Composition, Associated Tissue Methyltransferase Activity, and Catabolic End Products of Transfer RNA from Carcinogen-induced Hepatoma and Normal Monkey Livers


Summary

This investigation was designed to explore transfer RNA (tRNA) methyltransferase activity, urinary excretion levels of RNA degradation products, and tRNA base composition in normal and hepatocellular carcinoma-induced tissues. After the development of the tumor, 24-hr urine specimens were collected, and the livers were removed or tRNA isolation and methyltransferase activity studies. The tRNA methyltransferase activity and capacity and the urinary excretion levels for selected tRNA degradation products (pseudouridine, N²,N²-dimethylguanosine, 1-methylhydantoin, 7-methylguanine, and β-aminosuberic acid) were elevated for the hepatoma-bearing monkeys when compared to those with normal liver. The isolated tRNA pools were analyzed by high-resolution liquid chromatography, and similar base compositions were found for the hepatoma-bearing and normal monkeys. With the use of methyl-deficient Escherichia coli tRNA as the methyl receptor and the analytical procedure for tRNA analysis, the methylating ability of the tRNAs and their degradation products were investigated. The isolated tRNA pools were analyzed by high-resolution liquid chromatography, and similar base compositions were found for the hepatoma-bearing and normal monkeys. The methylating ability of the tRNAs and their degradation products were investigated. The isolated tRNA pools were analyzed by high-resolution liquid chromatography, and similar base compositions were found for the hepatoma-bearing and normal monkeys.

Introduction

tRNA (2, 3, 12, 24, 25, 29, 32) and variations in its cellular interactions and structure associated with neoplastic change have been of intense interest and study. In particular, the methylation of the intact tRNA macromolecule by methyltransferases of malignant cells has been investigated extensively, primarily by in vitro techniques using heterologous tRNA's (4, 8, 13, 14, 30, 33). Also, the urinary excretion levels of methylated degradation products and other minor base constituents derived predominantly from tRNA have been determined (10, 17–20, 22, 23, 34–37). It has been suggested that aberrant tRNA methylation and altered tRNA structure may be factors in the oncogenic process (3, 30). In 1971, Borek (3) reported that high tRNA methyltransferase activity had been found in more than 30 different malignant tumors when compared to their corresponding normal tissue of origin. Since then, these findings have been extended and verified in a variety of different tumor types involving both animal and human neoplasms (8, 13, 14, 33, 38). However, 20 benign tumors examined by Sheid et al. (28) had normal methyltransferase activity.

Initial investigations by Berquist and Matthews (2) and by Viale et al. (32) indicated that the tRNA's isolated from tumor cells contain increased amounts of specific methylated nucleic acid derivatives when compared to their normal tissue counterpart. Recently, further analytical techniques have become available (17, 21, 26, 27); a report by Randerath et al. (25) indicated few, if any differences in the methylated tRNA base content between certain normal and related tumor tissues. More extensive comparative studies, however, are desirable in order to gain a better perspective regarding the relationship between alterations in tRNA-methylated base content and malignant change.

Increased urinary excretion of methylated and minor base nucleic acid derivatives that originate predominantly from tRNA as degradation products has been reported for a number of different malignant diseases (9, 10, 16, 19, 20, 22, 23, 34, 35, 37). Some of these compounds promise to be potential biological markers for diagnosis and of possible value in monitoring the effectiveness of therapy (31, 36). This investigation was designed to define more clearly the role of methylated and minor base nucleic acid derivatives as diagnostic and monitoring markers.
possible relationship between increased tumor cell tRNA methyltransferase activity, the increased urinary excretion of minor base tRNA-catabolic end products, and the content of these bases in tRNA pools isolated from the same tumor cells. Previous preliminary study had shown that monkey hepatoma (hepatocellular carcinoma) cells, induced by N-nitrosodimethylamine, had increased tRNA methyltransferase activity (33) when compared to the cells of normal monkey liver. These original observations are being extended to determine the predominant individual methylated and minor nucleic acid base compounds and their levels found in urine from monkeys with hepatomas and, for comparative purposes, to isolate, purify, and degrade tRNA's from these hepatomas to determine their base composition. For control purposes, similar determinations were carried out using normal monkeys and tRNA isolated from their livers.

MATERIALS AND METHODS

Apparatus

A Mark II UV analyzer (Oak Ridge National Laboratories, Oak Ridge, Tenn.) equipped with a 150- x 0.455-cm (i.d.) column containing Aminex 27 anion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.), a 2-channel gradient generating system (7), and a programmable temperature control system were used for separation and analysis of the major and minor bases present in tRNA's and for the analysis of the urine excretion levels of the minor nucleosides and 7-MeGua. The eluate from the chromatographic column was monitored at 254 and 280 nm by a doo Monitor (Laboratory Data Control, River Beach, Fla.), and a Servo/ Riter II (Texas Instruments, Houston, Texas) dual-pen recorder was used to record the chromatograms.

A Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, Ill.) was used to measure tRNA methyltransferase activities and to determine the extent of methylation of tRNA by the methyltransferases.

A sand bath with a variable temperature control (±2°) was used for hydrolysis of the tRNA samples.

A Gilford 2400-S spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ottawa) was used to determine tRNA levels and protein content of tissue homogenates.

Samples and Sample Preparation

Animals and Induction of Cancer. N-Nitrosodimethylamine was administered to normal healthy rhesus (Macaca mulatta) monkeys (male and female) as previously reported (1, 33). Periodic liver biopsies were done to determine the status of the organ, and monkeys with clinical and histo-pathological evidence of hepatocellular carcinoma were selected for study. The animals were killed by decapitation, the livers were removed, and tumor tissue was dissected free of surrounding liver. Portions of tumor were also taken for confirmatory histological examination. Normal control monkey livers were prepared at the same time. All tissues were stored at —170° for later study. Just prior to decapitation, 24-hr urine specimens from each hepatoma-bearing and normal control monkey were collected without preservative in containers chilled by surrounding ice. Aliquot samples were frozen and maintained at —170° until analysis.

tRNA Methylase Activity. The tRNA methyltransferase activity of each liver tissue (4 hepatoma and 3 normal controls) was determined by the method reported earlier by Waalkes et al. (33). After thawing, the tissues were homogenized in 3 volumes of a solution containing 0.3 m sucrose, 1 mm dithiothreitol, 0.01 m MgCl2, and 0.01 m Tris (pH 8.0), with a Teflon-glass motor-driven homogenizer. Tissue extracts were centrifuged at 105,000 x g for 1 hr at 4°, and the supernatant fraction was filtered through glass wool. The filtrate was divided into aliquot samples and stored at —170° for later enzyme assays.

Protein content of the tissue extracts were determined by the method of Lowry et al. (18).

For the measurement of the tRNA methyltransferase-specific activities (rates) of tissue homogenates, the reaction mixture contained 6.8 nmoles S-adenosyl-L-[14C]methionine (New England Nuclear, Boston, Mass.; 57.7 mCi/mmmole), 25 μmoles Tris-HCl (pH 9.0), 70 μmoles ammonium acetate, 0.3 μmoles dithiothreitol, variable amounts of tissue homogenates, and 60 μg undermethylated Escherichia coli tRNA prepared from a relaxed E. coli mutant K12W6, by the method of Fleissner and Borek (11). For determination of tRNA capacities (maximal extent of methylation), the reaction mixtures were the same except that only 1 μg of tRNA was used. For the latter assays, tRNA was shown to be limiting and the methyltransferases were in excess. Control samples without tRNA were run for each assay. Reactions were carried out at 37° for 1 hr and terminated by immersion in ice water. Carrier tRNA, 0.05 ml of a 10-mg/ml solution, was added to each tube followed by 1.0 ml 95% ethanol. After 1 hr at —30°, the tubes were centrifuged at 7000 rpm for 20 min at 4°, and the ethanol was decanted off. To extract the tRNA from the precipitate, 1 ml of 1 m NaCl solution was added and incubated at 40° for 45 min. After centrifugation at 12,000 rpm for 20 min, the supernatant was removed and mixed with 3 ml of 10% TCA. After 20 min in ice, the contents of each tube were filtered through type HA Millipore filters (pore size, 0.45 μm) and the precipitate was washed with 30 ml ice-cold 5% TCA. The filters were dried and put in vials containing 10 ml toluene phosphor, and radioactivity was determined in a Packard liquid scintillation counter.

Urinary Excretion Levels of Nucleic Acid Components. The urinary excretion levels of the nucleic acid components, pseudouridine m2G, m1, 7-MeGua, β-AIBA, and β-alanine, were determined for the normal and hepatoma-bearing monkeys. The determination of the urinary excretion level of the minor nucleic acid derivatives was accomplished by a method previously reported, using high-resolution liquid chromatography for separation and quantitation.

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The abbreviations used are: i.d., inside diameter; 7-MeGua, 7-methylguanine; TCA, trichloroacetic acid; μg, m1G, m4G,m4-dimethylguanosine; m1I, 1-methylinosine; β-AIBA, β-aminoisobutyric acid; MeB, methylated base; 2-MeGua, N4,N5-dimethylguanine; 1-MeGua, 1-methylguanine; S-MeCyt, S-methylcytosine.
The urine sample was first filtered through a 0.45-
μm Millipore filter and then placed directly on a 125- x
0.455-cm (i.d.) Aminex 27 anion-exchange column. The
sample was eluted with a 0.015 M ammonium acetate buffer,
pH 4.7, and the eluate was monitored at 254 and 280 nm.
The urinary excretion levels of β-AIBA and β-alanine were
determined by a modification (C. W. Gehreke, and K. K. Kuo,
unpublished observations) of the method previously
reported (35). The analysis of mI by the liquid chromato-
graphic method was not possible due to an interfering
urinary component. The excretion levels for mI were deter-
mined by the gas-liquid chromatographic method reported
by Chang et al. (6).

Transfer RNA Isolation and Analysis. tRNA was isolated
from the hepatoma and normal monkey liver tissues by the
method described by Gallo and Pestka (12) with an addi-
tional final purification by chromatography on DEAE-cellu-
lose as described by Yang and Novelli (39). After thawing,
the tissue was homogenized with a solution containing 0.01
M Tris, 0.15 M NaCl, 1 mM EDTA, 1 mM MgCl₂, and 1% bentonite (1 g tissue per ml solution). Following centrifuga-
tion, the supernatant was extracted with phenol (freshly
distilled), saturated with the homogenizing solution, and
then with chloroform. The aqueous phase was mixed with
2.2 volumes 95% ethanol and 0.1 volume 20% potassium
acetate and allowed to stand overnight at —30°. After cen-
trifugation at 10,000 rpm, the ethanol was decanted and the
tRNA precipitate was dissolved in the homogenizing solu-
tion. After 2 chloroform extractions and precipitation with
ethanol-potassium acetate, the tRNA precipitate was disso-
volved in a solution of 1 M NaCl, 1 mM EDTA, and 10 mM
MgCl₂. The extract was centrifuged at 10,000 rpm, and the
supernatant was removed. The residue was extracted again
and centrifuged, and the supernatants were combined and
precipitated with ethanol:potassium acetate. The tRNA pre-
cipitate was dissolved in the homogenizing solution and
subjected to DNase at 37° for 15 min. The enzyme was
removed by extraction with phenol, and the aqueous layer
was mixed with ethanol:potassium acetate to precipitate the
tRNA. The sample was centrifuged at 10,000 rpm, and the
precipitate was dissolved in deionized water and cen-
trifuged at 40,000 rpm for 1 hr. The supernatant was removed,
shell frozen, and lyophilized. The tRNA was dissolved in
deionized water and placed on a 12.5- x 1.0-cm (i.d.) DEAE
column. The column was washed with 50 ml of 1.25 M NaCl
at 1 ml/min, and the tRNA was eluted with 50 ml 0.7 M NaCl
at 1 ml/min. The fractions containing the tRNA were com-
bined and precipitated with ethanol. The precipitate was
dissolved in water, shell-frozen, and lyophilized. The tRNA
was stored at —170° for later use.

The analytical procedure used to determine the methyl-
atated base content of the normal and hepatoma monkey liver
tRNA pools was reported earlier (17). The tRNA pools were
first hydrolyzed to the free bases with 400 μl of trifluoroaa-
cetic acid:formic acid (1:1, v/v) heated in a closed vial at 200°
for 1.5 hr (15). The hydrolyzing agents were removed by
simple evaporation, and the sample was dissolved in 1.0 ml
0.1 N NaOH. The sample was placed on the Mark II UV
Analyzer, and the major and minor bases separated using a
2-chamber gradient system (7). The 1st chamber contained
0.015 M ammonium acetate, pH 9.2, and the 2nd contained
1.2 M ammonium acetate, pH 9.2. The flow rate was approx-
imately 35 ml/hr, and the eluate was monitored at 254 and
280 nm. The temperature was at ambient for the 1st 1.75 hr
and then programmed at 0.5°/min to 60° for the remainder
of the analysis. To measure quantitatively the MeB content
of the individual tRNA’s, duplicate samples containing 200
to 300 μg tRNA were hydrolyzed and analyzed. The level of
each MeB was calculated by measuring the peak height at
254 or 280 nm and comparing it to the peak height of a
known amount of that MeB on a standard chromatogram. A
similar procedure was used to determine the major base
levels of each tRNA, except that 70 to 80 μg of tRNA were
hydrolyzed.

Since pseudouridine was found to be degraded during
the hydrolysis of the tRNA to the free base level, it was
necessary to analyze the tRNA’s at the nucleoside level to
determine the pseudouridine content. The tRNA samples
were enzymatically hydrolyzed to the nucleoside level in 0.1
ml 0.2 M sodium acetate and 0.02 M magnesium acetate (pH
8.8) containing 80 μg snake venom phosphodiesterase and
1 unit alkaline phosphatase (27). The samples were incu-
bated at 37° for 4 hr, and the enzymes were removed by
precipitation with ethanol. The resulting nucleosides were
placed on the analyzer and separated with a 0.02 m ammo-
nium acetate buffer, pH 3.2, and the pseudouridine level
was determined from its extinction coefficient at 254 nm.

Assessment and Comparison of Degree of Methylation.
To evaluate further and compare the possible differences in
the individual activities and methylating ability of the tRNA
methyltransferases present in normal liver and hepatoma
homogenates, undermethylated E. coli tRNA, as substrate,
was reacted in vitro with S-adenosyl-L-[C14]methionine in
the presence of the individual tissue extracts. The amounts
of the methyltransferase reaction described earlier were
increased 10-fold each, the total protein content for each
tissue homogenate was adjusted to exactly 20 mg, and the
methyl-deficient E. coli tRNA content was 200 μg. The iso-
lation and purification of the E. coli tRNA were as previously
indicated. The final tRNA precipitate, however, was not
collected on a Millipore filter after the 10% precipitation but
was washed first with 5% TCA, centrifuged, and then pre-
cipitated with ethanol. The tRNA was hydrolyzed and subse-
quently analyzed by the high-resolution liquid chromato-
graphic method discussed earlier. The eluate was collected
in 5-ml fractions, dried, redissolved in 0.5 ml 0.1 N acetic
acid, and counted in 10 to 15 ml Aquasol liquid scintillation
fluid in a Packard Tri-Carb spectrometer. The identification
of the various MeB was verified by the addition of 2.5 μg of
each individual base to the hydrolyzed tRNA sample prior to
liquid chromatographic analysis. For determination of the
amount of the 14C methyl group present in the isolated tRNA
as methylated bases, an aliquot was taken and counted
prior to hydrolysis; the prehydrolysis count was then com-
pared to the summation of the MeB counts obtained after
analysis.

For further study of the individual tRNA methyltransferase
activities and methylating abilities, E. coli β tRNA and nor-
mal monkey liver and hepatoma tRNA was used as the
methyl receptors. The methyltransferase reaction condi-

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tissues, tRNA isolation, and liquid chromatographic separation and analysis were the same as for the undermethylated E. coli tRNA.

RESULTS

tRNA Methyltransferase Activity and Capacity. In Chart 1A, the results obtained when undermethylated E. coli tRNA was used as the methyl acceptor for varying amounts of tissue homogenates from hepatoma and normal monkey livers are shown. In these experiments, the 60 μg tRNA per sample were in excess, and the enzyme was limiting. The tRNA methyltransferase activities of the 4 hepatoma homogenates were 1.5 to 2.0 times higher than those of the 3 normal livers. Chart 1B depicts the curves obtained when the tRNA was limiting (1 μg assay) with the same level of enzyme. The tRNA methyltransferase activities of the 4 hepatoma homogenates were 1.5 to 2.0 times higher than those of the 3 normal livers. Chart 1B depicts the curves obtained when the tRNA was limiting (1 μg assay) with the same level of enzyme.

Urinary Excretion Levels. The average urinary excretion levels of pseudouridine, m1G, m22G, and 7-MeGua expressed as uracil are presented in Table 1. Two 24-hr collections were obtained and analyzed for hepatoma-bearing monkeys 1, 2, and 3 and for normal Monkey 3. Also in Table 1, the average excretion levels of β-AIBA and β-alanine in μmoles/kg/24 hr volume for the hepatoma and normal monkeys are presented, along with the β-AIBA:β-alanine ratio. The standard error for each of the compounds was calculated and is given in Table 1. The excretion levels of the tRNA degradation products for the hepatoma-bearing monkeys were all higher than the levels of the corresponding compound from the normal monkeys. The degree of elevation varied among the hepatoma monkeys and for individual compounds. The average level of excretion for pseudouridine, m22G, and β-AIBA for the hepatoma-bearing monkeys was more than twice the average level for the normal monkeys.

Isolation and Analysis of tRNA. Using the procedure outlined in "Materials and Methods" for tRNA isolation, the livers of 3 normal and 3 hepatoma-bearing monkeys were homogenized and the tRNA was purified. A typical chromatogram obtained from the final DEAE column purification step is shown in Chart 2. Each isolated tRNA sample gave a single chromatographic peak indicating a homogeneous mixture of the various individual tRNA's. Charts 3 and 4 present chromatograms for the analysis of MeB in hepatoma and normal monkey liver tRNA pools, respectively. The average mole % composition and the standard error for the normal and hepatoma tRNA's are given in Table 2. The pseudouridine content of the tRNA's was determined at the nucleoside level; chart 5 presents a typical chromatogram for pseudouridine analysis. The average μmole pseudouridine per μmole uracil % and the standard error for the normal and hepatoma tRNA pools are also given in Table 2.

Individual tRNA Methyltransferase Capacity. The methyltransferase capacity of the hepatoma and normal monkey tissue extracts as previously used. At saturation, the 4 hepatoma homogenates produced a greater level of methyl group incorporation when compared to those of the normal livers.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normala</th>
<th>S.E.</th>
<th>Hepatomaa</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudouridine</td>
<td>1.49</td>
<td>0.12</td>
<td>3.06</td>
<td>0.18</td>
</tr>
<tr>
<td>m1G</td>
<td>0.11</td>
<td>0.01</td>
<td>0.21</td>
<td>0.01</td>
</tr>
<tr>
<td>m22G</td>
<td>0.08</td>
<td>0.01</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>7-MeGua</td>
<td>0.16</td>
<td>0.02</td>
<td>0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>β-AIBA</td>
<td>0.544</td>
<td>0.064</td>
<td>1.43</td>
<td>0.17</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>10.45</td>
<td>0.69</td>
<td>11.36</td>
<td>0.58</td>
</tr>
<tr>
<td>β-AIBA:β-Alanine</td>
<td>0.052</td>
<td>0.006</td>
<td>0.132</td>
<td>0.018</td>
</tr>
</tbody>
</table>

* Average normal monkey excretion levels. Single 24-hr collections for two monkeys and two 24 hr collections for 1. All samples analyzed in duplicate.

† Average hepatoma monkey excretion levels. Two 24-hr collections for 3 monkeys. All samples analyzed in duplicate.
The results in Chart 6 show that by in vitro interaction the methyltransferases of the hepatoma and normal monkey livers possess different methylating abilities for specific bases in the same tRNA. The hepatoma methyltransferases, when compared to the average of the normal enzymes, gave increased levels of thymine, 2-Me2Gua, 1-MeGua, and 7-MeGua; decreased N2-methylguanine content; and similar levels for 5-MeCyt, 1-methylhypoxanthine, and 1-methyladenine:N6-methyladenine.

The average methyltransferase activity with undermethylated \textit{E. coli} tRNA as substrate, the mole % of the isolated tRNA's and the urinary excretion levels are compared in Table 3 for the hepatoma-bearing and normal monkeys. Increased hepatoma methyltransferase activity for thymine, 2-MeGua, and 7-MeGua correlated well with the increased excretion of these compounds by the hepatoma-bearing monkeys; however, the hepatoma tRNA content for these

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**Chart 3.** High-pressure liquid chromatography analysis of methylated bases in hepatoma monkey liver tRNA. Column, 150 x 0.455 cm i.d.; Aminex A-27 anion-exchange resin. First chamber, 0.015 M ammonium acetate (pH 9.2); 2nd chamber, 1.2 M ammonium acetate (pH 9.2). Temperature, ambient for 1st 1.75 hr, then programmed to 60° for remainder of analysis. Flow rate, 35.0 ml/hr (3.6 ml/sq cm/min). UV output, 0.04 absorbance unit full scale. Peak identification: 1, 3-methylcytosine; 2, cytosine; 3, 5-MeCyt; 4, 1-methyladenine; 5, 1-MeGua; 6, thymine; 7, uracil; 8, 7-MeGua; 9, guanine; 10, 2-methyladenine; 11, Adenine; 12, 2-methylguanine; 13, 1-methylhypoxanthine; 14, 2-MeGua; 15, N6-methyladenine; R, ribose degradation product; U, unidentified component.

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**Table 2.** Mole % composition of monkey liver tRNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normal (\text{Av.})</th>
<th>S.E.</th>
<th>Mole % composition of monkey liver tRNA</th>
<th>Normal (\text{Av.})</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methylcytosine</td>
<td>0.42</td>
<td>0.03</td>
<td>0.28</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>0.05</td>
<td>0.28</td>
<td>27.99</td>
<td>1.29</td>
<td></td>
</tr>
<tr>
<td>5-MeCyt</td>
<td>1.91</td>
<td>0.11</td>
<td>1.57</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>1-Methyladenine:N6-</td>
<td>1.28</td>
<td>0.10</td>
<td>1.24</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>methyladenine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Methylguanine</td>
<td>0.70</td>
<td>0.05</td>
<td>0.49</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Thymine(^{e})</td>
<td>1.38</td>
<td>0.11</td>
<td>5.67</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>19.33</td>
<td>0.53</td>
<td>21.46</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>7-MeGua</td>
<td>0.57</td>
<td>0.04</td>
<td>0.41</td>
<td>0.03</td>
<td></td>
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<tr>
<td>Guanine</td>
<td>24.90</td>
<td>0.96</td>
<td>22.32</td>
<td>0.72</td>
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<tr>
<td>2-Methyladenine</td>
<td>0.37</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>16.99</td>
<td>0.22</td>
<td>17.29</td>
<td>1.05</td>
<td></td>
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<tr>
<td>2-Methylguanine</td>
<td>1.44</td>
<td>0.09</td>
<td>1.18</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>1-Methylhypoxanthine</td>
<td>0.40</td>
<td>0.06</td>
<td>0.44</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>2-MeGua</td>
<td>0.51</td>
<td>0.04</td>
<td>0.49</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Pseudouridine(^{d})</td>
<td>17.3</td>
<td>0.70</td>
<td>12.4</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Mole/composition = nmole base/nmole total base × 100.

\(^{b}\) Average of 3 normal and 3 hepatoma tRNA pools. Each tRNA pool analyzed in duplicate.

\(^{c}\) Possible DNA contamination.

\(^{d}\) Pseudouridine as \(\mu\)mole/\(\mu\)mole uracil %.
Comparison of methyltransferase activity, tRNA content, and urinary excretion level.

<table>
<thead>
<tr>
<th>Methyltransferase activity</th>
<th>tRNA content</th>
<th>Urinary excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.0</td>
<td>1.38</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>3.2</td>
<td>5.67</td>
</tr>
<tr>
<td>2-MeGua</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>17.3</td>
<td>0.51</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>22.7</td>
<td>0.48</td>
</tr>
<tr>
<td>1-Methylhypoxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.8</td>
<td>0.40</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>0.7</td>
<td>0.44</td>
</tr>
<tr>
<td>7-MeGua</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>12.1</td>
<td>0.57</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>19.9</td>
<td>0.41</td>
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<tr>
<td>Pseudouridine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.16</td>
<td>1.46</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>0.74</td>
<td></td>
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</tbody>
</table>

Chart 5. High-pressure liquid chromatography analysis of pseudouridine in hepatoma monkey tRNA. Column, 125 x 0.465 cm i.d.; Aminex A-27 anion-exchange resin. Eluent, 0.02 M ammonium acetate (pH 3.2). Temperature, as given for Chart 3. Flow rate, 20 ml/hr (1.4 ml/sq cm/hr). UV output 0.04 absorbance until full scale. Peak identification: 1, N'-methylcytosine, N'-methyladenine, N'-methylguanine; 2, cytosine, N'-methylcytosine; 3, pseudouridine; 4, uracil, 5, adenine; 6, N-methylguanine; 7, m2G; 8, guanine, N'-methylguanine, N'-methyladenine.

Chart 6. Distribution of methylated bases in undermethylated E. coli tRNA after methyltransferase reaction. Experimental conditions for methyltransferase reaction, tRNA purification and hydrolysis, liquid chromatographic separation and collection, and liquid scintillation counting given in text. —, average of normals; ‒ ‒ ‒ ‒ ‒ ‒, 1 S.D. from normal average; ○, normals; △, Hepatoma 1; ○, Hepatoma 2; △, Hepatoma 3; △, Hepatoma 4. Total counts: 10, 10,000; 20, 20,000. 1-MeAde, 1-methyladenine; 6-MeAde, N'-methyladenine; Thy, thymine; 1-MeHypo, 1-methylhypoxanthine.

When E. coli β tRNA was the methyl receptor for the methyltransferases, the profiles for the MeB were similar to those obtained for undermethylated E. coli tRNA. The hepatoma methyltransferases for 5-MeCyt showed increased activity with E. coli β tRNA as substrate, whereas incorporation of 7-MeGua in E. coli β tRNA was greatly reduced for normal and hepatoma methyltransferases. Also, a methyltransferase for 2-methyladenine was detected with E. coli β tRNA.

Table 3: Comparison of methyltransferase activity, tRNA content, and urinary excretion level.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normal</th>
<th>Hepatoma</th>
<th>Normal</th>
<th>Hepatoma</th>
<th>Normal</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymine</td>
<td>1.0</td>
<td>3.2</td>
<td>1.38</td>
<td>5.67</td>
<td>0.544</td>
<td>1.43</td>
</tr>
<tr>
<td>2-MeGua</td>
<td>17.3</td>
<td>22.7</td>
<td>0.51</td>
<td>0.48</td>
<td>0.10</td>
<td>0.21</td>
</tr>
<tr>
<td>1-Methylhypoxanthine</td>
<td>0.8</td>
<td>0.7</td>
<td>0.40</td>
<td>0.44</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>7-MeGua</td>
<td>12.1</td>
<td>19.9</td>
<td>0.57</td>
<td>0.41</td>
<td>0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>Pseudouridine</td>
<td>1.16</td>
<td>0.74</td>
<td>1.46</td>
<td>3.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Average of values obtained from 3 hepatoma-bearing and 3 normal monkeys.
b Methyltransferase activity as percentage of total counts with undermethylated E. coli tRNA as methyl receptor. No activity for pseudouridine.
c tRNA content as mole % composition. Pseudouridine as μmole/μmole uracil %.
d Urinary excretion: thymine as μmole/kg/24 hr; 1-MeGua as mg/kg/24 hr; 2-MeGua as mg/kg/24 hr m2G; 1-methylhypoxanthine as mg/kg/24 hr; pseudouridine as mg/kg/24 hr.

Chart 7. Distribution of methylated bases in Normal 2 and Hepatoma 3 tRNA using their respective tRNA methyltransferases, calculated as percentage of total MeB. Bars: H-H, Hepatoma 3 tRNA and methyltransferases; H-N, Normal 2 tRNA and Hepatoma 3 methyltransferases; N-H, Hepatoma 3 tRNA and Normal 2 methyltransferases; N-N, Normal 2 tRNA and methyltransferases.
prior saturation of the methylation sites or a difference in the methyl receptor sites between the monkey or E. coli tRNA's. As shown in Chart 7, only minor variations were found by using the normal monkey methyltransferases with either the hepatoma or normal tRNA as substrate. Similar MeB incorporation levels were observed when the hepatoma methyltransferase-normal tRNA substrate were interchanged except for an increased 2-methylguanine content. The hepatoma methyltransferase-hepatoma tRNA substrate gave increased thymine incorporation and reduced levels of 5-MeCyt and 1-MeGua.

**DISCUSSION**

The increased tRNA methyltransferase activity and capacity of the monkey hepatoma tissue homogenates confirm the results of an earlier report (33). The increased urinary excretion levels of specific tRNA degradation products for patients, including those with a number of different types of malignant disease, have been described before (16, 20, 33-37). The results presented here show a similarly increased excretion of pseudouridine m5'G, 7-MeGua, and β-AIBA by monkeys with N-nitrosodiethylamine-induced hepatomas compared to normal control monkeys. The analysis of the isolated tRNA from hepatoma tissue and normal livers from these same monkeys showed similar, or slightly decreased, levels of tRNA-methylated bases. Randerath et al. (25) have reported similar or decreased levels of MeB's in rat liver hepatoma tRNA which agrees with the data presented for the monkey liver tRNA's. The values reported for thymine in the tRNA pools varied greatly, which could be caused by DNA contamination (5). Although the tRNA isolation procedure contained a DNase step and final tRNA purification by DEAE-cellulose chromatography, the thymine tRNA values are considered questionable and were not used in the evaluation of the results.

As presented in "Results" when undemethylated E. coli tRNA was used as the methyl receptor, the hepatoma liver homogenates were able to synthesize greater amounts of thymine, 2-MeGua, 1-MeGua, and 7-MeGua than the normal liver extracts with the same tRNA substrates. Similar ratios of the MeB, using the hepatoma and normal homogenates, were obtained when E. coli β tRNA was the substrate, except for 5-MeCyt, which was increased by the hepatoma methyltransferases. The ratio of 7-MeGua incorporation for the 2 E. coli tRNA's as a substrate was similar; however, a much lower level of synthesis of 7-MeGua was obtained with E. coli β tRNA. The increased methyltransferase activity for 2-MeGua, 1-MeGua, and 5-MeCyt does not show increased tRNA content in the hepatoma-bearing monkeys although an increased excretion level for M22G was noted. When the hepatoma methyltransferases were reacted with their own isolated tRNA, reduced levels of 5-MeCyt and 1-MeGua were found, correlating with the hepatoma tRNA composition data and suggesting different methyl receptor sites for the E. coli and monkey liver tRNA's.

Comparison of the urinary excretion levels, tRNA base composition, and individual tRNA methyltransferase activity for the hepatoma-bearing and normal monkeys indicates both increased tRNA turnover and aberrant tRNA methylation in the hepatoma-bearing monkeys. The increased total tRNA methyltransferase activity and capacity and the greater urinary excretion levels for the catabolic tRNA end products without a corresponding increase in the tRNA composition suggest a more rapid tRNA turnover. This is further substantiated by increased pseudouridine excretion by the hepatoma-bearing monkeys, although pseudouridine content is actually decreased in the hepatoma-bearing monkeys. Also, Borek et al. (9) have recently found very high turnover of tRNA in rats with tumors of the bladder. The variations in the individual tRNA methyltransferase activities of the hepatoma and normal liver homogenates point to differences in the methylation of the tRNA's. Since there are at least 60 tRNA species present in the mammalian cells (12) and since the tRNA base composition was determined on the total tRNA pool, it is possible that aberrant methylation occurs on only a few of the species, and these changes are not detected when the total tRNA pool is analyzed. Further fractionation of the tRNA pool into individual tRNA's will be necessary to determine whether alterations occur on only a few of the tRNA's that are present.

**REFERENCES**


