Uridine Nucleotide Incorporation into Pigeon Liver Microsome Ribonucleic Acid

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The role of nucleotides as precursors of ribonucleic acid in animal tissues has been well documented (1-4). Aside from the formation of polymers by the enzyme polynucleotide phosphorylase (5), only one preliminary report of net synthesis of polynucleotides in cell-free systems has been made (6). Hilmoe and Heppel (7) have demonstrated the phosphorolysis of added polyadenylic acid by a partially purified enzyme system from guinea pig liver nuclei but were unable to obtain incorporation of adenosine diphosphate into RNA. The incorporation of mononucleotides into RNA of nuclear (7-11) microsomal (3, 11), and supernatant (11-13) fractions of cell-free systems has been described. All of these systems apparently utilize nucleoside triphosphates as the direct precursors of RNA.

Some of these systems incorporate nucleotides nonterminally (3, 8, 9, 12) whereas the others (10, 11, 13) involve terminal addition of nucleoside triphosphates to RNA. The nonterminal incorporation of adenosine monophosphate, under conditions of oxidative phosphorylation, into cytoplasmic RNA of pigeon liver has previously been reported by one of us (1).

Herbert et al. (3) have shown that orotic acid is first converted to uridine monophosphate and the latter is then incorporated into rat liver microsomal RNA presumably after conversion to uridine diphosphate or UTP. It seemed pertinent to determine the phosphorylation level of the nucleotide which was the direct precursor of microsomal RNA since most cytoplasmic RNA is in a nonterminal form. It is possible, however, that uridine nucleotides may serve as templates for the biosynthesis of proteins. In this study the conditions required for nucleotide incorporation into pigeon liver microsomal RNA have been determined and a comparison made between UMP, UDP, and UTP as RNA precursors. The quantities of terminal and nonterminal incorporation have been determined, as well as some of the other properties of the system.

EXPERIMENTAL PROCEDURE

Materials—Orotic acid-6-C14 was purchased from the Volk Radiochemical Company. H3PO4 was obtained from Oak Ridge National Laboratory. Crystalline bovine plasma albumin, crystalline trypsin, and recrystallized RNase were obtained from Armour and Company. Recrystallized lysozyme was purchased from Pentex Biochemicals, and pyruvate kinase from C. F. Boehringer and Sons. All other reagents were the best commercially available grade.

Protein Determination—The method of Lowry et al. (14) was used for protein determination. Crystalline bovine plasma albumin was used as the standard.

RNA Determination—RNA was determined by the orcinol method of Dische and Schwartz (15) modified in that samples were kept at 100° for 15 minutes. The standard used was RNA isolated from pressed bakers' yeast by the method of Crestfield et al. (16).

Aliquots of microsome suspensions were precipitated in the cold and washed 5 times with 0.5 M HClO4. Lipids were removed by extraction with ethanol, ether, and chloroform 2:2:1 (volume for volume), and the residue dried before addition of 0.3 M KOH. Hydrolysis was carried out at 37° for 18 hours, after which the acid-insoluble portion and K+ ions were removed by precipitation with cold HClO4, and the acid-soluble fraction analyzed for RNA as mixed 2'(3')-nucleotides.

Synthesis of UMP2 and UMP-6-C14—UMP2 was synthesized by a new method, the reaction of H3P-O4 with 2',3'-O-isopropylidene uridine and N,N'-dicyclohexylcarbodiimide in a homogeneous p-dioxane system. The specific radioactivity of the UMP2 was 5.0 × 108 c.p.m. per mole. Details of this synthesis will be described elsewhere.

Orotic acid-6-C14 (1.5 mg per mole) was enzymically converted to UMP-6-C14 by use of mixed orotidin-5'-P pyrophosphorylase and carbamylase in the 25 to 57% ethanol fraction of an autolysate of dried brewers' yeast according to the method of Lieberman et al. (17). The UMP-6-C14 was isolated by ion exchange chromatography on a Dowex 1-Cl- column.

Synthesis of Labeled UDP and UTP—ATP was used as the donor for the enzymic phosphorylation of UMP2 by the nucleotide kinase mixture isolated from brewers' yeast autolysates according to the method of Lieberman et al. (18). The reaction mixture, containing both adenosine and uridine nucleotides was fractionated on Dowex 1-Cl- with the use of 0.003 M HCl and increasing concentrations of NaCl as eluants. UDP2 and UTP2 were collected in tubes containing about 150 pmoles of NH4OH (final pH 8).1 The uridine nucleotides were converted to the potassium salt and concentrated on small columns of Dowex 1-Cl- at 4° (19). These columns were eluted with 0.01 M HCl-0.35 M KCl; the eluant volume used gave a 2500:1 ratio of chloride to nucleotide. Eluates were neutralized with KOH, concentrated, and analyzed spectrophotometrically.

For the C14-labeled UMP, another phosphorylation method was used.1 In this study, nucleotide P32 was always in the ester position.
necessary. UMP-6-C\textsuperscript{14} was converted to the pyridium salt and this salt phosphorylated with tri-n-butylammonium phosphate and N\textsubscript{7},N\textsubscript{7}'-dicyclohexylcarbodiimide according to the method of Smith and Khorana (20). The product mixture was fractionated on Dowex I-Cl\textsuperscript{-}. UTP-6-C\textsuperscript{14} was concentrated as described for the \textsuperscript{15}N labeled uridine nucleotides.

All labeled nucleotides were characterized spectrophotometrically and by cochromatography with authentic standards with the use of 2:1 isopropanol-1% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} as solvent and Whatman No. 1 paper previously soaked in 1% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and dried (21). Radioautographs of the chromatograms were made and in all cases radioactivity was found to be associated exclusively with the ultraviolet quenching spot of the appropriate standard; no other radioactive areas were found on the chromatograms.

The chemical phosphorylation of nucleoside monophosphates has not been successful, in our hands, with quantities of starting material less than about 20 \mu moles. The enzymic phosphorylation, in contrast, requires the separation of ATP, used in excess, from the desired nucleotides; this is difficult when more than about 20 \mu moles of monophosphate starting material are used. Despite the more complex separation problem, the enzymic phosphorylation is much faster than the chemical synthesis. Both methods give comparable yields of UTP (50 to 70%) and UDP (20 to 30%) based on UMP.

**Isolation and Washing of Pigeon Liver Microsomes**—Pigeons were decapitated, the livers rapidly removed, weighed, placed on ice, and minced. Minces were mixed with 4 volumes of homogenization medium,\textsuperscript{2} in a hydraulic homogenizer (22) and the suspension rapidly forced through a 42 \mu m annular orifice. Initial homogenates were rapidly rehomogenized to obtain maximal cell breakage.

Nuclei, unbroken cells, erythrocytes, and connective tissue were sedimented by centrifugation at 600 \times g for 10 minutes. The cytoplasmic fraction was then centrifuged for 15 minutes at 15,000 \times g to sediment mitochondria. Microsomes, contaminated with glycogen, were sedimented by centrifugation of the 15,000 \times g supernatant for 60 minutes at 90,000 \times g.

The supernatant fraction was carefully decanted and tubes wiped dry while still inverted. The microsomes were washed by suspending the residue in fresh homogenization medium followed by recentrifugation for 25 minutes at 90,000 \times g. Other subcellular fractions were similarly washed. All operations were carried out at 0-4\degree C after weighing the liver.

Homogenates and nuclear and cytoplasmic fractions were stained and examined microscopically. No evidence could be found for disruption of nuclei, and identifiable nucleoli were absent from the cytoplasmic fraction.

The washed microsomal fraction was homogenized in a Potter-Elvehjem homogenizer with rehomogenization medium to give 0.8 to 1.2 g equivalents per ml. In some cases the rehomogenization medium was the same as that used for initial homogenization whereas in others this medium contained MgCl\textsubscript{2} buffer at the same concentration, and pH used for incorporation experiments.

**Removal of Bound Nucleotides from Microsomes**—Evidence has been obtained showing that microsomes bind nucleotides (see \textsuperscript{3}

\textsuperscript{2}The homogenization medium was 0.004 M MgCl\textsubscript{2}, 0.033 M KCl, 0.25 M sucrose, 0.04 M Tris-HCl, pH 7.6.

\textsuperscript{3}All gravitational fields refer to the average radius at which the field was applied.

\textsuperscript{3}The ATP-pyrophosphate washing procedure removes most of the bound nucleotides as well as 20 to 40% of the total RNA, and 10 to 20% of total protein; the RNA-protein ratio is lowered from 0.16 to 0.12. Removal of ribonucleoprotein particles and RNA from rat liver microsomes with more concentrated pyrophosphate buffers has previously been reported by Sachs (23).

**Incorporation Experiments**—Except where otherwise indicated, incubation mixtures were 0.05 M Tris-HCl buffer at various pH values, and 0.25 M sucrose; they contained 0.1 \mu mole labeled uridine nucleotides and, usually, 0.1 \mu mole each of the corresponding cytidine, adenosine, and guanosine nucleotides. An ATP-generating system consisting of 1.0 \mu mole P-enolpyruvate and 5 \mu g pyruvate kinase was used in most cases. Details of each incubation mixture are given in the tables describing the experiments. Mixtures were made up complete except for microsomes, or other subcellular fraction, and labeled uridine nucleotide. After a brief preincubation at 37\degree C, microsomes were added, followed within 10 seconds by labeled uridine nucleotide at zero time. At the end of the incubation period, samples were placed in an ice bath and made 0.5 M with perchloric acid. Zero time control samples were made up and processed similarly except that HC\textsubscript{1}O\textsubscript{4} was added before the microsomes and they were kept at 0\degree C. The acid-insoluble residues were washed with HC\textsubscript{1}O\textsubscript{4} and lipids were removed as described above.

**Extraction of RNA from Protein Nucleates by Pyrophosphate**—The extraction of RNA from microsome protein nucleates by the method of Davidson and Smellie (24) gave low and varying recoveries of RNA and could not be used. It was found, however, that extraction of protein nucleates with 0.1 M Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, pH 7.7, gave nearly quantitative separation of RNA and protein.

Protein nucleates were suspended in 0.1 M pyrophosphate buffer, pH 7.7, and incubated 60 minutes at 37\degree C followed by 5 minutes at 100\degree C. Samples were cooled and centrifuged to remove protein. The average recovery of RNA in the supernatants was 97% in a large number of experiments. Protein contamination was estimated to range from 10 to 20 \mu g per mg of RNA although some nonprotein contaminant might have contributed significantly to the very slight absorbancy in the Lowry method (14).

RNA and P\textsubscript{i} were separated by precipitating the RNA with 3.5 volumes of 8:1 (volume for volume) absolute ethanol-70% HC\textsubscript{1}O\textsubscript{4} at -18\degree C, allowing 1 to 2 hours before centrifugation. After two washings with ethanol-perchloric acid and two with acetone, recoveries of RNA were about 70 to 80%.

**Separation of Bound Nucleotides from RNA**—Zero time control acid-insoluble fractions were frequently found to be more radioactive than corresponding incubated samples. It seemed clear that all radioactivity in the zero time samples resulted from the binding of labeled 5'-uridine nucleotides to the precipitated microsomes. Many different procedures were tried in attempts to eliminate these bound nucleotides.

Repeated washing of acid-insoluble protein nucleates with HC\textsubscript{1}O\textsubscript{4} (0.25 to 1.0 M) does not remove bound radioactivity.

\textsuperscript{'Results'}). To remove these nucleotides, the 15,000 \times g supernatant was made 1.6 \times 10^{-4} M in ATP and 8 \times 10^{-4} M in pyrophosphate buffer, pH 7.7, before the initial sedimentation of microsomes. The microsomes were washed 3 times with ATP and P\textsubscript{i} (8 \times 10^{-4} M ATP and 1.0 \times 10^{-4} M P\textsubscript{i}, buffer, 8 \times 10^{-4} M ATP and 8 \times 10^{-4} M P\textsubscript{i}, buffer, 8 \times 10^{-4} M P\textsubscript{i}, buffer). The final pellets were rinsed several times with small volumes of homogenization medium and then rehomogenized as described for microsomes.

The ATP-pyrophosphate washing procedure removes most of the bound nucleotides as well as 20 to 40% of the total RNA, and 10 to 20% of total protein; the RNA-protein ratio is lowered from 0.16 to 0.12. Removal of ribonucleoprotein particles and RNA from rat liver microsomes with more concentrated pyrophosphate buffers has previously been reported by Sachs (23).
Extraction of RNA from protein nucleates with 10% NaCl (24), or pyrophosphate buffer, pH 7.7, solubilizes the bound nucleotides as well as the RNA. However, the nucleotides could not be completely separated from RNA in such extracts by: (a) dialysis against water, (b) dialysis against 1% water suspensions of Dowex 1-Cl-, (c) electrodialysis, (d) precipitation and washing of extracted RNA with ethanol or ethanol-HClO₄, (e) differential adsorption of nucleotides onto Norit A (11), and (f) differential elution of RNA from Norit A with phenol (25). All of these procedures lower the ratio of zero time radioactivity to incubated samples except at the expense of large losses of RNA sample radioactivity but none eliminate zero time bound nucleotides or even reduce the zero time radioactivity to the level of the incubated samples except at the expense of large losses of RNA (70 to 90%). The failure of dialysis and ethanol precipitation procedures indicated that the 5'-nucleotides in NaCl and pyrophosphate extracts of microsome protein nucleates were present bound to some larger molecule. In model experiments, virtually all labeled nucleotide could be separated from yeast RNA by these procedures but considerable radioactive remained bound to such proteins as bovine plasma albumin. Probably the nucleotides are bound to traces of protein which are solubilized in the extraction procedures. A suitable, isolable, RNA derivative or a more effective RNA purification procedure was needed. Methods of achieving both aims were developed which eliminate zero time radioactivity. Phosphorus from nucleotides incorporated into RNA appears in 2'(3')-nucleotides after alkaline hydrolysis of RNA; separation of these RNA derivatives from any 5'-nucleotides in the alkaline hydrolysate affords an unambiguous determination of nucleotide incorporation into RNA. A paper chromatographic solvent system, 6:3:1 absolute ethanol-2% (weight for volume) H₃BO₃-10% NH₄OH (density 0.9), was developed which separates 2'(3')-nucleotides from 5'-nucleotides. Pertinent RF values are (Whatman No. 3MM, descending): 0.55, 0.52, 0.54, 0.45 for 2'(3')-UMP, 2'(3')-AMP, 2'(3')-CMP, and 2'(3')-GMP; 0.34, 0.29, 0.34 and 0.25 for the corresponding 5'-nucleotides; 0.24 for UDP; and 0.21 for UTP.

The total RNA, derived from protein nucleates by pyrophosphate extraction and acid-alcohol precipitation, was hydrolyzed with 0.100 ml of 0.3 M KOH (18 hours, 37°), neutralized with Dowex 50W-H+, and chromatographed with the borate solvent. The 2'(3')-nucleotide area, readily visible under ultraviolet light, was cut out and eluted overnight in 0.01 M HCl. Aliquots of the eluates were analyzed for radioactivity and RNA (as mixed 2'(3')-nucleotides). Alternatively, protein nucleates were hydrolyzed directly, the hydrolysates acidified with HClO₄, centrifuged, and then neutralized with KOH or tri-n-heptylamine in chloroform before chromatography. Perchlorate ion retards migration of nucleotides in the borate solvent and its removal as KClO₄ or tri-n-heptylammonium perchlorate is necessary.

The chromatographic separation of 2'(3')- and 5'-nucleotides with the borate solvent has been carried out on alkaline hydrolysates of RNA (Procedure 1-A) and protein nucleates (Procedure 1-B).

The second method (Procedure 2) for eliminating zero time radioactivity was based on the observation of Metzenberg (26) that RNA formed an insoluble salt with the cationic detergent cetyltrimethylammonium bromide. Protein nucleates were extracted with pyrophosphate and the RNA-free acid precipitated with ethanol-HClO₄ as described. The RNA residue was mixed with 2.0 ml of 0.01 M cetyltrimethylammonium chloride and the flocculent precipitate which formed was collected by centrifugation. The residue was washed twice with 0.01 M detergent, twice with water, and once with acetone. At this stage the zero time cetyltrimethylammonium RNA was still slightly radioactive, but bound nucleotide was almost completely removed by dissolving the residue in ethanol followed by reprecipitation of the RNA-free acid with ethanol-HClO₄. After removing HClO₄ with acetone the residue was dissolved in 0.05 M NH₄OH and aliquots taken for determination of RNA and radioactivity.

When freshly prepared, cetyltrimethylammonium chloroform solutions may gel in the cold, therefore the procedure must be carried out at room temperature. However, this tendency to gel disappears on aging, and the work may be carried out at 4°. The recovery of RNA with the use of the complete procedure was 70 to 80% and was not affected by the use of aged detergent solutions. Perchlorate ion also forms a water-insoluble, ethanol-soluble, cetyltrimethylammonium salt and must be completely removed before addition of detergent.

Radioactivity Determinations—Aliquots of samples containing P⁳² were dried in aluminum cups, and counted using a proportional type gas flow counting tube (10% methane, 90% argon) with an aluminized Mylar window. C¹⁴ samples were counted as infinitely thin layers (<0.1 mg per cm²) in aluminum cups with an internal gas flow Geiger counter. The counting error for both C¹⁴ and P⁳² determinations was 3% or less.
Zero time binding of UMP, UDP, and UTP to microsomes

In addition to the standard components, samples contained:
A. Eight and six-tenths micromoles of MgCl₂, 10 μmoles of MnCl₂, 325 pmoles of KCl, and 0.9 g equivalent of microsomes. UMP₃² and UDP₃² (0.08 μmole) and UTP₃² (0.1 μmole) were used; specific activity of all uridine nucleotides was 8.5 × 10⁶ c.p.m. per μmole. Final volume was 2.14 ml, pH 7.6. PPi extracts of zero time protein nucleates analyzed for RNA and radioactivity.

B. Nucleotides as indicated, 0.1 μmole of UTP₃² (1.8 × 10⁶ c.p.m. per μmole), 9.4 μmoles of MgCl₂, 10.0 μmoles of MnCl₂, the ATP-generating system, 10.0 μmoles of spermidine-HCl, 330 pmoles of KCl, and 1.0 g equivalent of microsomes in a total volume of 2.34 ml, pH 7.6. KOH hydrolysates of zero time protein nucleates analyzed for RNA and radioactivity.

C. Nucleotides as indicated, 0.1 pmole of UTP₃² (4.2 × 10⁶ c.p.m. per pmole), 20 pmoles of MgCl₂, the ATP-generating system, 336 pmoles of KCl, and 0.8 g equivalent of ATP-PPi washed microsomes (3 washes) in a total volume of 2.47 ml, pH 7.6. PPi extracts of zero time protein nucleates analyzed for RNA and radioactivity.

# Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nucleotides</th>
<th>Specific binding of uridine nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>UMP + 3 NMP</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>UDP + 3 NDP</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>UTP + 3 NTP</td>
<td>17.0</td>
</tr>
<tr>
<td>B</td>
<td>UTP + 3 NTP</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>UTP alone</td>
<td>16.5</td>
</tr>
<tr>
<td>C</td>
<td>UTP + 3 NTP</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>UTP alone</td>
<td>7.8</td>
</tr>
</tbody>
</table>

* NMP, NDP, and NTP refer to nucleoside mono-, di-, and triphosphates, respectively.

Comparison of nucleoside mono-, di-, and triphosphates as precursors of RNA in pigeon liver microsomes

Incubation mixture the same as in Table II, A. The ATP generating system used as indicated. Incubation for 10 minutes at 37°. One μmole additional P-enolpyruvate added after 5 minutes. Procedure 1 A used to process samples.

<table>
<thead>
<tr>
<th>Precursors</th>
<th>Uridine nucleotide incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specfic</td>
</tr>
<tr>
<td></td>
<td>μmoles/mg RNA</td>
</tr>
<tr>
<td>UMP + 3 NMP</td>
<td>0.007</td>
</tr>
<tr>
<td>UDP + 3 NDP</td>
<td>0.10</td>
</tr>
<tr>
<td>UTP + 3 NTP</td>
<td>0.36</td>
</tr>
<tr>
<td>UTP + 3 NTP + generating system</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Results

Binding of Nucleotides to Subcellular Fractions from Pigeon Liver The zero time binding of UTP to the various subcellular fractions from pigeon liver and of all three uridine nucleotides to microsomes was measured and the results are presented in Tables I and II.

It is clear that all subcellular fractions bind UTP₂₃ firmly under these experimental conditions. Nuclei and mitochondria bind much less UTP₂₃ than microsomes or supernatant and the two former fractions appear to lower the binding of the two latter when mixed with them. The mixture of microsomes and supernatant shows a greater total binding than the sum of the binding of individual fractions suggesting that there is some interaction between them.

The microsome fraction binds UTP most extensively, followed in order by UDP and UMP. The labeled substances bound were positively identified as UTP, UDP, and UMP by chromatography in the borate solvent and radioautography. Other experiments showed that zero time binding is constant between pH 7.0 and 9.0 but is much reduced at pH 6.1 and 5.5, indicating that the extent of binding is dependent on the valence of the nucleotide.

The increased binding of UTP which occurs when other nucleoside triphosphates are omitted from the zero time samples suggests that a given nucleotide is not bound to a site specific for that nucleotide. Microsomes show a doubling in binding of UTP when CTP, ATP, and GTP are omitted; ATP-pyrophosphate-washed microsomes show a quadrupling when these nucleotides are omitted. This fact and the nonspecific binding suggest that ATP and pyrophosphate displace nucleotides bound to native microsomes.

In addition to nucleotide binding, Sachs (27) has reported that rat liver microsomes bind pyrophosphate and we have found that the binding of orthophosphate to pigeon liver microsomes is quite extensive. As much as 5 μmoles of added P₃² could be detected in the acid-washed, defatted, protein nucleate from 1 g equivalent of microsomes in a system containing 50 μmoles of P₃² at pH 7.5. The binding of these polyvalent anion derivatives of phosphoric acid suggests that microsomes may bind...
other polyanions of biological importance such as amino acid transfer RNA (28, 29).

Comparison of UMP, UDP, and UTP as Precursors of Microsomal RNA—The incorporation of P3*-labeled UMP, UDP, and UTP, with mixtures of the corresponding cytidine, adenosine, and guanosine nucleotides, into microsomal RNA was measured. Results are presented in Table III. It is clear that nucleoside triphosphates are the best precursors of RNA in this system. UTP incorporation is markedly increased by the addition of an ATP-generating system and this is taken as further evidence that the triphosphates are the direct precursors of RNA in microsomes. Simultaneous by the ATP-generating system suggested that enzyme(s) are present in microsomes which degrade the triphosphates and we found that microsomes do convert ATP to ADP; the initial specific activity is about 80 μmoles of P1 produced per mg of protein per hour.

We do not know whether the incorporation of UDP32 requires initial conversion to UTP32 or whether it also occurs by an independent route.

Time Course of UTP Incorporation into Microsomal RNA—The incorporation of UTP32 increases linearly for 10 minutes and then the specific activity of the microsomal RNA decreases precipitously to less than 15% of the maximal incorporation by 60 minutes (Fig. 1). Since the specific activity passes through a maximum, the newly labeled RNA must be degraded and released to the acid-soluble fraction more rapidly than the remainder of microsomal RNA.

Incubation of microsomes in homogenization medium for 2 hours at 37° results in a 12% loss of acid-insoluble RNA which is completely recovered as acid-soluble nucleotides and nucleosides. Pigeon liver microsomes contain a nuclease and microsomal RNA does not appear to be a homogeneous substrate for this enzyme.

The nuclease activity explains the decrease in RNA radioactivity after 10 minutes but, since this enzyme is present at all times and may be active during the first 10 minutes of incubation, the actual incorporation reaction must proceed at a rate less than that of the degradative reaction after the first 10 minutes have elapsed. The synthesizing system must have used up or destroyed some essential component. Nucleoside triphosphates other than CTP, ATP, UTP, or GTP, such as pseudouridine triphosphate (90, 91) or other minor constituents of RNA, small quantities of which may be bound to microsomes, could be used up by 10 minutes, or those RNA molecules required for incorporation (see below) may be degraded or used up.

Dependence of UTP Incorporation into RNA on the Presence of RNA—Preincubation of microsomes with pancreatic RNase strongly inhibits the incorporation of UTP32 into RNA (Table IV). No attempt was made to remove RNase before UTP32 addition to the incorporation system and it is possible that the inhibitory effect of the enzyme is due to rapid hydrolysis of the newly synthesized RNA and not to removal of RNA molecules required for incorporation. The data presented in Table IV show about the same inhibition of incorporation in Experiments A and B but the loss of RNA due to RNase in Experiment A was about 20%, whereas in Experiment B was only about 4%. These data suggest, but do not definitely establish, that pancreatic RNase inhibits incorporation of UTP32 into microsomal RNA by degrading biologically active RNA molecules required for the process of incorporation.

It has been noted (32) that some of the effects of RNase may be due to its relatively high isoelectric point (pl) rather than to its specific enzymic activity. In this system, preincubation with lysozyme, which has an even higher pl, sometimes stimulates incorporation. This stimulation has not been reproducible but lysozyme has never been found to inhibit nucleotide incorporation into RNA.

UTP32 incorporation into microsomal RNA is completely inhibited by pyrophosphate but only inhibited 40% by the same concentration of orthophosphate. Since nucleoside triphosphates are the direct precursors of RNA in microsomes, a pyrophosphorylsis of biologically active RNA would be expected. Pyrophosphate, added at the point of maximal incorporation, causes a reduction of incorporated UTP-6-C41 over and above the reduction due to the endogenous RNase. This reduction is

### Table IV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Specific Labeled UTP Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total mmoles/mg RNA</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>RNase</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>K+PO4 buffer, pH 7.6</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Na2HPO4 buffer, pH 7.6</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Heated microsomes (5 minutes, 100°)</td>
<td>0.005</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>RNase</td>
<td>0.04</td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>0.20 ml water at 10 minutes</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>30 μmoles Na2HPO4 buffer, pH 8.0</td>
<td>0.05</td>
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</table>

* Calculation of total incorporation not justifiable because of the loss of RNA to the acid soluble fraction.
in a total volume of 1.84 ml, pH 8.0. Samples incubated 10 minutes at 37°; 1.0 pmole of P-enolpyruvate added after 5 minutes. Samples processed by Procedure 2.

**Table V**

<table>
<thead>
<tr>
<th>Washes with ATP-PPi</th>
<th>Nucleotides</th>
<th>Labeled UTP Incorporation</th>
<th>Specific</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>UTP-32 + 3 NTP</td>
<td>0.28</td>
<td>0.18</td>
<td>18</td>
<td></td>
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<tr>
<td></td>
<td>UTP-32 alone</td>
<td>0.35</td>
<td>0.22</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>UTP-32 + 3 ATP</td>
<td>0.05</td>
<td>0.06</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UTP-32 alone</td>
<td>0.07</td>
<td>0.08</td>
<td>+14</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>UTP-6-C14 + 3 NTP</td>
<td>0.06</td>
<td>0.07</td>
<td>-50</td>
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<tr>
<td></td>
<td>UTP-6-C14 alone</td>
<td>0.04</td>
<td>0.08</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Dependence of UTP Incorporation into RNA on Protein**—

Preincubation of microsomes with trypsin greatly inhibits incorporation of UTP-32. There is no incorporation when boiled microsomes are used (Table IV). These facts show that the incorporation is most likely an enzymic process. Purification of the enzyme responsible for RNA formation in microsomes is now being attempted.

**Dependence of UTP Incorporation into RNA on Nucleotides**—

Early attempts to show that UTP-32 incorporation into RNA was dependent on the presence of all four nucleoside triphosphates were unsuccessful (e.g., Table V, 0). The finding of zero time nucleotide binding suggested that the microsomes might contain sufficient bound nucleoside triphosphates to synthesize RNA without requiring the addition of other nucleotides. The ATP-pyrophosphate washing procedure was designed to eliminate such bound nucleotides and experiments with these microsomes show that the incorporation of UTP is not great, although the stimulation of incorporation by added CTP, ATP, and GTP is not great. ATP-pyrophosphate-washed microsomes have a lower incorporation of UTP-32 than microsomes isolated from the same homogenate but not so washed; this may be due to removal of required RNA (see above).

The demonstration of nucleotide dependence with ATP-pyrophosphate-washed microsomes, in contrast to the inability to demonstrate this with microsomes not washed in this way, confirms the previous conclusion, based on zero time binding, that native microsomes do bind nucleotides and that ATP and pyrophosphate can displace bound nucleotides.

**pH Optimum**—The incorporation of UTP-32 into microsomal RNA as a function of pH is presented in Fig. 2. The optimal pH is about 8.0. One half of the maximal activity is found at pH 7.5 and about 9.2.

**Divalent Cation Requirement**—Chung et al. (12) have reported that Mn+2 stimulates ATP-8-C14 incorporation into embryonic chicken liver supernatant RNA in the presence of an optimal quantity of Mg+2. Both manganous and magnesium ions have been tested with the pigeon liver microsome system (Table VI). The stimulatory effect of these ions appears to be equal under the conditions used. The incorporation of UTP-6-C14 is inhibited 85% when EDTA, in amounts equivalent to the amount of Mg2+ used, is added to the system; this amount of EDTA would not remove magnesium originally present in the microsomes. The EDTA inhibition suggests that there is an absolute requirement for a divalent cation, either Mg+2 or Mn+2. No other divalent cations have been tested in this system.

**Effect of Spermidine HCl on UTP Incorporation into RNA**—Chung et al. (12) also report that spermidine stimulates ATP-8-C14 incorporation into embryonic chicken liver supernatant RNA. The addition of spermidine-HCl to the pigeon liver microsome system at a concentration of 0.004 M resulted in a 44% inhibition of UTP-32 incorporation.

**Distribution of Isotope in Alkaline Hydrolysates of RNA Labeled by UTP-32 and UTP-6-C14 Incorporation**—Nucleoside triphosphate ester phosphorus incorporated into RNA is recovered from an alkaline hydrolysate esterified in the 2′ or 3′ position of the nucleoside moiety which was adjacent to the incorporated nucleotide. A distribution of P32 among all the 2'(3')-nucleotides derived from RNA labeled by incorporation of a single ester-labeled nucleotide suggests nonterminal incorporation (1, 8), and labeling of only one 2'(3')-nucleotide suggests terminal incorporation (2). Neither of these findings can be considered de-
to a particular nucleoside moiety would appear as terminal addition. Nonterminal incorporation of a nucleotide in a position adjacent to a particular nucleoside moiety would appear as terminal addition by the randomization criterion.

Frog liver microsome RNA was labeled by incorporation of UTP, the RNA isolated by Procedure 2, hydrolyzed with KOH, and then fractionated by chromatography on Dowex 1-Cl- by a modification of the method of Cohn (19). was found in all 2'(3')-nucleotides (Table VII). This finding is consistent with some nonterminal incorporation but the high proportion of the total incorporated radioactivity in 2'(3')-UMP is not consistent with random incorporation as found with polynucleotide phosphorylase (33).

The use of purine- or pyrimidine-base-labeled nucleotides, such as UTP-6-C, allows unequivocal determination of terminal and nonterminal incorporation (13). Uridine isolated from alkaline hydrolysates of RNA would be labeled if UTP-6-C were added uniformly whereas C would appear as 2'(3')-UMP if the nucleotide was incorporated nonterminally. Microsome RNA, labeled by incorporation of UTP-6-C, was isolated, hydrolyzed, and fractionated as for RNA-32. Most of the C was recovered in 2'(3')-UMP showing that the incorporation is largely nonterminal and not random.

The unidentified fraction indicated in Table VII was eluted by 0.002 M HCl just before 2'(3')-CMP and after the column had been washed with 100 resin bed volumes of water, the last 80 volumes of which showed no absorption at 290 mp. The ion exchange properties of this fraction suggest that it contains nucleotides since this volume of water usually elutes all nucleosides. Paper chromatography of this fraction with carrier uridine in the isopropanol-HCl solvent of Wyatt (34) showed that the radioactivity migrated with the uridine. Insufficient material remained, however, to confirm this possible identity of the unidentified fraction.

The unidentified fraction was found by Hecht et al. (13) in the ion exchange chromatogram of the alkaline hydrolysate of supernatant RNA labeled terminally by incorporation of ATP-32. They suggested that this fraction was the dinucleotide adenyl-(5',3')-cytidine phosphate which was more resistant to alkaline hydrolysis than other phosphodiester. The unidentified fraction found in the present work must be derived from UTP with microsomal RNA as a required intermediate. Work is in progress to determine the nature of this fraction.

**DISCUSSION**

All cell-free systems isolated from the tissues of higher animals which synthesize RNA (8-13) utilize nucleoside triphosphates as direct precursors. The differences which exist between them, apart from species and cytological source of the enzymes, are chiefly with reference to nucleotide dependence, RNA dependence, and terminal or nonterminal addition. The synthesis of amino acid transfer RNA (13) is not dependent on the presence of four nucleoside triphosphates, utilizing only CTP and ATP.

---

**TABLE VI**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mg++</th>
<th>Mn++</th>
<th>EDTA, pH 8.0</th>
<th>Specific incorporation of labeled UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.4</td>
<td>0</td>
<td>2.0</td>
<td>0.20</td>
</tr>
<tr>
<td>B</td>
<td>8.6</td>
<td>10.0</td>
<td>18.6</td>
<td>0.46</td>
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<tr>
<td>C</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>0.18</td>
</tr>
<tr>
<td>D</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

**Specific incorporation**

<table>
<thead>
<tr>
<th>Substance</th>
<th>RNA-P32</th>
<th>RNA-C14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoside</td>
<td>22 5</td>
<td>366 93</td>
</tr>
<tr>
<td>Unidentified</td>
<td>112 26</td>
<td>12 3</td>
</tr>
<tr>
<td>2'(3')-CMP</td>
<td>72 18</td>
<td>2.1 10</td>
</tr>
<tr>
<td>2'(3')-AMP</td>
<td>34 9</td>
<td>1.0 15</td>
</tr>
<tr>
<td>2'(3')-UMP</td>
<td>218 56</td>
<td>6.3 50</td>
</tr>
<tr>
<td>2'(3')-GMP</td>
<td>60 15</td>
<td>1.8 50</td>
</tr>
</tbody>
</table>

**Recovery**

- RNA-P32 (380 c.p.m.), 1.92 mg, used
- RNA-C14 (266 c.p.m.), 2.90 mg, used
- Listed in order of elution from a Dowex 1-Cl- column.
- The frequency of nucleotide pairs, . . . XpU . . . , in the labeled fraction of microsomal RNA relative to . . . ApU . . . in this fraction. X is cytidine (C), adenosine (A), uridine (U), or guanosine (G).
- This fraction chromatographed with carrier 5'-UMP with the use of the borate solvent. All radioactivity was found in the 2'(3')-UMP spot.
but is dependent on RNA which undergoes terminal addition; the calf thymus nuclei system of Hurwitz et al. (10) and the rat liver microsome system of Herbert (11) (which requires the presence of either nuclei or supernatant) are similar. RNA-synthesizing systems from embryonic chicken liver supernatant (12), and the pigeon liver microsome system are nucleotide dependent, RNA dependent, and they show nonterminal incorporation; the rat liver microsome system described by Herbert et al. (3) may be similar. The rat liver nuclei system of Weiss (8) is nucleotide dependent, shows nonterminal incorporation, and is apparently DNA dependent. The calf thymus nuclei system of Edmonds and Abrams (9) is not nucleotide or RNA dependent and shows nonterminal incorporation.

The pigeon liver microsome system is clearly different from the embryonic chicken liver system of Chung et al. (12) in that the effect of divalent cations, pH optima, and the effects of spermidine are different. Except for the DNA dependence, there are no apparent qualitative differences, with regard to the criteria listed, between the microsome system and the rat liver nuclei system of Weiss (8).

There appear to be several RNA-synthesizing systems in cells of higher animals and even within certain subcellular particulate fractions.

The firm binding of nucleotides (11, 35), pyrophosphate (27), and orthophosphate to rat and pigeon liver microsomes may have physiological significance in that such binding would provide a means of concentrating biologically active polyamions at enzyme sites where they are utilized. In view of the binding of nucleotides to all subcellular fractions, caution must be used in the interpretation of experiments measuring the incorporation of precursors into RNA based on simple determination of radioactivity in the acid-insoluble protein nucleate fraction; such binding must also be considered in work involving other phosphate esters and anhydrides.

Zalokas (36) has concluded that all RNA in Neurospora crassa in synthesized in the nucleus on the basis of autoradiographs of centrifuged hyphae incubated with tritiated uridine. Goldstein and Micou (37) have come to the same conclusion from similar experiments with cultured human amnion cells using tritiated cytidine. The results of the experiments reported here, on pigeon liver cytoplasmic fractions, show that RNA synthesis does occur in the microsome fraction. The work of others (1–4, 6, 11–13) may be interpreted to show that RNA synthesis takes place in the cytoplasm. The conclusion that RNA synthesis occurs only in the nucleus cannot be universally valid.

**SUMMARY**

Uridine triphosphate was found to be a better precursor of pigeon liver microsomal ribonucleic acid than either uridine diphosphate or monophosphate. The incorporation of uridine triphosphate: (a) requires a divalent cation (magnesium or manganese); (b) is stimulated by an adenosine triphosphate generating system and by addition of cytidine, adenosine, and guanosine triphosphates; (c) is inhibited by preincubation of microsomes with ribonuclease or trypsin, by heating the microsomes, or by addition of pyrophosphate to the incubation system; and (d) increases linearly for 10 minutes after which the ribonucleic acid specific radioactivity falls rapidly.

All four 2'(3')-nucleotides in an alkaline hydrolysate of microsomal ribonucleic acid labeled by incorporation of ester phosphorus uridine triphosphate are radioactive; over 50% of the isotope in such hydrolysates occurs as 2'(3')-uridine monophosphate. About 60% of C in microsomal ribonucleic acid labeled by incorporation of uridine triphosphate-6-C is found in 2'(3')-uridine monophosphate after alkaline hydrolysis. Nucleotides were found to be firmly bound to microsomes and other subcellular fractions from pigeon liver; methods are described for the removal of these bound nucleotides.

**REFERENCES**

Uridine Nucleotide Incorporation into Pigeon Liver Microsome Ribonucleic Acid
David B. Straus and Eugene Goldwasser


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