Table 1. Reversal of methotrexate and carboxypeptidase G_1 inhibition of L5178Y tissue culture cells. L5178Y cells in logarithmic growth were diluted to 150,000 cells per milliliter in Fischer's medium containing 10 percent horse serum. The medium supplements consisted of thymidine ($4 \times 10^{-6}M$), hypoxanthine ($2 \times 10^{-5}M$), L-serine ($1.4 \times 10^{-3}M$), and glycine ($1.4 \times 10^{-3}M$). The cells were incubated for 30 minutes at 37°C prior to the addition of either methotrexate or carboxypeptidase G_1. One day later, cell counts were performed on a model B Coulter counter.

Methotrexate (M)	Carboxypeptidase (units/ml)	Medium supplement	Cell growth	
			No.* (cells/ml)	Percentage of control
		−	702,300	100.0
		+	609,100	86
1×10^{-7}		−	85,500	12
1×10^{-7}		+	439,700	63
	0.012	−	161,500	32
	0.012	+	534,300	76

* Average of duplicate cultures.

of L1210 leukemia in vitro, both a parent line sensitive to methotrexate, and a strain resistant to high levels of methotrexate, was observed with approximately comparable levels of this enzyme. The growth of the Walker carcinoma and a human lymphoblastoid line (RPMI 4265), also propagated in culture, was also inhibited by this enzyme. Leucovorin, at one-hundredth of the molar concentration of folic acid, satisfied the folate requirements for growth of L5178Y cells (*9*). When carboxypeptidase G_1 was added to cells grown in this medium, similar inhibition of growth was observed.

In order to demonstrate that the inhibition of cell replication was indeed due to enzymic folate depletion, a reversal study was carried out as illustrated in Table 1. The end products, resulting from folate coenzyme-requiring reactions, were supplied the cells in the absence and presence of carboxypeptidase G_1 or in the absence and presence of methotrexate. It can be seen that carboxypeptidase G_1 inhibition of cell growth was reversed by the addition of thymidine, hypoxanthine, and serine in a manner similar to the ability of these compounds to reverse methotrexate toxicity (*10*).

Values of K_m (the Michaelis constant) for folic acid, methotrexate, leucovorin, and 5-methyltetrahydrofolate are 1.1 μM, 3.9 μM, 18.1 μM, and 12.9 μM, respectively. Thus this enzyme would be expected to deplete serum folates, since the major folate form in serum is 5-methyltetrahydrofolate.

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References and Notes

1. R. H. Adamson and S. Fabro, *Cancer Chemother. Rep.* **52**, 617 (1968); R. L. Capizzi, J. R. Bertino, R. E. Handschumacher, *Annu. Rev. Med.* **21**, 433 (1970).
2. R. W. Heinle and A. D. Welch, *J. Clin. Invest.* **27**, 539 (1948).
3. F. Rosen and C. A. Nichol, *Cancer Res.* **22**, 495 (1962).
4. We thank Dr. A. von Graevenitz for identifying the organism.
5. We thank Dr. J. Fruton for help in naming this enzyme.
6. C. C. Levy and P. Goldman, *J. Biol. Chem.* **242**, 2933 (1967); P. Goldman and C. C. Levy, *Proc. Nat. Acad. Sci. U.S.* **58**, 1299 (1967).
7. B. Chabner, J. L. McCullough, J. R. Bertino, *Proc. Amer. Assoc. Cancer Res.*, in press.
8. A unit of enzyme activity is defined as that amount capable of converting 1 μmole of substrate (methotrexate) per minute at 37°C and at *p*H 7.3.
9. Fischer's medium contains 10 mg of folic acid per liter.
10. M. T. Hakala and E. Taylor, *J. Biol. Chem.* **234**, 126 (1959).
11. Supported by NIH grant CA 08010. J.R.B. holds a career development award from NIH (5-K3-Ca-8853); J.L.M. is a predoctoral fellow, PHS grant 5-T01-GM-0059-09.
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New Class of Purine Mutants of Chinese Hamster Ovary Cells

Abstract. *Mutants of Chinese hamster ovary cells (CHO/Pro⁻) were isolated after mutagenesis with* N*-methylnitrosoguanidine and selection by the bromodeoxyuridine technique. Six of these were mutants classified as purine-requiring. The metabolic block appears to be early in the purine biosynthetic pathway. The mutants do not appear to be genetically identical.*

A major limitation in the study of mammalian cell genetics at the molecular level has until recently been the paucity of well-characterized, stable gene mutations. Multiply marked cell strains are an essential prerequisite for studying genetic exchange mechanisms in any cell system. A number of different mutants of mammalian cells have been reported (*1*).

The possibility of isolating such mutants has been made more promising with the increased availability of defined mediums and suitable cell lines. Kao and Puck (*2*) utilizing the incorporation of bromodeoxyuridine (BUdR) into prototrophic cells and the resulting photoinactivation of such cells have described a selective technique for the isolation of mutants. Auxotrophs which do not incorporate BUdR survive this treatment.

We now describe the isolation and characterization of a new class of mutants from Chinese hamster ovary cells (CHO/Pro⁻). These mutants are purine-requiring and, we believe, form a new class of mutants not reported before.

The Chinese hamster cell line CHO/Pro⁻ was supplied by Dr. T. Puck. Karyotypic analysis confirmed that it contained a model number of 20 chromosomes. This cell line has an absolute requirement for proline. At low cell populations, this strain also requires asparagine. Cells were maintained in Eagle minimal essential medium (MEM) supplemented with nonessential amino acids, fetal calf serum (5 percent), streptomycin (100 μg/ml), penicillin (100 unit/ml), and fungizone (5 μg/ml). The F12 medium was a modification of that described by Ham (*3*), and contained double concentrations of amino acids (*4*). To screen for mutants, F12 was prepared without essential amino acids (F12-IX) or without hypoxanthine, thymidine, and myoinositol (F12-X).

The selection technique for the isolation of mutants was a modification of that described by Kao and Puck (*2*). Sparse cell monolayers were incubated in F12 medium in the presence of 0.05 μg of *N*-methylnitrosoguanidine per milliliter for 16 hours. Cells were then washed free of the mutagen and reincubated for 6 days in fresh F12. The surviving cells were trypsinized, transferred at 10^4 cells per petri dish (60 mm in diameter), and starved, after attachment to the petri dish surface, in MEM. After 24 hours the medium was replaced with MEM supplemented with 10 percent dialyzed calf serum and $10^{-5}M$ BUdR. Cells were further incubated for 16 hours, washed with saline, and placed under visible light for 2 hours. This was followed by trypsinization and the transfer of the cells to F12 containing 5 percent calf serum. Surviving clones were picked,

transferred once more, and tested for growth on MEM supplemented with asparagine and proline, on MEM supplemented with nonessential amino acids, on unsupplemented F12 (F12-IX, F12-X), and on complete F12. Cells from clones that did not grow in minimal mediums or unsupplemented mediums were further examined.

Of the large number of clones isolated, six were found that did not grow in MEM supplemented with asparagine and proline, in MEM with nonessential amino acids, or in F12 deficient in purines.

Each of the mutants was examined for response to the addition of nutritional supplements. All six grew well when any of the mediums were supplemented with hypoxanthine, while no growth occurred when thymidine was added. However, hypoxanthine could be replaced by other purines or purine derivatives, such as adenosine monophosphate, adenosine triphosphate, and guanosine monophosphate.

The mutants isolated are very stable, and no spontaneous revertants have been found. Since this class of mutants responds to hypoxanthine, the genetic block must occur prior to inosinic acid formation in the purine biosynthetic pathway. Consistent with this is the observation that these mutants are insensitive to HAT medium (hypoxanthine, aminopterin, and thymidine) (*5*), which inhibits growth of mutants lacking the enzyme hypoxanthine guanine pyrophosphorylase. Initial tests for growth of these mutants on purine intermediates suggest that these mutants are blocked between glycinamide ribotide and aminoimidazole carboxamide ribotide (AICR) (Table 1). There are at least six enzymatic steps between glycinamide ribotide and AICR (*6*).

The following observations suggest that these mutants are not identical mutational events. (i) Both 104/2b and 102/2b undergo a few divisions in very low concentrations of hypoxanthine (0.1 μg/ml); (ii) both 17/1 and 102/2b grow more slowly in MEM supplemented with AICR than other isolates; (iii) we have noted complementation as a result of heterokaryon formation between all six mutants under selective conditions. Heterokaryons are formed by mixing two mutant cell lines together at high population densities in minimal medium for 48 hours, and then by plating 10^4 cells into petri dishes containing minimal medium. Only heterokaryons or revertants should form clones under these conditions. The frequency of heterokaryon formation under such conditions is fairly high. This does not appear to be a characteristic of the mutant type, but of the Chinese hamster cell line. We assume that the growth of heterokaryons in mediums lacking hypoxanthine is due to genetic complementation, although we cannot rule out gene dosage effects or other regulatory mechanisms (*7*).

Table 1. Growth of mutants of Chinese hamster ovary cells (CHO/Pro$^-$) in the presence of different purines and purine intermediates. Numbers refer to cultures grown from different clones isolated following mutagenesis and selection. ++, Good growth; +, poor growth; −, no growth. Abbreviations: ATP, adenosine triphosphate; PRPP, phosphoribosyl phosphate; Gly R, glycinamide ribotide; AIC, aminoimidazole 4-carboxamide; AICR, aminoimidazole 4-carboxamide ribotide.

Mutant	Guanidine	Adenine	ATP	Hypoxanthine	PRPP	Gly R	AIC	AICR
130/1b	++	++	++	++	−	−	++	++
17/1	++	++	++	++	−	−	++	+
104/2b	+	+	++	++	−	−	++	++
102/2b	++	++	++	++	−	−	++	+
121/2b	++	++	++	++	−	−	++	++
1c	++	++	++	++	−	−	++	++
CHO-PI	++	++	++	++	++	++	++	++

Other mutants of the purine biosynthetic pathway of mammalian cells have been reported (*8*). The Lesch-Nyhan syndrome of man is associated with hyperuricemia and excess uric acid synthesis. The purine accumulation results from loss of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Littlefield (*5*), using azaguanine resistance as a selective marker, has isolated strains of L cells and human fibroblast deficient in HGPRT; these strains are mutants that occur late in the purine biosynthetic pathway. However, the mutants reported here occur relatively early in the purine biosynthetic pathway and may be utilized to study regulation in mammalian cells. Since these mutants now carry three genetic markers (proline, asparagine, and purine), they should be useful in studying mechanisms of gene recombination in cells of higher animals.

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References and Notes

1. R. S. Krooth, G. A. Darlington, A. A. Velasquez, *Annu. Rev. Genet.* **2**, 141 (1968);
→ E. H. Y. Chu and H. V. Malling, *Proc. Nat. Acad. Sci. U.S.* **61**, 1306 (1968).
→ F. Kao and T. T. Puck, *Proc. Nat. Acad. Sci. U.S.* **60**, 1275 (1968).
→ R. G. Ham, *ibid.* **53**, 288 (1965).
4. H. G. Coon and M. C. Weiss, *ibid.* **62**, 825 (1969).
5. J. Littlefield, *Science* **145**, 709 (1964).
6. A. White, P. Handler, E. L. Smith, *Principles of Biochemistry* (McGraw-Hill, New York, 1963), pp. 559–566.
7. J. Littlefield and S. Goldstein, *In Vitro* **6**, 21 (1970).
8. M. Lesch and W. L. Nyhan, *Amer. J. Med.* **36**, 561 (1964); F. M. Rosenbloom, J. F. Henderson, I. Caldwell, W. N. Kelley, J. E. Seegmiller, *J. Biol. Chem.* **243**, 1166 (1968).
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2-Thiophenecarboxylic Acid: Inhibitor of Bone Resorption in Tissue Culture

Abstract. *In tissue culture 2-thiophenecarboxylic acid inhibited both spontaneous resorption of bone and that induced by parathyroid hormone. Its action was to inhibit calcium transfer out of bone.*

Fang first discovered the hypoglycemic and antilipolytic actions of 2-thiophenecarboxylic acid (2-TCA) in rats made diabetic with alloxan (*1*). 2-Thiophenecarboxylic acid also has strong hypocalcemic and hypophosphatemic actions in intact and thyroparathyroidectomized rats (*2*), and bone appears to be a principal site of these actions (*3*). We now present further evidence, derived from experiments with mouse calvaria in culture, showing that 2-TCA inhibits metabolically mediated calcium transfer out of bone and thus