Early Hypomethylation of 2′-O-Ribose Moieties in Hepatocyte Cytoplasmic Ribosomal RNA Underlies the Protein Synthetic Defect Produced by CCl_4

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 (CCl_4) 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 CCl_4-induced methylation defect is specific for the 2′-O-ribose position, and a corresponding proportional increase in m^7G base methylation occurs in vivo. Undermethylated ribosomal subunits (rRNA) from CCl_4-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 m^7G, 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 CCl_4 disrupts protein synthesis by inhibiting this 2′-O-ribose methylation.

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CARBON tetrachloride (CCl_4)[1] has long served as a model compound for study of hepatic injury (9, 36, 37, 39, 46). Like many other agents, CCl_4 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 CCl_4 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 CCl_4 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 CCl_4-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 CCl_4 administration.

Address correspondence and reprint requests to Dr. Clawson at Department of Pathology, 502 Ross Hall, George Washington University, 2300 Eye Street N.W., Washington DC 20037.

1. *Abbreviations used in this paper*: AdoMet, S-adenosyl-methionine; CCl_4, carbon tetrachloride; ER, endoplasmic reticulum.

Materials and Methods

Male Sprague-Dawley rats that weighed 250–300 g were used. CCl_4 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 CCl_4; 0.5 h later, they were given 1.6 mCi [^{3}H]methyl-L-methionine (New England Nuclear, Boston, MA, 70–85 Ci/mol in H_2O; 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 [^{3}H]methylmethionine at 0.5 h after CCl_4 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-

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crose of molarity as specified, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol). The homogenate was centrifuged at 10,000 *g* for 10 min at 0°C. The supernatant fractions were further centrifuged at 105,000 *g* for 90 min at 4°C; the supernatant resulting from this centrifugation, designated as S100, was adjusted to 30 or 5 mg protein/ml (19) 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 120 min at 4°C. The resultant polysomal pellet was resuspended in 25 mM Tris-HCl, pH 7.4, 5 mM KCl, 1 mM $MgCl_2$, 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 (4). The subunits were harvested by centrifugation and separately pooled.

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 mM $MgCl_2$, 100 mM NH_4Cl, 3.1 mM dithiothreitol (DTT), 0.31 mM ATP , 0.31 mM GTP, and [^{14}C]phenylalanine (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 liquid scintillant. Translation of poly(A)$^+$ mRNA purified from S100 fractions was performed with a nuclease-treated rabbit reticulocyte lysate system (Bethesda Research Laboratories, Bethesda, MD) and [^{35}S]methionine.

For methylation studies, cytoplasmic RNA from the 10,000 *g* supernate was isolated by centrifugation through 5.7 M CsCl (8, 11); recovery of tRNA 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 centrifugation at 117,000 *g* for 16 h at 4°C. The 28S and 18S ribosomal RNAs were collected and precipitated with ethanol.

Crude nuclear pellets from the initial 10,000 *g* centrifugation were resuspended by homogenization in 0.25 M STKM2, nuclei were purified (1), and nucleoli were isolated (26). RNA was initially extracted with chloropane (chloropane consists of equal volumes of phenol and chloroform, 0.1% 8-hydroxyquinoline, saturated with ANE buffer, which consists of 0.01 M Na acetate, pH 6.0, 0.1 M NaCl, and 1 mM EDTA), precipitated with ethanol, then digested with DNase I (Worthington Biochemical Corp., Freehold, NJ) and subsequently with proteinase K (23), then reextracted and precipitated. Nucleolar and cytoplasmic RNA preparations were free of detectable DNA and protein, as assessed using standard assays (10, 19).

Purified RNA fractions were then digested to nucleosides (6); 3 mg of RNA preparations were resuspended (2 ml) in 5 mM sodium acetate (pH 5), heated to 95°C for 5 min, and then rapidly cooled. 125 U of RNase T_2 (Bethesda Research Laboratories) and 40 U of RNase P_1 (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 nucleosides (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 redigested 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 assortment of methylated nucleosides (in these cases, the counts were summed).

In other experiments, gradient-purified ribosomal subunits were prepared as described; 20 pmol were mixed with various quantities of S100 and 2.5 µCi [^{3}H]methyl-labeled s-adenosyl-methionine (AdoMet; New England Nuclear, 55–85 Ci/mmol, diluted to a specific activity of 0.5 Ci/mmol [13, 43, 49, 52]; standard aliquots contained 12 µg or 24 µg ribosomal subunits, 1.5 µg or 7.5 µg S100 protein, and 1 nmol [^{3}H]AdoMet). After various periods (0–40 min) at 37°C, the mixtures were extracted with chloropane, and TCA-precipitable material was collected on filters, rinsed extensively, and counted. In other experiments, after methylation of isolated ribosomal subunits with [^{3}H]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 [^{14}C]phenylalanine.

To examine the specificity of the in vitro methylation, purified 40S and 60S ribosomal subunits from treated or control preparations were incubated in 10× [^{3}H]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 CCl_4 intoxication (Fig. 1). No significant changes in methylation of poly(A)$^+$ RNA were observed (Fig. 1, Table I), although the "degranulation" 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 maximal (60%) decrease in methylation of cytoplasmic poly(A)$^-$ RNA occurred by 1.5 h after CCl_4 administration; this decrease was maintained at 2.5 h, and the methylation level was returning toward control levels at 4.5 h. The methylation defect paralleled the defect in protein synthesis noted under these conditions (see Fig. 1).

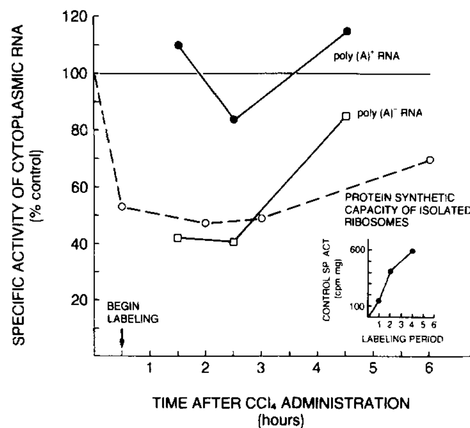

Figure 1. Methylation of cytoplasmic RNA fractions after CCl_4. [^{3}H]Methylmethionine was administered intraperitoneally 0.5 h after oral CCl_4 administration and animals were killed after various labeling intervals. Inset shows the linearity of incorporation of [^{3}H]methyl groups into cytoplasmic poly(A)$^-$ RNA over the initial 4-h period. Cytoplasmic RNA was fractionated into poly(A)$^+$ and poly(A)$^-$ fractions, and the specific activities of these fractions were assessed. The fluctuations in poly(A)$^+$ labeling were not significantly different whereas those in poly(A)$^-$ were. Also shown are the marked reductions in protein synthetic capacity of ribosomes isolated at various intervals after CCl_4 intoxication.

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Table I. Incorporation of [^{3}H]Methyl Groups into 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 CCl_4 and 0.5 h later, they received [^{3}H]methylmethionine. After an additional 1-h labeling period, they were killed and cytoplasmic and nucleolar RNAs were prepared.

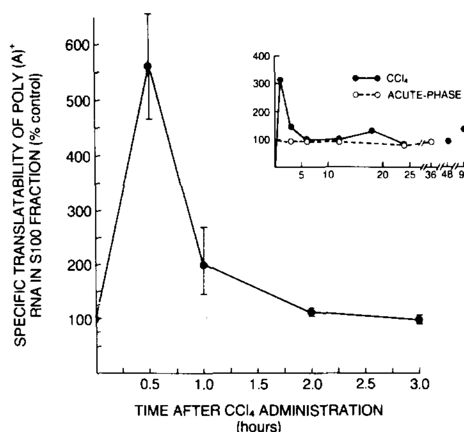

Figure 2. Translatability of poly(A)$^+$ RNA released into the S100 fraction after CCl_4 intoxication. CCl_4 was administered orally and rats were killed after various intervals. Poly(A)$^+$ RNA was prepared from S100 fractions and equivalent amounts were translated in a standard in vitro protein synthesis assay. The increased translatability was significantly different from controls at 0.5 and 1 h after treatment. Inset shows the translatability of S100 poly(A)$^+$ RNA over 1–96 h after treatment; also shown is the lack of fluctuation of preparations after induction of the acute-phase response. Translation of control poly(A)$^+$ RNA yielded 48,000 cpm/μg.

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 rRNA 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 m^{7}G in the treated fractions. Subsequent experiments showed that the m^{7}G 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 CCl_4-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. Our results seem inconsistent with a simple precursor–product relationship. In two sets of experiments, we observed increased methylation of nucleolar pre-rRNA in treated preparations (Table I). Perhaps more surprisingly, the specific activities of control nucleolar RNA were significantly lower than those of cytoplasmic poly(A)$^-$ RNA from the same preparations in the absence of any "chase," while they were higher in treated nucleolar preparations (Table I).

To examine the specificity of this increased nucleolar RNA methylation, we subjected nucleolar RNA preparations from CCl_4-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 m^{7}G, analogous to that noted in treated cytoplasmic preparations (Fig. 3, Table II), and a somewhat lower percentage of m^{6}A.

We next isolated ribosomal subunits, incubated them with S100 fractions and [^{3}H]AdoMet, and quantitated the radioactivity incorporated into the rRNA. Incorporation was significantly greater with treated preparations (Fig. 5), thus suggesting a substantial undermethylation of treated ribo-

Table II. Distribution of Incorporated [^{3}H]Methyl Groups in Poly(A)$^-$ Cytoplasmic RNA

Sample	Percent of incorporated label in						
	2′OmeC	2′OmeU	2′OmeG	2′OmeA	me^{6}A	me$^6_2$A	me^{7}G
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.

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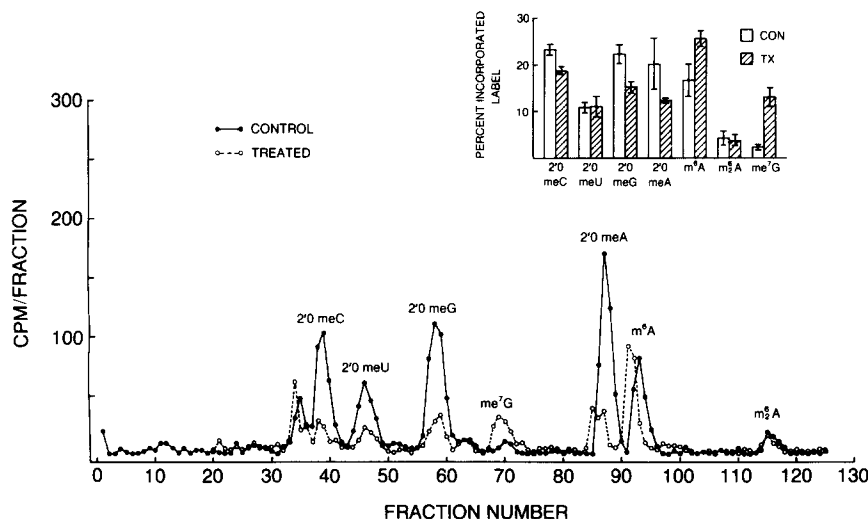

Figure 3. HPLC analysis of nucleoside digests of cytoplasmic poly(A)$^-$ RNA. 0.5 h after administration of CCl_4 (or mineral oil), rats were given 1.67 μCi[^{3}H]methylmethionine. After a 1-h labeling, rats were killed and poly(A)$^-$ cytoplasmic RNA was prepared. The preparations were digested to uncharged nucleosides, and separated in HPLC as described. Inset shows percentages of radioactivity associated with the various constituents. A marked increase was noted in me^{7}G, and also m^{6}A.

somes. Increased incorporation into treated 40S or 40 + 60S ribosomal subunits was observed with either control or treated S100 fractions (Fig. 5).

To assess the ability of S100 fractions to demethylate methylated ribosomal subunits, ribosomes were prelabeled with [^{3}H]AdoMet in vitro, precipitated, resuspended in S100 fractions, and incubated for various periods of time. We observed a decline in methylation which was similar with both treated and control S100 fractions; the decline amounted to ~25% under the conditions used. These data indicate that "demethylation" of ribosomal subunits proceeds at ~20% of the rate of methylation, and suggest that the net undermethylation of treated ribosomes does not result from increased demethylation of methylated rRNA.

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 remethylation 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 m_2^6A component, which is a well-described "late" cytoplasmic methylation (2, 17, 41, 55). In contrast, while control preparations also showed a prominent m_2^6A component, there was little or no labeling at 2′-O-ribose moieties. Instead, most incorporation occurred at me^{7}G. 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 CCl_4 intoxication that occurs concurrently 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 CCl_4 to the ribosomes themselves.

Table III. Protein Synthetic Capacity of Ribosomes after CCl_4 Administration

Sample	Incorporation of [^{14}C]phenylalanine (percent control) −Poly(U)	+Poly(U)
Experiment I		
Control	100 ± 14	388 ± 100
Treated	53 ± 02	132 ± 05
Experiment II		
Control	100 ± 13	224 ± 30
Treated	49 ± 14	257 ± 50
Microsome Source/S100 Source		
Control/control	100	89
Control/treated	95	111
Treated/control	48	55
Treated/treated	36	44

Rats were given 0.25 ml CCl_4 as described and killed 1 h later. Ribosomes were prepared and in vitro protein synthesis was assessed using [^{14}C]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.

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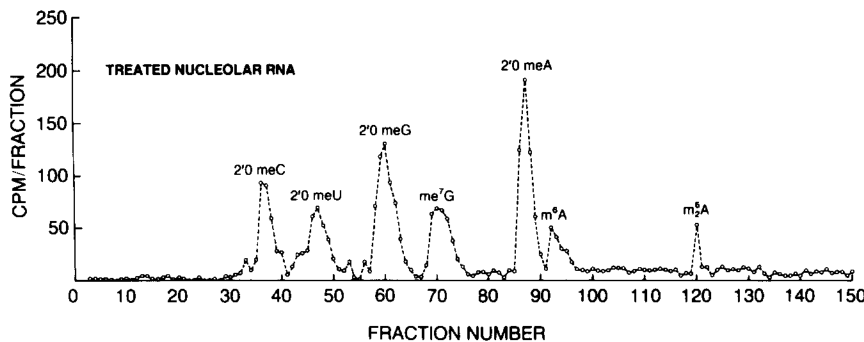

Figure 4. HPLC analysis of nucleoside digest of nucleolar RNA. Nucleolar RNA was labeled (as in Fig. 3), purified as described, digested to nucleosides, and analyzed via HPLC. The radioactivity associated with the various 2'-O-ribose moieties was essentially identical to that found in control cytoplasmic RNA preparations. The increased me^7G content noted (Fig. 3) was present in treated nucleolar preparations, and a proportional decrease in m^6A labeling was noted (m^6A labeling has been reported to be largely cytoplasmic).

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 specific 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 [^{3}H]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

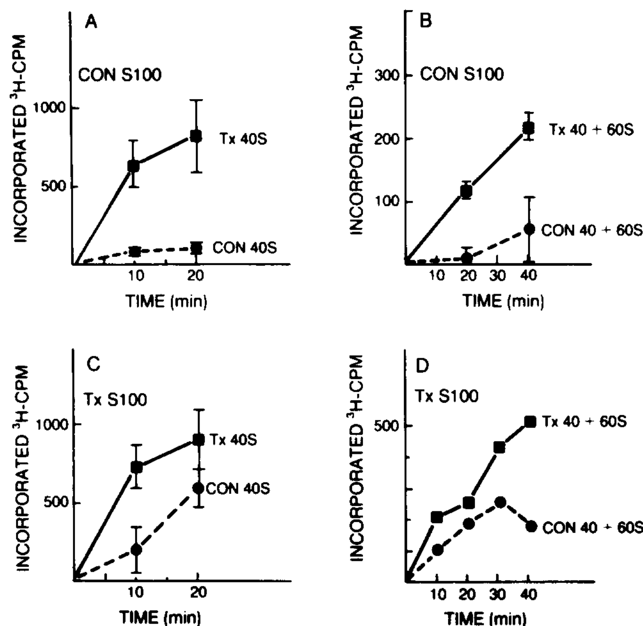

Figure 5. In vitro methylation of ribosomal subunits at various interludes after CCl_4 intoxication. Rats were given CCl_4 (or mineral oil) and sacrificed 1.5 h later. Ribosomal subunits were isolated and resuspended in S100 fractions from treated or control animals with [^{3}H]AdoMet. After various intervals, aliquots were precipitated with TCA collected on filters, rinsed extensively, and counted. The differences in quantity of added S100 protein reflect titration with individual preparations. Values shown are means ± SE from three separate preparations. (*A*) Methylation of 40S ribosomal subunits in control S100. 60 µg 40S subunits were incubated with 30 µg S100 protein and [^{3}H]AdoMet. (*B*) Methylation of 40 + 60S ribosomal subunits on control S100. 100 µg 40 + 60S subunits were incubated with 240 µg S100 protein and [^{3}H]AdoMet. (*C*) Methylation of 40S ribosomal subunits in S100 from treated animals. 60 µg 40S subunits, 30 µg S100 protein. (*D*) Time course of methylation of 40 + 60S ribosomal subunits in S100 from treated animals. 200 µg 40 + 60S subunits, 240 µg S100 protein. The values show a significantly increased methylation of ribosomal subunits from treated preparations in both control and treated S100 fractions.

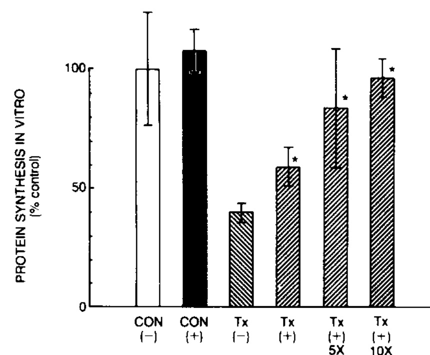

Figure 6. Protein synthetic capacity of 40 + 60S ribosomal subunits after in vitro methylation with AdoMet. Ribosomal subunits were isolated and methylated in vitro in the presence (or absence) of unlabeled AdoMet (1× = 170 µM AdoMet, 5× = 850 µM, 10× = 1.7 mM). After 40 min, reactions were diluted and chilled to 4°C, and the ribosomal subunits were harvested by centrifugation. The subunits were then assessed for protein synthesis in the presence of control S100 fractions as described. No significant differences were noted in control preparations incubated with or without AdoMet. The differences observed with treated preparations were significant with either 1×, 5×, or 10× AdoMet (compared with treated preparations incubated without AdoMet); the protein synthetic capacity of treated preparations methylated with 5× AdoMet was not significantly different from concurrent control preparations incubated similarly. There was nearly 100% restoration of protein synthetic capacity of treated ribosomal subunits after in vitro methylation with 10× AdoMet (97 ± 8% of that for similarly incubated control preparations).

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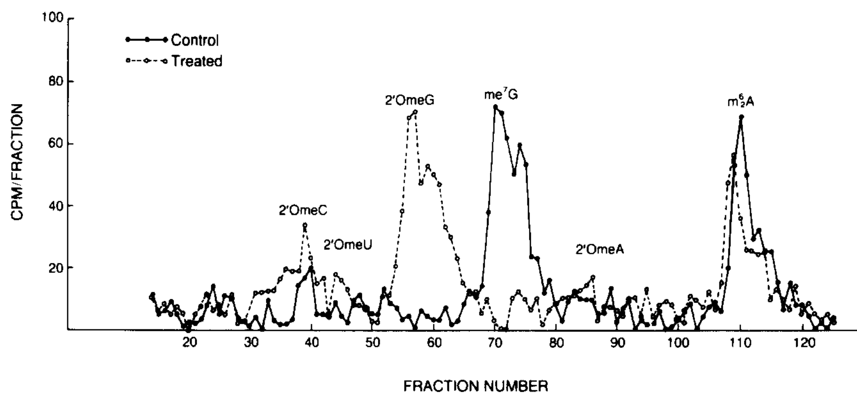

Figure 7. HPLC analysis of nucleoside digests of ribosomal subunit RNA after in vitro methylation with [^{3}H]AdoMet. Purified 40S and 60S ribosomal subunits from treated or control preparations were incubated with 10× [^{3}H]AdoMet (250 μCi) with control S100 fractions for 30 min at 37°C. The mixtures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

Treated preparations, which showed restored functional activity, showed methylation at the 2′-O-ribose positions, mostly at G residues, along with considerable methylation as m$^6_2$A, which is a well described "late" cytoplasmic methylation. There was no labeling of me^{7}G in treated preparations (presumably because me^{7}G methylation occurred in vivo). In contrast, there was no significant labeling of 2′-O-ribose positions in control preparations (these are apparently fully methylated in vivo), whereas there was a marked labeling of me^{7}G along with m$^6_2$A. The significance of methylation at me^{7}G is not clear, although it does not appear to affect protein synthetic capacity.

ribosome RNA (subunits), presumably in the cytoplasm (although the defect cannot be attributed to S100 fractions). In this regard, "late" cytoplasmic methylations of rRNA have been described (2, 17, 41, 55), but they largely involve base methylations, which are relatively unaffected here. Furthermore, dimethylation of A residues (m$^6_2$A) is a prominent component of the in vitro methylation we observed (Fig. 7).

The bases for resistance to various antibiotics have been ascribed to differences in methylation of rRNA (13, 18, 31–33, 43, 49, 52). In some instances the defects are associated with altered interactions between the subunits (31–33), and a specific conserved methylated sequence has been implicated (7, 13, 31–33, 52). The methylation defects have generally been associated with defects in rRNA maturation (18, 28, 35), although uncoupling of methylation 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 selective 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 me^{6}A methylations may participate in processing reactions (14). Furthermore, our data suggest the functional importance of the methylation, because 2′-O-methylation of undermethylated 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*) CCl_4 treatment disrupts protein synthesis by inhibiting cytoplasmic 2′-O methylations at a very early stage in cellular injury. Teleologically speaking, just at 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. Smuckler, 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.

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