 200 μl. At various times of incubation at room temperature, 20-μl samples were spotted onto PEI cellulose sheets ("Polygram" CEL300 PEI, Brinkman Instruments, Inc, Westbury, NY). The sheets were dried and washed four times with water and the spots were cut out and counted for radioactivity. The velocities of phosphoribosylation were estimated from the linear portions of the progress curves.

Assay for Acid and Alkaline Phosphatase Activity

The final reaction mixtures contained 4 mM p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO), 40 mM citrate buffer, pH 4.8, or glycine buffer, pH 10.5, and appropriate amounts of unheated cell sap in a total volume of 0.6 ml. After 30 min of incubation at 37°C, the reaction mixtures were diluted tenfold with NaOH to yield a final concentration of 0.02 N and analyzed for absorbance at 410 nm for an estimate of the amounts of p-nitrophenol formed.

Assay of Total Nucleotidase Activity

The final reaction mixture contained 200 mM Tris-HCl (pH 7.4), 10 μM [^{3}H]UMP, 5 mM $MgCl_2$, and cell lysate from 1×10^7 cells/ml. At 1, 2.5, 10, and 20 min of incubation at 37°C, samples of the mixture were deproteinized and analyzed for radioactivity in UMP and uridine (uracil) by chromatography with solvent 28.

Quantitation of Ribonucleotides

Ribonucleotides were extracted from cells with 0.5 M perchloric acid and the extract neutralized by shaking with trioctylamine in freon, according to Khym [14]. Separation of ribonucleotides in the extracts was achieved on a 2.5 × 250-mm column of Whatman Partisil-10 SAX, eluted with a phosphate gradient as described by Webster and Whaun [15].

Quantitation of P-Rib-PP

About 1×10^8 cells were collected by centrifugation and were suspended in 1 ml of 1 mM EDTA (pH 7.0) at 100°C and the suspension was further heated in a boiling water bath for 1 min. The suspension was centrifuged and the supernate analyzed for P-Rib-PP by an enzymatic method using purified HPRT and 0.2 mM [^{14}C]Hyp as described previously [16].

Materials

Materials were purchased as follows: [2-^{14}C]AzaGua from ICN Pharmaceuticals, Inc. (Irvine, CA) and Research Products International Corp. (Elk Grove Village, IL); [2-^{3}H]Hyp and [8-^{14}C]Hyp from Moravek Biochemicals (Brea, CA); [8-C^{14}]Gua and L-[1-^{3}H]glucose from New England Nuclear (Boston, MA); [5-^{3}H]cytosine and [^{3}H]actinomycin D from Schwarz/Mann (Spring Valley, NY); 5,5-dimethyl[2-C^{14}]oxazolidine-2,4-dione ([^{14}C]DMO) from Amersham (Arlington Heights, IL);

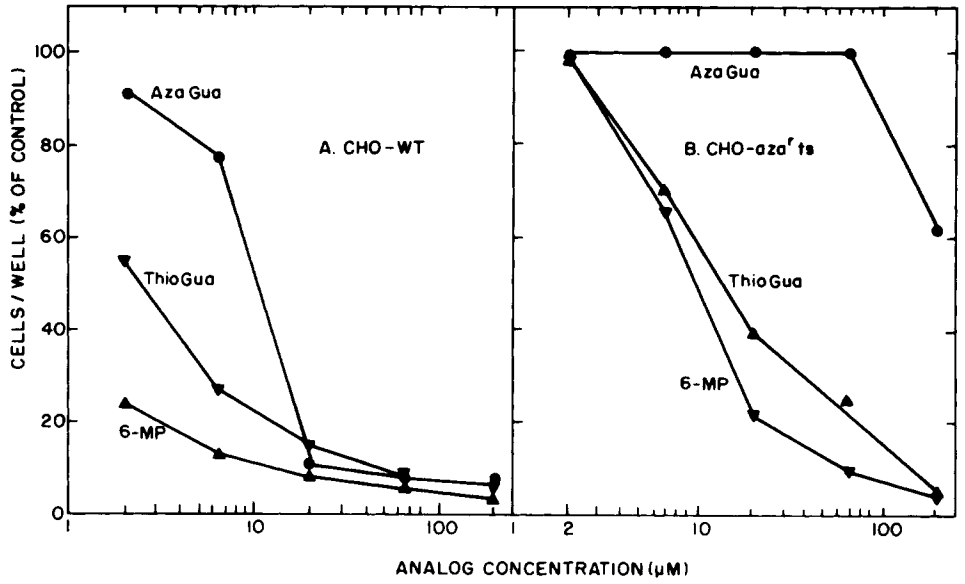

Fig. 1. Effect of AzaGua, ThioGua, and 6-MP on the growth of aza^{r}ts and wild-type CHO cells. Suspension cultures of both types of cells grown at 38°C were diluted with fresh growth medium to 2 × 10^4 cells/ml and seeded into 24-well Linbro culture plates, 1 ml/well. The wells were supplemented with the indicated concentrations of AzaGua and the plates incubated at 37–38°C for 4 days. Then the cells of each well were removed by treatment with EDTA-trypsin and enumerated in a Coulter counter. All values represent averages of duplicate wells. Wells not treated with AzaGua contained about 9 × 10^5 aza^{r}ts of wild-type cells (100%).

6-mercapto[8-^{14}C]purine from Research Products; and unlabeled nucleobases from Sigma Chemical Co.

RESULTS

Resistance of Cells to Purine Analogs

A comparison of the data in Figure 1A and B shows that the aza^{r}ts variant of CHO cells was 20- to 50-fold more resistant to AzaGua than wild-type CHO cells. These results are similar to those reported by Harris and Whitmore [3]. Similar results were obtained in repeated analyses and the aza^{r}ts variant retained its resistance during about 3 years of propagation in the absence of AzaGua. The aza^{r}ts cells were also significantly more resistant (about 10 times) to both 6-thioguanine (ThioGua) and 6-mercaptopurine (6-MP) than wild-type CHO cells (Fig. 1).

Uptake of AzaGua and Other Purine Analogs by Whole Cells

Figure 2 illustrates the slowness of uptake of AzaGua relative to that of Hyp by CHO cells, which is comparable to that previously reported for Novikoff cells [7]. The finding that the rate of AzaGua uptake by HPRT-deficient Novikoff cells was not affected by high concentrations of Hyp, but rather a function of its lipid solubility, indicated that entry of AzaGua into cells is nonmediated [7]. ThioGua and 6-MP, on the other hand, were found to be efficiently transported by the Hyp transporter [7].

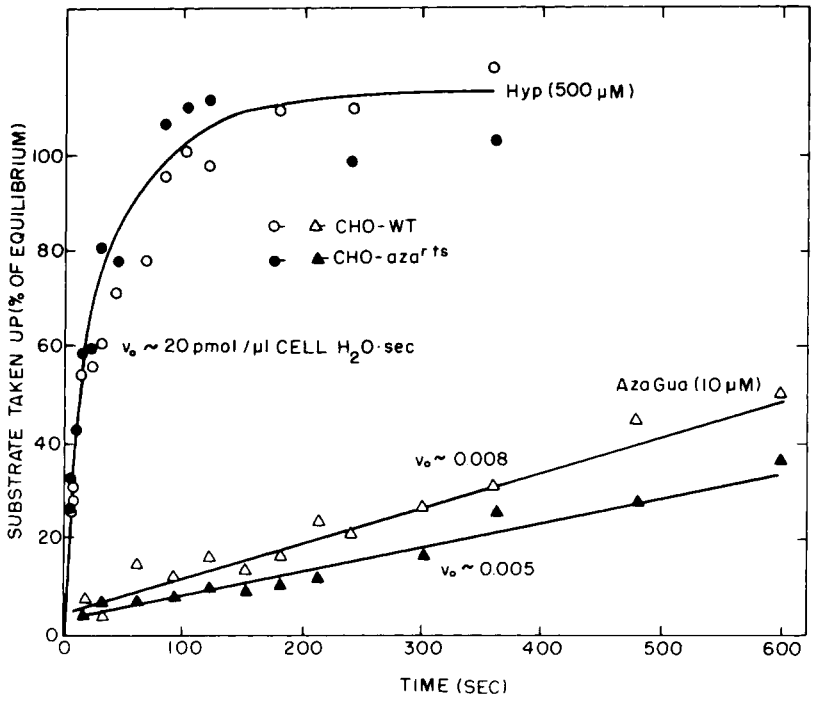

Fig. 2. Comparison of initial time courses of uptake of 500 μM Hyp and 10 μM AzaGua by aza^{r}ts and wild-type CHO cells. Samples of suspensions of both types of cells propagated at 38°C (about 1 × 10^7 cells/ml) were supplemented with 500 μM [^{3}H]Hyp (1 cpm/pmole) or 10 μM [^{14}C]AzaGua (13.5 cpm/pmole) and the uptake of labeled substrate was measured at 25°C by a rapid kinetic technique (see Materials and Methods). Uptake (intracellular radioactivity) is expressed as percent equilibrium with the extracellular concentration. v_o, Initial velocity of uptake in pmole/μl cell water · sec.

In the experiment illustrated in Figure 2, the initial rate of AzaGua uptake by aza^{r}ts cells was about 60% that exhibited by wild-type cells. In other experiments the differences in initial rate of AzaGua uptake between wild-type and aza^{r}ts cells was even less, indicating that AzaGua resistance was not due to reduced membrane permeability, which has been reported to be responsible for the resistance of CHO variants to various lipophilic drugs such as colchicine, actinomycin D, and adryamycin [17,18]. This conclusion is further supported by the finding that aza^{r}ts cells took up labeled cytosine and L-glucose, hydrophilic substances whose entry, like that of AzaGua, is believed to be nonmediated [7,19], at about the same rate as wild-type cells (data not shown).

The net uptake of AzaGua by aza^{r}ts cells, however, ceased after a few minutes of incubation of 37°C, whereas it continued throughout the 2-hr incubation period in wild-type cells (Fig. 3A). Furthermore, chromatographic analysis of cell-associated radiolabeled material showed that none of the AzaGua taken up by aza^{r}ts cells became phosphorylated, whereas >50% (up to 85% in other experiments) of the radioactivity in wild-type cells was associated with AzaGTP (Fig. 3B). This explains why little if any AzaGua was incorporated into acid-insoluble material in aza^{r}ts cells (Fig. 3A). In contrast, there was continuous AzaGua incorporation into acid-insoluble material in wild-type cells. Small amounts of AzaGua became converted to 8-azaguanosine (AzaGuo), but the amounts were only slightly higher in aza^{r}ts than wild-type cells (Fig. 3B).

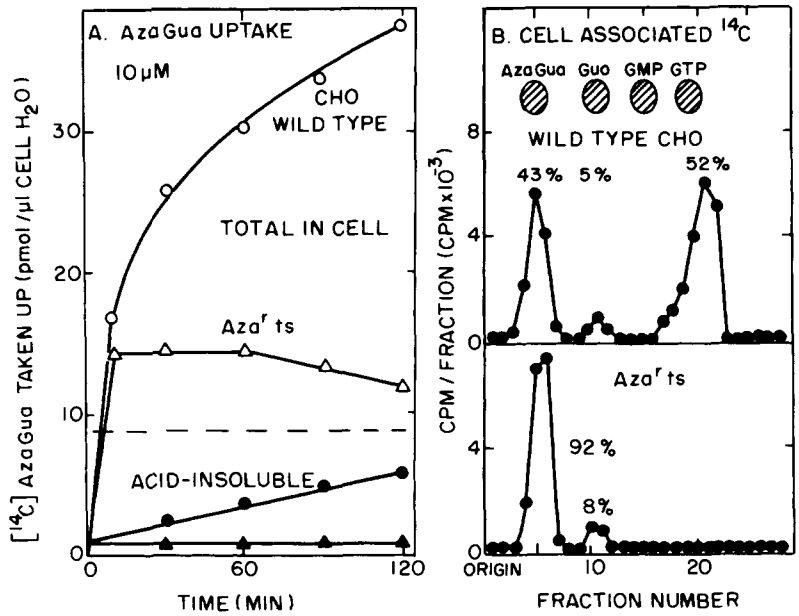

Fig. 3. Uptake of [^{14}C]AzaGua by wild-type and aza^{r}ts cells (A) and chromatographic analysis of cell-associated radioactivity (B). A) cells of both types propagated at 38°C were suspended to about 1.5 × 10^7 cells/ml in BM42B in which bicarbonate was replaced with 30 mM HEPES and which was adjusted to pH 6.0. Samples of the suspensions were supplemented with 10 μM [^{14}C]AzaGua (44 cpm/pmole). At various times of incubation at 38°C cells from 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. Radioactivity per cell pellet was corrected for that trapped in extracellular space. Other 0.5-ml samples of suspension were analyzed for radioactivity in acid-insoluble material. All values are averages of duplicate samples. The broken line indicates the intracellular concentration of substrate equivalent to that in the medium at 0 time. B) cell-associated radioactivity was extracted from 120-min labeled cells with trichloroacetic acid and the acid extracts were chromatographed with solvent 9.

The levels of unmodified AzaGua accumulating in aza^{r}ts cells varied with the extracellular pH (pH_e, data not shown). We have shown previously [7] that the plasma membrane is practically impermeable to ionized AzaGua (pK_a = 6.6 [20]), as is true for the ionized forms of other weak acids or bases [21, 22], because of their low lipid solubility. It follows that the intracellular steady-state level of unphosphorylated AzaGua decreases with an increase in pH_e [7]. The slight decrease in intracellular concentration of AzaGua in aza^{r}ts cells with time of incubation (Fig. 3A) reflected an increase in pH_e from about 6.2 to 6.6 during the course of the experiment.

We interpret the data in Figure 3 to indicate that the AzaGua resistance of aza^{r}ts cells is due to a lack of its phosphoribosylation. The following experiments were designed to inquire into reasons for the lack of phosphoribosylation of AzaGua in these cells. The most obvious reason could have been a lack of HPRT or a change in its kinetic properties but neither was detected.

Kinetics of Hyp Phosphoribosylation In Situ and in Cell-Free Extracts and of Hyp Transport

We compared the kinetics of Hyp phosphoribosylation by aza^{r}ts and wild-type cells both in situ and in cell-free extracts. We have demonstrated previously that when Novikoff cells are exposed to radiolabeled Hyp, an intracellular steady-state level of

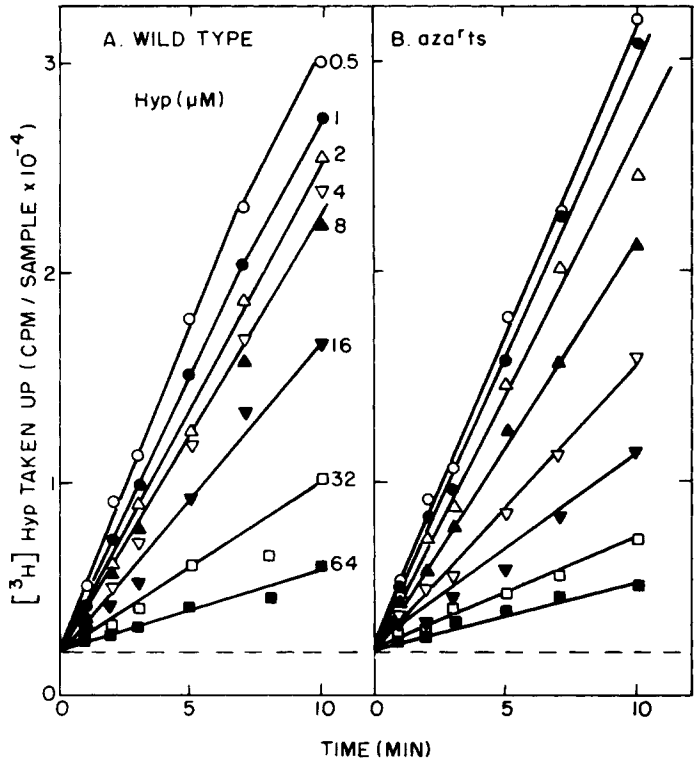

Fig. 4. Kinetics of in situ phosphoribosylation of Hyp by aza^{r}ts and wild-type CHO cells. Cells were suspended in BM42B to 1 × 10^7/ml. Samples of the suspensions were supplemented with the indicated concentrations of [^{3}H]Hyp (280 cpm/μl, irrespective of concentration) and incubated at 25°C. At the indicated times, the cells from 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. All values are averages of duplicate samples and are corrected for substrate trapped in extracellular space of cell pellets (1,100 cpm/sample). The broken lines indicate the intracellular concentration of radioactivity equivalent to that in the medium at 0 time. Velocities of ^{3}H uptake were estimated from the linear portions of the uptake curves and converted to pmole/μl cell water·sec (μM/sec) on the basis of experimentally determined cell water spaces. The Michaelis-Menten equation was fitted to the data and the best-fitting parameters are summarized in Table I (first line).

unmodified Hyp is attained within less than 1 min due to rapid influx and that the subsequent time course of intracellular accumulation of radioactivity reflects the conversion of Hyp to nucleotides [4,5]. The same held for both wild-type and aza^{r}ts CHO cells, and we have utilized this fact for estimating the rates of the in situ phosphoribosylation of Hyp (Fig. 4). In the experiment shown, the concentration of [^{3}H]Hyp was held constant while the absolute concentration was adjusted with unlabeled Hyp. Thus, the decrease in rate of uptake of ^{3}H reflected saturation of the uptake system, that is, of phosphoribosylation.

The Michaelis-Menten equation was fitted to the rates estimated from the data in Figure 4 and other similar experiments and the best-fitting parameters are summarized in Table I. The apparent maximum velocities were consistently somewhat lower for aza^{r}ts than wild-type cells whether grown at 33°C or 39°C. This finding is in agreement with the results of Harris and Whitmore [3], but, contrary to their results and those of Prasad et al [6], we find that the K_m values for wild-type and aza^{r}ts cells did not differ significantly whether the cells were propagated at 33–34°C or 38–39°C

TABLE I. Kinetic Parameters for the In Situ Phosphoribosylation of Hyp by Wild-Type and aza^{r}ts CHO Cells*

Growth temp (°C)	Test temp (°C)	Wild-type		aza^{r}ts	
		K_m (μM)	V_{max} (μM/sec)	K_m (μM)	V_{max} (μM/sec)
33–34	25	7.4 ± 1.7	0.22 ± 0.02	5.8 ± 0.2	0.15 ± 0.02
38–39	25	7.6 ± 3.0	0.20 ± 0.03	7.7 ± 1.0	0.18 ± 0.01
38–39	38	10.2 ± 1.6	0.25 ± 0.01	13.7 ± 3.6	0.23 ± 0.02

*The kinetic parameters ± SE of estimate listed in the first line pertain to the data illustrated in Figure 2. The other values were obtained in the same manner as described for this data set in the legend to Figure 2 except that, where indicated, the cells had bee propagated at 38–39°C rather than 33–34°C and uptake was measured at 38°C. To facilitate comparison to transport data, V_{max} was expressed as pmol of Hyp converted to nucleotides per μl cell water·sec (= μM/sec). One microliter cell H_2O is equivalent to about 7×10^5 cells.

TABLE II. Kinetic Parameters for the Phosphoribosylation of AzaGua and Hyp by Cell-Free Extracts of aza^{r}ts and Wild-Type CHO Cells*

Substrate	Growth temp (°C)	Wild-type		aza^{r}ts	
		K_m (μM)	V_{max} (μM/sec)	K_m (μM)	V_{max} (μM/sec)
Azagua	33	11.3 ± 3.7[a]	4.6 ± 0.5	10.4 ± 3.9	2.3 ± 0.4
	38	5.5 ± 1.2	3.2 ± 0.2	10.9 ± 2.2	2.0 ± 1.1
Hyp	38	22.5 ± 7.2	1.4 ± 0.3	11.1 ± 2.8	1.3 ± 0.1

*Cell extracts were prepared from cells propagated at 33°C or 38°C and assayed for HPRT activity with AzaGua or Hyp as substrate at room temperature as described in Materials and Methods. To facilitate comparison to transport data, V_{max} was expressed as pmol substrate phosphoribosylated per μl cell water·sec (= μM/sec).
[a]SE of estimate.

and tested at 25°C or 38°C. Similar results were obtained for the uptake of [^{14}C]Gua (data not shown). That the HPRT of aza^{r}ts cells per se is functionally unaltered with Hyp as substrate is indicated by the finding that the kinetics of phosphoribosylation of Hyp were also about the same in cell extracts from wild-type and aza^{r}ts cells grown at 38°C (Table II).

The kinetics of Hyp transport were also about the same for wild-type and aza^{r}ts cells. Figure 2 showed typical time courses of uptake of 500 μM Hyp at 25°C and illustrated the similar rapidity of Hyp accumulation by both types of cells; the $t_{1/2}$ for transmembrane equilibration was about 15 sec. At this concentration of Hyp the amount of Hyp phosphoribosylated during the 360-sec experimental period was insignificant (data not shown). The Michaelis-Menten constants and maximum velocities for Hyp transport determined in independent experiments were 2,393 ± 221 and 2,716 ± 327 μM, and 41.2 ± 1.8 and 37.2 ± 2.0 pmol/μl cell water·sec for aza^{r}ts and wild-type CHO cells, respectively.

Kinetics of AzaGua Phosphoribosylation in Cell-Free Extracts

The failure of AzaGua to be phosphoribosylated in intact aza^{r}ts cells, on the other hand, suggested that the substrate specificity of HPRT might be altered in these cells. However, this was not the case. The kinetics of phosphoribosylation of AzaGua

by cell extracts of wild-type and aza^{r}ts cells at pH 6.2 were about the same whether the cells were propagated at 33°C or 38°C (Table II). Because nonionized AzaGua is the substrate for HPRT [20], the rate of its phosphoribosylation decreased progressively with an increase in pH above 6.5, but the decrease was about the same with extracts from wild-type and mutant cells (data not shown).

The results show that the lack of phosphoribosylation of AzaGua in whole aza^{r}ts cells is not due to a lack of HPRT per se or to alterations in its affinity for nonionized AzaGua. We also ruled out the possibility that it results from a difference in intracellular pH regulation in the two types of cells. We have estimated pH_i of the cells as a function of pH_e by determining the intracellular-extracellular distribution of the weak acid DMO at steady state [23]. As previously observed for Novikoff [23], pH_i increased progressively from about 7.0 to 8.0 with an increase in pH_e from 6.0 to 8.0 (data not shown). The relationship between pH_i and pH_e was the same for wild-type and aza^{r}ts cells.

Intracellular Concentrations of P-Rib-PP

The kinetic behavior of HPRT in situ may be influenced by numerous factors in addition to structural changes in the enzyme itself; the cytoplasmic concentration of P-Rib-PP is prominent among these [14]. That the in situ kinetics of Hyp phosphoribosylation are similar in aza^{r}ts and wild-type cells supports the conclusion that none of these possible factors is crucially different in the variant cells propagated at either temperature. In agreement with this conclusion, we found the P-Rib-PP levels of aza^{r}ts cells and wild-type cells very comparable, varying between 15 and 90 μM, higher concentrations being present in cultures progressing into stationary phase (data not shown), similar to the situation observed for Novikoff cells [16].

Degradation of AzaGua or AzaGMP

The resistance of cells to AzaGua has been attributed in some cases to rapid degradation of AzaGua or of AzaGMP due to high levels of guanine deaminase and 5′-nucleotidase or alkaline phosphatase activities, respectively [1,24–27], although in the case of alkaline phosphatase changes in activity in some cell systems have been found to be unrelated to drug resistance [28,29]. Such enzyme activities result in the formation of 8-azaxanthine and AzaGuo. We have not detected any accumulation of significant amounts of 8-azaxanthine and only small amounts of AzaGuo in the culture fluid of suspensions of 1×10^7 wild-type or aza^{r}ts cells per ml of basal medium during 2 hr of incubation at 37°C with 10 μM [^{14}C]AzaGua. Over 90% of the remaining radioactivity, which made up at least 80% of that added to the cultures, still comigrated with authentic AzaGua in solvents 9 or 28 or in HPLC.

We also did not detect any 8-azaxanthine inside cells incubated with [^{14}C]AzaGua for 2 hr (Fig. 3B; azaxanthine is recovered in fractions 2 and 3). As discussed already, small amounts of AzaGuo were present intracellularly but in similar amounts in both aza^{r}ts and wild-type cells. The results from in vitro assays suggest that this AzaGuo resulted from direct conversion of AzaGua by purine nucleoside phosphorylase rather than from degradation of AzaGMP. In the presence of P-Rib-PP, AzaGua was converted to AzaGMP in cell lysates of both types of cells without significant formation of AzaGuo (Fig. 5B). Upon a 1-hr incubation of completed reactions (>90% conversion to AzaGMP) at 37°C, no significant decrease in labeled AzaGMP was observed (data not shown). Substitution of ribose-1-phosphate for P-Rib-PP in

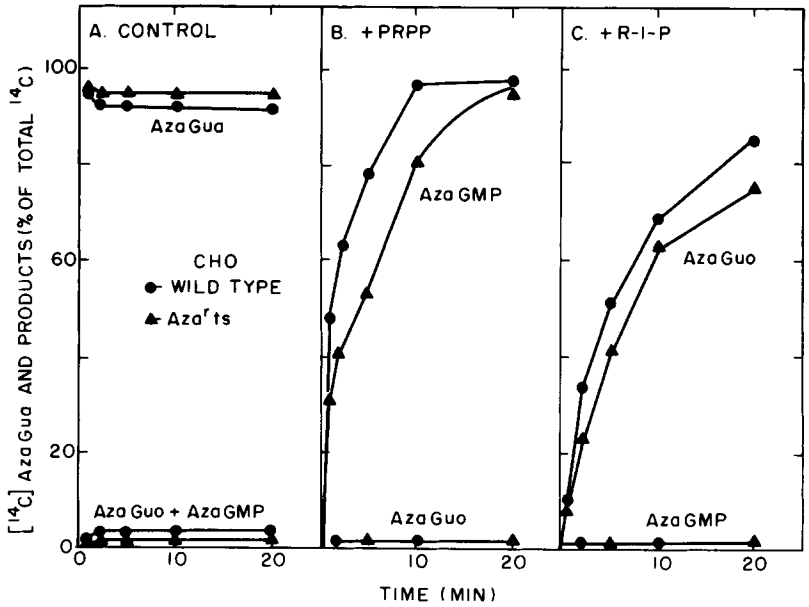

Fig. 5. Conversions of [^{14}C]AzaGua in cell-free lysates of aza^{r}ts and wild-type CHO cells. The phosphoribosylation of [^{14}C]AzaGua (B) was measured at 37°C as described in Materials and Methods. The reactions in A and C were the same, except that the 1 mM P-Rib-PP was omitted or replaced by 1 mM ribose-1-phosphate, respectively. The final reaction mixtures all contained 80 mM phosphate buffer, pH 6.1, 5 mM $MgCl_2$, 50 μM [^{14}C]AzaGua, 0.2 mg bovine serum albumin/ml, and cell lysate from 2 $\times$ 10^7 cells/ml. AzaGua and its products were separated by chromatography with solvent 9.

the reaction, on the other hand, resulted in a substantial formation of AzaGuo in lysates of both aza^{r}ts and wild-type cells (Fig. 5C). In the absence of ribose-1-phosphate or P-Rib-PP, AzaGua was highly stable during incubation at 37°C in lysates of both types of cells (Fig. 5A). Similar results were obtained whether the pH of the reaction was 6.1 (Fig. 5) or 7.4 (data not shown).

In addition, both alkaline and acid phosphatase activities of wild-type and aza^{r}ts cells were about the same: 0.15 $\pm$ 0.01 and 0.12 $\pm$ 0.02, and 33 $\pm$ 0.4 and 33 $\pm$ 0.4 nmol/10^6 cells·min, respectively (values are means of at least three analyses $\pm$ SEM). The same was true for total nucleotidase activity of wild-type and aza^{r}ts cells as measured by the degradation of 10 μM [^{3}H]UMP in whole cell lysates (about 140 pmol/10^7 cells·min). The nucleotide content of the cells also did not differ significantly and consistently (ATP = 2.5–4.3 mM; ADP = 170–210 μM; AMP = 540–550 μM; GTP = 320–470 μM; UTP = 650–700 μM; CTP = 220–260 μM).

DISCUSSION

We have confirmed the high resistance of aza^{r}ts CHO cells to AzaGua reported by Harris and Whitmore [3]. Our results indicate that resistance is not due to a defect in Hyp-Gua transport as initially postulated [3,6] but rather to a lack of accumulation of AzaGua nucleotides in the aza^{r}ts variant. However, the reasons for this block in aza^{r}ts cells are obscure. Our results show that it is not due to a decreased permeability of the plasma membrane to AzaGua or a lack of HPRT. Nor does it result from an

altered affinity of HPRT in these cells for natural purines or AzaGua. Also, the pH dependence of the AzaGua phosphoribosylation in vitro by HPRT is unaltered. In addition, lack of AzaGua nucleotide accumulation in aza^rts cells is not due to deamination of AzaGua or rapid degradation of AzaGMP once it is formed. Thus we conclude that the AzaGua resistance of aza^rts cells is due to a block in AzaGua phosphoribosylation per se that prevents its incorporation into nucleic acids. The aza^rts variant differs from other guanine-analog–resistant variants of a number of cell lines that incorporate Gua or Hyp into nucleic acids at a reduced rate in spite of possessing normal levels of HPRT activity [1,2,30]. In the case of the aza^rts variant the block is specific for AzaGua phosphoribosylation and, in addition, is apparent only in whole cells; phosphoribosylation of AzaGua in cell-free lysates is normal. This discrepancy between in situ and in vitro results is not due to differences in intracellular P-Rib-PP, nucleotide levels, or intracellular pH in wild-type and mutant cells.

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REFERENCES

1. Thompson LH, Baker RM: Meth Cell Biol 6:209, 1973.
2. Gillin FD, Roufa DJ, Beaudet AL, Caskey CJ: Genetics 72:239, 1972.
3. Harris JF, Whitmore GF: J Cell Physiol 83:43, 1974.
4. Marz R, Wohlhueter RM, Plagemann PGW: J Biol Chem 254:2329, 1979.
5. Plagemann PGW, Wohlhueter RM: Curr Top Membr Transp 14:225, 1980.
6. Prasad RD, Shopsis G, Hochstadt J: Biochim Biophys Acta 643: 306, 1981.
7. Plagemann PGW, Marz R, Wohlhueter RM, Graff JC, Zylka JM: Biochim Biophys Acta 647:49, 1981.
8. Plagemann PGW, Swim HE: J Bacteriol 91:2317, 1966.
9. Schneider EL, Stanbridge EF, Epstein CJ: Exp Cell Res 84:311, 1974.
10. Plagemann PGW: J Cell Physiol 77:213, 1971.
11. Wohlhueter RM, Marz R, Graff JC, Plagemann PGW: Meth Cell Biol 20:211, 1978.
12. Plagemann PGW, Wohlhueter RM: Biochim Biophys Acta 688:505, 1982.
13. Kong CM, Parks ER Jr: Mol Pharmacol 10:648, 1974.
14. Khym JY: Clin Chem 21:1245, 1975.
15. Webster HK, Whaun JM: J Chromatog 209:283, 1981.
16. Wohlhueter RM, Godfrey J, Plagemann PGW: J Biol Chem 257: 12691, 1982.
17. Bech-Hansen NT, Till JE, Ling V: J Cell Physiol 88:23, 1976.
18. Debenham PG, Kartner N, Siminovitch L, Riordan JR, Ling V: Mol Cell Biol 2:881, 1982.
19. Graff JC, Wohlhueter RM, Plagemann PGW: J Biol Chem 252:4185, 1977.
20. Parks RE Jr, Agarwal KC: In Sartorelli AC, Johns DG, (eds) "Antineoplastic and Immunosuppressive Agents." 1975, part 2, Springer-Verlag, New York-Heidelberg-Berlin p 458.
21. Cohen RD, Isles RA: Crit Rev Clin Lab Sci 6:101, 1975.
22. Waddel NJ, Bates RG: Physiol Rev 49:285, 1979.
23. Wohlhueter RM, Plagemann PGW: J Biol Chem 256:869, 1981.
24. Van Diggelen OP, Donahue TF, Shin SI: J Cell Physiol 98:59, 1979.
25. Berman JJ, Tong C, Williams GM: In Vitro 16:661, 1980.
26. Meyers MB, Shin S: Cytogenet Cell Genet 30:118, 1981.
27. Wolpert JK, Damle SD, Brown JE, Szyncer E, Agarwal KC, Sartorelli AC: Cancer Res, 31:1620, 1971.
28. Lee SH, Sartorelli AC: Biochem Pharmacol 30:3109, 1981.
29. Nikaido O, Fox M: Mutat Res 35:279, 1976.
30. Brockman RW: In Sartorelli AC, Johns DG (eds): "Antineoplastic and Immunosuppressive Agents." 1974, part 1, Springer-Verlag: New York, Heidelberg-Berlin, p 352.