Intracellular 5-Phosphoribosyl-1-Pyrophosphate: Decreased Availability During Glutamine Limitation

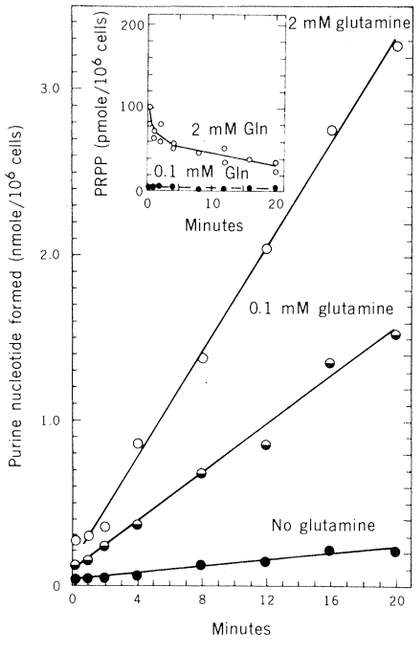
Abstract. *Culture of human lymphoblasts in medium containing limited glutamine (less than 0.2 millimolar) effects an inhibition of purine base reutilization and synthesis de novo. Both processes appear to be inhibited by a decreased availability of the common substrate 5-phosphoribosyl-1-pyrophosphate. Under conditions of glutamine limitation, the maintenance of capacity for purine base utilization and synthesis de novo and consequently growth was dependent on supplementation of cultures with inosine. The purine nucleoside provides a source of ribose phosphate for synthesis of 5-phosphoribosyl-1-pyrophosphate. Glutamine limitation may result in a decreased availability of ribose-5-phosphate.*

Glutamine occupies a central role in cellular metabolism (*1*) as a precursor in protein synthesis, a substrate in many nitrogen transfer reactions, including purine synthesis de novo, and as a major oxidative substrate and carbon source (*2, 3*). Our research has been primarily concerned with the role of glutamine in the regulation of purine biosynthesis (*4*). Glutamine enters the purine de novo synthetic pathway directly at three steps (*5*, pp. 74–77). Thus, extracellular variations in the concentration of this amino acid could have direct effects upon nucleotide biosynthesis. Chinese hamster cells (*6*) accumulate in the G_1 portion of the cell cycle when glutamine is depleted from the medium. Glutamine concentrations greater than 0.1 m*M* were required for maximal reinitiation of DNA synthesis.

The influence of extracellular glutamine concentration on purine base reutilization and synthesis de novo was examined in the human splenic lymphoblast line WI-L2 (*7*) and lines deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) selected from WI-L2 for resistance to 8-azaguanine (*8*). Cells were cultured, unless otherwise indicated, in suspension at 37°C in autoclavable minimum essential medium containing 5.5 m*M* glucose (*8*) and supplemented with 2 m*M* L-glutamine and 10 percent dialyzed fetal calf serum in sealed plastic flasks purged with a mixture of 5 percent CO_2 and 95 percent air. Purine synthesis was measured by incubating cultures with [^{14}C]formate for 30 to 60 minutes, followed by acid hydrolysis of cell pellets at 100°C and ion exchange chromatography to isolate the ^{14}C-labeled purine bases (*9*). Within two population doublings, cell growth and purine synthesis were inhibited with 0.1 m*M* glutamine but not with 2 m*M* glutamine in the medium.

In studies in which de novo purine synthesis was blocked either chemically (*10*) or by mutation (*11*), supplementation of cultures with hypoxanthine or adenine was used to allow growth. Hypoxanthine is converted by HGPRT to inosinic acid, the end product of the de novo pathway. Supplementation of WI-L2 cultures with hypoxanthine did not allow growth under glutamine limitation. However, supplementation of WI-L2 cultures with the ribonucleoside form of hypoxanthine, inosine, did permit growth. Cultures of the HGPRT-deficient line required supplementation with adenine in addition to inosine for growth under glutamine limitation. Adenine alone or in combination with uridine was not sufficient for growth of either cell line.

The WI-L2 cultures under glutamine limitation have diminished capacity for conversion of exogenous adenine to nucleotide (Fig. 1). The initial step in adenine utilization is conversion to adenosine monophosphate by adenine phosphoribosyltransferase (APRT), which requires Mg^{2+} and 5-phosphoribosyl-1-pyrophosphate (PRPP). A comparison of the acid-soluble adenine nucleotide pools (*12*) of cultures supplemented with 0.1 and 2 m*M* glutamine indicated essentially no difference in the composition of adenine nucleotide pools, that is, adenylate charge ratio (*13*). The PRPP pools (*14*) were markedly different within several hours under glutamine limitation: approximately 100 pmole/10^6 cells in WI-L2 cultures supplemented with 2 m*M* glutamine and 5 to 7 pmole/10^6 cells with 0.1 m*M* glutamine. If a value for cell water



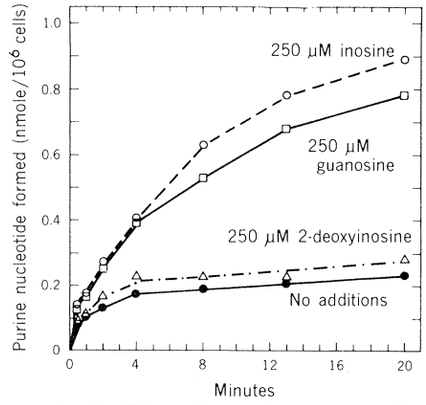


Fig. 1 (left). Effect of glutamine concentration on rate of conversion of adenine to nucleotide. A 50-ml culture of exponentially growing WI-L2 was harvested at a cell density of 10^6 cells per milliliter and resuspended at 0.7×10^6 cells per milliliter in medium without glutamine. The culture was incubated for 12 to 16 hours, harvested, and resuspended at 10^6 cells per milliliter in fresh medium without glutamine. After 30 minutes of incubation the culture was divided into three portions, and glutamine was added at the concentrations indicated. The subcultures were incubated for 2 hours more and assays were initiated by addition of [8-^{14}C]adenine (59 μc/μmole) to a final concentration of 50 μM. At the times indicated, conversion of adenine to nucleotide was determined by transferring 50 μl of sample to 2 by 2 cm squares of diethylaminoethyl (DEAE) cellulose paper (DE-81, Whatman). After 5 seconds the squares were transferred to a 1 m*M* ammonium formate wash solution, approximately 40 squares per liter of wash solution. After three 5-minute washes to remove unreacted [^{14}C]adenine the squares were transferred to 95 percent ethanol, removed, and dried on a hot plate. The radioactivity remaining bound to the filter, that is, nucleotide, was determined by scintillation counting in 2 ml of toluene-based scintillation solution; *Gln*, glutamine. Fig. 2 (right). Purine nucleoside-dependent stimulation of adenine conversion to nucleotide. A 50-ml culture of exponentially growing HGPRT-deficient lymphoblasts was harvested at 10^6 cells per milliliter and the cells were washed twice in 10 ml of minimal Hepes (MH) buffer (*19*). After the final centrifugation, the cells were resuspended at 2×10^6 cells per milliliter in MH buffer. One minute before initiation of the assay by the addition of 20 μl of 1 m*M* [8-^{14}C]adenine (59 μc/μmole), 50 μl of MH buffer only or buffer containing the purine nucleoside indicated at a concentration of 2 m*M* were added to 330 μl of the cell suspension (0.4 ml total). At the times indicated conversion of adenine to purine nucleotide was determined as described for Fig. 1.

of 1.0 μ1/10^6 cells is used (*15*), the estimated PRPP concentration in cells incubated with 0.1 m*M* glutamine is in the range of the Michaelis constant (K_M) for PRPP, 7 μM, observed with purified APRT (*16*). Thus, a subsaturating concentration of PRPP could account for the diminished rate of adenine conversion to nucleotide indicated in Fig. 1. Most other PRPP-utilizing enzymes involved in purine and pyrimidine reutilization and de novo synthesis have higher K_M values for PRPP than does APRT (*5*, pp. 105, 128, 178). This suggests that glutamine limitation may result in a decrease in availability of PRPP for these reactions.

In studies with an HGPRT-deficient line similar to those with WI-L2 described in Fig. 1, 50 μM inosine stimulated adenine conversion to nucleotide in cultures supplemented with 0.1 m*M* glutamine to rates equivalent to those in cultures with 2 m*M* glutamine. The specificity of stimulation of adenine conversion is indicated in Fig. 2. Only substrates of purine nucleoside phosphorylase, namely, inosine and guanosine, stimulated adenine conversion to nucleotide. Guanosine could not overcome the glutamine growth limitation in WI-L2, probably because of toxicity specifically related to guanosine or guanine (*17*). As with other mammalian cells (*5*, pp. 87–88), an active pathway for conversion of these purine nucleosides to PRPP via the intermediates ribose-1-phosphate and ribose-5-phosphate can be observed in supernatants prepared by 100,000*g* centrifugation of extracts from cultured human lymphoblasts. Since (i) purine nucleosides alone are sufficient for maximal stimulation of adenine conversion to nucleotide in glutamine-limited cultures and (ii) in other studies purine synthesis de novo in an HGPRT-deficient cell line was nearly maximal at concentrations of 0.1 to 0.2 m*M* (*18*) if an inosine supplement greater than 10 μM was also present, glutamine limitations may give rise to a lack of availability of a precursor of PRPP, such as ribose-5-phosphate.

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Cholera Toxin–Peroxidase: Changes in Surface Labeling of Glioblastoma Cells with Increased Time in Tissue Culture

Abstract. *Cholera toxin coupled to peroxidase yielded a highly specific ultrastructural marker of plasma membrane monosialogangliosides. Studies with cultures of brain and brain tumors suggested that long-term culture of tissue in monolayers results in eventual loss of surface monosialogangliosides.*

Fractionation of cells both from brain and from other organs suggests that gangliosides are a constituent of membranes and possibly serve as receptor molecules on the cell surface. Gangliosides are abundant in the central nervous system (CNS). The ultrastructural localization of gangliosides to the plasma membranes of intact CNS cells has not been investigated. In our study we have coupled peroxidase directly to cholera toxin, which specifically binds to the monosialoganglioside G_{M1} (*1*), in order to investigate the distribution of G_{M1} at the surfaces of normal cells and of tumor cells derived from the CNS. We now describe a human glioblastoma tumor line that showed alterations in cholera binding with increasing time in tissue culture.

Highly purified cholera toxin (*2*) was coupled to peroxidase (type VI, Sigma) with glutaraldehyde as the cross-linking agent (*3*). The conjugate (cholera-peroxidase) displayed a single peak on G-22 Sephadex chromatography, and neither free peroxidase nor free cholera toxin was detectable.

Glioblastoma TC 593 cell line was initiated from a human surgical specimen and was maintained in monolayer culture for 14 months (*4*), the cells were frozen and stored in liquid nitrogen for 7½ years and started again in monolayer culture for our experiments. Cholera binding was first tested 2 months after cells were taken from storage, again tested after 7 months and 8 months. Conjugated cholera-peroxidase was added to monolayers of cells in the log phase of growth at a density of about 80 percent after they had been washed several times in phosphate-buffered saline (PBS). Intact cells were exposed to the conjugate for 15 minutes at 22°C, and unbound material was washed off with excess PBS (three washings for 5 minutes each) prior to fixation of the intact monolayer with glutaraldehyde (*5*). In order to demonstrate specificity of the conjugated cholera toxin, the following experiments were done. (i) Excess human ganglioside or purified fractions of human ganglioside (G_{M1} and G_{D1a}) were added to the incubation medium containing cholera-peroxidase in order to prevent binding of cholera-peroxidase to the cell surface. The G_{D1a} was used because it is a disialoganglioside, and thus it should not be as effective as G_{M1} in binding cholera-peroxidase. (ii) Unlabeled toxin was added to the incubation medium to test whether all cholera-peroxidase could be prevented from binding to the cell surface. (iii) The TC 593 cells were first treated with ganglioside fractions and then washed with PBS in order to enhance the binding of cholera-peroxidase when cells were subsequently exposed to the conjugate. This experiment was based on studies showing that fat cells can take up into their plasma membranes exogenously applied ganglioside (*6*). (iv) ^{125}I-Labeled cholera toxin (specific activity 15 μc/μg) was also used to see whether autoradiographic findings