NUCLEOTIDE POOLS IN NOVIKOFF RAT HEPATOMA
CELLS GROWING IN SUSPENSION CULTURE

III. Effects of Nucleosides in Medium on Levels
of Nucleotides in Separate Nucleotide Pools for
Nuclear and Cytoplasmic RNA Synthesis

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ABSTRACT

This study was undertaken to measure the absolute levels of nucleoside pools in Novikoff
rat hepatoma cells (subline N18S-67) during growth in suspension culture in the presence
of high concentrations of various nucleosides in the medium, and to obtain further evidence
for the compartmentalization of the nucleotides in independent cytoplasmic and nuclear
pools. The levels of nucleotide pools were measured by growing the cells in medium supple-
mented with inorganic phosphate-32P. The nucleotide pool levels (mostly in the form of
triposphates) ranged from about 1 nmole of cytidine nucleotides to 8 nmole of adenosine
nucleotides per 10^6 cells. The presence of 1 mM uridine, cytidine, guanosine, or adenosine
in the medium resulted in marked increases in the intracellular levels of the corresponding
nucleoside phosphates of at least 3-4 nmole/10^6 cells. These increases were partially com-
pensated for by decreases in the levels of other nucleotides. Evidence is presented to indicate
that it is the cytoplasmic pool that expands during incubation with high concentrations of
nucleosides in the medium, whereas the nuclear pool remains constant and very small in
size. Preincubation of cells with 1 mM uridine-5H for 5.5 hr, which resulted in a threefold
increase in the total intracellular level of uridine nucleotides, had no effect on the subsequent
incorporation of uridine-14C into cellular nucleic acids in the nucleus, whether present at a
1 μM or 1 mM concentration in the medium. In contrast, the incorporation of uridine-14C
into cytoplasmic viral-specific RNA by mengovirus-infected Novikoff cells was reduced
60-70% as a result of preincubation of the cells with high concentrations of uridine-3H.
Further, within 1-2 min upon addition of 2.5 or 6.5 μM 3H-labeled uridine, cytidine, adeno-
sine, guanosine, or inosine to cultures of Novikoff rat hepatoma cells, the incorporation
of label into nucleic acids reached a constant and maximum rate, in spite of the presence of
high intracellular concentrations (0.4-3 mM) of the corresponding unlabeled nucleoside
triposphates. Marked differences were also observed in the relative incorporation of the
various nucleosides into the different nucleotides of the acid-soluble pool, and of mengovirus
RNA and cellular RNA.
INTRODUCTION

Results from previous studies with Novikoff rat hepatoma cells growing in suspension culture (10-12) have shown that the presence of uridine or adenosine in the medium at concentrations of 0.2 mM and above completely suppresses the de novo synthesis by the cells of pyrimidine and adenosine nucleotides, respectively. Further, within minutes of the addition of the nucleosides to the medium, all uridine monophosphate (UMP) and adenosine monophosphate (AMP) used for RNA synthesis are derived from the respective nucleosides in the medium. The finding that the rate of incorporation of these nucleosides into the acid-soluble nucleotide pool is appreciably greater than the rate of their incorporation into nucleic acids, even after several hours of labeling, suggested that the exposure of cells to high concentrations of nucleosides results in an expansion of the uridine and adenosine nucleotide pools, respectively. An expansion of the pools was also indicated by the finding that actinomycin-treated cells continue to incorporate the nucleosides into the nucleotide pool at an undiminished rate for several hours, in spite of the complete inhibition of RNA synthesis.

The results also indicated that most of the uridine and adenosine nucleotides derived from the respective nucleoside in the medium accumulates in a pool that is not readily available for nucleic acid synthesis in the nucleus and that the pool used for nucleic acid synthesis is very small. This conclusion was supported by the finding that upon removal of the labeled nucleosides from the medium, the transfer of label from the nucleotide pool into nucleic acids ceases within 5-10 min, in spite of the presence of a large intracellular pool of labeled uridine triphosphate (UTP) or adenosine triphosphate (ATP), respectively. Upon readdition to the cell suspension of labeled uridine or adenosine, even when added at very low concentrations, the nucleosides begin to be incorporated into nucleic acids without delay and at a constant rate predetermined by the nucleoside concentration in the medium and without affecting the fate of the nucleotides synthesized during the first labeling period. Thus, isotope equilibration between the extracellular nucleosides and the intracellular nucleotides used for nucleic acid synthesis in the nucleus is very rapid. Further, adenosine is incorporated into both guanosine monophosphate (GMP) and AMP, and uridine into both cytidine monophosphate (CMP) and UMP of RNA, though significant amounts of adenosine-derived guanosine nucleotides and of uridine-derived cytidine nucleotides do not accumulate in the cells. We suggested that most of the nucleotides of the cell are compartmentalized in the cytoplasm and, therefore, are not available for nucleic acid synthesis in the nucleus (11). This conclusion was supported by the finding that the nucleotides incorporated into mengovirus RNA, which is synthesized in the cytoplasm (16), are supplied by a different pool that is much larger than that used for cellular RNA synthesis (11).

However, the absolute sizes of the pools or their relative increase due to the presence of uridine or adenosine in the medium could not be estimated from these experiments, nor was it clear whether the expansion of the uridine and adenosine nucleotide pools has any effect on the levels of other nucleotides in the cell. In the present investigation, we determined the absolute levels of various nucleotides in exponentially growing Novikoff cells by labeling the cells for two generations with inorganic phosphate-32P (3, 20) and then separating the nucleotides of the acid-soluble pool by two-dimensional electrophoresis chromatography. We also determined the effect of the presence of high concentrations of guanosine, uridine, cytidine, inosine, and adenosine in the medium on the levels of various nucleotides in the cells. The finding that the absolute size of the UTP pool expands at least threefold during incubation of the cells with high concentrations of uridine allowed further studies on the effect of this pool expansion on the incorporation of labeled uridine from the medium into cellular RNA in the nucleus and mengovirus in the cytoplasm. The results further support the concept that the nucleotides are compartmentalized in separate, relatively independent pools in the cytoplasm and the nucleus and that only the former expands during incubation with extracellular nucleosides. Although more direct evidence is required to establish the exact location of the nucleotides in the cells, and other explanations of our data might still exist, for convenience we will refer to the different pools used for cellular and viral RNA synthesis as nuclear and cytoplasmic, respectively.
MATERIALS AND METHODS

Cell Culture

The cultivation of Novikoff rat hepatoma cells (subline N1S1-67) in suspension culture in Swim's medium 67 and the method of enumerating cells have been described previously (15, 19). Cultures were frequently tested for contamination with mycoplasma by cultivation in broth or on agar plates, with negative results. Independent tests by Dr. George K. Kenny were also negative.

Incorporation of Labeled Nucleosides or Inorganic Phosphate-\(^{32}\)P

Suspensions of cells were supplemented with \(^{3}\)H-labeled nucleosides or inorganic phosphate-\(^{32}\)P plus various unlabeled nucleosides as indicated in the appropriate experiments. Replicate 0.5-ml samples of suspension were analyzed (14) for: (a) total radioactivity associated with the cells. Two samples were centrifuged at 500 g for 2 min at 0°C, and the cells were washed in 5 ml of cold balanced salt solution (BSS, 14). Cells labeled with nucleosides-\(^{3}\)H were washed once, and those labeled with phosphate-\(^{32}\)P twice. Then the cells were suspended in 0.2 ml of 0.5 M trichloroacetic acid, and the mixture was heated at 70°C for 30 min and analyzed for radioactivity. (b) radioactivity in acid-insoluble material. Another two samples were quickly frozen in a bath of solid CO\(_2\) in ethanol. Later, the samples were thawed, mixed with perchloric acid, and the precipitate was washed repeatedly with perchloric and trichloroacetic acid as described previously (14). The washed precipitates were heated in 0.1 ml of 0.5 M trichloroacetic acid as under a and analyzed for radioactivity. The radioactivity in acid-soluble material was calculated by subtracting the radioactivity values for b from those for a. The total radioactivity in suspensions was estimated as follows: duplicate 0.1-ml samples of suspensions were mixed with 0.1 ml of 1 M trichloroacetic acid, heated at 70°C for 30 min, and analyzed for radioactivity.

Preparation of Acid-Extracts from Cells and RNA Hydrolysates

Samples of 8 ml of cell suspension were centrifuged, and the culture fluid was saved for chromatographic analysis. The cells were washed twice with BSS and then mixed with 0.3 ml of 0.5 M perchloric acid. After 30 min at 0°C, the mixture was centrifuged and the supernatant was removed. The residue was reextracted with 0.2 ml of 0.5 M perchloric acid, and the pooled supernatants were mixed at 0°C with 0.05 ml of 5 n KOH and 0.1 ml of 1 M Tris-HCl (pH 7.4). The solution was clarified of precipitated potassium perchlorate, and the supernatant fluid (cell extract) was stored at -20°C. RNA hydrolysates were prepared from the acid-extracted residue as described previously (10).

Chromatography and Electrophoresis

Acid-soluble extracts from cells labeled with \(^{3}\)H-labeled nucleosides were analyzed by ascending chromatography on 3 MM Whatman paper (10). The paper was developed with solvents 281 or 191 (10) at 30°C for 18 hr. All developed chromatograms were cut at right angles to the direction of migration into 1-cm segments, and these were rocked with 1 ml H\(_2\)O for 1 hr at 37°C, and the paper and the eluate together were analyzed for radioactivity.

Acid-extracts from \(^{32}\)P-labeled cells were analyzed by two-dimensional electrophoresis chromatography (see Fig. 2). Along with 10 µl of a solution containing 5 mg/ml each of ATP, AMP, guanosine triphosphate (GTP), GMP, cytidine triphosphate (CTP), CMP, UTP, UMP, and uridine diphosphate (UDP)-glucose, 5 µl of acid extract were spotted onto one corner of a 35 X 40 cm sheet of 3 MM Whatman paper (see Fig. 2 B). The paper was subjected to high voltage electrophoresis on a Pherograph (L. Hormuth, Heidelberg, Germany) at 65 v/cm for 90 min using a pH 5.5 buffer (0.05 M formic acid plus 0.02 M ammonium tartrate), 7. After electrophoresis, the paper was dried and then developed by ascending chromatography at 30°C for 12-14 hr with a solution composed of 4.5 M (NH\(_4\))\(_2\)SO\(_4\), 0.1 M NaHPO\(_4\), and 2% (v/v) l-propanol and adjusted to pH 6.8 with H\(_3\)PO\(_4\) (solvent 351). The positions of the nucleotide standards were located by examining the paper under ultraviolet light. Then the paper was cut into segments as indicated in Fig. 2 B, and the segments were placed directly into scintillation fluid and analyzed for radioactivity. Close to 100% of the radioactivity placed on papers was recovered in the segments, but only 50-60% of the radioactivity was recovered in the nucleotide spots. Most of the remainder of all acid extracts was associated with inorganic phosphate and another component the position of which coincided with that of phosphorylcholine (compare Fig. 2 A and B). However, most of the label could not have been in phosphorylcholine, since other experiments have shown that N1S1-67 cells in the late exponential phase contain only 2-3 nmole/10\(^6\) cells of phosphorylcholine (13). We have

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\(^{1}\)The number refers to the solvents described in the 1967 catalogue (p. 123) of Schwarz Bio Research Inc., Orangeburg, N.Y.
not identified this 32P-labeled intermediate, but a component at this position also becomes highly labeled from glucose-14C (unpublished data). Small amounts of 32P might also be associated with fructose-6-phosphate, ribose-5-phosphate, or glucose-6-phosphate (compare Fig. 2 A and B). The presence of label in deoxyribonucleotides was disregarded, since results with other mammalian cells have indicated that the levels of these nucleotides are at least one order of magnitude lower than those of the ribonucleotides (3, 20).

Nucleoside monophosphates released from RNA by alkali hydrolysis were separated by high-voltage paper electrophoresis (65 v/cm; 2.5 hr, at pH 3.5) as described previously (17).

Radioactivity Determinations

Radioactivity was determined by liquid-scintillation counting using a modified Bray's solution (15). The efficiency of counting was estimated by the external standard method. Values were corrected for background.

Materials

Materials were purchased as follows: all unlabeled nucleosides, guanosine-8-3H, adenosine-8-3H, cytidine-5-3H, uridine-2-14C, and carrier-free inorganic phosphate-32P from SchwarzBio Research Inc., Orangeburg, N.Y.; inosine-G-3H from Amer- sham-Searle Corp., Arlington Heights, Ill.; and uridine-5-3H from Dohm Products, Ltd., Hollywood, Calif. Solutions of lower radioactivity concentrations and lower specific radioactivity were prepared by addition of the appropriate unlabeled nucleosides and 0.14 M NaCl. The specific radioactivities of the nucleosides were corrected for the presence of small amounts of labeled contaminants (10). Actinomycin D was a gift from Merck, Sharp, & Dohme, Rahway, N.J.

RESULTS

Incorporation of 3H-Labeled Nucleosides by N1S1-67 Cells during Two Generations of Growth in Suspension

In a preliminary experiment, we investigated the incorporation of various nucleosides into the acid-soluble pool of the cells under experimental conditions similar to those used subsequently for estimating the nucleotide levels of the cells by labeling with inorganic phosphate-32P. Cells were grown for about two generations in the presence of 1 mm 3H-labeled uridine, cytidine, guanosine, inosine, or adenosine. At various time intervals, samples of cells were analyzed for the amounts of intracellular acid-soluble nucleotides derived from the respective nucleosides in the medium (see legend to Table I). The results were similar to those reported previously (10). The cells accumulated approximately 11 nmole/10^6 cells of nucleoside-derived, acid-soluble nucleotides when grown in the presence of 1 mm uridine, guanosine, or inosine and about 8 nmole/10^4 cells when grown with cytidine. With each of these nucleosides, the maximum intracellular nucleotide level was attained within 10-20 hr of growth. The maximum level of newly synthesized adenosine nucleotides in the cells was higher (20 nmole/10^4 cells) and was reached more rapidly than with the other nucleosides.

Chromatographic analysis of the acid-soluble material extracted from labeled cells showed that the nucleosides accumulated mainly as nucleoside triphosphates (Table I). In agreement with previous reports (10, 18), uridine was found only in uridine nucleotides, in spite of the fact that CMP and UMP of RNA became equally labeled from uridine, and inosine and adenosine accumulated as adenosine nucleotides, although both GMP and AMP of RNA became labeled from these nucleosides (Table I). The incorporated cytidine and guanosine were confined to cytidine and guanosine nucleotides, respectively, both in the nucleotide pool and in RNA (Table I). These labeling patterns were confirmed by results (not shown) from two-dimensional electrophoresis chromatography (see Fig. 2) of the acid extracts. Most of the radioactivity from each nucleotide was recovered in the corresponding nucleoside triphosphate, and little, if any, radioactivity from uridine-3H was found in acid-soluble cytidine nucleotides or from adenosine-3H or inosine-3H in guanosine nucleotides. Significant amounts of labeled nucleosides were not recovered in acid extracts from cells that had been washed with BSS before extraction.

Incorporation of Inorganic Phosphate-32P into the Acid-Soluble Pool in Presence and Absence of High Concentrations of Unlabeled Nucleosides

The experimental conditions for the experiment illustrated in Fig. 1 were about the same as those for the experiment just described, except that the
TABLE I

Chromatography of Acid-Extracts and Electrophoresis of RNA Hydrolysates from Cells Grown for 28 hr in the Presence of 1 mM of Various \(^3\)H-Labeled Nucleosides*

<table>
<thead>
<tr>
<th>Nucleoside-(^3)H</th>
<th>Acid extract</th>
<th>RNA hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>XTP</td>
</tr>
<tr>
<td>Uridine</td>
<td>18,820</td>
<td>62</td>
</tr>
<tr>
<td>Cytidine</td>
<td>13,480</td>
<td>95</td>
</tr>
<tr>
<td>Guanosine</td>
<td>15,560</td>
<td>96</td>
</tr>
<tr>
<td>Inosine</td>
<td>20,540</td>
<td>96</td>
</tr>
<tr>
<td>Adenosine</td>
<td>13,800</td>
<td>95</td>
</tr>
</tbody>
</table>

* Swim's medium 67 was inoculated with cells from an exponential phase culture to 6 \(\times\) 10\(^6\) cells/ml. Samples of the culture were supplemented with 1 mM of uridine-\(^3\)H (1.25 cpm/pmole), cytidine-\(^3\)H (1.88 cpm/pmole), guanosine-\(^3\)H (1.54 cpm/pmole), inosine-\(^3\)H (1.45 cpm/pmole), or adenosine-\(^3\)H (1.0 cpm/pmole). The suspensions were incubated on a gyratory shaker at 37°C and monitored as a function of time of incubation for cell concentration and radioactivity in intracellular acid-soluble nucleotides, as described in Materials and Methods. After 28 hr of incubation, 2 \(\times\) 10\(^7\) cells were harvested from each suspension and washed in BSS. Acid extracts were prepared from the cells and RNA hydrolysates from the residue as described under Materials and Methods.

† UDP glucose (UDP-G) refers to those components which migrate in chromatography and electrophoresis identical to authentic UDP-G (18).

§ Total radioactivity associated with nucleotides after chromatography of 50 \(\mu\)l of acid-soluble extract with solvent 28 at 30°C for 18 hr.

¶ Per cent of total radioactivity associated with individual nucleotides. XTP and XMP refer to uridine, cytidine, and guanosine nucleotides where labeled with uridine, cytidine, and guanosine, respectively, and to adenosine nucleotides where labeled with both inosine or adenosine (see 12, and Fig. 4). Less than 10% of the total radioactivity was associated with nucleoside diphosphates, and these are included in the triphosphate fraction.

‖ Total radioactivity in nucleotides after electrophoresis of 20 \(\mu\)l of RNA hydrolysate at pH 3.5, 65 v/cm for 2.5 hr.

** Per cent total radioactivity associated with individual nucleotides.

cells were grown for two generations in medium containing 1 mM unlabeled uridine, cytidine, guanosine, inosine, or adenosine plus inorganic phosphate-\(^32\)P (1 mM, 3.3 \(\mu\)Ci/ml). The suspensions were monitored for cell concentrations (Fig. 1 C), and radioactivity in total cell material (acid-soluble plus acid-insoluble) and in acid-insoluble material. The radioactivity values were converted to absolute amounts of phosphate incorporated per 10\(^6\) cells (Fig. 1 A), and the amounts of incorporated phosphate in the acid-insoluble pool per 10\(^6\) cells (Fig. 1 B) were calculated by subtracting the amounts in acid-insoluble material from the total amounts in the cells. The presence of a 1 mM concentration of the various nucleosides in the medium had no significant effect on the growth of the cells, except that guanosine caused a slight reduction in growth during the latter part of the experiment (Fig. 1 C). The nucleosides also had no significant effect on the incorporation of phosphate into total and acid-insoluble material (Fig. 1 A) and thus into the acid-soluble pool (Fig. 1 B). The values for the different suspensions did not differ significantly, and their ranges are indicated by the bars. The cells accumulated a maximum of 60-70 nmole/10\(^6\) cells of phosphate in the acid-soluble pool, and the maximum level was attained after about 8 hr of growth. About 30% of the phosphate present in the medium had been incorporated into total cell material at the end of the experiment. The presence of the nucleosides in the medium also had no effect on the labeling of RNA. The proportion of radioactivity in each nucleotide of RNA was about the same at 6, 14, and 24 hr of labeling whether the medium contained unlabeled nucleosides or not; 28.0-29.3% of the label was in CMP, 18.1-19.6% in AMP, 31.9-33.5% in GMP, and
FIGURE 1 Growth of NIS1-67 cells (Fig. 1 C) and incorporation of inorganic phosphate-32P into total cell material and acid-insoluble material (Fig. 1 A) and into the acid-soluble pool (Fig. 1 B) in the presence and absence of various unlabeled nucleosides. Swim's medium 67 supplemented with 3.3 μCi/ml of carrier-free inorganic phosphate-32P was inoculated with cells from an exponential phase culture to 8 × 10⁵ cells/ml. Samples of 50 ml of this culture were supplemented with 1 mM unlabeled uridine, cytidine, guanosine, inosine, or adenosine, or no additional nucleoside. The suspensions were incubated on a gyratory shaker at 37°C, monitored for cell concentration (Fig. 1 C), and duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in total cell material and acid-insoluble material as described under Materials and Methods. The amounts of phosphate (in nanomoles) incorporated per 10⁶ cells (Fig. 1 A) were calculated from the averages of the duplicate radioactivity values and the corresponding cell concentration of the suspension. The specific radioactivity of the phosphate was estimated by counting samples of total cell suspension and from the phosphate content of Swim's medium 67 (1 mM). Both the cell concentrations and the amounts of phosphate incorporated by the cells were about the same in all six suspensions, except that the cell concentration of the guanosine-supplemented suspension was somewhat lower than those of the other suspensions. This is indicated by the broken line in Fig. 1 C. The bars encompass the range of all other values observed. The amounts of phosphate in acid-soluble material (Fig. 1 B) were calculated by subtracting the amounts in acid-insoluble material from the total amounts associated with the cells. At 6, 14, and 24 hr of incubation, cells were collected from 8 ml of each suspension and acid-soluble extracts and RNA hydrolysates were prepared as described in Materials and Methods (see Table II and text).

19.3–20.6% in UMP. This base composition was similar to that of total stable RNA of other mammalian cells (4).

In contrast, the presence of the nucleosides in the medium had a marked effect on the composition of the nucleotide pools (Table II). Acid extracts were prepared from cells after 6, 14, and 24 hr of growth in the presence of inorganic phosphate-32P. Approximately 100% of the incorporated phosphate in the acid-soluble pool (50–70 nmole/10⁶ cells), as estimated from the data in Fig. 1 B, was recovered in the acid extracts. The acid extracts were analyzed by two-dimensional paper electrophoresis chromatography (see Materials and Methods). The general pattern of separation of the ribonucleotides, some other phosphorylated metabolites, and inorganic phosphate by this procedure is illustrated in Fig. 2 A. The nucleotides were well separated except for UMP and UDP and CMP and CDP. Since only relatively small amounts of nucleoside diphosphates accumulate in NIS1-67 cells (Table I, see Fig. 4 and references 10, 12), label in UDP and CDP was neglected. In routine analyses, 5-μl samples of acid extract were coanalyzed with 10 μl of a standard mixture of ribonucleoside mono- and...
Nucleotide Content of Cells as a Function of Time of Labeling with Phosphate-\(^{32}\)P in the Presence and Absence of Various Unlabeled Nucleosides*  

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>UXP</th>
<th>CXP</th>
<th>GXP</th>
<th>AXP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hr</td>
<td>14 hr</td>
<td>24 hr</td>
<td>6 hr</td>
<td>14 hr</td>
</tr>
<tr>
<td>None</td>
<td>3.5</td>
<td>2.7</td>
<td>2.3</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Uridine</td>
<td>7.9</td>
<td>8.7</td>
<td>6.6</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Cytidine</td>
<td>3.2</td>
<td>2.7</td>
<td>2.1</td>
<td>3.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Guanosine</td>
<td>3.6</td>
<td>1.5</td>
<td>1.3</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Inosine</td>
<td>2.8</td>
<td>1.5</td>
<td>1.3</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.2</td>
<td>1.1</td>
<td>1.2</td>
<td>0.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The details of the experiment are described in the legend to Fig. 1. At the indicated times (hr) of growth in Swim's medium supplemented with 3.3 \(\muCi/ml\) of carrier-free orthophosphate-\(^{32}\)P and a 1 mm concentration of the indicated unlabeled nucleosides, acid extracts were prepared from the cells contained in 8 ml of culture as described in Materials and Methods. Samples of 5 \(\mul\) of each acid extract were analyzed by two-dimensional electrophoresis chromatography as described in Materials and Methods (see Fig. 2). The papers were cut into segments as indicated in Fig. 2 B, and the segments were analyzed for radioactivity. The amounts of the various nucleotides per 10\(^6\) cells were calculated from the radioactivity values, the corresponding cell concentration (Fig. 1 C), and the specific radioactivity of the phosphate. The latter was estimated from the radioactivity values of samples of total cell suspension and the phosphate content of the medium (1 mm). Radioactivity in the nucleotide mono- and triphosphate segments was assumed to be associated with these nucleotides. Radioactivity in diphosphates was disregarded, and the radioactivity in the segments between CMP and CTP, GMP and GTP, and AMP and ATP (see Fig. 2 B) was added in equal portions to the radioactivity values of the appropriate mono- and triphosphate segments. All values are averages of the sums of the appropriate nucleoside mono- and triphosphates (X) from two or three electrophoretic-chromatographic separations of each acid extract. Values from duplicate analyses fell within 5-10% of each other.  

Triphosphates (see Fig. 2 B). The nucleotides were located by examining the paper under ultraviolet light, and the paper was cut into segments as indicated in Fig. 2 B and the segments were analyzed for radioactivity. Fig. 2 B illustrates the results of a typical analysis of an acid extract from cells after 14 hr of growth with phosphate-\(^{32}\)P in the medium but without added nucleosides. The total radioactivity recovered from the paper was equivalent to 10.5 nmole of phosphate, or 62 nmole/10\(^8\) cells, a value in good agreement with the levels of phosphate in the acid-soluble pool estimated by the direct analysis of the cells (Fig. 1 B). A similar good agreement of the data was obtained with the other acid extracts prepared from the cells. About 50-60% of the phosphate was recovered in the nucleotide spots and was associated mainly with the triphosphates and UDP-G as expected from the results obtained by labeling the cells with \(^{3}H\)-labeled nucleosides (Table I). Since this was the case with all acid extracts from \(^{32}\)P-labeled cells, we have summarized the results from this experiment in Table II by listing the total amounts of uridine, cytidine, guanosine, and adenosine nucleotides per 10\(^6\) cells as estimated from the radioactivity values.  

The results in Table II show the following: (a) the levels of the various nucleotides in cells grown in the absence of added nucleosides (first row) were similar to those reported for 3T3 mouse cells (20), but appreciably higher than those of chick embryo fibroblasts (3). On the basis of a total volume of 2.5 \(\mul/10^8\) cells (18), the average over-all intracellular concentrations of adenosine, uridine, guanosine, and cytidine nucleotides were about 3.2, 1.2, 0.5, and 0.4 mm, respectively. However, it has not been excluded entirely that the nucleotide spots were free of all other non-nucleotide phosphorylated intermediates. Particularly, a slight overlap of these components with UTP and CTP is possible (see Fig. 2) and the levels of the latter, therefore, might be slightly...
overestimated. The relative amounts of inorganic phosphate and of label recovered in the other upper segments and the lower part of the paper (see Fig. 2 B) were very similar for all of the acid extracts examined.

(b) The presence of 1 mM uridine, cytidine, or guanosine in the medium resulted in a three to fourfold increase in the intracellular levels of the corresponding nucleotides. The relative increase in adenosine nucleotides due to the presence of adenosine or inosine in the medium was much smaller (about 50%), but the increases in absolute amounts of the respective nucleotides (2-3 nmole/10⁶ cells) were about the same for each of the nucleosides. An increase in the adenosine nucleotide content due to the presence of adenosine or inosine in the suspension medium has also been observed with Krebs-2 ascites cells incubated in vitro (22). The absolute levels of nucleotides induced by the nucleosides in the medium estimated from this experiment were somewhat lower than those estimated by the incorporation of ³²P-labeled nucleosides. The reasons for this discrepancy are not known. It may be related to the fact that, for the analysis of the total radioactivity associated with the cells, ³²P-labeled cells were washed twice with BSS, whereas nucleoside-³H-labeled cells were washed only once. We have noted previously (10) that repeated washing of the cells by centrifugation, even at minimal force and time to sediment the cells, tends to damage some cells and thus causes the loss of their nucleotides. However, we decided to wash ³²P-labeled cells twice with BSS to ensure the removal of all extracellular phosphate which could have interfered in the separation of the nucleotides by two-dimensional electrophoresis chromatography (see Fig. 2).

(c) In spite of the marked increase in the level of specific nucleotides due to the presence of high concentrations of nucleoside in the medium, the

Figure 2: Two-dimensional electrophoresis chromatography of various nucleotides and other phosphorylated metabolites (Fig. 2A) and of a sample of acid extract from ³²P-labeled cells (Fig. 2 B). Fig. 2 A is a composite of several analyses. A total of 20 µl of solutions of various standards was placed at the origin and separated by two-dimensional electrophoresis chromatography as described under Materials and Methods, except that for the separation of nonelectric light-absorbing materials the phosphate buffer in solvent B was replaced by Tris-HCl so as to allow the subsequent localization of the organic phosphates by treating the paper with molybdate reagent as described by Zweig and Whitaker (21). The nucleotides were located by examining the paper under ultraviolet light. Fig. 2 B: The details of the experiment are described in the legend to Fig. 1; 2 µl (80,000 cpm) of acid extract prepared from cells after 14 hr of growth in the presence of inorganic phosphate-³²P without added nucleosides and 10 µl of a mixture of nucleotide standards were analyzed by two-dimensional electrophoresis chromatography as described in Materials and Methods. The standards were located by examining the paper under ultraviolet light, and the paper was then cut into segments as indicated, and these segments were analyzed for radioactivity. The radioactivity values are indicated for each segment. A total of 51,370 cpm were recovered (10,500 pmole of phosphate) or 62 pmole phosphate/10⁶ cells.
total level of nucleotides in the cells was relatively little affected (last three columns of Table II). In part, this was due to the fact that the absolute increase in the concentration of a specific nucleotide was relatively small compared to the total nucleotide concentration of the cells, but, in some cases, the increase in a specific nucleotide was compensated for by a decrease in the level of other nucleotides. For instance, the presence of guanosine, inosine, and adenosine resulted in a lowering of the levels of the uridine and cytidine nucleotides. Uridine and cytidine had no effect on the levels of the other nucleotides, but guanosine also caused a marked decrease in the level of the adenosine nucleotides. This decrease may have been due to feedback inhibition of inosine monophosphate (IMP) formation by the guanosine nucleotides (5), but it is not clear whether this decrease was related to the slight growth inhibitory effect of guanosine (Fig. 1 C). On the other hand, inosine and adenosine had no effect on the concentration of guanosine nucleotides. It is also of interest that the presence of uridine in the medium had no effect on the level of cytidine nucleotides, in spite of the fact that the level of UTP increased about threefold. Results similar to those summarized in Fig. 1 and Table II have been obtained in a second experiment of this type.

**Figure 3** Time-courses of incorporation of various $^3$H-labeled nucleosides into total cell material and into acid-insoluble material. Samples of a suspension of $2 \times 10^6$ N151-67 cells/ml of BM42 were supplemented with the following: (Fig. 3A) 6.25 µM uridine-5-$^3$H (9 cpm/pmol; ○—○, ○—○) or cytidine-5-$^3$H (14 cpm/pmol; △—△, △—△); (Fig. 3B) 2.5 µM adenosine-8-$^3$H (100 cpm/pmol; ○—○, ○—○); (Fig. 3 C) 2.5 µM guanosine-8-$^3$H (100 cpm/pmol; ○—○, ○—○) or inosine-G-$^3$H (100 cpm/pmol; △—△, △—△). The suspensions were incubated on a gyratory shaker at 37°C, and duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in total cell material (○—○, △—△) or for radioactivity in acid-insoluble material (○—○, △—△). All points represent averages of the duplicate samples. Acid extracts and RNA hydrolysates were prepared from samples of cells as described in the legend to Fig. 4.

**Relationship between Incorporation of Various Nucleosides into Nucleotide Pools and into Nucleic Acids**

One line of evidence which supports the conclusion that the uridine and adenine nucleotide pools used for cellular nucleic acid synthesis are very small, was the finding that the incorporation of uridine and adenosine into nucleic acids commences at an approximately constant rate almost immediately upon their addition to the medium.

P. G. W. Plagemann  Nucleotide Pools in Novikoff Cells. III  139
whether the nucleosides are added in concentrations of 0.5 or 100 µM (10, 11). The results in Fig. 3 illustrate that there was also no significant delay in the incorporation of other nucleosides into acid-insoluble material, even when used at relatively low concentrations (2.5 and 6.5 µM), in spite of the fact that the cells contained between 0.4 and 3 mM of the corresponding unlabeled nucleosides (Table I). The results indicate that all of these extracellular nucleosides equilibrated very rapidly with the intracellular pool of nucleoside triphosphates used for nucleic acid synthesis.

The data in Fig. 3 also show that the relative proportion of nucleosides incorporated into total cell material (acid-soluble plus acid-insoluble) and into nucleic acids varied greatly for each nucleoside. The initial rates of incorporation of uridine, cytidine, adenosine, guanosine, and inosine into the nucleotide pool were, respectively, 6.0, 3.6, 1.3, 3.4, and 7.0 times greater than the rates of their incorporation into nucleic acids. These differences may reflect differences in the relative proportion of nucleosides incorporated into the nuclear and cytoplasmic pools. The data
suggest that much smaller proportions of cytidine or guanosine are incorporated into the cytoplasmic nucleotide pool than of adenosine or uridine.

In the experiment illustrated in Fig. 3, the acid-soluble pools were extracted from samples of cells after 10 and 120 min of labeling with the various 3H-labeled nucleosides, and the acid extracts were analyzed chromatographically. Alkali-hydrolysates were prepared from the acid-washed residues, and the ribonucleotides were separated electrophoretically. In agreement with previous results (10, 12; see Table I), uridine was incorporated into both GMP and AMP of RNA (Fig. 4 B), whereas most of the label in the acid-soluble pool was in ATP whether the cells were labeled for 10 min (Fig. 4 A) or 120 min (not shown). The proportion of the inosine-label in GMP of RNA decreased to about 60% after 2 hr of labeling (not shown), and reached a relatively constant level of about 50% during long periods of incubation of cells with inosine-3H (Table I). In contrast, less than 10% of the inosine-3H incorporated into mengovirus-specific RNA which is synthesized in the cytoplasm (16) was recovered in GMP, whether the actinomycin-treated infected cells were labeled for 10 or 150 min (Table III). These results support the conclusion that the nucleotides used for cellular RNA synthesis in the nucleus are supplied by a pool that is different from the main nucleotide pool of the cells which seems to supply the nucleotides for mengovirus RNA synthesis.

Effect of Preloading Cells with Uridine Nucleotides on Subsequent Incorporation of Uridine from the Medium by Uninfected and Mengovirus-Infected Cells

Results from preliminary experiments reported previously (11) indicated that preincubation of N1S1-67 cells with high concentrations of unlabeled uridine (0.5-1 mM) had little or no effect on the subsequent rate and time-course of incorporation of labeled uridine into the nucleotide pool and into RNA, even when added at micromolar concentrations. In the meantime, we have shown that exposure of the cells to 1 mM uridine for 6 hr results in at least a threefold increase in the absolute size of the pool of uridine nucleotides (Table II). In the following experiment, one sample of a suspension of cells in BM42 was supplemented with 1 mM uridine-3H (zero time) and monitored during 5.5 hr of incubation at 37°C for radioactivity in total cell material and acid-insoluble material (Fig. 5 A). At the end of the incubation period, the cells contained approximately 12 n mole/10^6 cells of acid-soluble nucleotides derived from the uridine-3H in the medium or about 4.8 n mole uridine nucleotides. As indicated from chromatographic analysis of an acid extract prepared from a sample of cells, about 65% of the 3H was located in UTP, about 20% in UDP sugars,
and the remainder in UMP. Another sample of the same cell suspension was incubated for 5.5 hr in the absence of uridine. Then the cells from both suspensions were collected by centrifugation, washed, and suspended in fresh BM42. The suspensions were further incubated, and 20 min later (at 6 hr) one portion of each suspension was supplemented with 1 mM uridine-5-3H (0.35 cpm/p mole). The suspension was incubated on a gyratory shaker at 37°C and monitored for radioactivity in total cell material and acid-insoluble material. Another sample of the same culture was incubated without added uridine. After 5.5 hr of incubation, the cells from both suspensions were collected by centrifugation, washed once in BM42, and suspended to the original concentration in BM42. After 20 min of further incubation, uridine-2-14C was added to one-half of each suspension (Fig. 5 A) to a concentration of 1 mM (0.085 cpm/p mole) and to the other half (Fig. 5 B) to a concentration of 1 µM (50 cpm/p mole). The suspensions were further incubated and monitored for 3H and 14C in total cell material and acid-insoluble material. All points represent averages of values from duplicate 0.5-ml samples of each suspension.

The time-course of incorporation of uridine-14C into total cell material was the same whether or not the cells had been preincubated with 1 mM uridine-3H. Further, the incorporation by both cell populations was the same whether uridine-14C was present in the medium at a concentration of 1 mM (Fig. 5 A) or 1 µM (Fig. 5 B). More importantly, however, the time-course of incorporation of uridine-14C into acid-insoluble material was also not affected by the uridine nucleotide pool expansion resulting from preincubating the cells with 1 mM uridine-3H, even when the uridine-14C was added at 1 µM concentration (Fig. 5 B). Thus, the presence of 12 nmole of labeled-3H uridine nucleotides/10^6 cells had no effect on the fate of the 0.3 nmole of newly formed uridine-14C nucleotides with respect to their incorporation into RNA. Further, the incorporation of uridine-14C into nucleic acids commenced at a constant rate upon its addition to the medium (Fig. 5).

Quite different results were obtained in similar experiments with mengovirus-infected cells in which the incorporation of uridine-14C into viral RNA was measured (Fig. 6). Infected cells were
labeled with uridine in the presence of actinomycin D which prevents incorporation into cellular RNA without significantly affecting uridine incorporation into viral RNA which is synthesized in the cytoplasm (16). A sample of a suspension of actinomycin-treated infected cells were incubated with 500 µM uridine-3H until 3 hr after infection, and another sample of suspension was incubated without uridine. The cells were then further incubated in fresh medium containing 1 µM uridine-3H and monitored for 3H and 14C in total cell material and acid-insoluble material. Preincubation of infected cells with 500 µM uridine-3H resulted in a 20–30% decrease in the subsequent incorporation of uridine-3H into total cell material (Fig. 6). This effect has been observed in two other experiments of the same type, but the reason for it is not entirely clear. The incorporation of uridine-3H...
DISCUSSION

The results of the $^{32}$P-labeling experiments indicate that the presence of high concentrations of nucleosides in the medium leads to a marked increase in the intracellular level of the corresponding nucleotides and may result in the lowering of the levels of other nucleotides. The absolute increase in the nucleoside-induced levels of nucleotides is about the same for all four types of nucleotides. The relative increase, however, varies with each nucleotide since the basic level differs for each.

The present results also support our previous conclusions (11) that the ribonucleotides of Novikoff hepatoma cells are compartmentalized into independent cytoplasmic and nuclear pools. The nuclear pool seems to be relatively small, since labeled nucleosides added to the medium even at low concentrations will, without delay, be incorporated into cellular nucleic acids, even though the cells contain large amounts of unlabeled ribonucleotides (0.4–3 mm). It can be readily calculated that these amounts would be sufficient to completely satisfy the nucleotide requirements of the cells for RNA synthesis for at least several hours. Another explanation for the above finding would be that the nucleotides formed from nucleosides in the medium, although passing through a single intracellular pool, are preferentially used for cellular RNA synthesis. However, the mechanism of such preferential utilization would be difficult to explain. Nevertheless, it has been observed that various amino acids, when added to the medium, also seem to be incorporated into cellular proteins without passing through the main intracellular amino acid pool (6). A process by which a certain proportion of the nucleosides taken up by the cells is directly transported to the nucleus and phosphorylated there could account for all of our results (11). The existence of two independent nucleotide pools, one small nuclear and one large cytoplasmic pool, is also supported by the finding that the labeling pattern of CMP in cellular RNA from uridine and of GMP from inosine does not reflect the pattern of labeling of the main cellular nucleotide pool, whereas the labeling pattern of viral RNA does (Fig. 4, Table III). It is further supported by the differences in the results from pulse-chase experiments (11) and preloading experiments (Fig. 5 and 6) with uninfected and mengovirus-infected cells. The results are consistent with the conclusion that the nucleotides for viral RNA synthesis are supplied by the pool that expands during incubation of the cells with high concentrations of uridine. Although it is difficult to calculate this exactly, the degree of reduction of uridine-¹⁴C incorporation into viral RNA due to preincubation with uridine-²H was about what would be expected from a two- to threefold increase in the uridine nucleotide content of the cells during the preincubation period.

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The present results also support our previous conclusions (11) that the ribonucleotides of Novikoff hepatoma cells are compartmentalized into independent cytoplasmic and nuclear pools. The nuclear pool seems to be relatively small, since labeled nucleosides added to the medium even at very low concentrations will, without delay, be incorporated into the cytoplasmic nucleic acid pool. Although it is difficult to calculate this exactly, the degree of reduction of uridine-¹⁴C incorporation into viral RNA due to preincubation with uridine-²H was about what would be expected from a two- to threefold increase in the uridine nucleotide content of the cells during the preincubation period.
the basis of this finding, and the observation that
the alterations in ATP metabolism induced by
various treatments of the animals were similar
whether analyzed in the whole organs or isolated
nuclei, Ove et al. (9) concluded that liver nuclei
can neither concentrate nor exclude ATP and
that the nucleotides in the nucleus and cytoplasm
are in rapid equilibrium. Our results indicate that
such rapid equilibration does not occur in cul-
tured Novikoff rat hepatoma cells. The two pools
of N1S1-67 cells, however, are not entirely inde-
pendent, since upon labeling of cells with trace
amounts of uridine most of the label is eventually
(after one population doubling time) incorporated
into nucleic acids (11).

In contrast to the ribonucleotides, the deoxy-
ribonucleotides of mammalian cells seem to be
largely confined to the nucleus (1, 8). These ob-
servations suggest that the nucleus might contain
similar concentrations of both ribo- and deoxy-
ribonucleotides, in spite of the fact that the total
content of ribonucleotides of animal cells in culture
seems to be 50–100 times higher than that of the
deoxyribonucleotides (3, 20). The GTP derived
from inosine in the medium and the CTP derived
from uridine also seem to be largely confined to
the nuclear pool and rapidly used for nucleic
acid synthesis, since significant amounts of labeled
GTP or UTP do not accumulate in cells during
incubation with inosine-3H or uridine-3H, re-
spectively, in spite of the fact that after 10 min of
labeling over 80% of the inosine label in RNA is in
GTP, and uridine is a precursor of both CMP and
UMP of RNA. It seems possible, therefore, that
the conversion of IMP to GMP and of UTP to
CTP occurs mainly in the nucleus. This conclusion
is supported by the finding that uridine (11) and
inosine (Fig. 4, Table III) are much more rapidly
incorporated into CMP and GMP, respectively,
of cellular RNA than into CMP and GMP of
mengovirus RNA. It could also explain the finding
that the presence of high concentrations of uridine
in the medium causes a threefold increase in the
uridine nucleotide level without affecting the total
cytidine nucleotide content of the cells (Table II)
or delaying the incorporation of uridine into CMP
of RNA. On the contrary, the higher the uridine
concentration in the medium, the more rapidly
does the CMP of RNA become labeled from uridine
(10). Further, these observations raise the
possibility that the cytidine nucleotides in the
cytoplasmic pool are synthesized in a manner
other than by amination of UTP (2), whereas the
CTP used for RNA synthesis is exclusively derived
from UTP (Table I). However, more information
and direct evidence are needed for a clear under-
standing of the interrelationship between the
nucleotide pools, and of the mechanism by which
mammalian cells compartmentalize nucleotides
and how this process is regulated.

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P. G. W. PLAGEMANN Nucleotide Pools in Novikoff Cells. III 145


