The Continuous Growth of Vertebrate Cells in the Absence of Sugar*

(Received for publication, April 9, 1980, and in revised form, March 11, 1981)

Burton M. Wice, Lawrence J. Reitzer, and David Kennell

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

Cultured HeLa cells were grown indefinitely in the complete absence of sugar (<10 \(\mu\)M) when the medium was supplemented with specific nucleosides at \(\geq 1\) mM. Uridine or cytidine alone was sufficient and gave growth rates comparable to those on 10 mM glucose, while most purine ribosides and all deoxyribosides tested were ineffective. Uridine could also substitute for sugar to support continuous growth of mouse L, early passage human fibroblast, primary chicken embryro fibroblasts, and numerous other cell lines, including mouse NCTC 2071 which grew in a completely defined medium, lacking serum as well as sugar.

Uridine was depleted from the sugar-free medium of HeLa cells at 2 nmol/min/mg of protein; this rate is about 10 times faster than the depletion rate of hexose during growth on 2 mM fructose and one-fourth the rate on 10 mM glucose. About 40% of the metabolized \([U-^{14}C]uridine\) carbon was lost to the medium as a result of the release of about 90% of the uracil moiety. All four nucleotide components of RNA were labeled by \([U-^{14}C]uridine\) but only pyrimidines were labeled by \([2-^{14}C]uridine\). Close to 6% of the \(^{14}C\) from either \([U-^{14}C]\) or [2-\(^{14}C\)]uridine was found in the pyrimidine nucleotides; this correspondence suggests that \(de\) \(novo\) pyrimidine synthesis is at least partially shut off. However, the incorporation of formate or glycine into nucleic acids was at normal rates suggesting that purines were synthesized by the normal pathway from \(\alpha\)-D-5-P-ribosyl-P.

All of the uridine carbon depleted from the medium could be accounted for in the various 5% trichloroacetic acid fractions of the cells (cold soluble pools, hot soluble and precipitable macromolecules) plus nonuridine compounds in the medium and released CO\(_2\). While the uracil moiety was only present in pyrimidine nucleotides or medium uracil, the ribose moiety was found in all fractions. Only about one-quarter of this ribose ended in nucleic acids. Most of the remainder was found in small molecules in the cell or media with only about 6% present in protein and lipid. About 14% of the ribose carbon was released as CO\(_2\). The very low level of \(^{14}C\) in lactate, which is derived from pyruvate, as well as the low concentration of glucose-6-P in sugar-free cells strongly suggested that this CO\(_2\) was derived exclusively from the oxidative arm reactions of the pentose cycle rather than from the oxidation of pyruvate carbon in the citrate cycle. From the amount of CO\(_2\) released, it was calculated that about half of the ribose-5-P derived from uridine makes one or more complete cycles back to ribose-5-P to give an oxidative arm flux of about one-half the depredation rate of uridine. Using the kinetic equations for the two dehydrogenase reactions of the pentose cycle and the measured concentrations of glucose-6-P and 6-P-glucose as well as the NADP\(^+\)/NADPH ratio, the flux through these reactions was calculated to be equal to that on 10 mM glucose growth; this flux is also one-half the rate of uridine depletion.

In these cells, uridine apparently provides all necessary functions of sugar as well as glucose does and probably better than any other sugar does.

Carbohydrates are a major component in the diets of all animals. Probably the most abundant carbohydrate subunit is the hexose sugar glucose; it is found in the sap of plants and at high concentrations (5 to 10 mM) in the blood of mammals and other animals. Usual tissue culture media contain these high levels of glucose and it is probably metabolized more rapidly by mammalian cells than is any other component of the medium, most to lactic acid (1-4). However, it also provides much of the carbon for small molecules of the cell as well as for the subunits of macromolecules and until recently had been assumed to be the major, if not the only, source of energy (2-4). It has not been known which, if any, of these functions of sugar metabolism is essential. That it is essential is shown by the disintegration of cells incubated in medium lacking only sugar.

When glucose is replaced by other hexoses or when the concentration of the supporting sugar in the medium is decreased, there is a dramatic decline in glycolytic flux in HeLa cells (about 900-fold when 2 mM fructose replaces 10 mM glucose) (1). In this case, all cell energy is derived from glutamine metabolism (1). However, the flux of sugar carbon through the oxidative arm of the pentose cycle changes much less (4- to 5-fold) and can be related to the rate of nucleic acid synthesis (5). It was concluded that the only essential function of sugar metabolism in HeLa cells is to provide ribose-P, a primary precursor for nucleic acid. Zielke et al. (6) observed a 2- to 3-fold increase in the mass of human fibroblasts cultured in a medium containing a very low concentration of glucose (17 \(\mu\)M) when it was supplemented with glycine and a mixture of purine and pyrimidine bases and nucleosides. However, the cells stopped growing, and they concluded that sugar metabolism must be necessary for other functions as well. In this paper, we report that human HeLa cells, as well as a diverse variety of other cell types, can grow indefinitely in the complete absence of sugar if the medium is supplemented with a high concentration of a pyrimidine riboside.

**EXPERIMENTAL PROCEDURES**

**Cells and Growth Conditions**—In all experiments, cells were grown as monolayers in T-flasks as described (1). All media were specially prepared by Kansas City Biologicals with antibiotics but lacking NaHCO\(_3\), glutamine, and glucose. NaHCO\(_3\) was added to give 2.5 g/
litter and glutamine was added to give 4 mM at the time the media were prepared for growth. Sugar or nucleosides were added as described earlier (5). Dialyzed fetal calf serum (Gibco) or dialyzed bovine calf serum (Kansas City Biologicals) was used only if it contained <200 µM glucose (<10 µM in final medium containing 5% serum) and supported growth with uridine in place of sugar. Different lots of dialyzed sera gave somewhat different growth rates on uridine, and 1 lot out of more than 10 gave no growth of HeLa cells on uridine or fructose, although growth on non-glucose sugar substrates was normal. This suggests a nondialyzable serum component that may be important when glyco-
lytic activity is low as is the case on fructose (1) or uridine ("Results").

Prior to 1979, all studies were made with media containing DFC, but as it became increasingly unavailable, it was replaced by DBC. There are no fundamental growth differences between these sera in terms of any major conclusions of this paper, and the results with one or the other are used interchangeably as noted in the text. A significant advantage of DBC over DFC (besides cost) is the absence of uridine phosphorylase activity in the former with measurable activity in DFC.

HeLa cells were grown in Joklik's modified minimum essential medium described earlier (5) with either 5% DFC or 5% DBC. Mouse L-cells were grown in Eagle's MEM, Earle's base, with 5% DBC. Normal human diploid fibroblast, strains GM37 and GM42 (from The Human Genetic Mutant Cell Repository, Camden, NJ) were grown in Eagle's base, with 5% DBC. Normal human diploid fibroblasts, strains GM37 and GM42 (from The Human Genetic Mutant Cell Repository, Camden, NJ) were grown in Eagle's base, with 5% DBC. Normal human diploid fibroblasts, strains GM37 and GM42 (from The Human Genetic Mutant Cell Repository, Camden, NJ) were grown in Eagle's base, with 5% DFC. Rat hepatoma strain NISI-67 (kindly provided by P. G. W. Plagemann) was adapted to monolayer growth by repeated subculture in Eagle's MEM, Earle's base, and 15 mM glucose or 10 mM uridine plus 5% DBC. 4-[(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (20 mM) buffer plus 6 mM glutamine were added for growth.

Mouse L-cells, strain NCTC 2071 (ATCC CCL 1.1) (kindly provided by Dr. D. Silber) had been derived from strain L-299 to grow in various serum-free media (8). The most simple medium reported (NCTC medium 126) was still very complex (8). We have been able to eliminate 12 amino acid compounds including d-glucosamine (a possible energy source), as well as d-glutamine, deoxyuridylates, ribo-
gluconolactone (a possible energy source), and acetate. The one essential component needed to supplement Eagle's MEM, Earle's base, was choline chloride and it was added to 0.33 mM. Cells were maintained in MEM plus 5% DBC and were first adapted to serum-
free growth for several generations in the richer Higuchi medium (9) (no serum) before growth in Eagle's MEM, Earle's base (no serum). T-flask cultures were split after washing twice with phosphate-
buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM NaHPO4, pH 7.3) followed by treatment with 1 to 4 ml (depending on cell type) of trypsin (EC 3.4.21.4) (0.05%) plus EDTA (0.02%) (no serum) before growth in Eagle's MEM, Earle's base, and 15 mM glucose or 10 mM uridine plus 5% DBC. 4-[(2-Hydroxyethyl)-1-piperazineeth-
anesulfonic acid (20 mM) buffer plus 6 mM glutamine were added for growth.

Mouse L-cells, strain NCTC 2071 (ATCC CCL 1.1) (kindly provided by Dr. D. Silber) had been derived from strain L-299 to grow in various serum-free media (8). The most simple medium reported (NCTC medium 126) was still very complex (8). We have been able to eliminate 12 amino acid compounds including d-glucosamine (a possible energy source), as well as d-glutamine, deoxyuridylates, ribo-
gluconolactone (a possible energy source), and acetate. The one essential component needed to supplement Eagle's MEM, Earle's base, was choline chloride and it was added to 0.33 mM. Cells were maintained in MEM plus 5% DBC and were first adapted to serum-
free growth for several generations in the richer Higuchi medium (9) (no serum) before growth in Eagle's MEM, Earle's base (no serum). T-flask cultures were split after washing twice with phosphate-
buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM NaHPO4, pH 7.3) followed by treatment with 1 to 4 ml (depending on cell type) of trypsin (EC 3.4.21.4) (0.05%) plus EDTA (0.02%) (no serum) before growth in Eagle's MEM, Earle's base, and 15 mM glucose or 10 mM uridine plus 5% DBC. 4-[(2-Hydroxyethyl)-1-piperazineeth-
anesulfonic acid (20 mM) buffer plus 6 mM glutamine were added for growth.

The Fate of Uridine—HeLa cells were pregrown for several generations in 75 cm² T-flasks containing about 20 ml of sugar free medium supplemented with 2 mM uridine. About 16 h before the start of the measurements, the cells were washed twice with phosphate-
buffere d buffer and split to give about 10 ml to each of a sufficient number of 25-cm² T-flasks (Costar). The next morning, the media were removed by aspiration and replaced with fresh media containing 2 mM uridine (or as indicated) and [U-14C]uridine at 0.2 µCi/ml or [2-14C]uridine at 0.1 µCi/ml. CO2 traps were inserted in flasks designated for [14C] CO2 measurement as described (1). Other T-
flasks were used for acid extractions. Media samples were taken to measure contents of [14C]uridine, [14C]uracil, [14C]lactic acid, and other compounds.

[14C]Uridine, [14C]uracil, and [14C]lactate concentrations were measured by applying 5 µl of medium to Gelman ITLC-SA sheets (20 X 1.5 cm) and separated by ascending chromatography with 1-butanol/glacial acetic acid (50/50). The approximate Rf values were 0.70, 0.58, and 0.40 for lactate uracil, and uridine, respectively. After drying, the sheets were dipped in 0.4% 2,5-diphenyloxazole in 2-methylph-
ol and dried and then spots were detected by fluorography after exposure of Kodak XR-5 film at ~80 °C (9). The sheets were hung up to allow the 2-methylphenylthiazole to sublime and spots were cut out and eluted for counting.

Before acid extraction, the cells were washed twice with phos-
phate-buffered saline (0 °C). They were then covered with 0.1 M perchloric acid or 0.1 M perchloric acid (1 ml EDTA) at 0 °C and the remains were scraped off and the contents transferred to a 12 ml centrifuge tube. The flask was rinsed with acid, the rinse was added to the centrifuge tube, and after 30 min at 0 °C the tube was cen-
trifuged. The precipitate was washed and resuspended in 5% trichlo-acetic acid and heated at 90 °C for 30 min and centrifuged to extract nucleic acids.

The 0 °C perchloric acid precipitate could be treated with 0.3 N KOH for separation of the RNA nucleotides as described (5). Radio-
activity in a precipitate was measured by collection on glass fiber filters and washing with acid and ethanol before drying. The precip-
itate was solubilized in Protosol (New England Nuclear) and counted using an aqueous-tolerant scintillation mixture (10). In order to cor-
rect for relative counting efficiencies between various fractions, an aliquot of the original medium was always counted under the same conditions and used to correct for efficiencies, e.g. spotted onto a glass fiber filter before Protosol treatment to calculate the fraction of counts incorporated into the precipitate.

Cell-free Activities toward Uridine—In all experiments that fol-
lowed uridine metabolism, parallel T-flasks were incubated without cells. With DFC but not with DBC, there was a noncellular compo-
nent of uridine breakdown due to serum activity with production of uracil plus ribose-1-P that indicated a high activity of pyrimidine nucleoside phosphorylase (EC 2.4.2.2) (Fig. 1). The urine depleted and uracil accumulated without cells (about 20% of the cell activity) were subtracted from the corresponding values with cells at each sampling time to give the changes due to cell metabolism. Most of the data presented here on the fate of uridine carbon were measured in cultures growing on DBC serum so that this correction was not necessary.

Since uridine phosphorylase is a mycoplasma enzyme, the sensitive and reliable assay of Sauer and Schneider et al. (11) was used to show the absence of mycoplasma contamination. A uridine/uracil incorporation ratio of 500/1 was observed indicating no mycoplasma cells.

Assay for Major Components—Ribose was assayed by the proce-
dure of Meijbaum (12) and DNA was assayed by the method of Ceriotti (13). The standards were ribose or DNA, respectively. The measurements of protein, accumulated [14C]CO2, and radioactivity have been described (1). All chemicals were reagent grade. Radioactive compounds were [5,6-3H]uridine, 40 Ci/mmol; [U-14C]uridine, 400 Ci/mmol; [U-14C]uracil, 250 µCi/0.11 mg from New England Nu-
clear; [2-14C]uridine, 65 mCi/mmol; [1-14C]glycine, 55 mCi/mmol from ICN; [U-14C]ribose, 260 mCi/mmol from Research Products International Corp.; and [6-3H]ribose, 9.6 Ci/mmol (no longer available from stock) from Amersham/Searle.

RESULTS

Growth in the Absence of Sugar—HeLa cells stop growing and start to disintegrate in normal growth medium lacking sugar. However, the same medium, supplemented with the four ribosides (≥1 mM each), supported growth at a rate similar to that observed in the normal medium containing 10 mM glucose. In fact, not all four nucleosides were necessary, and the cells grew almost as fast in a medium containing only uridine or cytidine in place of sugar (Fig. 2). With serial transfers, we have maintained HeLa cells at this same growth rate indefinitely (>20 generations) on either pyrimidine ribosi-
de. The deoxynucleosides did not support growth either in combination or alone.

The purine nucleosides adenosine or guanosine did not support growth of HeLa cells. However, inosine or xanthosine at appropriate concentrations were able to promote some growth. As noted in "Experimental Procedures," ribose-P, Pursuant to accumulation in cell-free medium containing DFC, presumably as a result of a serum phosphorylase activity acting on uridine and P1. Also, uracil is a major product of the

1 The abbreviations used are: DFC, dialyzed fetal calf serum; DBC, dialyzed bovine calf serum; MEM, minimum essential medium.

2 Unpublished observations.
**Life without Exogenous Sugar**

**Fig. 1. Outline of the major HeLa cell metabolic pathways considered in this paper.** The superscripts on the CO2 refer to the carbon number of the hexose. If after releasing the 1-carbon in the pentose cycle the remaining 5-carbon moiety was randomized to the 1, 2, or 3 positions of the regenerated glucose-6-P in the reactions of the nonoxidative arm (22). The three known routes of uridine metabolism are shown: 1) uridine kinase converts uridine to uridine-5'-P and uracil phosphoribosyltransferase converts UMP to α-D-5-P-riboosyl-P1 (PRPP) plus uracil; 2) uridine nucleosidase favors formation of uracil plus ribose and ribose can be phosphorylated with ribokinase (EC 2.7.1.15); and 3) uridine phosphorylase activity would produce uracil plus ribose-1-P. Further details are presented in Fig. 1 of Reitzer et al. (1). Fru-1,6-P, fructose 1,6-bisphosphate; GAP, glyceraldehyde-3-P; DHAP, dihydroxyacetone-P; X-5-P, xylulose-5-P; Sed-7-P, sedoheptulose-7-P.

---

**Fig. 2. HeLa cell growth in sugar-free media (with 5% dialyzed calf serum).** Cells were pregrown in 75-cm² T-flasks in sugar-free medium containing 10 mM uridine for several generations. Equal cell aliquots were dispensed to 25-cm² T-flasks containing the same medium. The next morning the cells were washed twice with 10 ml of phosphate-buffered saline, refed with the designated media, and incubated at 37°C (time zero). a, each point represents the cell number or amount of RNA, DNA, or protein in a T-flask harvested at the indicated time. Cell numbers were determined by hemocytometer count with trypan blue. The RNA-ribose has not been corrected to total RNA which accounts for the apparently lower RNA than DNA. O, 10 mM glucose; Δ, 5 mM uridine; □, no sugar or nucleoside. The latter cultures had nondetectable amounts of any components or cell numbers at the last sampling time. b and c, protein/flask as a function of nucleoside supplement replacing glucose. The specific supplements are noted for each curve. Glucose, 10 mM (O); guanosine, 1 mM (□); deoxyuridine (●) and adenosine (◆); inosine, 1 mM (▲) or 10 mM (●); xanthosine, 1 mM (▲) or 10 mM (●); uridine, 2 mM (▲) or 10 mM (●); no sugar or nucleoside (□). Deoxycytidine and thymidine gave curves identical with deoxyuridine.

---

The Fate of Uridine Carbon—Two nanomoles of uridine were depleted from sugar-free medium/min/mg of protein. This rate can be compared to the rates of utilization of sugars measured earlier (1). Even in normal tissue culture medium containing 10 mM glucose, the copious rate of sugar metabolism is only about 5 times faster. On 2 mM fructose, about 10 times fewer molecules of sugar are metabolized/min/mg of protein than are uridine molecules in sugar-free medium; the cells grow almost twice as fast on uridine as they do on 2 mM fructose.

Very little of the uracil moiety of uridine was incorporated into cell components. Only the pyrimidine residues of RNA contained uracil carbon while all four nucleotides were labeled from [U-14C]uridine (Fig. 3). However, about 6% of the 14C from both [U-14C]uridine as well as [2-14C]uridine was incorporated into pyrimidine nucleotides ("Discussion"). The very slow labeling of the AMP residues must result from a large cell metabolism of uridine (see below). Neither ribose-1-P nor uracil together or separately could support HeLa cell growth in sugar-free media. As noted, the test for mycoplasma itself showed that uracil incorporation into cells was barely measurable.
pool of adenosine compounds in the cell which requires a long time to saturate.

The fates of uracil and ribose carbons from uridine were studied further by measuring \(^{14}C\) in acid-extracted fractions of cells that had been growing in either [2-\(^{14}C\)]uridine (uracil label) or [U-\(^{14}C\)]uridine (Fig. 4). The total \(^{14}C\) retained by whole cells was accounted for by the \(^{14}C\) recovered in the cold soluble plus precipitable fractions. The macromolecular components accumulated \(^{14}C\) continuously and at a constant rate which paralleled cell growth. The cold acid-soluble fraction (small molecules) accumulated \(^{14}C\) at a decreasing rate, as expected for a more rapidly saturated compartment. It was noted earlier that a large fraction of metabolized ribose carbon is found in this cold acid-soluble fraction of cells growing on either glucose or fructose (5). Its relative size was the same in cells growing on uridine, 60 to 80% of the ribose mass in total precipitable RNA. Carbon from the uracil moiety was found to a lesser extent fractionally in this small molecule pool and not at all in the hot acid precipitate or in CO\(_2\). Besides the small molecules in the cell, uracil carbon was only found in the nucleic acid fraction and in the medium. However, ribose carbon was found in all fractions including a significant amount in CO\(_2\) ("Discussion"). The fraction of CO\(_2\) from exogenous [U-\(^{14}C\)]ribose, relative to incorporation into whole cells, was about twice that from [U-\(^{14}C\)]uridine. This would be expected since the ribose moiety contains about half (½) the total uridine carbon.

The curves in Fig. 4 were not normalized to uridine-depleted. When this was done, only 20 to 30% of the depleted uridine could be accounted for in cellular components with only 10 to 15% found in nucleic acids. This is shown in Table I in which all products are normalized to uridine depletion. Since only a minor fraction of the uridine carbon was being used for nucleic acids, we attempted to determine its other metabolic products. Exogenous uridine at 0.4 mM gave a larger and thus more accurate measurement of fractional depletion, but the results were similar with 2 mM. The exact fraction of ribose carbon that ends in any component can be calculated from the fractions of \(^{14}C\) that are derived from the utilized [2-\(^{14}C\)]uridine and [U-\(^{14}C\)]uridine. If \(X\) is the percentage of \(^{14}C\) from [U-\(^{14}C\)]uridine that is found in a component and \(Y\) that from the [2-\(^{14}C\)]uridine, the percentage of utilized ribose carbon (\(R\)) from uridine in that component is

\[
R = \left( X - \frac{4}{9} \right) \left( \frac{9}{5} \right)
\]

Using Equation 1, the values for the fraction of ribose carbon incorporated into each component in Table I has been derived; the values of \(R\) from the incorporation rates in 2 mM uridine cultures are in good agreement but not shown. Only about 23% of the utilized uridine ribose carbon was incorporated into nucleic acids. Thus, there was a 4- to 5-fold excess of ribose produced from uridine beyond that found in the nucleic acids.

While 80 to 90% of the uracil carbon was released to the medium as free uracil, a large fraction of the ribose carbon (about 25%) was also lost to the medium, and none of it was in uracil. Most of it could be resolved into three components which have yet to be identified. The values in the "Total" column (Table I) for \(^{14}C\) in the medium differ from the sum of the \(^{14}C\) in uracil plus nonuracil compounds because they include \(^{14}C\) in the medium which has not been resolved as discrete compounds with our thin layer chromatography separations.

About twice the fractional amount of \(^{14}C\) from [U-\(^{14}C\)]uridine than that from [2-\(^{14}C\)]uridine was incorporated into nucleic acids. This is consistent with most or all of the nucleic
acid pyrimidines being derived from incorporation of the intact uridine, i.e. with little or no de novo pyrimidine synthesis ("Discussion"). About 5% of the ribose carbon and very little of the uracil were present in the hot precipitate which is mostly protein with some lipid and polysaccharide. Finally, 12 to 15% of the ribose carbon (and insignificant uracil carbon) was released as CO₂. With DFC, this fraction was even higher (13% of the uridine or 24% of the ribose carbon; data not shown). Of the [¹⁴C]uridine depleted, the total ¹⁴C recovered in cells, media, or CO₂ was between 87 and 104% in the two media.

A compound prominent in the media of sugar-grown cells is lactic acid, an indicator of glycolytic activity. We would have detected [¹⁴C]lactate by thin layer chromatography if as much as 0.5% of the depleted [U-¹⁴C]uridine were converted to it. The compounds in the medium containing ribose or uracil carbon do not include lactate, indicating very little pyruvate from uridine in uridine-grown cells.

Purine Biosynthesis—The preceding results showed that the purine nucleotides of RNA contained carbon only from the ribose moiety of uridine. The normal pathway of purine biosynthesis uses α-D-5-P-ribosyl-P₇ derived from ribose-5-P. That the cells are using the normal de novo pathways for purine nucleotide biosynthesis was shown by the following experiment. In early steps subsequent to the synthesis of α-D-5-P-ribosyl-P₇, glycine and formate residues are incorporated into the growing skeleton of the purine nucleotide. The incorporation of the exogenously supplied precursors is a measure of the activity of this pathway. In uridine-supported HeLa cells, the ratio of formate incorporation into nucleic acids/leucine incorporation into protein was virtually identical with that in sugar-growing cells (Fig. 5). This incorporation of formate was completely dependent upon the presence of either sugar or uridine in the medium. At the same time, protein synthesis, as measured by leucine incorporation, was not affected by their absence during the period of this experiment.

Concentrations of Key Metabolites in Cells Growing without Sugar—The flux of carbon through glycolysis is about 900 times lower in HeLa cells growing on 2 mM fructose than it is with 10 mM glucose (1). The cells continue to grow exponentially with all energy derived from glutamine oxidation. As is the case in 2 mM fructose cells, the level of ATP was maintained at about the same level as it is in normal (10 mM glucose) media (Table II). In these cells, relative flux rates between glycolysis and the pentose cycle are probably determined by the relative concentrations of the common starting intermediate, glucose-6-P (5). In 10 mM glucose, it was about 25 times its level in 2 mM fructose media, in agreement with earlier measurements (5). In uridine-supported cultures also, glucose-6-P is much lower than is its level in 10 mM glucose.

---

**Table I**

The distribution of metabolized uridine carbons in cells, medium, and CO₂

HeLa cells were grown in sugar-free medium containing either [2-¹⁴C]uridine or [U-¹⁴C]uridine at 0.4 mM or 2 mM concentration as indicated. Media samples were taken at 24 h and the cells were washed and extracted in 5% trichloroacetic acid (0°C) as described.

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Cells</th>
<th>5% trichloroacetic acid fractions</th>
<th>CO₂ Total recovereda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uracil</td>
<td>Nonuracil</td>
<td>Total*</td>
</tr>
<tr>
<td>2-¹⁴C (0.4 mM)</td>
<td>85.5</td>
<td>2.6</td>
<td>92.2</td>
</tr>
<tr>
<td>U-¹⁴C (0.4 mM)</td>
<td>37.0</td>
<td>12.4</td>
<td>56.8</td>
</tr>
<tr>
<td>Uridine-riboseb</td>
<td>0</td>
<td>28.5</td>
<td>28.5</td>
</tr>
<tr>
<td>2-¹⁴C (2 mM)</td>
<td>87.0</td>
<td>&lt;0.5</td>
<td>78.1</td>
</tr>
<tr>
<td>U-¹⁴C (2 mM)</td>
<td>36.1</td>
<td>10.6</td>
<td>64.3</td>
</tr>
</tbody>
</table>

* The uracil and nonuracil values were ¹⁴C in discrete spots (24 h) detected by thin layer chromatography. The "Total" column gives the total nonuridine carbon (t) when the uridine would have been completely exhausted. It was calculated from the following: if y = total ¹⁴C in the medium at the sampling time (24 h) and u = the ¹⁴C in uridine at 24 h, then (y - u)/(100-y) = t where all values can be expressed as a percentage of the initial ¹⁴C uridine counts per min. For this experiment, u = 40.0% for 0.4 mM uridine cultures and u = 73.1% for 2 mM uridine. For the 0.4 mM cultures, y = 95.3% for [2-¹⁴C]uridine and 74.1% for [U-¹⁴C]uridine, while the 2 mM were 94.1% for [2-¹⁴C] and 90.4% for [U-¹⁴C].

This total is noticeably higher than the sum of the uracil + nonuracil values for [U-¹⁴C]uridine because there were unresolved compounds containing ribose carbon in the medium (see text).

b The uridine-ribose line gives the percentage of the carbon from the ribose moiety that ends in each component. It is calculated from the values for total [2-¹⁴C]uridine and [U-¹⁴C]uridine in the preceding lines (see Equation 1 in text).
cells (Table II) (about the same as in 10 mM fructose). The concentration of the second intermediate in the oxidative arm of the pentose cycle, 6-P-glucuronate, is also very low and close to its level in 2 mM fructose-growing cells. However, as is the case with 2 mM fructose cells, the levels of xylulose-5-P (probably in equilibrium with ribose-5-P) in uridine cells are only 3-fold lower than they are in glucose cells.

The flux of carbon through the oxidative arm is a function of the hexose-P substrate concentrations and to the concentrations of the coenzymes NADP\(^+\) and NADPH (5). The effective ratio of NADPH/NADP\(^+\) was significantly lower (about 3-fold) in uridine-grown compared to sugar-grown cells (Table II).

**Growth of Other Vertebrate Cells in the Complete Absence of Sugar**—HeLa cells were derived from a cervical carcinoma and have been cultured for many years. It is possible that the metabolic capacity to grow without sugar is not shared by more differentiated cell lines or by other species of vertebrate cells. Fig. 6 shows the growth of another undifferentiated cell line, this one derived from mouse (d) as well as two primary lines of fibroblasts. The chick embryo fibroblasts were cultured directly from embryos and show that the capacity to grow without sugar extends to avian cells. Like them, the normal human fibroblasts (in passages 5 to 15) also had the typical morphology of differentiated fibroblasts, gave a continually decreasing rate of growth as a function of cell density, and had a finite life span of about 20 passages on uridine as well as sugar. They were able to grow for many passages in the absence of sugar, although as opposed to all other cell lines tested, these cells did grow at a somewhat slower rate on uridine compared to glucose. Note that the uridine concentration must be above a minimal level to support growth; 100 \(\mu M\) was not sufficient. This latter observation accounts for the earlier failure to observe more than a marginal mass increase of human fibroblasts incubated in sugar-free medium supplemented with nucleosides (6). Other human fibroblast strains, as well as rat hepatoma cells, have been cultured as monolayers on uridine in place of sugar (not shown). The capacity to grow on specific purine nucleosides in place of sugar is somewhat cell-specific. Of those tested, only human fibroblast strains GM37 (Fig. 6) and GM412 could also grow on 10 mM inosine ("Discussion").

The only strain of many tested that could not grow on uridine in place of sugar was an SV40-transformed human fibroblast (GM637); however, this strain could not grow on fructose either, implying a defect in metabolism other than the production of ribose-P from uridine.

The only undefined components of the tissue culture media are those in the dialyzed serum. That one of these is not substituting for sugar was shown by the death of cells when incubated in media lacking sugar or uridine (Figs. 2 and 6). Mouse strain NCTC 2071 could be grown with uridine in place of sugar in a completely defined medium lacking serum (Fig. 6, e).

**DISCUSSION**

**Growth on Nucleosides Is Specific**—Nearly all vertebrate cell lines examined could grow on uridine or cytidine in place of sugar, while HeLa did not grow on any deoxyribonucleosides, adenosine, or guanosine and minimal growth, or only maintenance, was observed with 1 mM or 10 mM xanthosine.

---

**TABLE II**

Concentrations of intermediates in the pentose cycle and the cytoplasmic NADPH/NADP\(^+\) ratio in HeLa cells as a function of exogenous sugar or uridine in place of sugar

<table>
<thead>
<tr>
<th>Sugar (10 mM)</th>
<th>Fructose (10 mM)</th>
<th>Fructose (20 mM)</th>
<th>Uridine (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>416 ± 38</td>
<td>49.0 ± 7.6</td>
<td>17.2 ± 1.9</td>
</tr>
<tr>
<td>6-Phosphogluconate</td>
<td>272 ± 18</td>
<td>41.9 ± 6.3</td>
<td>17.5 ± 2.1</td>
</tr>
<tr>
<td>Xylulose-5-P</td>
<td>75.2 ± 8.7</td>
<td>—</td>
<td>24.0 ± 1.9</td>
</tr>
<tr>
<td>NADPH/-</td>
<td>32.2 ± 4.2</td>
<td>—</td>
<td>11.7 ± 1.7</td>
</tr>
<tr>
<td>ATP</td>
<td>4500 ± 320</td>
<td>—</td>
<td>5460 ± 230</td>
</tr>
</tbody>
</table>

*— not determined.

---

**Fig. 6. Growth of cultured vertebrate cells in media containing nucleosides in place of sugar.** Cells were pregrown in 10 mM uridine (Urid) except the inosine (Inos) cultures which were pregrown on 1 mM inosine before dispensing to 25-cm² T-flasks. Each time point gives the amount/flask. Uridine was 10 mM except as indicated. Human diploid fibroblasts, strain GM37 (protein in a), RNA in b, and mouse L-292 cells (d) were pregrown as described in "Experimental Procedures." DCS, 5% dialyzed calf serum, None, no added sugar or nucleoside.

**Fig. 2. Chick embryo fibroblasts (c) were prepared from embryos ("Experimental Procedures") and grown for 2 days in medium containing glucose before washing twice and subculturing to the indicated media. Mouse L-cell, strain NCTC 2071, growth in serum-free media is shown in e. This strain had been pregrown as described under "Experimental Procedures." DCS, 5% dialyzed calf serum, None, no added sugar or nucleoside.
Inosine supported good growth at 1 mM but was non-supportive at a 10 mM concentration (Fig. 2). However, several lines of human diploid fibroblasts grew on either 1 mM or 10 mM inosine. Other nucleosides were not tested to support growth except with HeLa cells. Why do some nucleosides support growth while others do not? It is not surprising that deoxyribosides cannot do so since the purine and pyrimidine deoxyriboside diphosphates are synthesized in the complex and irreversible reductase reactions from the corresponding riboside nucleotides. Furthermore, thymidine-5'-P is synthesized in another complex and irreversible reaction utilizing a tetrahydrofolic derivative. Thus, the cells cannot grow because they have no known mechanism to synthesize the RNA nucleotides from the deoxynucleosides.

It is not so clear why most purine nucleosides cannot support growth; they do enter readily (14). Adenosine itself is toxic to mammalian cells; however, it is probably deaminated rapidly in the medium to inosine (15). Thus, the suitability of a specific nucleoside to replace sugar in the growth medium can differ from cell to cell. Since on a purine nucleoside, α-D-5-P-ribose-P or ribose-5-P would have to be synthesized for pyrimidine synthesis, just as is the case for purine synthesis when growth is on uridine, it is possible that the activities of these initial reactions of nucleoside metabolism to generate α-D-5-P-ribose-P or ribose-5-P would determine if a particular nucleoside can support growth. Alternatively, a purine riboside could inhibit pyrimidine biosynthesis. Until these reactions are identified (Fig. 1), it will not be possible to understand the basis for nucleoside specificity in supporting sugar-free growth.

Pyrimidine Biosynthesis May Be Shut Off—The normal de novo pathway of pyrimidine biosynthesis starts from aspartate plus carbamoyl-P to generate the pyrimidine base structure. The ribose-P moiety is then added to this structure. The former compounds must be derived from unlabeled amino acids in the medium (particularly glutamine and arginine) while the ribose is derived from the exogenous uridine. Thus, if the only synthesis were by this pathway, then no 14C should be incorporated into RNA from [2-14C]uridine but there would be incorporation from [U-14C]uridine. While the synthesis was inoperative and all incorporation were from the intact uridine, then the fractions of 14C incorporated into pyrimidine nucleotides from [2-14C]uridine and [U-14C]uridine would be equal. Within the limits of experimental error, the latter pattern was observed, i.e., in both cases 6% of the 14C was recovered as RNA pyrimidines.

The Origin of CO2 from Uridine—Since almost 90% of the metabolized uridine released its uracil moiety and only about 23% of its ribose carbon was found in nucleic acids (Table I), a large excess of ribose is produced beyond its obvious function as a precursor for nucleic acids. A significant fraction of the ribose carbon was recovered as CO2 (about 13% in DBC and 24% in DFC). Identifying the origins of CO2 from uridine metabolism would help clarify cell metabolism in this sugar-free medium. There are two distinct pathways for its evolution: 1) release of CO2 in the entry into and subsequent oxidation of pyruvate carbon in the citrate cycle and 2) release of the hexose 1-carbon to CO2 in the oxidative arm of the pentose cycle (Fig. 1).

The observations suggest that virtually all the CO2 is derived from the latter pentose cycle reactions for the following reasons. 1) Pyruvate is not even produced from uridine metabolism as evidenced by the absence of detectable lactate from uridine. For comparison, note that, on 2 mM fructose, almost all hexose carbon passes through the pentose cycle and glycolytic flux is 900 times lower than on 10 mM glucose; even then some lactate from fructose can be measured (1). This difference in flux patterns can be correlated with the greatly reduced level of glucose-6-P in fructose cells; the level of this key substrate is similarly low in uridine cultures (Table II). However, lactate continued to be produced from pyruvate carbon but it was derived from glutamine; as in sugar-supported cells, about 15% of the glutamine carbon was found in lactate acid (not shown). All energy in fructose or uridine cultures is derived from aerobic oxidation of glutamine carbon with no energy from glycolysis (1). ATP levels are maintained even in the absence of both sugar and nucleoside but only in the presence of O2 (1). Note that, even on 10 mM glucose in which 85% of the hexose carbon becomes pyruvate, about two-thirds of all the CO2 from glucose is derived from the 1-carbon in the pentose cycle (1). Thus, although more direct evidence might be possible, it seems unlikely that any CO2 from uridine could come from the citrate cycle.

2) In contrast, when the measured reactant concentrations (Table II) are inserted into the equations derived for both dehydrogenases of the oxidative arm (20, 21), the calculated flux values can account for all the CO2 from uridine; these equations had given flux values that agreed with the in vivo rates from CO2 evolution on fructose- or glucose-grown cells (5). For the glucose-6-P dehydrogenase reaction, the calculated reaction rates were virtually identical in uridine and glucose-grown cultures. The flow is very sensitive to the NADPH/NADP ratio (16–19).

This calculated pentose cycle flux can be compared to the total uridine metabolism in the cell. The measured in vivo flux (from CO2) in glucose-growing cultures is 0.9 nmol/min/mg of protein (5). Since the rate calculated above is approximately the same in uridine-growing cells and since 2 nmol of uridine are depleted/min/mg of protein, this means that close to half as many molecules of uridine ribose are flowing through the oxidative arm reactions as the total uridine metabolized at any instant. This represents an extremely high flux through these reactions, sufficient to account for all the CO2 from uridine ribonucleotides.

Carbon Probably Recycles in the Pentose Cycle—If all CO2 is derived from the pentose cycle reactions, it is probable that ribose carbon cycles around the cycle and the extent of recycling can be estimated. For every CO2 molecule released, 5 ribose carbons must complete the cycle back to ribose-5-P. Thus, consider a given 100 ribose-5-P carbons from uridine; during their metabolic life, a total of 75 (5 × 15% to CO2) return to ribose-5-P (some of these will have made the cycle more than one time). Each ribose-5-P molecule has the same probability as does any other ribose-5-P molecule of ending in any given component shown in Table I, e.g., nucleic acids (more specifically, a specific carbon of the ribose-5-P does, but for this argument it is just as well to consider the average of the 5 carbons). However, only 100 ribose-5-P carbons can possibly end in products from 100 at the start. Thus, a total of 175 ribose-5-P carbons will exist during the population’s lifetime, and in the first round 100/175 (57%) of the 100 molecules will end in final products in the same proportions as measured, but almost half (43%) will cycle their ribose back to ribose-5-P. In the next round, 0.43 × 43 or 19% of these molecules would recycle to ribose-5-P.

This estimated recycling can be used to calculate a flux rate through the oxidative arm and this value can then be com-
pared to that derived in the preceding section. Since 90% of the metabolized uridine (2 nmol/min/mg of protein) loses its uracil moiety, 90% of the utilized uridine enters metabolism as a ribose-P (Fig. 1). Since 43% of the ribose-5-P carbon is passing up the oxidative arm at any instant, the flux through this arm is (0.43)/(0.9)(2 nmol/min/mg of protein) or 0.77 mmol/min/mg of protein calculated in the preceding section from the kinetic equations. These values were derived from entirely independent measurements and their agreement is remarkable and provides further independent evidence that the CO₂ production is probably entirely from the pentose cycle reactions.

In HeLa cells growing on 10 mM glucose or 2 mM fructose, there is little, if any, recycling of pentose carbon around the pentose cycle (5). On 2 mM fructose, it does not recycle because ribose-5-P synthesis is limiting for the needs of nucleic acids and all of it is diverted to this product. On 10 mM glucose, excess ribose-5-P is generated and the excess (about %) returns down the nonoxidative arm. But, when it returns as fructose-6-P plus glyceraldehyde-3-P, these molecules become a part of very high levels of all the glycolytic intermediates in cells growing on 10 mM glucose, and they are “swept” down glycolysis which accounts for 85% of the flow of hexose carbon (5). Uridine growth, reported here, represents a third carbon (5). Uridine growth, reported here, represents a third growth. Since the only essential function of sugar in HeLa cells (besides minor flow to sugar nucleotides and other derivatives) appears to be to provide ribose-5-P for nucleic acids (5), uridine provides this function as well as glucose and better than fructose does. Ironically, its ribose moiety provides a large excess of carbon beyond its need for nucleic acids and this carbon can be found in many components and products of the cell and satisfies many other requirements for cell growth.

Acknowledgments—James G. Cushman and Dr. Jolanta Stanisz participated in several experiments.

REFERENCES