a population of chromosomes all incompletely replicated becomes 1.8 rather than 2.0. Then the estimate of incomplete BCW 503 chromosomes increases to 89 percent. This estimate may further increase, since *B. subtilis* growing in the glucose-salts medium, with a generation time of 70 to 80 minutes, may have a resting period for DNA synthesis during its cell cycle (*14*). Another factor which would increase this estimate is that the *met*B marker may not lie at the terminus of all replicons in *B. subtilis*, a subject which will be dealt with in a subsequent report.

Among the 89 percent of chromosomes that were incomplete, replication appears to have stopped in a random fashion with regard to position on the chromosome. This viewpoint is supported by the decreasing gradient of marker frequency ratios from the origin to the terminus. The pattern of replication after amino acid starvation of BCW 503 also indicated a random distribution of replication activity along the chromosome (*10*). Cells in balanced growth have a random distribution of replication forks on the chromosome (*10*). These replication forks apparently stop with the same random distribution during amino acid starvation of BCW 503 cells. On restart of growth, replication merely resumes from sites where it had stopped.

Regulation at the initiation step alone cannot account for the effects of amino acid starvation on chromosome replication in *B. subtilis*. Recent findings with *E. coli* and *Salmonella typhimurium* may indicate that regulation only at the initiation step is not sufficient to account for the effects of amino acid starvation on chromosome replication in all of the strains of these organisms either (*6, 15*). My results along with others concerning the ratio of DNA to mass during and after amino acid starvation (*16*) suggest that a second regulatory circuit acts to control chromosome replication in *B. subtilis*. This second regulatory circuit appears to act independently of the initiation control circuit during amino acid starvation.

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Inhibition of Growth of Leukemia Cells by Enzymic Folate Depletion

Abstract. *A new bacterial enzyme, designated as carboxypeptidase G_1, inhibited growth of the L5178Y and L1210 murine leukemias, as well as the Walker carcinoma and the human lymphoblastoid line (RPMI 4265), propogated in vitro. This enzyme hydrolyzes the glutamate moiety from both oxidized and reduced folate forms, and thus it may prove to be of value in creating rapid folate depletion in vivo.*

The successful use of the enzyme L-asparaginase to produce a rapid depletion of the amino acid L-asparagine, necessary for the growth of certain human lymphoblastic leukemias (*1*), has stimulated the search for other enzymic therapies for neoplastic disease. Encouraged by the early results of Heinle and Welch (*2*) that demonstrated a beneficial result of dietary folate depletion on human leukemia, and the finding that an experimental tumor, the Walker 256 carcinoma, was markedly inhibited by dietary folate deficiency (*3*), we initiated the studies described below. Leucovorin (5-formyltetrahydrofolate), a close congener of the less readily available naturally occurring major folate coenzyme 5-methyltetrahydrofolate, was used as the sole carbon-nitrogen source to select an organism that would be capable of hydrolyzing reduced folate coenzymes. A pure culture of a Gram-negative rod, identified as a strain of *Pseudomonas stutzeri* was isolated (*4*). An enzyme, designated as carboxypeptidase G_1 (*5*), capable of cleaving terminal glutamate or aspartate residues from folate coenzymes or peptides was purified some 600-fold by ammonium sulfate fractionation, gel filtration on Sephadex G-100, and chromatography on diethylaminoethyl (DEAE)-cellulose. This enzyme differs from the previously described carboxypeptidase G (*6*) in several respects, the most important of which is the ability of the G_1 enzyme to hydrolyze reduced folate coenzymes (*7*).

The highly purified enzyme was tested for its ability to inhibit the growth of murine L5178Y cells, propogated in vitro (Fig. 1). An I_{50} (inhibition of 50 percent of the cells) was obtained with 0.012 unit of enzyme activity (*8*). Inhibition of the growth

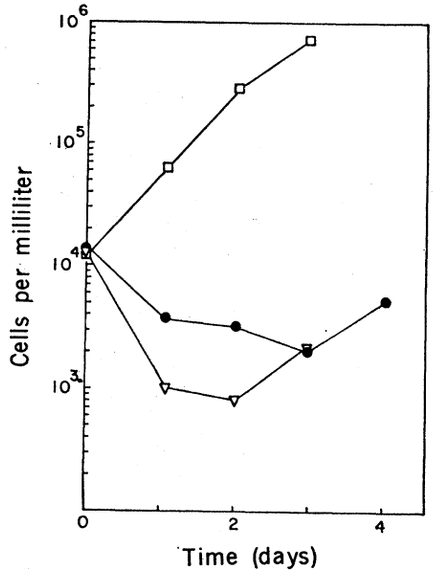

Fig. 1. Growth curve of 5178Y cells in the presence or absence of carboxypeptidase G_1. Cells were grown at 37°C and sampling for cell counting was performed each day on a model B Coulter counter. □, Tris buffer control; ●, 0.025 unit of enzyme per milliliter; ▽, 0.25 unit of enzyme per milliliter.

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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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,