8-AZAGUANINE RESISTANCE IN MAMMALIAN CELLS
I. HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE

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ABSTRACT

Chinese hamster cells were treated with ethyl methanesulfonate or N-methyl-N'-nitro-N-nitrosoguanidine, and mutants resistant to 8-azaguanine were selected and characterized. Hypoxanthine-guanine phosphoribosyltransferase activity of sixteen mutants is extremely negative, making them suitable for reversion to HGPRTase+. Ten of the extremely negative mutants revert at a frequency higher than 10^{-7} suggesting their point mutational character. The remaining mutants have demonstrable HGPRTase activity and are not useful for reversion analysis. Five of these mutants have < 2% HGPRTase and are presumably also HGPRTase point mutants. The remaining 14 mutants utilize exogenous hypoxanthine for nucleic acid synthesis poorly, and possess 20–150% of wild-type HGPRTase activity in *in vitro*. Their mechanism of 8-azaguanine resistance is not yet defined.

IN order to examine the biochemical alterations of mutations in cultured animal cells, we have induced and isolated a number of Chinese hamster cell mutants affecting hypoxanthine-guanine phosphoribosyl transferase (HGPRTase; E.C. 2.4.2.8). The gene for this enzyme is X-linked in man (SEEGMILLER, ROSENBLOOM and KELLEY 1967; HENDERSON *et al.* 1969), but its chromosomal location is not established in Chinese hamster cells. This gene appears suitable for a detailed analysis of point mutation in animal cells similar to that performed with bacterial tryptophan synthetase (YANOFSKY, ITO and HORN 1966) and yeast iso-1-cytochrome C (SHERMAN *et al.* 1970).

Using phosphoribosylpyrophosphate (PRPP), HGPRTase catalyzes the synthesis of 5′ mononucleotides from hypoxanthine, guanine, or certain guanine analogues (8-azaguanine, 6-thioguanine, 6-mercaptopurine and others) (KRENITSKY, PAPAIOANNOU and ELION 1969). Many of these guanine analogues are toxic to mammalian cells. Furthermore, HGPRTase activity is required for cell growth in media containing hypoxanthine, aminopterin, and thymidine (SZYBALSKI, SZYBALSKA and RAGNI 1962). Since aminopterin blocks *de novo* purine biosynthesis, cells must utilize the hypoxanthine in this medium as their source of purines. Thus, culture media are available for selecting mammalian cells which are HGPRTase$^-$ and for counterselecting HGPRTase+ revertants.

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An established cell line (V-79) derived from the lung of a male fetal Chinese hamster (FORD and YERGANIAN 1958) has been used in these studies. This cell line has a stable karyotype (2n = 21), grows rapidly (10 hr generation time), and is cloned with high efficiency (60–90%). Although others have isolated 8-azaguanine (8-AG)-resistant animal cells (LITTLEFIELD 1963; SUBAK-SHARPE 1965; CHU and MALLING 1968; HARRIS 1971; and MORROW 1969) we report here extensive characterization of 35 Chinese hamster cell mutants resistant to 8-AG, isolated for the purpose of characterizing mechanisms of mutation and reversion in animal cells.

METHODS

Growth and storage of Chinese hamster cells: The male Chinese hamster lung cells used in these studies were originally provided by Dr. E. CHU. All mutants were derived from a subclone of V-79 designated A-3. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum in the absence of antibiotics. All cells were stored in ampules containing 5×10^6 to 10^7 cells in 50% DMEM with 10% fetal calf serum and 50% cryoprotective media (Microbiological Assoc., 15% DMSO) in liquid nitrogen. All cell lines are routinely checked for mycoplasma contamination by the methods of HAYFLICK (1965) and are discarded if found defective. None reported carry detectible mycoplasm.

Selection of mutants: Azaguanine-resistant Chinese hamster mutants were isolated by modifications of the methods described by CHU and MALLING (1968). Monolayers (70–90% confluent) of A-3 grown in 250 ml flasks were exposed to either 10^{-5} M N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 10^{-2} M ethyl methanesulfonate (EMS) in Earle's balanced salt solution for two hours at 37°C in a 10% CO_2 atmosphere. The mutagen solution was removed and the monolayers were rinsed with 12 ml of Earle's salts. Monolayers were dispersed to single cells with a 0.05% buffered trypsin solution containing 10^{-4} M EDTA, cell counts were determined with a hemacytometer, and cells were distributed in DMEM at densities of 10^4–10^5 cells per 60 mm Petri plates. After 48 hr of growth, the medium was removed and DMEM containing 30 μg/ml 8-AG was added. Mutant cell colonies were apparent after 7–10 days in selective medium. Mutant cell colonies were recloned in microtiter plates (COOPER 1970). No more than a single clone was isolated from a selection plate. Clones from microtiter plates were taken from wells with a single focus of growth. Microtiter plates with > 30% of their wells developing colonies were discarded to further assure an effective recloning.

Selection of revertants of HGPRTase$^-$ clones was performed as also described by CHU and MALLING (1968). Azaguanine resistant mutants were maintained in 8-AG until exposure to the chemical mutagens described above. Monolayers were trypsinized and distributed at cell densities of 10^3–10^6 to 100 mm Petri plates in DMEM. Following 48 hr of growth in DMEM, revertant selection was initiated in medium containing 10^{-4} M hypoxanthine, 10^{-5} M aminopterin and 10^{-5} M thymidine (HAT medium). The medium was changed every 3–4 days. Revertant colonies were apparent by 10–14 days. Revertant colonies were isolated, recloned in HAT medium, and stored as described above. Routinely all mutants and revertants are cultured in the presence of appropriate selective agents.

The calculation of mutation frequency is complicated by colony splitting which occurs with Chinese hamster cells. This splitting increases the apparent mutational frequency if calculated by the number of mutant colonies divided by the total number of cells. For this reason we estimated mutational frequencies by the method of LURIA and DELBRÜCK (1943). This method assumes a Poisson distribution of mutants on a series of selection plates. If the cell number per plate is chosen properly, the mutational frequency can be accurately calculated on the basis of the fraction of plates which do *not* possess mutant colonies. This method does not utilize the observed number of mutant colonies per plate and therefore avoids the error introduced by colony splitting. Since cell density can also affect the observed mutational frequency, all studies were performed at cell concentrations which yield the highest mutation frequencies.

Chinese hamster cell extract preparation: Cells were grown in their respective media on 150 mm Petri plates to 70–80% confluence, rinsed with Earle's salt solution, and scraped from the plates in 1.5 ml 0.05 M phosphate buffer pH 7.0 containing 10^{-2} M β-mercaptoethanol. Cell suspensions were frozen and thawed three times, centrifuged at 30,000 $\times$ *g* for 15 min, and supernatants were removed for storage in liquid nitrogen. These extracts were used for all enzyme and protein determinations. Extract protein concentrations ranged from 1 to 2.0 mg/ml as determined by the method of LOWRY (LOWRY *et al.* 1951).

Hypoxanthine-guanine phosphoribosyltransferase assay: Each reaction was incubated at 37°C and contains in 0.05 ml; phosphoribosylpyrophosphate (PRPP) 10^{-3} M; (^{14}C) hypoxanthine, 10^{-4} M; magnesium chloride, 5×10^{-3} M; Tris-HCl, 5×10^{-2} M, pH 7.4; and 1–50 μg extract protein. Reactions were terminated by dilution with 1.0 ml cold buffer containing Tris-Cl 10^{-3} M pH 7.0; potassium chloride 10^{-3} M; and EDTA 1.5×10^{-3} M. Reactions which contained EDTA from zero time were terminated with the above buffer without EDTA. Since crude extracts often contain PRPP, the HGPRTase activity reported here is the difference between IMP formed in the above reactions compared to reactions containing 3×10^{-2} M EDTA from zero time. Partially purified HGPRTase has little endogenous PRPP (see Table 2). Adenine phosphoribosyltransferase activity has been determined under identical conditions except that (^{14}C) adenine, 10^{-4} M, was substituted for (^{14}C) hypoxanthine.

The method used to assay (^{14}C)IMP and (^{14}C)AMP formed was a modification of that described by ATKINSON and MURRAY (1965). Reactions were applied to 25 mm diameter DEAE filter discs (Whatman DE-81) held in a Millipore sampling manifold; reaction tubes were rinsed twice with 1.0 ml of 10^{-3} M TrisCl, pH 7.0, containing 10^{-3} M potassium chloride, and filters were washed by two 10 ml aliquots of the same buffer. The filters were dried under an infrared lamp, and then radioactivity was determined by scintillation counting of the dried filter in toluene with POPOP-PPO. The counting efficiencies for (^{14}C) and (^{3}H) isotopes on DEAE filter discs were 73% and 6% respectively.

Product identification was performed by electrophoretic analysis of the radioactive product. Known purine nucleotide markers were used for comparison. The radioactivity retained on the DE-81 filters was eluted by triethylamine bicarbonate buffer, lyophilyzed, and subsequently electrophoresed on Whatman 3 mm paper in sodium borate 0.05 M, pH 9.0, containing EDTA 0.05 M, 30 min at 1500 volts and 250 ma. The radioactivity recorded had an electrophoretic migration identical to the 5′ mononucleotide of the radioactive base added to the enzyme reactions. No radioactive nucleosides or free bases were recovered from the DE-81 filter.

Cellular incorporation of radioactive adenine and hypoxanthine: Incorporation of radioactive purines into nucleic acid was determined with cells grown in 60 mm Petri plates in DMEM. At zero time the medium was replaced with 2.0 ml DMEM containing aminopterin 10^{-5} M and (^{14}C) hypoxanthine 10^{-5} M. Simultaneous adenine and hypoxanthine incorporation utilized DMEM containing (^{3}H) hypoxanthine 10^{-7} M, (^{14}C) adenine 10^{-6} M, and where indicated aminopterin 10^{-5} M. All plates were incubated at 37°C in a 10% CO_2 atmosphere for times up to 6 hrs. Following the incubation period, radioactive medium was removed. Each plate was rinsed with 4.0 ml of 0.9% sodium chloride, and cells lysed by addition of 1.0 ml of 1% SDS. Plates were then rinsed twice with 1.0 ml of deionized water. SDS and deionized water rinses were pooled (3.0 ml), and nucleic acids precipitated by addition of 3.0 ml 10% TCA at 4°C. Radioactive nucleic acid precipitates were recovered on Millipore filters and quantitated by scintillation counting.

MATERIALS

The powdered DMEM and fetal calf serum were obtained from Grand Island Biological Company. Tap water was processed via Hydro Services Ultra Pure Water System, Model C Type 18. The two mutagens, MNNG and EMS, were supplied by Aldrich and Eastman Chemical Companies respectively. Thymidine, 8-azaguanine, 6-thioguanine, and 8-azahypoxanthine were supplied by Sigma. Adenine, guanine, and hypoxanthine were supplied by Pabst Laboratories. The (^{14}C) adenine (40 mC/mM), (^{14}C) hypoxanthine (50 mC/mM) ,and (^{3}H) hypoxanthine (12 C/mM) were purchased from Schwarz/Mann. Disposable sterile tissue culture plates and

flasks were supplied by Microbiological Associates and Falcon. Microtiter plates were supplied by Linbro and Falcon.

RESULTS

We have selected Chinese hamster cell mutants which are resistant to 30 μg/ml 8-AG in an effort to obtain lines with altered HGPRTase cistrons. Two base changing mutagens, MNNG and EMS, have been employed to enhance mutational frequency (FREESE and FREESE 1966). Under the mutagenic conditions employed 22 and 95% of the cells survived treatment with MNNG and EMS respectively. The spontaneous mutation frequency to 8-AG resistance (10^{-5}) was enhanced approximately 70 fold (MNNG, 6.8×10^{-4}; EMS, 6.9×10^{-4}) by mutagenesis. Thus, most mutants cloned from cultures treated in the above manner have arisen as a result of EMS or MNNG and have been designated by E or N preceding the clone number. These observations are in substantial agreement with those reported earlier by CHU and MALLING (1968).

Although all mutants are resistant to 30 μg/ml of 8-azaguanine, they differ phenotypically in their ability to grow in media containing 6-thioguanine or HAT components. Thioguanine requires HGPRTase activity for cellular toxicity and kills cells at lower concentrations than 8-AG (SUBAK-SHARPE 1965). Growth of cells in HAT medium requires HGPRTase activity for utilization of exogenous hypoxanthine. Thus growth in these media provided additional criteria for the absence or presence of HGPRTase in each mutant cell line. These growth characteristics have been assessed by the growth of visible colonies in 7 days from single cells in the various media. The wild-type cell, A-3, is sensitive to both 8-AG and 6-thioguanine but grows normally in HAT (Table 1). Seventeen mutants differ from A-3 since they grow in both guanine analogues but not in HAT. These growth characteristics are in agreement with the expected drug sensitivities of cells which lack HGPRTase activity. Twelve mutants grow in all three selective media and therefore differ phenotypically both from the first class of mutants and from A-3. The mechanism(s) of their analogue resistance is not yet clear. Six additional mutants grow in HAT but not in 6-thioguanine, suggesting they possess a low level of HGPRTase activity.

The HGPRTase activity of all mutants has been directly quantitated *in vitro*. The characteristics of the assay and Chinese hamster HGPRTase are given in Table 2. Highly purified HGPRTase (BEAUDET, manuscript in preparation) recognizes hypoxanthine and guanine but not adenine, requires Mg^{++} (not shown), and is inhibited totally by EDTA. The synthesis of (^{14}C)IMP from (^{14}C) hypoxanthine is inhibited by addition of non-radioactive guanine or 6-thioguanine; but not by addition of 8-AG, 8-azahypoxanthine, or adenine at 3 times and 10 times (not shown) the level of (^{14}C) hypoxanthine. The substrate specificity of Chinese hamster HGPRTase is similar to that described for human HGPRTase (KRENITSKY, PAPAIOANNOU and ELION 1969). We have found this assay suitable for determination of specific activity (Figure 1A) of unfractionated Chinese hamster cell extracts. Crude extracts, as used in the assay, neither degrade (^{14}C) IMP or (^{14}C) AMP nor reduce the measurable HGPRTase purified from

TABLE 1

Chinese hamster 8-azaguanine-resistant mutants

Clones	Colonies formed in selective media			HGPRTase activity	(^{14}C) Hypoxanthine incorporation
	8-Azaguanine	6-Thioguanine	HAT		
A-3	0	0	168	2.21	54.2
N-1	111	116	0	0.00	0.6
N-5	119	114	0	0.01	0.1
N-6	142	160	0	0.00	0.6
N-10	106	98	0	0.00	0.8
N-14	122	126	0	0.00	0.1
N-15	127	128	0	0.00	0.4
N-17	78	89	0	0.00	0.3
N-19	148	131	0	0.00	0.3
N-20	102	105	0	0.00	0.7
E-31	91	85	0	0.00	0.3
E-33	300	300	0	0.00	0.3
E-36	300	300	0	0.00	0.2
E-37	94	44	0	0.00	0.2
E-43	188	122	0	0.00	0.4
E-26	32	42	0	0.01	0.1
E-27	133	144	0	0.02	0.2
N-22	35	34	0	0.02	7.5
N-2	134	120	106	0.01	54.0
N-3	112	103	103	0.64	33.0
N-7	100	74	41	0.68	32.0
N-8	76	66	63	0.64	3.9
N-11	100	70	80	0.38	11.0
N-12	39	45	29	0.73	16.2
E-28	46	32	42	0.00	8.1
E-29	178	127	196	0.76	7.0
E-39	300	300	300	0.02	1.0
E-44	300	300	300	3.44	5.0
E-47	185	71	142	2.30	16.1
E-49	171	93	110	0.54	5.4
N-9	27	0	40	2.48	34.0
N-13	108	6	106	0.37	16.2
N-16	70	0	89	0.47	34.0
N-18	139	0	68	0.02	8.6
N-21	108	0	58	0.07	10.2
N-24	66	0	96	0.57	5.4

The specific activities of (^{14}C) IMP formation (nmoles IMP formed/min/mg protein) are determined under assay conditions with unfractionated cell extracts as described in METHODS. The specific activity for (^{14}C) hypoxanthine incorporation in culture is equal to (^{14}C) Hx cpm $\times$ 10^{-3}/hr/mg protein when the specific activity of (^{14}C) Hx is 50 mc/mmol and incorporation is determined after 6 hr in medium containing 10^{-5} M (^{14}C) hypoxanthine and 10^{-5} M aminopterin. Growth in selective media is evaluated after 7–10 days by hematoxylin staining of duplicated plates and subsequent colony counting. Concentration of 8-azaguanine is 10^{-4} M, and 6-thioguanine 10^{-5} M, in the indicated selective media.

wild-type cells. There is no detectible conversion of IMP to inosine under these assay conditions. The rate of (^{14}C) IMP synthesis is constant for at least 40 min when less than 40 μg of extract protein is added.

TABLE 2

Chinese hamster hypoxanthine-guanine phosphoribosyl transferase

Components	(^{14}C) IMP
Study 1	
Complete	727
—PRPP	6
—Enzyme	7
Complete + EDTA	0
Study 2	
Complete	1168
+Guanine	358
+6-Thioguanine	214
+8-Azaguanine	1171
+8-Azahypoxanthine	1113
+Adenine	1169

Study 1: Each reaction is incubated for 40 min at 37°C and contains in 0.05 ml 6.5 μg purified A-3 HGPRTase and, where indicated, 3×10^{-2} M EDTA; as well as the other components described in METHODS. A zero time value (50.0 pMoles) is subtracted from all values. Study 2: Each reaction contained in 0.05 ml 24 μg of A-3 extract, 0.08 mM (^{14}C) hypoxanthine, other components described in METHODS, and as indicated, the following purine base competitors: guanine, 0.14 mM; 6-thioguanine, 0.22 mM; 8-azaguanine, 0.17 mM; 8-azahypoxanthine, 0.18 mM; and adenine, 0.15 mM. Reactions were incubated at 37°C for 20 min, and a zero time (EDTA) value of 15 pMoles is subtracted from all values.

The HGPRTase activity of each 8-AG-resistant mutant has been determined from crude cell extracts. Specific activities were determined at limiting extract concentrations with 2×10^{-4} M (^{14}C) hypoxanthine and 10^{-3} M PRPP. These concentrations exceed their KM's by a minimum of 10 fold. The spectrum of HGPRTase activity for these mutants is indicated in Figure 2, and listed for each mutant in Table 1. Many MNNG- and EMS-induced 8-AG-resistant clones have significant activity. Furthermore, examination of the HGPRTase activity extracted from additional mutants selected by resistance to 6-thioguanine (10^{-5} M) and to a combination of 6-thioguanine and 8-AG yielded a similar distribution of HGPRTase activity (not shown). All extracts devoid of HGPRTase activity have normal APRTase activity and do not degrade ^{14}C IMP. All mutants examined thus far which are resistant to both guanine analogues and which do not grow in HAT media have no HGPRTase activity detectable *in vitro* (Table 1). Several sublines (E-28, E-39, and N-18) grow well in HAT media but have little to no detectable *in vitro* HGPRTase activity. Furthermore, cell lines resistant to both guanine analogues are found with considerable HGPRTase (N-3, N-7, N-8, N-12, E-29, E-44, E-47, and E-49). In order to obtain a better understanding of these drug-resistant cells, we have examined their utilization of exogenous (^{14}C) hypoxanthine for *in vivo* nucleic acid synthesis.

The presence of HGPRTase can be assessed indirectly by radioactive hypoxanthine incorporation (Figure 1B), since (^{14}C) IMP synthesis is required for

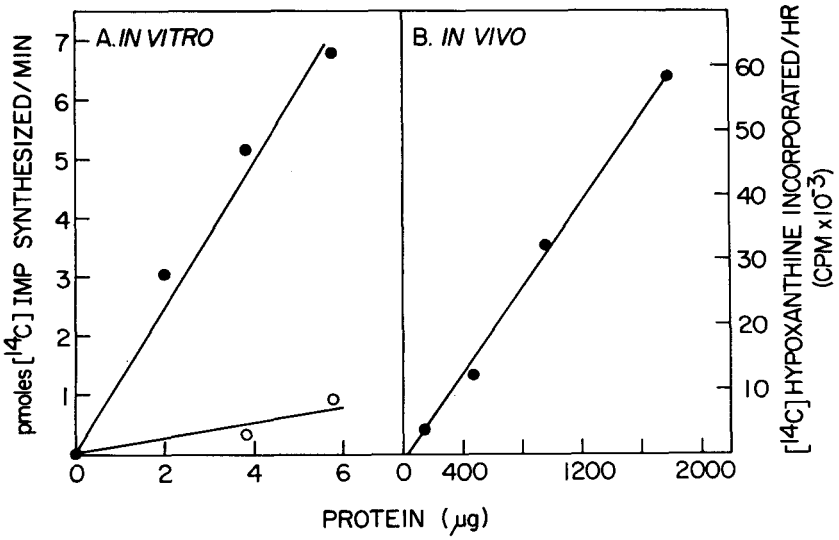

FIGURE 1.—*In vitro* and *In vivo* HGPRTase activity. A. each reaction is incubated 40 min at 37°C and contains in 0.05 ml: indicated A-3 extract; EDTA 3×10^{-2} M (○) as indicated; and additional components as described in METHODS. Formation of (^{14}C)IMP is determined by DEAE filter retention as described in METHODS. A zero time background of 38 pmoles has been subtracted from all values which are the average of duplicate determinations. B. Each determination is made from duplicate 60 mm Petri dish incubated 6 hr at 37°C in a 10% CO_2 atmosphere and contains 2 ml DMEM medium with 10^{-5} M aminopterin and 10^{-5} M (^{14}C)hypoxanthine. Incorporation into nucleic acids and protein is determined as described in METHODS. Background (200 CPM) is subtracted from all values.

the incorporation of (^{14}C) hypoxanthine into nucleic acids. The rate of hypoxanthine incorporation into nucleic acid is proportional to cell protein (Figure 1B). While this incorporation does not specifically measure HGPRTase activity, it provides a physiological measurement of exogeneous hypoxanthine utilization and is our most sensitive index of the presence of HGPRTase. For example, wild-type cells (A-3) incorporate hypoxanthine equally well in the presence or absence of aminopterin (Figure 3). Mutant N-1 (Figure 3) cannot utilize hypoxanthine, lacks *in vitro* HGPRTase activity, and therefore appears to lack a functional enzyme. Two other mutants, N-3 and N-7, incorporate hypoxanthine poorly under normal growth conditions. However, hypoxanthine incorporation by N-3 and N-7, but not N-1, is stimulated over 200 fold at 5 hr by the addition of aminopterin. This effect of aminopterin has been observed by others (CHU *et al.* 1969) and is in contrast to the effect of 6-TG reported by SUBAK-SHARPE (1965). The HGPRTase specific activity of N-3 extracts prepared at several time intervals following aminopterin addition is unchanged. A similar aminopterin effect is seen during incorporation of (^{14}C) guanine and inosine (data not shown) while (^{14}C) adenine incorporation is affected very little. These results indicate the presence of HGPRTase in N-3 and N-7 and correlate with the *in vitro* measurements (Table 1). In view of the striking stimulation of hypoxanthine incorporation by aminopterin, we have assessed (^{14}C) hypoxanthine incorporation by all sublines

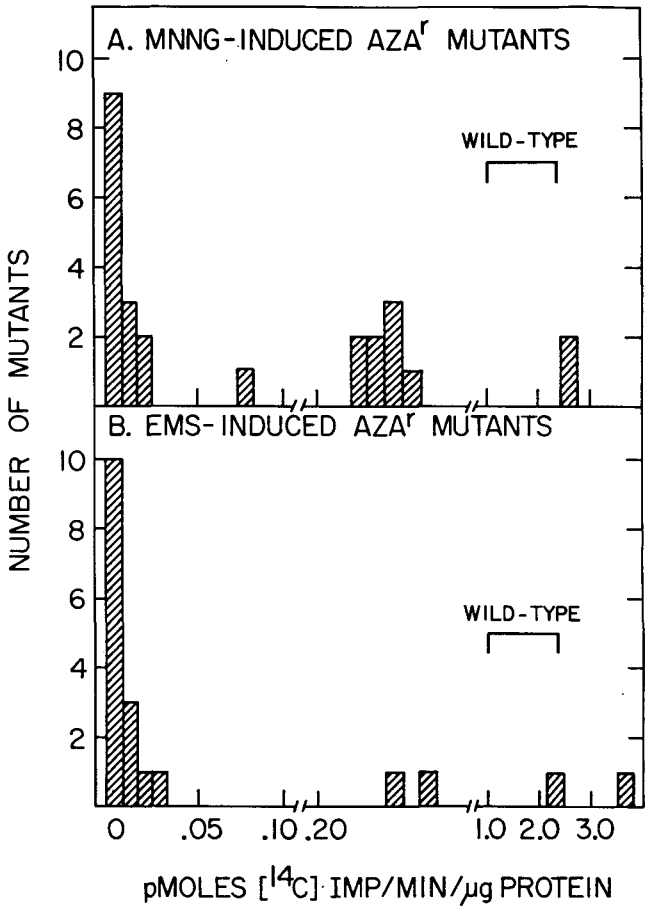

FIGURE 2.—*In vitro* HGPRTase of 8-AG resistant mutants. The specific activity of HGPRTase is determined *in vitro* as described in METHODS and footnote to Table 1. Mutants were selected following a 2 hr mutagenesis with 10^{-5} M MNNG (A) or 10^{-2} M EMS (B) as detailed in METHODS.

in its presence (Table 1) and absence (not reported). Mutants exhibiting 2% of the hypoxanthine incorporation of A-3 and also lacking HGPRTase as measured *in vitro* are unable to grow in HAT and are therefore considered extreme negative mutants. The remaining cell lines incorporate hypoxanthine in the presence but not absence of aminopterin, and grow in HAT media. This group includes N-2, N-18, E-28, and E-39 which have < 1% HGPTase activity *in vitro* as well as clones which have near wild-type levels of HGPRTase enzyme activity (E-44, E-49, and N-9). While the cell lines with low HGPRTase activity would appear to be mutants in that gene, we cannot determine from the present data whether the cell lines with near normal *in vitro* HGPRTase activity also represent mutations in that gene. If mutants have arisen in other genes which control purine metabolism, their frequency of occurrence is high.

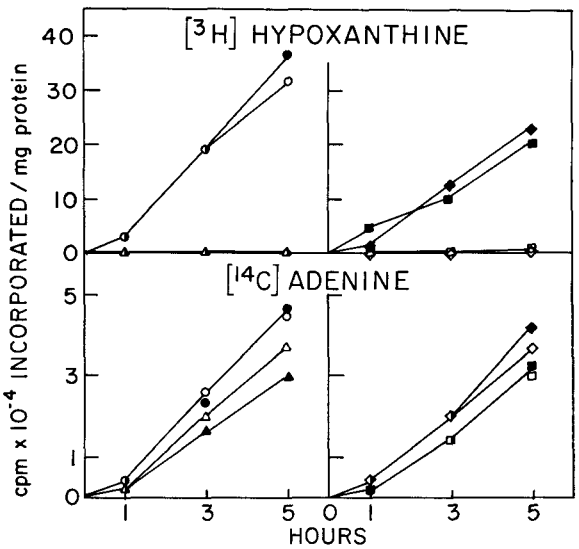

FIGURE 3.—The (^{3}H)hypoxanthine and (^{14}C)adenine incorporated into nucleic acid is determined from 60 mm Falcon plates with approximately 70% confluent growth incubated at 37°C in a 10% CO_2 atmosphere for the indicated times. All cells were incubated in the presence of DMEM; (^{3}H)hypoxanthine 10^{-7} M, (^{14}C)adenine 10^{-6} M, and as indicated by the closed symbols aminopterin 10^{-5} M. The cell lines studied are A-3 (○), N1 (△), N3 (□) and N7 (◇). Radioactive incorporation into nucleic acid is determined as indicated in METHODS by double label scintillation counting in a Beckman LS-233 counter.

A significant number of the 8-AG-resistant mutants clearly have reduced HGPRTase activities and therefore are mutants in the HGPRTase gene or in genes which govern its regulation. We have examined a number of these mutants by an additional genetic parameter—reversion to HGPRTase$^+$. We have studied only 8-AG-resistant mutants which do not grow in HAT media. With one exception, N-22, these HAT-sensitive mutants have no detectable HGPRTase activity. The reversion frequency of each mutant (HGPRTase$^-$, HAT sensitive) to HAT resistance has been determined as it occurs spontaneously or as affected by a chemical mutagen. Revertant clones have been selected and their cell-free HGPRTase activity verified. Thus far the following mutants have been reverted to HGPRTase$^+$ at a frequency greater than 10^{-7}: N-1, N-5, N-6, N-10, N-14, N-15, N-17, N-19, N-20, N-22, E-31, and E-43. These mutants differ markedly in their reversion frequencies (10^{-7}–10^{-4}) and thus appear to be a heterogenous group of point mutations. We have determined the cell-free extract HGPRTase specific activity of a number of revertants derived from each of several HGPRTase$^-$ mutants and found them to differ considerably (Figure 4). Since point mutations can be phenotypically corrected in three ways in bacteria: 1) intragenic reversion, 2) second-site intragenic reversion (FREESE and FREESE 1966), and 3) extragenic (suppressor) mutation (GAREN and SIDDIQI 1962), we are currently analyzing our HGPRTase$^-$ mutants and revertants in an attempt to categorize these animal cell mutations and their mechanisms of reversion.

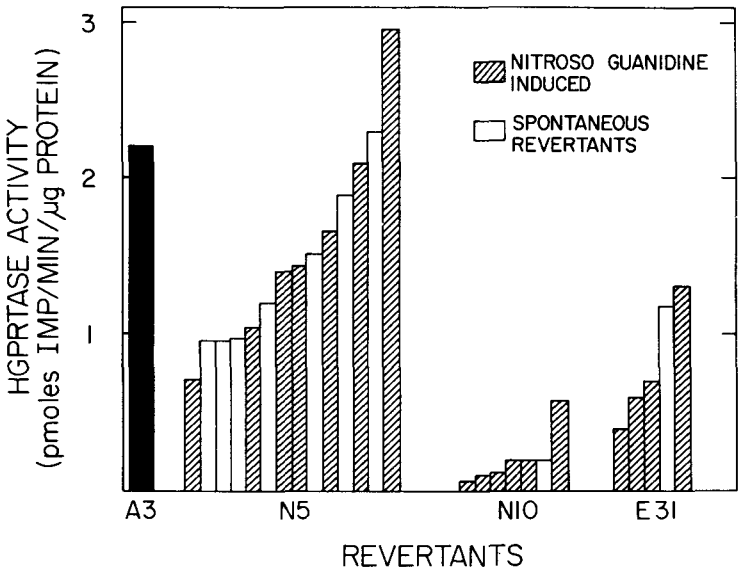

FIGURE 4.—The HGPRTase specific activities derived from three 8-AG-resistant mutants (N-5, N-10, and E-31) are determined from extracts as described in METHODS. The shaded areas represent values from clones which developed following treatment with MNNG while those developing spontaneously are not shaded.

DISCUSSION

Sixteen of our 8-AG-resistant mutants have no detectable HGPRTase activity. The genetic mechanisms responsible for such extreme negative mutants have been carefully examined in culture for a number of bacterial and yeast proteins, but never for a mammalian protein. Deletions, frameshift, missense and nonsense mutations all are capable of producing extreme negative enzyme mutations. However, the choice of mutagen significantly affects the type of mutations induced (FREESE and FREESE 1966). EMS and MNNG have been shown in other studies to be primarily base-changing mutagens (WHITFIELD, MARTIN and AMES 1966; FREESE and FREESE 1966; EISENSTARK, EISENSTARK and VAN SICKLE 1965). Since our mutants have been induced by these same mutagens and are highly revertible, they are likely to be point mutants.

Drug resistance has been suggested by several investigators (HARRIS 1971; MEZGER-FREED 1972) to occur by events other than mutation (epigenetic events). The 8-AG-resistant lines reported here which are of the extreme negative type are most consistent with mutational alterations since: (1) They lack detectible HGPRTase activity; (2) extracts of these mutants possess no inhibitor of HGPRTase; (3) study of somatic cell hybrids between these mutants and a line which is HGPRTase$^+$ and thymidine kinase$^-$ revealed expression of HGPRTase$^+$ by their ability to grow in HAT media (ROUFA *et al.* 1972). Thus the HGPRTase$^-$ characteristic is recessive. (4) Immunologically active but enzymically inactive, HGPRTase protein has been found in extracts of the extreme negative mutants.

The immunologic specific activity of the different mutants varies considerably (7–8 fold) suggesting the mutants produce an altered HGPRTase protein (BEAUDET, manuscript in preparation).

The frequency of nonsense mutations is high (30–90%) among extreme negative mutants of several bacterial genes (WHITFIELD, MARTIN and AMES 1966; GAREN 1968; FOWLER and ZABIN 1968) and is affected by the choice of chemical mutagen (WHITFIELD, MARTIN and AMES 1966). Both EMS and MNNG effectively induce bacterial nonsense mutations (GAREN and SIDDIQI 1962). Since missense mutations may result in mutant proteins with residual activity, and nonsense mutations usually result in total loss of enzyme activity, the percentage of nonsense mutants in our extreme negative group of mutants is expected to be high (WHITFIELD, MARTIN and AMES 1966; GAREN 1968; YANOFSKY 1963). Some naturally occurring mutants of man (Lesch-Nyhan syndrome) have no detectable erythrocyte HGPRTase activity, but extracts of these cells react readily with antibody (CRM^{+}) prepared to normal human red cell HGPRTase (RUBIN *et al.* 1971).

Five of our 8-AG-resistant mutants have very low but detectable (2%) HGPRTase activity which is adequate to support their growth in HAT media. Naturally occurring mutants of this type have also been described in fibroblasts from certain patients with Lesch-Nyhan syndrome (KELLEY and MEADE 1971).

The remaining fourteen mutants possess significant HGPRTase activity (20–150% of wild type), are resistant to 8-AG, and grow normally in HAT media. Resistance to guanine analogues has been reported to occur by a variety of cellular mechanisms which include mutant phosphoribosyltransferase (MURRAY 1971). All of our mutants which possess significant levels of HGPRTase incorporate exogenous hypoxanthine and guanine into nucleic acids very poorly. Since both guanine analogues are incorporated into nucleic acids through the same biochemical pathways (MURRAY 1971), poor utilization of exogenous guanine seems to be an adequate physiological explanation for their drug resistance. The precise biochemical basis for this phenotype, however, is not yet clear. It is apparently not a total defect in the transport of guanine since all of these mutants incorporate hypoxanthine when *de novo* purine biosynthesis is inhibited by aminopterin and all such mutants grow in HAT medium. We cannot exclude the possibility of partial transport defects since transport has yet to be examined quantitatively. Mutants with significant *in vitro* HGPRTase and low hypoxanthine incorporation (N-8, E-44, and E-49) are obvious candidates for such defects.

One naturally occurring human HGPRTase mutant (MCDONALD and KELLEY 1971) has been identified with altered substrate Km's. The specific activity of this enzyme varies with substrate concentrations which do not affect the wild-type enzyme. More detailed studies of our mutants are required to identify similar subtle changes. Km alterations in HGPRTase could explain poor utilization of exogenous hypoxanthine and guanine. Hypoxanthine utilization may increase as a result of changes in cellular levels of HGPRTase substrates (PRPP) and products (IMP and GMP) during aminopterin inhibition of *de novo* synthesis.

Alterations in the regulation of *de novo* purine biosynthesis could also bring

about the observed changes in exogenous hypoxanthine utilization characteristic of our mutants. The first enzyme of *de novo* purine biosynthesis, glutamine: phosphoribosylpyrophosphate amidotransferase (EC 2.42.14) is inhibited by purines (WYNGAARDEN and ASHTON 1959; CASKEY, ASHTON and WYNGAARDEN 1964) and purine analogue ribonucleotides (MCCOLLISTER, GILBERT and ASHTON 1964), and is the site of feedback control for the *de novo* purine biosynthetic pathway. An 8-AG-resistant mutant of *Schizosaccharomyces pombe* (NAGY 1970) which appears defective in amidotransferase feedback inhibition has now been identified. Furthermore, a 6-methyl-mercaptopurine resistant Ehrlich ascites mutant is relatively insensitive to suppression of *de novo* purine biosynthesis by guanine although its capacity to synthesize ribonucleotides is normal (HENDERSON, CALDWELL and PATERSON 1967). Further study of our 8-AG mutants is needed to assess their regulation of *de novo* biosynthesis and the possibility that among them are also amidotransferase mutants.

The biochemical characterization of 8-AG-resistant mutants offers the opportunity for determining mutational mechanisms in the gene for HGPRTase and examining cellular regulation of purine utilization and biosynthesis. The mutants described here appear adequate for such studies.

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