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CHEMICAL AND MOLECULAR BIOLOGICAL ASPECTS OF
ALKYLHYDRAZINE-INDUCED CARCINOGENESIS IN
HUMAN CELLS IN VITRO

Donald T. Witiak
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^{14}C-labeled 1,1-DMH and 1,2-DMH of high specific activity (≥ 100 mCi/m mol) have been prepared and utilized, along with ^{14}C-labeled MMH, to study alkylation of DNA and proteins in low passage human neonatal foreskin derived fibroblasts. When human fibroblasts were treated under conditions which would be expected to lead to a negligible number of transformation events, i.e. cells labeled were in a non-growing environment and the dose of methylhydrazine (1.6 μg/ml) was well below the ED_{50} value (62-100 μg/ml), significant differences in DNA and
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protein methylation were observed. For both protein and DNA, 1,2-DMH produced a higher degree of methylation than 1,1-DMH, with MMH demonstrating the lowest level. The relative quantity of $^{14}CO_2$ released into the atmosphere above the treated fibroblast monolayers increased in order: MMH > 1,1-DMH > 1,2-DMH. The data suggests that in these cells there are significant differences in the metabolic and/or chemical transformation of the methylhydrazines to the proposed active metabolite, methyl diazonium ion. The majority of label in DNA isolated from cells treated with 1,2- and 1,1-DMH was found in the apurinic acid fraction. This is in contrast to that observed for MMH, when the majority of label was found in unmodified adenine (Ade) and guanine (Gua). Thus, in this latter case the carbon label appears to be more efficiently trapped in the 1-carbon pool than for the other methylhydrazines. While 3-MtAde, 7-MtGua and O^6-MtGua were not detected in DNA from MMH treated cells, they were found in DNA from 1,2-DMH and 1,1-DMH. While there was no significant difference in the amount of 3-MtAde, there was over a 2-fold increase in the amount of 7-MtGua and O^6MtGua detected in DNA from 1,2-DMH treated cells over that in 1,1-DMH treated cells. In 1,2-DMH treated cells the average O^6-MtGua/ 7-MtGua ratio was lower (0.5) than that in 1,1-DMH treated cells (0.8). These results, while consistent with the fact that the higher ratio was found in DNA of cells that were treated with the transforming isomer, 1,1-DMH, were unexpected since treatment conditions were such that transformation would not be expected. Thus, the significance of the persistence of O^6-MtGua to carcinogenesis appears to be in question.

PROGRESS REPORT TO

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CHEMICAL AND MOLECULAR BIOLOGICAL ASPECTS OF

ALKYLHYDRAZINE-INDUCED CARCINOGENESIS IN

HUMAN CELLS IN VITRO

September 1, 1980 – August 31, 1981

Submitted by:

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Chemical and Molecular Biological Aspects
of Alkylhydrazine-Induced Carcinogenesis
In Human Cells In Vitro

A. Introduction:

The role of hydrazine and its simple alkyl derivatives in carcinogenesis
has been reviewed (1). In several species of rodents 1,2-dimethylhydrazine
(1,2-DMH) has been shown to be a potent carcinogen, producing a variety
of neoplasms with tissue distribution of tumors being dependent upon
dosage and method of administration (2-9). The mechanism of 1,2-DMH
initiated carcinogenesis has been postulated to be analogous to that of
other alkylating carcinogens, in that the procarcinogen is metabolically
transformed into a highly reactive methylating agent (methyl diazonium ion)
within susceptible tissue and subsequently alkylates a variety of intra-
cellular macromolecules, e.g. DNA, RNA, protein.

Perturbations in nuclear function during carcinogenesis have generally
been attributed to aberrations or modifications of the DNA template.
Recently, attention has focused on one particular DNA lesion, O^6-alkyl-
hydrazine, produced by several alkylating carcinogens, including 1,2-DMH.
In 1969, Loveless suggested that the O^6-alkylguanine was a promutagenic
DNA modification (10). Support for this hypothesis has come from two
areas of investigation. Work with RNA and DNA polymerases utilizing
synthetic polynucleotides as templates has demonstrated that the
presence of O^6-methylguanine leads to incorrect base incorporation (11-13).
Alternatively, in in vivo studies utilizing 1,2-DMH, simple aliphatic
nitrosamines and nitrosamides a positive correlation has been found between
tumorigenesis and the formation and persistence of O^6-alkylguanine in
target tissues (14-20).

Evidence is accumulating which suggests that this abnormal purine is
enzymatically removed in some tissues, and it appears that its persistence

results from a lack of the enzyme system, its inhibition and/or saturation (21-23).

While DNA template damage or modification is largely accepted as being directly correlated with carcinogenesis, modification in the interactions between DNA and the closely associated chromsomal proteins has also been suggested as a mechanism for aberrations in nuclear function and loss of growth control in carcinogenesis. Indeed, a considerable amount of evidence has accumulated which indicates that there are differences in composition of the suspected regulatory proteins, the nonhistones, in normal and transformed cell nuclei (24-29). Specifically, in studies of nonhistone populations in colonic adenocarcinomas induced by 1,2-DMH an accumulation of two nuclear protein classes, MW 44,000 and MW 62,000 were found to be selectively accelerated within four weeks after the carcinogen was administered, long before the appearance of pathological indications of malignancy (29-30). The MW 44,000 nonhistones were found largely in nuclei from dividing cells, whereas the MW 62,000 proteins were enriched in the nondividing cells (31). In addition, neither protein class was detected in normal colonic epithelial cell nuclei. Subsequently, the MW 44,000 tumor specific nonhistones were found to have a much greater affinity for DNA than the MW 62,000 class and were found to be associated with putative template-active regions of the tumor nucleus DNA while the MW 62,000 proteins were not (32).

Studies by Milo et al. (33) have revealed that in human fibroblast cell cultures hydrazine and 1,1-DMH induce neoplastic transformation, whereas monomethyl hydrazine (MMH) and 1,2-DMH only elicited cellular toxicity. Also, hydrazine and 1,1-DMH have been found to be co-carcinogens in the feline sarcoma virus induced transformation system.

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To understand differences in toxicity or carcinogenicity relative
to alkylation of intracellular macromolecules we have undertaken analysis
of DNA and chromatin proteins in human fibroblast cell cultures exposed
to ^{14}C-radiolabelled MMH, 1,2-DMH and 1,1-DMH. The labelled alkyl-
hydrazines utilized in our studies are of high specific activity
($\geq$100 mCi/mmol), synthesized by methodologies developed in our laboratories
(34-36).

B. Results and Discussion:

   1. Synthesis of Radiolabelled Methylhydrazines:

     We have completed the synthesis of ^{14}C-1,1-DMH of high specific
activity (115 mCi/mmol) and the manuscript describing this methodology
has been submitted for publication (35). Scheme I illustrates steps
involved in the synthetic sequence.

<div align="center">

Scheme I

</div>

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-3-

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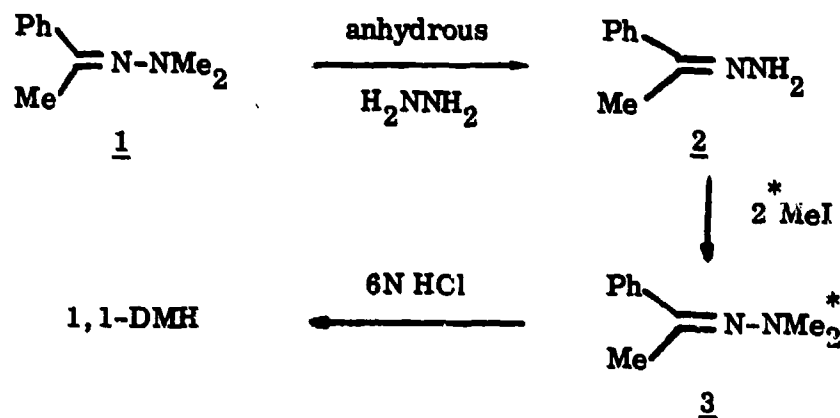
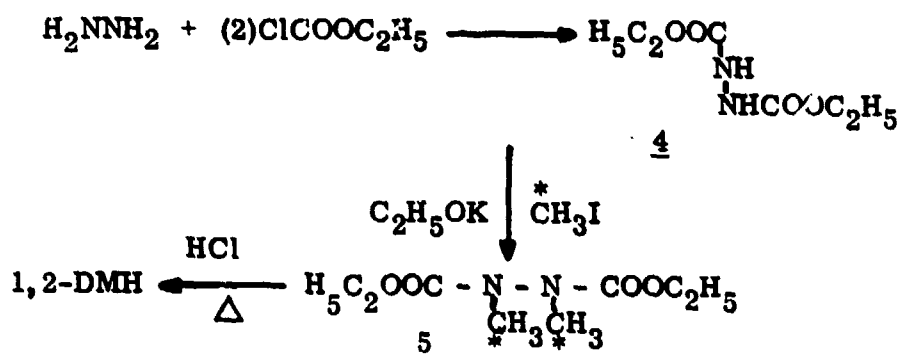The critical step entails dimethylation of acetophenone hydrazone $\underline{2}$ with ^{14}C-methyl iodide (sp. act. = 58 mCi/mmol) in the presence of potassium in liquid ammonia. Dialkylation of hydrazone $\underline{2}$ has been reported by Kaiser et al. (37). Using alkyl halides other than methyl iodide in the presence of potassium in liquid ammonia, hydrazone $\underline{2}$ is converted to its blue-green dianion using a slight excess of potassium in liquid ammonia. The solution color change from deep blue (color of the potassium in liquid ammonia) to blue-green serves as a reliable indicator of dianion quality. Addition of 2.2 equivalents of ^{14}C-methyl iodide converts the blue-green solution to a pale yellow color signifying the quenching of the dianion by methylation. Following removal of excess liquid ammonia, ether and methyl iodide, a yellow solution is obtained by dissolving the residue in 5-10 ml of ether. Removal of an aliquot by pipette separates the desired product from crystalline potassium iodide. Radiolabelled dimethylhydrazone $\underline{3}$ is converted to 1,1-DMH by stirring in an aqueous 6N HCl solution. The radiochemical yield of 1,1-DMH is consistently between 55 to 60%.

We have recently completed the synthesis of ^{14}C-1,2-DMH of high specific activity (_100 mCi/mmol). The methodology utilized is depicted in Scheme II.

Scheme II

$$H_2NNH_2 + (2)ClCOOC_2H_5 \longrightarrow \begin{matrix} H_5C_2OOC \\ NH \\ NHCOOC_2H_5 \end{matrix}$$

$$\underline{4}$$

$$C_2H_5OK \downarrow \overset{*}{C}H_3I$$

$$1,2\text{-DMH} \overset{HCl}{\underset{\Delta}{\longleftarrow}} \quad H_5C_2OOC - N - N - COOC_2H_5$$
$$\underset{\underline{5}}{\overset{}{\qquad}} \overset{}{\underset{*}{C}H_3} \overset{}{\underset{*}{C}H_3}$$

Thus, treatment of hydrazine hydrate with ethyl chloroformate affords ethylhydrazodicarboxylate 4 (38). Treatment of 4 with potassium ethoxide generates the dianion which, in turn, is methylated using 2 equivalents of methyl iodide in THF. Dimethylhydrazodicarboxylate 5 is obtained in 80 to 85% yield.. Acid catalyzed hydrolysis of 5 using concentrated HCl provides 1,2-DMH in 80 to 90% yield (39). The specific activity of ^{14}C-1,2-DMH preparations to date has been approximately 100 mCi/mmol.

2. <u>Alkylation of DNA and Proteins in Cultured Human Fibroblasts</u>:

Initially, our in vitro labelling experiments, with low passage human neonatal foreskin derived fibroblasts, have been conducted under conditions which would be expected to lead to a negligible number of transformation events, i.e. cells labelled were in a nongrowing environ-ment (4% FBS) and the dose of methylhydrazine (1.6 g/ml) was well below the ED_{50} values (62-100 g/ml). The rationale for performing such experiments is to first identify background or noncrical alkylation lesions which can later be "subtracted" from observations made under conditions which are known to lead to cellular toxicity and/or trans-formation of the particular cells in question. Thus, we would be able to identify specific alkylation events which directly or indirectly bring about alterations in genotypic expression of the cells.

Data for alkylation of DNA and total intracellular protein by 1,2-DMH, MMH and 1,1-DMH is shown in Table 1. These results demonstrate that the extent of methylation of the two species of macromolecules by the three methylhydrazines is significantly different. For both protein and DNA 1,2-DMH produced a higher degree of methylation than 1,1-DMH, with MMH demonstrating the lowest level. For all three methylhydrazines DNA was methylated more than protein. This data suggests that in these cells there

significant differences in the metabolic and/or chemical transformation of the methylhydrazines to the proposed active metabolite, methyl diazonium ion.

Table 1. Binding Data for MMH, 1,2-DMH and 1,1-DMH at Low Dose Levels in Cultured Human Fibroblasts.[a]

	dpm/mg		
FRACTION	1,2-DMH	MMH	1,1-DMH
Protein	8,399	2,375	5,226
DNA	35,060	15,255	31,800

[a]Routinely sixty 150 mm culture dishes with 70-80% confluent monolayers were treated for 24h with 1.6 µg/ml of either ^{14}C-MMH, ^{14}C-1,2-DMH or ^{14}C-1,1-DMH at 1-2 µCi/ml in Detroit Special media containing 4% FBS. (3.33 x 10^{7}dpm/plate).

[b]Each value is a mean determined from at least two experiments.

Indeed, evidence for the differential metabolism is observed in terms of the quantity of $^{14}CO_2$ generated during the 24 hour incubation (Table 2). The relative quantity of $^{14}CO_2$ released into the atmosphere above treated fibroblast monolayers increased in order: MMH > 1,1-DMH > 1,2-DMH.

However, inconsistent with this metabolic trend is the finding that, unlike that found in 1,2-DMH and 1,1-DMH treated cells, over 50% of the radiolabel in DNA from MMH treated cells was found in unmodified Ade and Gua. Presumably, this incorporation occurs as a result of the ^{14}C-methyl groups entering the 1-carbon pool (formate) which are then utilized in the de novo synthesis of these purines. Thus, the carbon label is more efficiently trapped in the 1-carbon pool in MMH treated cells than in either of the other methylhydrazine treated cells. Whether this is the result of a particular metabolite or the function of cellular metabolism after exposure to the hydrazine derivative is unclear.

Table 2.
Binding Data for MMH, 1,2-DMH and 1,1-DMH at Low Dose Levels
in Cultured Human Fibroblasts.[a]

	dpm/fraction		
FRACTION	1,2-DMH	MMH	1,1-DMH
CO_2	8.5×10^6	6.6×10^4	9.1×10^5
Apurinic Acid	30,300	8,420	27,836
7-MtGua	940	N/D[b]	404
O^6-MtGua	477[c]	N/D	348[d]
3-MtAde	1,000	N/D	1,011
Ade	898	3,100	1,237
Gua	844	3,250	952

[a]Each value is a mean determined from at least two experiments.

[b]N/D = Not Detected

[c]O^6-MtGua/7-MtGua ratio = 0.5.

[d]O^6-MtGua/7-MtGua ratio = 0.8.

As seen in Table 2, the majority of label in DNA for cells treated with 1,2- and 1,1-DMH was found in the apurinic acid fraction. This fraction presumably contains pyrimidine oligonucleotides labeled via de novo synthesis, methylation of bases and production of phosphotriesters.

Table 2 also shows the extent of methylation of Ade at the 3 position and Gua in the 7 and O^6 positions. Whereas these radiolabelled purines could not be detected in cells treated with MMH, they were detected in both 1,2-DMH and 1,1-DMH treated cells. Although there was no significant difference in the amount

of 3-MtAde, there was over a 2-fold increase in the amount of 7-MtGua and O^6-MtGua detected in DNA from 1,2-DMH treated cells over that in the 1,1-DMH treated cells. In 1,2-DMH treated cells the average O^6-MtGua/7-MtGua ratio was lower (0.5) than the ratio in 1,1-DMH treated cells (0.8). These results, while consistent with the fact that the higher ratio was found in DNA of cells that were treated with the transforming isomer, 1,1-DMH, were unexpected since treatment conditions were such that transformation would not be expected.

3. Electrophoretic Analysis of Nonhistone Prote From Methylhydrazine Treated Human Fibroblasts.

In separate studies we have treated cells in a similar manner as with the labelling studies, except non-radiolabelled 1,2-DMH and 1,1-DMH were used in the treatment with nonhistones being labelled with ^{3}H-leucine (1 μCi/ml). Nuclei were isolated from the control (no treatment) and methylhydrazine treated cells and the nonhistone proteins extracted by a 0.5% Triton X-100/0.8 M NaCl wash. The extract proteins were subjected to SDS gel electrophoresis and the gels sliced and measured for radioactivity. Figure 1 shows that under these conditions of treatment (24 h) there was no significant change in the nonhistone protein population due to treatment.

C. Conclusion:

Under the conditions of treatment, i.e. non-growing cells and at low doses of methylhydrazines, we have found significant differences, both quantitative and qualitative, in the methylation of DNA and protein in human fibroblasts. Most interesting was the finding that the O^6-MtGua/7-MtGua ratio in DNA isolated from cells treated with the transforming isomer, 1,1-DMH, was significantly higher than that found in DNA isolated from the non-transforming isomer, 1,2-DMH. This would seem to support the findings of thers (14-20). However, the treatment utilized would not have been expected to produce a significantly large

population of transformed fibroblasts. Thus, the significance of the persistence of O^6-MtGua to carcinogenesis appears to be in question.

All three experimental treatments produced a marked incorporation of ^{14}C-radiolabel into unmodified Ade and Gua, indicating a significant contribution to the intracellular 1-carbon pool. This phenomenon was particularly evident in cells treated with MMH. This suggests that one or more metabolites of MMH is more efficiently incorported than any metabolite of 1,1-DMH or 1,2-DMH, supporting the different metabolic routes of the three chemicals.

Further investigations planned using parallel treatments with higher doses of 1,1-DMH and 1,2-DMH and utilizing synchronized growing cells should provide more detailed information into the key metabolic events leading to chemical transformation. In addition to monitoring alkylation of DNA and protein, we are now initiating studies to identify intermediate metabolites of both methylhydrazines located both in the extracellular media and in the intracellular compartments. In conjunction with these studies we are attempting to synthesize radiolabelled putative metabolites of 1,1-DMH and 1,2-DMH.

An initial comparative analysis of the putative regulatory nonhistones from treated versus nontreated cells revealed no significant differences in the protein populations. Specifically, no new species in the MW range of 44,000 or 62,000 could be detected. Because of the nontransforming conditions and the short period of treatment, these results were not surprising. Studies are now in progress to determine if new species of nonhistones can be detected in fibroblasts after treatment of synchronized cells at higher doses of 1,1-DMH and 1,2-DMH and after several additonal population doublings.

D. Materials and Methods:

1. Materials:

Unlabelled methylhydrazines, MMH, 1,1-DMH and 1,2-DMH were purchased from Aldrich and distilled prior to use. The purities of the samples were routinely checked by TLC as described by Fiala and Weisburger (40). Bovine serum albumin, ovalbumin, pronase, ribonuclease A were purchased from Sigma. Ribonuclease A was preheated at $80^{\circ}C$ for 30 min in SSC buffer (0.15 M NaCl and 15 mM sodium citrate, pH 7.0) prior to use to destroy trace amounts of DNAase. 7-MtGua, 3-MtAde, Gua and Ade were purchased from either Sigma or Vega. O^6-MtGua was synthesized according to the method described by Balsinger and Montgomery (41). PCS and NCS were from Amersham. Methanol for HPLC was spectral grade purchased from Burdick & Jackson. All chemicals utilized for electrophoresis were purchased from Bio-Rad Laboratories.

2. Methods:

Tissue Culture - Human neonatal foreskin fibroblasts were grown in minimal essential medium (Eagle's) - 25.0 mM HEPES, pH 7.2, containing 2.0 mM glutamine, 1x non-essential amino acids, 1x sodium pyruvate, 0.5 µg per ml gentamicin, 25 mM sodium bicarbonate and 10% fetal bovine serum (FBS). The cells were routinely passaged 1 to 10 in this medium, seeded into 150 mm tissue culture dishes and placed in a 4% CO_2, high humidity incubator. When at a 70-80% confluent density, the cells (PDL 10) were treated with radioactive methylhydrazine in 10 ml of the above medium but with 2% FBS. After 24 hours the treated medium was removed, the cells washed with incomplete medium (no FBS) and harvested by scraping with a rubber policeman. After pelleting by centrifugation, the cells were frozen in liquid nitrogen and stored at $-20^{\circ}C$ until the biochemical analysis was carried out. Alternatively, for nuclear protein studies cells were labelled with 1 µCi/ml of ^{3}H-leucine (Amersham) and treated

with unlabelled methylhydrazine, then nuclei were isolated and proteins ex-
tracted.

DNA and Protein Isolation - To the total cell lysate in a 50 ml Sorvall
tube was added 8 ml of the SSC buffer, 0.8 ml of warm 10% SDS and 4 ml of Kirby's
phenol reagent (75 ml m-cresol, 0.5 g 8-hydroxyquinoline, 600 g phenol in 55 ml
H_2O). The mixture was vigorously vortexed for 5 min and centrifuged at 10,000
x g for 30 min. The aqueous layer was carefully removed by pipet and the phenol
layer was extracted with 8 ml of SSC buffer. The aqueous layer was removed and
to the pooled aqueous layers was added absolute ethanol (20 ml). After standing
at 4^oC overnight, the precipitated DNA was centrifuged at 10,000 x g for 20 min
and the pellet washed with ethanol (2 x 10 ml and ether (2 x 10 ml). The pellet
was dried under nitrogen, dissolved in 3 ml of SSC buffer and treated with pre-
heated ribonuclease A (50 µg/ml) for 30 min at 37^oC. After subsequent treatment
with pronase (100 µg/ml) for 60 min at 37^oC the DNA was isolated using two ex-
tractions with 3 ml of Kirby's phenol. The DNA was precipitated, washed and
dried as described above, then the DNA pellet was dissolved in double-distilled
H_2O (2 ml). Aliquots of this solution were taken for DNA analysis and radio-
activity measurements. Quantitation of DNA was conducted by utilizing UV ab-
sorptions at 260 and 280 nm according to the equation:

$$\mu g \ DNA/ml = \frac{A260 - A280}{0.01}$$

The initial phenol layer was mixed with 10 ml of ethanol and the precipitated
proteins were chilled at 4^oC for 48 hours, centrifuged at 10,000 x g for 20 min,
washed with ethanol and ether twice each, dried under nitrogen and dissolved in
10 ml of 0.1 N NaOH. Aliquots of the solution were used for the quantitation of
protein and radioactivity. Protein determination was by the method of Bradford (42).

<u>Chromatographic Analysis of Purines (Sephadex G-10)</u> - The purified DNA was
made 0.1 N with respect to HCl and heated at 70°C for 30 min. The hydrolysate
was made 0.05 M with respect to ammonium formate and the pH adjusted to 4.8.
Purine bases were separated by chromatography on a Sephadex G-10 column
(1.5 x 90 cm) using 0.05 M ammonium formate as eluant. Absorbance was monitored
at 254 nm (ISCO, Model UA-5 monitor), and 4 ml fractions were collected.
Radioactivity was determined by mixing the fractions with 10 ml of PCS. The
fractions were counted in a Beckman Model 355 Scintillation Counter at 75%
counting efficiency.

<u>Chromatographic Analysis of Purines (HPLC)</u> - After hydrolysis the hydrolysate
was neutralized with NaOH, then 1.0 ml of 3 mM Tris/HCl, pH 8.0 was added and
the mixture passed through a DEAE-Sephacel column (V_t - 1 cm^3) equilibrated with
30 mM Tris/HCl, pH 8.0. The column was washed with 10 ml of 10 mM Tris/HCl, pH
8.0. The eluate was combined, reduced in volume under reduced pressure and
methylpurines were added as UV markers. The sample with markers (0.5 - 0.8 ml)
was applied to the HPLC column and 1.0 min fractions were collected. Radio-
activity in the fractions was detected as described previously. Separation of
3-MtAde, 0^6-MtGua, 7-MtGua, Ade and Gua was achieved utilizing a Laboratory
Data Control (LDC) HPLC system with accessory module containing LDC gradient
master, constametric pumps, spectromonitor III and equipped with three Spherisorb
ODS 5 μ (25 cm) columns connected is series with an ODS-pellicular precolumn
(10 cm). Elution was with the following program: Solvent A, 10 mM NH_4PO_4 buffer
pH 5.1; Solvent B, 80% (v/v) methanol. A linear gradient from 5% B to 30% B
over a 60 min period followed by holding at 30% B for at least 60 min with a flow
rate of 0.5 ml/min.

<u>SDS Polyacrylamide Gel Electrophoresis</u> - SDS PAGE was performed according to
a modified procedure of Laemmli and Favre (43) in a 9 x 16 cm slab (0.7 mm thick).

The stacking gel, 1.0 cm in length, contained acrylamide (4%), bis-acrylamide (0.19%), 0.125 M Tris/HCl, pH 6.8 and 0.1% SDS. The separating gel, 14 cm in length, contained acrylamide (12%), bis-acrylamide (0.32%), 0.375 M Tris/HCl, pH 8.8 and 0.1% SDS. The electrode buffer contained 0.05 M Tris, 0.38 M glycine and 0.1% SDS, pH 8.8. Both gels were polymerized with 0.04% ammonium persulfate and 0.06% TEMED. Protein samples (0.01 - 0.05 ml) were applied in 0.125 M Tris/HCl, pH 6.8, 2% SDS, 20% glycerol, 50 mM 2-mercaptoethanol and 0.01% bromphenol blue. Before application, the protein solution was heated for 5 min in a boiling water bath to completely dissociate the proteins into their subunits. Electrophoresis was conducted at room temperature at 20 mA at constant current until the tracking dye reached 1.0 cm from the bottom of the separating gel. Proteins were visualized by staining essentially by the method of Weber and Osborn (44). The gel was immersed in a solution of 0.25% Coomassie Brilliant Blue R-250, 50% methanol and 9% acetic acid and stained a minimum of 5 hours. The gel was then destained in a solution of 50% methanol and 10% acetic acid and was subsequently stored in a solution of 5% methanol and 7.5% acetic acid. For measuring radioactivity associated with proteins in the gel, a channel was sliced into 2 mm sections, then the sections incubated with 0.5 ml of NCS tissue solubilizer for 24 hours at 50°C. Following incubation the slice and NCS mixture was added to 10 ml of PCS and placed in a scintillation counter.

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