Sugar-free Growth of Mammalian Cells on Some Ribonucleosides but Not on Others*

Burton M. Wice and David E. Kennell

From the Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

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It was shown earlier that a variety of vertebrate cells could grow indefinitely in sugar-free medium supplemented with either uridine or cytidine at \( \geq 1\) mM. In contrast, most purine nucleosides do not support sugar-free growth for one of the following reasons.

1. The generation of ribose-1-P from nucleoside phosphorylase activity is necessary to provide all essential functions of sugar metabolism. Some nucleosides, e.g. xanthosine, did not support growth because they are poor substrates for this enzyme.

2. De novo pyrimidine synthesis was inhibited \( >80\% \) by adenosine or high concentrations of inosine, e.g. 10 mM, which prevented growth on these nucleosides; in contrast, pyrimidine synthesis was inhibited only marginally on 1 mM inosine or guanosine, but normal growth was only seen on 1 mM inosine, not on guanosine.

3. The inhibition of de novo adenine nucleotide synthesis prevented growth on guanosine, since guanine nucleotides could not be converted to adenine nucleotides. Guanine nucleotides were necessary for this inhibition of purine synthesis, since a mutant blocked in their synthesis grew normally on guanosine. De novo purine synthesis was severely inhibited by adenosine, inosine, or guanosine, but in contrast to guanosine, adenosine and inosine could provide all purine requirements by direct nucleotide conversions.

In an earlier report, it was shown that a variety of cultured animal cells could be grown for many generations in the complete absence of sugar if the medium was supplemented with \( \geq 1\) mM uridine or cytidine (1). Purine ribonucleosides or deoxyribonucleosides could not replace sugar except for inosine which at certain concentrations could support growth of some cell types (1, 2). The essential function of sugar, as well as of the nucleoside that replaces it, is not to provide energy; the oxidation of glutamine carbon in the citrate cycle can supply all energy needs of the cell (3). What is essential is the provision of ribose-P primarily for the synthesis of nucleic acids and triose-P (4). In several mammalian cells growing on uridine this phosphorylation was met by production of ribose-1-P via phosphorylase of the nucleoside (5). A mutase activity then converted ribose-1-P to ribose-5-P whose metabolism could provide all the essential functions of sugar.

Deoxynucleosides could not support growth since cells have no known mechanism to synthesize the RNA nucleotides from the deoxynucleosides (or ribose-P from deoxyribose-P). A purine nucleoside could be nonsupportive either because it was a poor substrate for purine nucleoside phosphorylase (EC 2.4.2.1) or because the nucleoside or a nucleotide product inhibited the synthesis of other nucleotides. There is an extensive literature on the "toxicities" of adenosine and guanosine in animal cells and tissues (reviewed in Refs. 6 and 7). Since steady state growth in sugar-free medium has only been achieved recently (1), these earlier papers studied growth inhibition by nucleosides in the usual glucose-containing media. The two cases (with and without glucose) could differ for a given nucleoside, because the concentrations of many metabolic intermediates are very different (1).

The purpose of this paper is to identify which product is limiting growth when cells do not grow on a particular purine nucleoside. Upon transfer to a medium in which a nonsupportive nucleoside had completely replaced sugar, there usually was a transient synthesis of protein at normal rates for about 20 h (1). This synthesis provided a means to measure accurately the relative rates of de novo pyrimidine or purine nucleotide synthesis in order to determine if one of these pathways was limiting for growth.

**Experimental Procedures**

Cells and Growth Conditions—HeLa, LM, and LM/HGPRT (deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) activity) cells were grown as monolayer as previously described (1) using Eagle's minimal essential medium with 5% dialyzed calf serum and 10 mM glucose. In all experiments the cells were washed with phosphate-buffered saline (NaCl, 0.14 M; KCl, 2.7 mM; Na2HPO4, 8.1 mM; KH2PO4, 1.5 mM, pH 7.5) treated with trypsin (EC 3.4.21.4) + EDTA (1 mM), and dispensed into 25 cm² T-flasks containing complete medium minus sugar and nucleosides as previously described (1). The cells were incubated at 37 °C for 16-20 h and then refed with media containing the indicated additions at the start of an experiment. All experiments using adenine were in media containing 5% dialyzed horse serum in place of calf serum since adenine is rapidly deaminated by adenosine aminohydrolase (EC 3.5.4.4) present in calf and fetal calf sera (8). As a control, results with uridine, glucose, or no sugar-no nucleoside were similar in calf and horse sera.

Measurement of de Novo Purine and Pyrimidine Biosynthesis—The incorporation of \( [14C] \) glycine or \( [14C] \) aspartic acid was used to measure purine or pyrimidine synthesis, respectively. In all cases, the incorporation of into protein, as well as into nucleic acids, was determined in order to correct for any change in the intracellular specific activity of these precursors. This was necessary since their intracellular levels can change as a function of the supporting sugar or nucleoside, e.g. intracellular aspartic acid increases from \( \sim 10 \) to \( \sim 20 \) mM when uridine replaces glucose in the growth medium (not shown). Also, the glycolytic activity of HeLa cells growing on various sugars or uridine varies by as much as 500- to 1000-fold (1, 3), so that intracellular levels of glycine (derived from glycolytic intermediates) might also fluctuate.

The incorporation of label into nucleic acids was measured by counting an aliquot of the 5% trichloroacetic acid-precipitable \( (0 \degree C) \) fraction after it was resolubilized in 5% trichloroacetic acid at 80 °C (1); incorporation into protein was measured by collecting the 90 °C precipitate on glass fiber filters (1). In order to be sure that the \( 80 \degree C \)
Enzymes were from Boehringer Mannheim. [8-'4C]Guanosine, on polyethyleneimine cellulose thin layers as previously described (1, 4). Xanthosine; Urid, uridine; Gluc, glucose; Aden, adenosine. PRPP, a-D-5-P-ribosyl-P2; Inos, inosine; Guan, guanosine; Xanth, xanthosine; Urid, uridine; Gluc, glucose; Aden, adenosine.

RESULTS

Protein Synthesis on Nucleosides in Place of Sugar—The criterion for growth on a nucleoside in place of sugar is the exponential increase in cell protein at a constant rate with repeated subculturing. Uridine or cytidine over a wide concentration range (~1 to ≥10 mM) and inosine at lower concentrations only (1 mM here) do meet this criterion for HeLa cells. Other nucleosides (inosine at 10 mM, adenosine, guanosine, or xanthosine as well as all deoxynucleosides) do not (1). Except for inosine, all the results to be reported here were the same for any nucleoside from 0.5 to 10 mM and differences noted in concentrations in this range in different experiments have no significance. With the exception of xanthosine, even nucleosides that did not support continuous growth for many generations did support a transient increase in protein measured by mass (1) or [3H]leucine incorporation (Fig. 1) for the first 20 h at close to normal growth rates. However, on 1 mM xanthosine, leucine incorporation was no greater than it was in the absence of nucleoside or sugar.

Ribose-1-P Synthesis from Nucleosides—Ribose-1-P was shown previously to be the essential product of uridine metabolism for growth of animal cells in the absence of sugar (5). Therefore, the production of ribose-1-P from each nucleo-
side was compared to the ability of that nucleoside to support growth. The synthesis of ribose-1-P was not detectable in the absence of nucleoside even if glucose was present. However, ribose-1-P synthesis could be detected in cells incubated after 3 or 24 h with guanosine, 10 mM inosine, or adenosine even though these nucleosides did not support growth (Fig. 2). It was also produced by cells in medium containing 1 mM inosine or 10 mM uridine in which the cells could grow. Thus, lack of growth need not result from an inability to generate ribose-1-P from the purine nucleosides. The one possible exception was 1 mM xanthosine on which there was no detectable ribose-1-P in the cells.

Intracellular Ribose-1-P Levels—In the absence of growth the ribose-1-P generated by the phosphorolytic cleavage of a nucleoside could accumulate to high concentration in the cell. This was seen especially in the case of 10 mM inosine which did not support growth but on which ribose-1-P was synthesized even after 20 h with intracellular levels accumulating to about 25 times the level on 10 mM uridine (Fig. 3). Also, ribose-1-P concentrations were the same (~0.5 mM) on 0.5 mM guanosine and 1 mM inosine although the former did not support growth while 1 mM inosine did (1). On xanthosine there was a low level of ribose-1-P similar to that with uridine but this probably reflects a small accumulation without further metabolism since no 32P compounds other than those seen with no sugar or nucleoside were observed (Fig. 2). That xanthosine was not toxic was shown by the normal growth of glucose or uridine cells in the presence of xanthosine. Glucose- or fructose-supported cells contained a very low level of ribose-1-P (~5 mM), and more important, it was no higher than in cells starving for sugar and nucleoside.

De Novo Pyrimidine Biosynthesis in the Presence of Various Nucleosides—While leucine incorporation was close to normal for 20 h in all cases except xanthosine, the inability of a nucleoside to support growth was manifest much sooner in a reduced rate of nucleic acid synthesis. As seen in Fig. 4, the rate of de novo pyrimidine synthesis was reduced by only about 30%, when either 10 mM uridine, 1 mM inosine, or 0.5 mM guanosine replaced 10 mM glucose in the medium. However, when 10 mM inosine replaced glucose, de novo synthesis was almost completely shut off after a few hours, and it was reduced by about 80% when 2 mM adenosine replaced glucose.

FIG. 2. The synthesis of ribose-1-P (R-1-P) in HeLa cells incubating in growth media with the indicated nucleoside in place of sugar. The cells had been prestarved as described under “Experimental Procedures” and then labeled in [32P]orthophosphate (0.5 mCi) for 3 h starting at 3 h or at 20 h. Ribose-1-P was identified using a standard (5). Not shown is 2 mM adenosine in dialyzed horse serum which gave the same intensity of the ribose-1-32P spot as on guanosine. The nucleoside in each case is indicated in the panel. The nucleoside concentrations are the same as given in Fig. 1.

FIG. 3. The intracellular concentration of ribose-1-P in HeLa cells incubated in growth media with the indicated nucleoside, or glucose, or with no addition. Cells were extracted with formic acid at the indicated times and assayed for ribose-1-P as described under “Experimental Procedures.” No sug refers to medium lacking sugar and nucleoside. Cells growing on 2 mM fructose gave ribose-1-P levels similar to those on 10 mM glucose. The indicated nucleoside is at the same concentration as given in Fig. 1.

FIG. 4. The de novo biosynthesis of pyrimidine nucleotides by HeLa cells incubated in growth medium with the indicated sugar or nucleoside. The 31C from aspartic acid is plotted in nucleic acids versus protein in order to correct for the intracellular specific activity of aspartate, with the efficiency of the fractionation shown by the insignificant [3H]leucine into nucleic acids (b). Dialyzed horse serum was used in b in order to avoid the deamination of adenosine. The last points on the curves correspond to about 24 h. The indicated nucleoside is at the same concentration as given in Fig. 1.

Note that in the presence of glucose, adenosine blocked pyrimidine synthesis almost completely after about 10 h, compared to the rate with adenosine alone. Therefore, adenosine inhibition appears to be greatly enhanced by the addition of glucose. The inability to synthesize sufficient amounts of
pyrimidine nucleotides could account for the lack of growth when 2 mM adenosine or 10 mM inosine replaced glucose in the growth media. However, on guanosine the rate of pyrimidine biosynthesis was sufficient to support growth, since the same rate was seen on 1 mM inosine which did support continuous exponential growth.

De Novo Purine Biosynthesis on Various Nucleosides—Purine compounds can also inhibit de novo purine biosynthesis (13). This inhibition could prevent growth if the exogenous nucleoside could not be converted to all essential purine nucleotides in sufficient amount. Compared to its rate with glucose or uridine, purine synthesis was reduced by 80% or more by any of the purine nucleosides at all concentrations tested (Fig. 5). However, cells were able to grow with 1 mM inosine but not with 0.5 mM guanosine, suggesting that the lack of de novo purine synthesis, by itself, is not sufficient to prevent the growth of cells on guanosine.

Purine Nucleotide Interconversions—According to known enzyme activities in animal cells (and bacteria), nucleotides derived from both inosine and adenosine can be interconverted as well as converted to guanine nucleotides so that de novo purine synthesis is not necessary. However, the situation with guanosine and xanthosine is less clear. The synthesis of IMP by GMP reductase (EC 1.6.6.8) was observed in enteric bacteria by Magasanik and Karibian in 1960 (14), but its presence in rat liver has been based on very preliminary data (15). The incorporation of label from guanosine into AMP of RNA was compared to label from adenosine or inosine into GMP (labels were in the bases only). HeLa cells growing in glucose medium were labeled for 3 h (20 h gave the same results), RNA extracted and hydrolyzed, and its AMP and GMP separated (1). The ratio of counts per min into GMP versus AMP was 2 when either [8-14C]adenosine or [8-14C]inosine was added. However, in the presence of [8-3H]guanosine, this ratio rose to 30 (data not shown). If there is a GMP reductase activity in HeLa cells, it must be insufficient to supply adenylate compounds from guaninic acid. The absence of this activity coupled with an inhibition of de novo purine synthesis could account for the lack of growth on guanosine. Also, uridine did not give growth in the presence of guanosine; inhibition of pyrimidine synthesis could not account for lack of growth.

A Mutation That Allows Growth on Guanosine—Cells should grow on guanosine if de novo purine synthesis were not inhibited. The presence of guanosine kinase activity is questionable in many mammalian cells and if absent, the synthesis of GMP from guanosine would have to result from the hypoxanthine phosphoribosyltransferase reaction. Guanine nucleotides might be necessary for inhibition of de novo purine biosynthesis (16, 17). A mouse strain variant in this activity and its parent wild type were compared. The parent mouse LM cells behaved like HeLa cells in that de novo purine biosynthesis was greatly reduced by either guanosine or nucleoside. Also, uridine did not give growth in the presence of guanosine; inhibition of pyrimidine synthesis could not account for lack of growth.

**DISCUSSION**

**Requirements for Growth in Sugar-free Media**

We concluded that the only essential function of sugar for growth of cultured vertebrate cells was to provide substrates...
for biosynthesis, the major one being ribose-5-P (4). Besides ribose-5-P, *de novo* nucleotide synthesis requires glutamine, glycine, folate, aspartate, as well as bicarbonate and other salt ions. All of these are present as essential components in normal culture media except for ribose-5-P, glycine, and aspartate. High levels of glycine and aspartate are needed for protein as well as nucleotide biosynthesis. Glycine is derived from triose-P via glycolysis. Aspartate can be provided from exogenous glutamine. Thus, the only essential intermediate from a nucleoside is ribose-5-P which is derived from ribose-1-P (5). This need for ribose-P is a major one; with growth on uridine 1 molecule of uridine is metabolized compared to 5 molecules of glucose in glucose medium (1) with 90% of the nonessential uracil moiety excreted. Since only 10% of the glucose carbon becomes ribose-5-P (4), perhaps twice as much ribose-5-P is made from uridine than is made from glucose. On uridine most ribose-5-P passes down the nonoxidative pentose cycle arm (1) to provide glycolytic intermediates, especially the triose-Ps needed for serine and glycine as well as for lipid biosynthesis. On glucose, glycolytic intermediates are provided in great excess by the enormous glycolytic flux. Thus, one reason that a given nucleoside might not support growth would be insufficient enzymatic activities to generate the large amounts of ribose-5-P from it; the only nucleoside-specific reaction for this function would be the initial phosphorolysis. Such inactivity was not an explanation in the case of protein (Fig. 1) or nucleic acids, above the no sugar or nucleoside or pyrimidine synthesis.

There is an extensive literature on purine toxicity (reviewed in Ref. 6) dating back 40 years to the observed toxicity of adenine for *Drosophila* (21). Toxicity has generally been ascribed to an inhibition of either purine or pyrimidine *de novo* synthesis. However, the pathway that might be inhibited to account for toxicity by a nucleoside in cells incubated with glucose (all published cases) might not be the one limiting growth on the same nucleoside in sugar-free medium; the concentrations of many metabolites are radically different. In general, glucose metabolism amplifies an inhibition by a nucleoside alone, but to an extent that varies with each case. We observed a much more severe inhibition (8-fold) of pyrimidine synthesis from glucose plus uridine than from uridine alone and a lesser amplification in the case of adenosine. Also, the rapidity of cell death was faster with glucose present while cells were often maintained for several days without growth on a nucleoside, especially guanosine. These magnified inhibitions were not seen with addition of fructose instead of glucose, showing the significance of the much greater carbon flux on glucose.

### The Basis for Toxicity of Nucleosides in Glucose-supported Cells

**Adenosine**—The most extensive studies of toxicity have been made with adenosine and its related compounds. Since AMP can be converted to GMP, adenosine inhibition of pyrimidine biosynthesis can account for toxicity and can be overcome by uridine if the uridine/adenosine ratio is above some minimum value (8, 22-26). However, it was also observed that uridine did not protect against adenosine toxicity in adenosine kinase mutants (27, 28). It has been concluded that this case reflects an inhibition of methylation by high internal concentrations of free adenosine (26, 29).

**Guanosine**—Guanosine nucleotides appear to be the sole effectors for the toxicity of guanosine (see under “Results” and Refs. 16 and 17). Since GMP cannot be converted to AMP, guanosine toxicity could result from sufficient inhibition of either the *de novo* purine or pyrimidine pathway. While uridine has been observed to overcome guanosine toxicity in some cultures, e.g. Ref. 30, in other systems it has not been effective (Refs. 31 and 32 and this paper). It was more difficult to show directly in the earlier studies that the toxicity resulted from adenine starvation since adenine addition would itself lead to greater pyrimidine starvation. Measuring the levels of nucleotides in inhibited cells may not identify a limiting pathway either. Brenton et al. (16) observed comparable reductions (2- to 3-fold) in both adenine and pyrimidine nucleotides in human lymphoblast cells growing with 0.1 mM guanosine.

### Regulated Reactions in Nucleotide Synthesis

While it is not a purpose here to identify special reactions that might be growth-limiting, the major control points will be mentioned.

1) **PRPP** is an essential substrate in both pyrimidine and purine nucleotide syntheses and its availability can limit either pathway. This availability could be affected by its rate of synthesis in the ribose phosphate pyrophosphokinase (EC 2.7.6.1) reaction (33-35), and its rate of depletion in one pathway can limit the other (36, 37). A large number of studies have shown lower PRPP levels in cells with exogenous adenine or guanosine (8, 26, 28, 30, 38, 39). The reduction by adenosine occurs even in adenosine kinase mutants (28) although perhaps to a lesser extent (26).

2) **Specific Reactions in Pyrimidine Biosynthesis**—Both guanosine and adenosine have been observed to prevent induction and *in vitro* activity (40-43) of glutamine-dependent carbamoylphosphate synthetase (EC 6.3.4.16) as well as aspartate carbamoyltransferase (EC 2.1.3.2) (43). An intermediate step in pyrimidine biosynthesis that is sensitive to...
PRPP availability is the PRPP-dependent orotate phosphoribosyltransferase (EC 2.4.2.10) reaction. Both guanosine and adenosine have been reported to block synthesis at this step with an accumulation of orotate (8, 26, 30, 39, 44). Which reaction might become limiting in a given situation is probably a function of several variables. For example, it has been proposed that the later reaction could limit synthesis when the ATP level was sufficiently high to drive the preceding carbamoylphosphate synthetase step (26, 45).

3) Reactions in Purine Biosynthesis—There have been fewer studies on limiting reactions in purine biosynthesis since purine synthesis never limits growth on adenosine. However, in vitro studies showed that the glutamine-dependent phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14) for the first reaction in purine biosynthesis is feedback inhibited by purine nucleotides, especially ADP and ATP. This inhibition is competitive for PRPP binding (46) and thus would also be sensitive to PRPP availability. There is evidence that the activity of adenylosuccinate synthetase (EC 6.3.4.4) that catalyzes the last reaction to convert IMP to AMP is sensitive to guanine and guanosine in the medium (47, 48).

Identifying the Pathway That Limits Growth on a Specific Nucleoside

The relative rates of de novo synthesis on each nucleoside, normalized in each case to protein synthesis, can identify the limiting pathway (Table I). On guanosine the rate of pyrimidine biosynthesis cannot be limiting, since it was as high on 1 mM inosine which supported exponential growth indefinitely. Also, uridine did not allow growth on guanosine. Thus, lack of growth on guanosine must result from an adenine starvation that is a consequence of the inhibition of de novo purine biosynthesis by a phosphorylated derivative of guanosine coupled with the absence of an enzymatic activity to convert guanine compounds to adenine nucleotides. This latter limitation would also reduce the total purine nucleotide pools in the cell and probably account for the only marginal inhibition of pyrimidine synthesis by guanosine.

Inosine derivatives can be converted to adenine and guanine nucleotides, so that on inosine or adenosine, the severe inhibition of purine biosynthesis is irrelevant. Here there is an obvious direct correlation between growth and pyrimidine synthesis. Pyrimidine synthesis was sufficiently high on 1 mM inosine to give growth but was too low on 10 mM inosine to support growth. This case demonstrated directly that the extent of inhibition can be a function of the concentration of the exogenous nucleoside. Thus, it is possible that whether or not a particular nucleoside could substitute for sugar could depend on its exogenous concentration; if a very low concentration were required to avoid inhibition, it might be too low to support exponential growth. Uridine must be >0.1 mM to support continuous growth (1). Finally, the capacity of a vertebrate cell to utilize a given nucleoside in a way that provides all the requirements for growth, normally met by sugar, probably varies somewhat from cell to cell (1). However, in general, a wide variety of cells can grow on either pyrimidine ribose while growth on a purine ribose is the exception.

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| Nucleoside (or sugar) | Growth | Ribose-1-P production | Pyrimidine synthesis | Purine synthesis | Purine interconversion |
|-----------------------|--------|-----------------------|---------------------|----------------|---------------------|
| Glucose               | +      | +                     | +                   | +              | +                   |
| Fructose              | +      | -                     | -                   | -              | -                   |
| Uridine               | +      | +                     | +                   | +              | +                   |
| Adenosine             | +      | +                     | +                   | +              | +                   |
| Guanosine (1 mM)      | -      | +                     | +                   | -              | -                   |
| Xanthosine (10 mM)    | -      | -                     | -                   | -              | -                   |
| None                  | -      | -                     | -                   | -              | -                   |

TABLE I
HeLa cell growth and nucleotide synthesis on nucleosides or sugar
A (+) (as opposed to a (−) value) in the first column indicates continuous exponential growth and in the last four columns to a sufficient activity to support growth. See Figs. 2-5 for observed activities in each medium. A (○) indicates that this activity (or activities) is limiting growth in a particular medium according to the conclusions of this paper.
Nucleosides to Replace Sugar

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