Early Hypomethylation of 2'-O-Ribose Moieties in Hepatocyte Cytoplasmic Ribosomal RNA Underlies the Protein Synthetic Defect Produced by CCl₄

Gary A. Clawson, John R. MacDonald, and C. H. Woo
Department of Pathology, School of Medicine, University of California, San Francisco, California 94143

Abstract. Carbon tetrachloride (CCl₄) treatment of rats produces an early defect in methylation of hepatocyte ribosomal RNA, which occurs concurrently with a defect in the protein synthetic capacity of isolated ribosomes. The CCl₄-induced methylation defect is specific for the 2'-O-ribose position, and a corresponding proportional increase in mG base methylation occurs in vivo. Undermethylated ribosomal subunits (rRNA) from CCl₄-treated preparations can be methylated in vitro to a much greater extent than those from control preparations, and in vitro methylation restores their functional capacity. In vitro methylation of treated ribosomal subunits (which restores functional capacity) occurs at 2'-O-ribose positions (largely G residues). In contrast, in vitro methylation of control ribosomal subunits (which does not affect functional activity) represents base methylation as mTG, sites which are apparently methylated in treated preparations in vivo. Methylation/demethylation of 2'-O-ribose sites in rRNA exposed on the surface of cytoplasmic ribosomal subunits may represent an important cellular mechanism for controlling protein synthesis in quiescent hepatocytes, and it appears that CCl₄ disrupts protein synthesis by inhibiting this 2'-O-ribose methylation.

Carbon tetrachloride (CCl₄) has long served as a model compound for study of hepatic injury (9, 36, 37, 39, 46). Like many other agents, CCl₄ is metabolized via cytochrome P-450 (25, 39) to yield a free radical (34, 44, 45, 50, 51), which may mediate subsequent damage. A number of subcellular processes/compartments are affected (including lipid peroxidation and Ca⁺⁺ influx), and a well-described morphologic sequence ensues (39, 46), although the basic mechanisms underlying the cellular injury are not clear (3, 40, 53).

Early studies established the endoplasmic reticulum (ER) as the site of the first significant cellular changes (38, 39, 46). Altered ER structure occurs by 30 min after oral CCl₄ administration (46, 47) and consists chiefly of a marked loss of ribosomes from rough ER ("degranulation") and a disaggregation of polysomes. Slightly later, other morphological changes in ER (such as tubular aggregates) can be detected.

The major early functional defect involves protein synthesis (47); alterations in protein synthesis are specific for hepatocytes and parallel morphological alterations within the lobules (24). The nature of the protein synthetic defect is not clear. Microsomes and ribosomes isolated after CCl₄ intoxication show significantly decreased protein synthesis (47), while the respective soluble cell-sap fractions appear to be equally efficacious in supporting in vitro incorporation (46); the changes do not involve alterations in amino acid pools or uptake (48).

The CCl₄-induced breakdown of polysomes might involve alterations in mRNAs, or may reside in the ribosomal subunits. More specifically, translatability of mRNA is regulated by methylation in "cap" structures (27), and changes in methylation of ribosomal subunits have been related to functional changes in protein synthesis (13, 15, 18, 28, 31-33, 43, 49, 52). We therefore explored the possibility that alterations in RNA methylation may underlie the early defect in protein synthesis after CCl₄ administration.

Materials and Methods

Male Sprague-Dawley rats that weighed 250-300 g were used. CCl₄ was administered at 0.25 ml/100 g body weight with an equal volume of mineral oil by stomach tube. For methylation studies, rats were given CCl₄; 0.5 h later, they were given 1.6 mCi [3H]methyl-L-methionine (New England Nuclear, Boston, MA, 70-85 Ci/mol in H₂O; or Amersham Corp., Arlington Heights, IL, 87 Ci/mol in 70% ethanol) intraperitoneally. After an additional 1-h labeling period, they were killed. For initial time course experiments, rats were given 0.25 mCi [3H]methylmethionine at 0.5 h after CCl₄ administration, and labeling was for 1, 2, or 4 h. For some comparative studies using the acute-phase response, rats were given 0.25 ml turpentine/100 g body weight subcutaneously, and were killed after various intervals (maximal levels of acute-phase reactant mRNAs occur after 36 h).

Rat livers were removed and homogenized in 0.25 M STKM2 buffer (su-
of molarity as specified, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 g for 30 min at 0°C. The supernatant fractions were further centrifuged at 105,000 g for 90 min at 4°C; the supernatant remaining from this centrifugation, designated as S100, was adjusted to 30 or 5 mg protein/ml (S9) for subsequent use. The crude pellets were used for subsequent nuclear isolations.

For preparation of ribosomes, sodium deoxycholate was added to the 10,000 g supernate to a final concentration of 1%, and this was centrifuged at 105,000 g for 45 min at 4°C. The pellet was resuspended in 25 mM Tris-HCl, pH 7.4, 5 mM KCl, 1 mM MgCl₂, and adjusted to 6 mg protein/ml; treated and control suspensions contained equivalent quantities of poly(A)⁺ RNA and mRNA. For preparation of ribosomal subunits, polysomes were dissociated by incubation with puromycin (4). The dissociated subunits were then harvested by centrifugation at 60,000 g for 16 h at 0°C. Ribosomal suspensions were fractionated on 10–40% sucrose buffer gradients by centrifugation at 117,000 g for 2.5 h at 4°C (S4). The subunits were harvested by centrifugation and separated by HPLC.

For analysis of in vitro protein synthesis, 60 μl of the ribosomal suspension and 10 μl of S100 (at 5 mg/ml) were added to reaction mixtures (100 μl) containing Tris-HCl, pH 8.5, 5 mM MgCl₂, 100 mM NaCl, 3.1 mM dithiothreitol (DTT), 0.31 mM ATP, 0.31 mM GTP, and [³⁵S]methionine (in some experiments, poly(U) was added to 5 mg/ml). Incubations were for 30 min, during which time incorporation was linear. After incubation, mixtures were precipitated on ice for 30 min in 10% TCA and collected on filters. These were rinsed with cold 5% TCA, twice with absolute ethanol, once with ethanol/ether, once with ether; then dried, and counted in scintillant. Translation of poly(A)⁺ mRNA purified from S100 fractions was performed with a nu- clease-treated rabbit reticulocyte lysate system (Bethesda Research Laboratories, Bethesda, MD) and [³⁵S]methionine.

For methylation studies, cytoplasmic RNA from the 10,000 g supernate was isolated by centrifugation through 57.2 M CsCl (8, 11); recovery of rRNA is <1%. RNA was separated into poly(A)⁺ and poly(A)⁻ fractions using LiCl buffers and oligo(dT)-cellulose (23). In other experiments, cytoplasmic poly(A)⁺ RNA was fractionated on 5–25% STKM₂ gradients by centrifu- gation at 117,000 g for 16 h at 4°C. The 28S and 18S ribosomal RNAs were digested to nucleosides (6); 3 mg of methylated RNA was treated with chloropane (chloropane consists of equal volumes of phenol and chloroform, 0.1% tri- fluorotoluene (TFT), and 0.5% chloroform) and precipitated twice in ethanol, then digested with DNase I (Worthington Biochemical Corp., Freehold NJ), and subsequently with proteinase K (23), then reextracted and precipitated. Nuclease and cytoplasmic RNA preparations were free of de- tectable DNA and protein, as assessed using standard assays (80, 19).

Purified RNA fractions were then digested to nucleosides (6); 5 mg of RNase A was added to each sample, the mixture was heated to 95°C for 5 min, and then rapidly cooled. 125 U of RNase T₂ (Bethesda Research Laboratories) and 40 U of RNase P₁ (Bethesda Research Laboratories) were added. After overnight incubation at 37°C, the pH of the mixture was adjusted to 9 by addition of ammonium acetate to 10 mM, and 100 U of bacterial alkaline phosphatase (Bethesda Research Laboratories) were added and the mixture was again incubated overnight at 37°C. This digestion resulted in products which were 100% soluble in TCA or ethanol.

RNA digests were concentrated by lyophilization, resuspended in water, and fractionated using HPLC essentially as described for uncharged nucleo- sides (6). We used a model No. 1084 HPLC (Hewlett-Packard Co., Palo Alto, CA), a reverse-phase Partisil 5 ODS-3 column (Whatman Inc., Clifton NJ), and a linear 0–10% acetonitrile gradient in water over the initial 40 min. Either 0.5- or 1-ml fractions were collected. An initial peak consisting of residual oligonucleotides was obtained in some experiments. In many cases, this peak was collected, lyophilized, and redissolved as before, and then refractionated via HPLC. This resulted in conversion to nucleosides with chromatographic profiles analogous to the original patterns, showing that the variable quantity of oligonucleotides represented a random assort- ment of methylated nucleosides (in these cases, the counts were summed).

In other gradients, gradient-purified ribosomal subunits were prepared as described; 20 pmol were mixed with various quantities of S100 and 2.5 μCi [³²P]methylated s-adenosylmethionine (AdoMet; New England Nuclear, 35–85 Ci/mmol, diluted to a specific activity of 0.5 Ci/mmol [13, 43, 49, 52]); standard aliquots contained 12 μg or 24 μg of methylated ribosomal subunits, 1.5 μg or 7.5 μg S100 protein, and 1 nmol [³²P]AdoMet. After various periods (0–40 min) at 37°C, the mixtures were extracted with chloro- rane, and TCA-precipitable material was collected on filters, rinsed exten- sively, and counted. In other experiments, after methylation of isolated ribosomal subunits with [³²P]AdoMet, the subunits were precipitated in ethanol and resuspended in S100 fractions from control or treated animals. After various periods, the radioactivity remaining in the ribosomal RNA was quantitated as before.

In other experiments, aliquots of 40–60S ribosomal subunits (90 μg protein) were methylated in vitro with unlabeled AdoMet (at 170 μM for 1 × incubations, and also at 5 and 10 ×) in the presence of 300 μg of S100 protein. After incubation at 35°C for 40 min, the mixtures were chilled and methylated ribosomal subunits were harvested by centrifugation at 105,000 g for 1 h. Aliquots were then resuspended with 1 μg of purified poly(A)⁺ mRNA, and protein synthesis was assessed as previously described with [³⁵S]methionine.

To examine the specificity of the in vitro methylation, purified 40S and 60S ribosomal subunits from treated or control preparations were incubated in 10× [³²P]AdoMet (250 μCi, specific activity 60 Ci/mmol) with control S100 (200 μg protein) for 30 min at 37°C. The mixtures were then extracted with chloropane and the aqueous phase was precipitated twice in ETOH. The pelleted RNA was then digested as described and examined via HPLC.

Results

In initial studies, we examined the time course of methylation of cytoplasmic RNA fractions after various labeling periods, beginning 0.5 h after CCI₄ intoxication (Fig. 1). No significant changes in methylation of poly(A)⁺ RNA were observed (Fig. 1, Table I), although the “degration” of ER was associated with a marked flux of functional poly(A)⁺ RNA into the S100 fraction (Fig. 2). In contrast, we observed marked changes in methylation of poly(A)⁻ RNA. A max- imal (60%) decrease in methylation of cytoplasmic poly(A)⁻ RNA occurred by 1.5 h after CCl₄ administration; this decrease was maintained at 2.5 h, and the methylation level was returning toward control levels at 4.5 h. The methylation def- ect paralleled the defect in protein synthesis noted under these conditions (see Fig. 1).

![Figure 1. Methylation of cytoplasmic RNA fractions after CCl₄.](https://example.com/figure1.png)

The Journal of Cell Biology, Volume 105, 1987 706
We examined the methylation defect in cytoplasmic poly(A)− RNA in more detail (see legend to Table I). The decreased specific activity of methylation of rRNA was most marked in 28S rRNA (1.92 ± 0.29 vs. 0.82 ± 0.17 cpm/µg), and a significant decrease was also noted in 18S rRNA (2.10 ± 0.30 vs. 1.37 ± 0.14 cpm/µg); the specific activity of the smaller RNA fraction (which includes 5.8S RNA and presumably poly(A)− mRNA) was not changed (3.80 ± 0.58 vs. 3.43 ± 0.43 cpm/µg). We estimate that >90% of cytoplasmic poly(A)− RNA methylation is associated with rRNA (see legend to Table I).

We enzymatically degraded cytoplasmic poly(A)− RNA preparations, and analyzed the resulting products by HPLC (Fig. 3, Table II). The defect in methylation was specific for 2′-O-ribose methylation. Control preparations contained 76.7 ± 4.8% of label in 2′-O-ribose positions, whereas this decreased to 57.6 ± 3.5% in treated preparations; these data imply a 70% decrease in total 2′-O-ribose methylation. There was sparing of purine base methylation, with a greatly increased proportion of the methylation as m7G in the treated fractions. Subsequent experiments showed that the m7G was found only in the 18S fraction (see below).

When we assessed the protein synthetic capacity of microsomes and ribosomes isolated from a number of treated preparations, we found that they showed a deficit similar in magnitude to the methylation defect (Table III). Since addition of poly(U) has been reported to stimulate protein synthesis by ribosomes isolated from CCl4-treated preparations, we examined the effects of poly(U) addition. We found that the restoration of protein synthetic capacity was highly variable and not reproducibly obtained, and was observed only with isolated ribosomes (this may reflect altered binding properties; see Discussion). Addition of poly(U) to microsomal preparations was without significant effect (Table III).

Our initial interpretation was that the decreased methylation of cytoplasmic rRNA must reflect a defect in methylation of nucleolar precursors (20–22, 29, 54, 56). We therefore isolated nucleoli and extracted pre-rRNA from them.

We next isolated ribosomal subunits, incubated them with CCl4-treated animals to enzymatic digestion and HPLC separations. The observed methylation pattern showed a 2′-O-ribose content (73.9%), which was equivalent to that of control cytoplasmic poly(A)− RNA (or rRNA) digests (Fig. 4). That is, the 2′-O-ribose methylation defect was not found in treated nucleolar pre-rRNA preparations. We also observed a significantly increased content of m7G, analogous to that noted in treated cytoplasmic preparations (Fig. 3, Table II), and a somewhat lower percentage of m6A.

Table II. Incorporation of [3H]Methyl Groups into Cytoplasmic RNA Fractions

| Sample | Cytoplasmic poly(A)+ mRNA | Cytoplasmic poly(A)− RNA | Nucleolar RNA |
|--------|--------------------------|--------------------------|-------------|
|        | cpm/µg                  | cpm/µg                  | cpm/µg     |
| Control | 5.69 ± 0.03            | 3.91 ± 0.66            | 1.98 ± 0.57 |
| Treated | 5.27 ± 0.58            | 1.72 ± 0.88            | 4.68 ± 1.00 |

Rats were given 0.25 ml CCl4 and 0.5 h later, they received [3H]methylmethionine. After an additional 1-h labeling period, they were killed and cytoplasmic and nucleolar RNAs were prepared.

Table II. Distribution of Incorporated [3H]Methyl Groups in Poly(A)− Cytoplasmic RNA

| Percent of incorporated label in | 2′OmeC | 2′OmeU | 2′OmeG | 2′OmeA | m6A | m6A | m7G |
|--------------------------------|-------|-------|-------|-------|-----|-----|-----|
| Control                        | 23.4 ± 1.1 | 10.9 ± 1.1 | 22.2 ± 2.0 | 20.2 ± 5.5 | 16.7 ± 3.5 | 4.2 ± 1.6 | 2.3 ± 0.5 |
| Treated                        | 18.8 ± 0.8 | 11.1 ± 2.2 | 15.2 ± 1.2 | 12.4 ± 0.5 | 25.6 ± 1.8 | 3.8 ± 1.2 | 13.0 ± 2.0 |

After treatments as described, cytoplasmic poly(A)− RNA fractions were prepared, digested to nucleosides, separated via HPLC, and radioactivity corresponding to the methylated ribonucleosides was quantitated. Analyses of individual preparations showed an average of 76.7 ± 4.8% in 2′-O-ribose methylation in control preparations, compared with 57.6 ± 3.5 in treated preparations.
Undermethylated ribosomal subunits from treated preparations were methylated in vitro with unlabeled AdoMet and their capacity to direct protein synthesis was measured and compared to control preparations treated similarly. Remethylation of the treated ribosomal subunits resulted in significant increases in their synthetic capacity (Fig. 6) in an AdoMet concentration-dependent manner; at the highest AdoMet concentration tested, treated subunits showed 97 ± 8% of the control activity. Similar remethylolation of control preparations was without significant change.

In another series of experiments, the specificity of the in vitro methylation was examined (Fig. 7). With 40S and 60S ribosomal subunits from treated preparations, we observed a considerable methylation at 2'-O-ribose positions, most notably at G residues, along with a significant incorporation into m6A component, which is a well-described "late" cytoplasmic methylation (2, 17, 41, 55). In contrast, while control preparations also showed a prominent m5A component, there was little or no labeling at 2'-O-ribose moieties. Instead, most incorporation occurred at meG. This methylation corresponds to that observed in vivo with treated cytoplasmic (Fig. 3) and nucleolar (Fig. 4) RNA preparations, although it was not observed in control preparations in vivo (in vitro methylation of control preparations did not appear to affect protein synthetic capacity).

**Discussion**

We have described a substantial undermethylation of ribosomal RNA early after CCl4 intoxication that occurs currently with (or presages) the defect in the protein synthetic capacity of isolated ribosomes. No significant differences in methylation of poly(A)+ mRNA or small RNA fractions were found; further, these fractions appear to remain functional, as demonstrated by the in vitro translation of released mRNA (Fig. 2), suggestive of normal "capping" of mRNA (27), and the unchanged in vitro activities of the S100 fraction in supporting protein synthesis (Table III). Furthermore, addition of poly(U) did not affect the protein synthetic capacity of isolated microsomal preparations (Table III). Indeed, the apparent ability of poly(U) to restore the protein synthetic capacity of treated ribosomal preparations was highly variable, and probably reflects the greatly altered binding capacity of treated preparations (46). Thus, our findings with poly(U) also appear to localize the protein synthetic defect produced by CCl4 to the ribosomes themselves.

**Table III. Protein Synthetic Capacity of Ribosomes after CCl4 Administration**

| Sample | -Poly(U) (percent control) | +Poly(U) |
|--------|---------------------------|----------|
| Control | 100 ± 14 | 388 ± 100 |
| Treated | 53 ± 02 | 132 ± 05 |
| Control | 100 ± 13 | 224 ± 30 |
| Treated | 49 ± 14 | 257 ± 50 |

Rats were given 0.25 ml CCl4 as described and killed 1 h later. Ribosomes were prepared and in vitro protein synthesis was assessed using [14C]phenylalanine and control S100 fractions (as described). Specific activities of incorporation were 35-70 cpm/μg with control ribosomes, and 45 cpm/μg with control microsomes. As shown, results with purified ribosomes without poly(U) were highly reproducible, but the results obtained with poly(U) were very inconsistent and variable. No significant differences were observed between the ability of S100 fractions from treated or control preparations to support protein synthesis.

The Journal of Cell Biology, Volume 105, 1987

708
For both quantitative and qualitative reasons, the decreased rRNA methylation we observed does not appear to result from decreased nucleolar methylation of pre-rRNA and subsequent rRNA maturation. Quantitatively, treated nucleolar preparations show significantly greater activities compared with control nuclear preparations. Also, the much larger size of the cytoplasmic RNA pool makes it doubtful that most of the labeling could reflect maturation of unlabeled nucleolar RNA in the absence of a chase period, even given the twofold larger size of the initial pre-RNA transcript compared with 28/18S rRNA. Qualitatively, the 2'-O-ribose methylation defect was not present in the nucleolar pre-rRNA isolated from the same treated preparations (Fig. 4).

When intact ribosomal subunits were incubated with [3H]AdoMet, we observed a significantly greater incorporation of label into ribosomes from treated preparations (Fig. 5). This both substantiates the notion that rRNA in treated preparations is undermethylated, and implies that the "undermethylated" sites are exposed on the surface of the subunits. This supports previous studies which showed that about one-half of all 2'-O-methylation sites are exposed on the surface of the ribosomes (12, 16), and studies which document in vitro methylation of ribosomal subunits (13, 43, 49, 52). Since preliminary experiments did not indicate any differences in demethylation of ribosomal preparations (prelabeled in vitro) by S100 fractions from treated and control preparations, our results suggest that the net undermethylation results from a defect in methylation of mature RNA...
cytoplasmic methylation/demethylation of 2'-O-ribose sites probably represents a continuous step that is committed functionally by undermethylation. In some instances, the defects are associated with defects in rRNA maturation (18, 28, 43, 49, 52). In some instances, the defects are associated with defects in rRNA maturation (18, 28, 35), although unmasking of the polymerase and processing may occur (5, 15). However, comparison studies on methylation of nucleolar vs. cytoplasmic rRNA, in general, reflect a steady-state look at the populations. Our results suggest a turnover of late cytoplasmic methylations in hepatocytes, which is sensitive for exposed 2'-O-ribose moieties. In this regard, the late methylation in mRNA caps occurs in the cytoplasm and involves a 2'-O-methylation (30, 42), although internal meG methylations may participate in processing reactions (14). Furthermore, our data suggest the functional importance of the methylation, because 2'-O-methylation of under methylated ribosomal subunits largely restores the functional deficit.

We therefore suggest, in rat hepatocytes, that (a) an important component of ribosome function involves a continuous cytoplasmic methylation/demethylation of 2'-O-ribose sites exposed on the surface of mature ribosomal subunits, and (b) CCL7 treatment disrupts protein synthesis by inhibiting cytoplasmic 2'-O methylations at a very early stage in cellular injury. Teleologically speaking, just as the time that the cell must respond to toxic injury by increasing protein synthesis to replace damaged components, the ribosomes are compromised functionally by undermethylation.

We would like to dedicate this work to the memory of Edward A. Snodderly, former chairman of the Department of Pathology of the University of California, San Francisco, whose zest was an inspiration to us all. We thank David Geller from the Department of Pathology at the University of California, San Francisco for fine editorial assistance.

Figure 7. HPLC analysis of nucleoside digests of ribosomal subunit RNA after in vitro methylation with [3H]AdoMet. Purified 40S and 60S ribosomal subunits from treated or control preparations were incubated with 10X [3H]AdoMet (250 μCi) with control S100 fractions for 30 min at 37°C. The mixtures were then extracted with chloroform and the aqueous phase was precipitated twice with ETOH. The pelleted RNA was then digested and examined via HPLC.

Treated preparations, which showed reduced functional activity, showed methylation at the 2'-O-ribose positions, mostly at G residues, along with considerable methylation of 2'-O-ribose positions in control preparations (these are apparently fully methylated in vivo), whereas there was a marked labeling of meG along with meA. The significance of methylation at meG is not clear, although it does not appear to affect protein synthetic capacity.

This work was supported by United States Public Health Service grants, and a Research Career Development Award CA21141, AM19843, CA40145 (CA40145); and grants 1745R1 and 211S from the Council for Tobacco Research.

Received for publication 28 January 1987, and in revised form 21 March 1987.

References

1. Blobel, G., and V. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. Science (Wash. DC). 154:1662-1665.
2. Brand, R., J. Klootwijk, T. Van Steenbergen, A. Dekok, and R. Plante. 1977. Secondary methylation of yeast ribosomal precursor RNA. Eur. J. Biochim. 75:311-318.
3. Brattin, W., S. Pencil, R. Waller, E. Glende, and R. Recknagel. 1984. Assessment of the role of calcium ion in halocarbon hepatotoxicity. Environ. Health Perspect. 57:321-323.
4. Brown, G., A. Kolb, and W. Stanley. 1974. A general procedure for the preparation of highly active eukaryotic ribosomes and ribosomal units. Methods Enzymol. XXX:368-387.
5. Caboche, M., and J. Bachelier. 1977. RNA methylation and control of eukaryotic RNA biosynthesis. Eur. J. Biochem. 74:19-29.
6. Campers, S., R. Albers, J. Coward, and F. Rottman. 1984. Effect of undermethylation on mRNA cylindrical appearance and half-life. Mol. Cell. Biol. 4:538-543.
7. Charldorp, R., and P. Van Knippenberg. 1982. Sequence, modified nucleotide, and secondary structure at the 3'-end of small ribosomal subunit RNA. Nucleic Acids Res. 10:1149-1158.
8. Chirgwin, J., P. Przybyla, R. MacDonald, and W. Rutter. 1972. Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18:5294-5299.
9. Christie, G., and J. Judah. 1984. Mechanisms of action of CCL4 on liver cells. Proc. R. Soc. Lond. Ser. B. 142:241-257.
10. Erin, B., C. Stotscheck, and J. Florini. 1981. A rapid fluorometric method for the estimation of DNA in cultured cells. Anal. Biochem. 110:291-294.
11. Glisin, V., R. Crkvenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry. 13:2633-2637.
12. Goddard, P., and B. Maiden. 1976. Reaction of HeLa cell 18S-ribosomal RNA with sodium bisulphite: a conformational probe for methylated sequences. Nucleic Acids Res. 3:431-440.
13. Halderp, T., J. Davies, and J. Dahlberg. 1972. Mechanism of kasugamycin resistance in Escherichia coli. Nat. New Biol. 235:6-9.
14. Kane, S., and K. Beemon. 1985. Precise localization of meA in Rous sarcoma virus RNA reveals clustering of methylation sites: implications of RNA processing. Mol. Cell. Biol. 142:241-257.
15. Kelker, R., H., and A. O. Pogo. 1980. The stringent and relaxed phenomena in Saccharomyces cerevisiae. J. Biot. Chem. 255:1526-1535.
16. Khan, M., and B. Maiden. 1978. Conformation of methylated sequences in yeast 18S ribosomal RNA. Eur. J. Biochem. 74:19-29.
17. Klootwijk, J., and R. Planta. 1973. Analysis of the methylation sites in yeast ribosomal RNA. Eur. J. Biochem. 29:325-333.
18. Li, A., R. Singer, and G. Johnston. 1985. Effects of sinefungin on rRNA
production and methylation in the yeast Saccharomyces cerevisiae. Arch. Biochem. Biophys. 240:613–620.

18. Lowry, O. H., N. Rosebrough, A. Farr, and R. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275.

19. Maden, B. 1979. Eukaryotic ribosomal RNA methylation: summary and recent developments. In Transmethylation. E. Usdin, R. Borchardt, and C. Creveling, editors. Elsevier/North Holland, Amsterdam, New York. p. 381.

20. Maden, B., C. Lees, and M. Salim. 1972. Some methylated sequences and the numbers of methyl groups in HeLa cell rRNA. FEBS (Fed. Eur. Biochem. Soc.) Lett. 28:291–296.

21. Maden, B., M. Salim, and D. Summers. 1972. Maturation pathway for ribosomal RNA in the HeLa cell nucleus. Nat. New Biol. 237:5–9.

22. Mammis, T., E. Fritsch, and J. Sambrook. 1983. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY. 545 pp.

23. Monlux, G., and E. Smuckler. 1969. An autoradiographic study of protein synthesis in mouse liver parenchymal cells during CCl₄ intoxication. Am. J. Pathol. 54:73–83.

24. Moody, D., J. James, G. Clawson, and E. Smuckler. 1981. Correlations among the changes in hepatic microsomal components after intoxication with alkyl halides and other hepatotoxins. Mol. Pharmacol. 20:685–693.

25. Muramatsu, M., and H. Busch. 1964. Studies on nucleolar RNA of Walker 256 carcinosarcoma and the liver of the rat. Cancer Res. 24:1028–1034.

26. Muthukrishnan, S., B. Moss, J. Cooper, and E. Maxwell. 1978. Influence of 5'-terminal cap structure on the initiation of translations of vaccinia virus rRNA. J. Biol. Chem. 253:1710–1715.

27. Muthukrishnan, S., B. Moss, J. Cooper, and E. Maxwell. 1978. Influence of 5'-terminal cap structure on the initiation of translations of vaccinia virus rRNA. J. Biol. Chem. 253:1710–1715.

28. Ouette, A., E. Bandman, and A. Kumar. 1976. Regulation of ribosomal RNA methylation in a temperature-sensitive mutant of BHK cells. Nature (Lond.) 262:619–621.

29. Perry, R. 1976. Processing of RNA. Annu. Rev. Biochem. 45:605–629.

30. Perry, R., and D. Kelly. 1976. Kinetics of formation of 5'-terminal caps in mRNA. Cell 8:433–442.

31. Poldermans, B., H. Bakker, and P. Van Knippenberg. 1980. Studies on the function of two adjacent N₆,N₆-dimethyladenosines near the 3' end of 16S ribosomal RNA of Escherichia coli. IV. The effect of the methyl groups on ribosomal subunit interaction. Nucleic Acids Res. 8:143–151.

32. Poldermans, B., N. Goosen, and P. Van Knippenberg. 1979. Studies on the function of two adjacent N₆,N₆-dimethyladenosines near the 3' end of 16S ribosomal RNA of Escherichia coli. I. The effect of kasugamycin on initiation of protein synthesis. J. Biol. Chem. 254:9085–9089.

33. Poldermans, B., C. Van Buul, and P. Van Knippenberg. 1979. Studies on the function of two adjacent N₆,N₆-dimethyladenosines near the 3' end of 16S ribosomal RNA of Escherichia coli. II. The effect of the absence of the methyl groups on initiation of protein biosynthesis. J. Biol. Chem. 254:9090–9094.

34. Poyer, J., R. Floyd, P. McCay, E. Janzen, and E. Davis. 1979. Spinning of trichloromethyl radicals formed from CCl₄. Biochem. Pharmacol. 28:2231–2235.

35. Prince, D. M., K. Kott, and D. Dubin. 1986. Evidence that the methylation inhibitor cycloleucine causes accumulation of a discrete ribosomal RNA precursor in hamster mitochondria. Mol. Biol. Rep. 11:51–55.

36. Recknagel, R. 1983. A new direction in the study of carbon tetrachloride hepatotoxicity. Life Sci. 33:401–408.

37. Recknagel, R., and E. Glende. 1973. Carbon tetrachloride hepatotoxicity: an example of lethal cleavage. CRC Crit. Rev. Toxicol. 2:263–297.

38. Recknagel, R., and B. Lombardi. 1961. Studies of biochemical changes in subcellular particles of rat liver and their relationship to a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation. J. Biol. Chem. 236:564–569.

39. Reynolds, E. 1964. Liver parenchymal cell injury. Initial alterations in the cell following poisoning with carbon tetrachloride. J. Cell Biol. 19:139–157.

40. Reynolds, E. 1967. Liver parenchymal cell injury. IV. Pattern of incorporation of carbon and chlorine from carbon tetrachloride into chemical constituents of liver in vivo. J. Pharmacol. Exp. Ther. 155:117–126.

41. Salim, M., and B. Maden. 1973. Early and late methylations in HeLa cell ribosome maturation. Nature (Lond.) 244:334–336.

42. Shakar, A. 1976. Capping of eukaryotic mRNA. Cell. 9:645–653.

43. Skegg, P., J. Thompson, and E. Cundliffe. 1985. Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of Streptomyces lividans carrying DNA from Streptomyces tenjamiensis. Mol. Gen. Genet. 200:415–421.

44. Slater, T. 1973. Mechanism of protection against acute liver injury. Biochem. Soc. Trans. 1:222–226.

45. Slater, T. 1966. Norgenic activation of carbon tetrachloride in the rat: speculative mechanisms based on activation. Nature (Lond.) 209:36–40.

46. Smuckler, E. 1976. Alterations produced in the endoplasmic reticulum by carbon tetrachloride. Panminerva Med. 18:392–303.

47. Smuckler, E., and B. Lombardi. 1973. Early and late methylations in HeLa cell ribosome maturation. J. Biol. Chem. 243:564–569.

48. Tomasi, A., E. Albano, K. Lott, and T. Slater. 1980. Spin trapping of free radical products of CCl₄ activation using pulse radiolysis and high energy radiation procedures. FEBS (Fed. Eur. Biochem. Soc.) Lett. 123:303–306.

49. Trudell, J. B. Bosterling, and A. Trevor. 1982. Reductive metabolism of carbon tetrachloride by human cytochrome F-450 reconstituted in phospholipid vesicles: mass spectral identification of trichloromethyl radical bound to dioxyl phosphatidylcholine. Proc. Natl. Acad. Sci. USA. 79:2678–2682.

50. Van Buul, C., J. Damm, and P. Van Knippenberg. 1983. Kasugamycin resistant mutants of Bacillus stearothermophilus lacking the enzyme for the methylation of two adjacent adenosines in 16S ribosomal RNA. Mol. Gen. Genet. 189:475–478.

51. Waller, R., E. Glende, and R. Recknagel. 1983. Carbon tetrachloride and bromochloromethane toxicity. Dual role of covalent binding of metabolic cleavage products and lipid peroxidation in depression of microsomal calcium sequestration. Biochem. Pharmacol. 32:1613–1616.

52. Winicov, I., and R. Perry. 1974. Enzymological aspects of processing of mammalian rRNA. Brookhaven Symp. Biol. 26:201–213.

53. Zimmerman, E. 1968. Secondary methylation of ribosomal ribonucleic acid in HeLa cells. Biochemistry. 9:3156–3164.

54. Zimmerman, E., and B. Holler. 1967. Methylation of 4S ribosomal RNA precursor in HeLa cells. J. Mol. Biol. 23:149–161.