Apoptosis and proliferation under conditions of deoxynucleotide pool imbalance in liver of folate/methyl deficient rats

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Weanling male F344 rats were fed either a semi-purified diet low in methionine and lacking in choline and folic acid (folate/methyl deficient) or a supplemented control diet for periods of 2, 5, 7 days, 3 weeks, and 9 weeks. Two days after initiating the folate/methyl deficient diet in weanling F344 rats, the incidence of apoptotic bodies, identified by in situ end-labeling of 3'-OH DNA strand breaks, was significantly increased in liver sections from the deficient rats. Apoptotic cell death was confirmed biochemically by an increase in nuclear Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease activity that paralleled the increase in apoptotic bodies over the 9-week feeding period. There was no morphologic evidence of necrotic foci or necrosis-associated inflammatory response over the 9-week period. Confirming that cell turnover is chronically elevated in this model, the increase in apoptotic rate was accompanied by a sustained increase in the mitotic index (MI). The DNA repair-associated enzyme, poly(ADP-ribose) polymerase (PARP), was similarly elevated and was associated with significant decreases in the substrate for ADP-ribose polymer synthesis, nicotinamide adenine dinucleotide (NAD). Because folate metabolites are essential for de novo purine and thymidine biosynthesis, prolonged deficiency in folic acid can induce an imbalance in the deoxynucleotide precursors for DNA replication/repair and negatively affect the fidelity of DNA synthesis. Using an HPLC method, hepatic deoxyuridine triphosphate (dUTP) levels were increased at 3 and 9 weeks after initiation of the deficient diet and levels of thymidine triphosphate (dTTP) were reduced. An increase in dUTP/dTTP ratio is consistent with a block in folate-dependent de novo thymidylate biosynthesis and may predispose to uracil misincorporation and DNA repair-related DNA strand breaks.

Introduction

Chronic dietary insufficiency of the lipotropic nutrients, choline and methionine, has been reproducibly shown to be hepatocarcinogenic in the rat and certain mouse strains with or without chemical initiation (1–3). Both semi-synthetic and amino acid-defined diet formulations have been utilized by investigators since the initial observations of tumorigenesis with lipotrope deprivation by Copeland and Salmon in 1946 and 1949 (4). The diet preparations currently in use tend to vary widely between laboratories in terms of: (i) protein source and content; (ii) fat source and content; (iii) amino acid-defined versus semi-purified diet formulation; and (iv) additional deficiencies in the two lipotropic vitamins, folic acid and vitamin B12. The most commonly used semi-purified diet is the choline-deficient, low methionine (0.18%) diet (5). Methyl-deficient amino-acid (AA*)-defined diets have also been used to produce a more severe form of methyl insufficiency (6,7). Despite formulation differences, both semi-synthetic and AA-defined diet preparations have been demonstrated to be hepatocarcinogenic with chronic feeding although distinct differences exist in the onset and severity of tumorigenicity as well as in specific histopathology (6,7).

Because the metabolic pathways of choline, methionine and folic acid are mutually interdependent, a deficiency in one lipotrope will alter the requirement and metabolic priorities of the others in an attempt to maintain normal one-carbon metabolism (8). Consequently, single lipotrope deficiencies should not be discussed in metabolic isolation. For example, chronic deficiencies in choline and methionine increase the requirement for folate metabolites and reduce total folate levels in the liver (9). Similarly, low dietary methionine increases the requirement for exogenous choline such that overt choline insufficiency is difficult to achieve without marginal methionine (0.18%) (10). Thus, the carcinogenic effects of a‘choline deficient’ diet have not been demonstrated in the presence of adequate methionine (>0.6%) (11). These observations underscore the metabolic interdependence of the lipotropic nutrients in maintaining normal one-carbon metabolism and their interaction in promoting carcinogenesis with chronic deficiency.

Previous studies have established that lipotrope deficient diets induce acute cell death and apparent regenerative proliferation in hepatocytes (12,13). The increase in cell turnover becomes chronic with prolonged deficiency and is a basic premise for most theories of carcinogenesis in this model. Although an increase in hepatocyte cell death has been previously documented, the mechanism and type of cell death have not been clarified. In the present study, we have examined the progression of cell death and proliferation in weanling F344 rats at intervals from 2 days to 9 weeks after initiation of a folate/methyl deficient diet. Using an in situ end-labeling method to facilitate morphological identification of apoptotic bodies in histological preparations, we show that the mechanism of cell death appears to be primarily by apoptosis and is accompanied by a parallel increase in mitotic figures. In addition, we quantified alterations in hepatic deoxynucleotide pools with progressive folate/methyl deficiency as possible contributors to genomic instability under conditions of increased DNA synthesis and DNA repair activity.

Materials and methods

Rats and diets

At 4 weeks of age (50 g body weight), F344 rats were randomly allocated to either a low methionine (0.18%), choline defiord diet additionally lacking in...
replacement. Body weights and food consumption were recorded weekly. Four weeks, the liver weights of the deficient rats were almost circular (form l) plasmid DNA to open circular (form 2) or linear (form 3) the apoptotic index (ABs/1000 hepatocytes) was significantly increased in deficient rats (20%). By 9 weeks, however, the difference in liver weights between groups was considerably less (20%), consistent with significant regression of lipid accumulation as previously described (21). Associated with the peak in liver weight at 3 weeks was an increase in total protein, a decrease in total DNA content and a significant increase in protein–DNA ratio (\(P < 0.01\)). By 9 weeks, total protein and DNA were not different from control values. An increase in protein–DNA ratio at 3 weeks is consistent with a delay or arrest in DNA synthesis (S-phase) in situ labeling of apoptotic bodies and mitotic index
Formalin-fixed paraffin-embedded liver sections, obtained from control and folate/methyl deficient rats at intervals from 2 days to 9 weeks were processed to stain the nuclei of apoptotic bodies (ABs) utilizing the Apoptag detection system (Oncor, Gaithersburg, MD). Briefly, permeabilized cells were enzymatically labeled with digoxigenin nucleotide via terminal deoxynucleotidyl transferase (TdT) and subsequently exposed to horseradish peroxidase-conjugated anti-digoxigenin antibody. Staining was developed in diaminobenzidine and sections were counterstained with methyl green. Apoptotic bodies were quantified in 25 microscopic fields per sample using a 40× objective and binocular scope (American Optical) and expressed per 1000 cells. Background labeling in cells other than the condensed nuclei of apoptotic bodies in situ labeling of ABs is presented in Figure 1 in order to demonstrate the staining specificity and lack of background staining. Mitotic figures were enumerated in 25 fields and expressed per 1000 cells.

Nuclear endonuclease activity
Protein extracts were prepared from nuclei isolated from control and folate/methyl deficient frozen liver samples as previously described (15). The plasmid incision assay was used to assess relative levels of \(\text{Ca}^{2+}/\text{Mg}^{2+}\)-dependent endonuclease activity present in the nuclear extracts (16). Briefly, each reaction contained 1 \(\mu\)g plasmid pUC 19 DNA, 10 mM Tris–HCl, pH 7.7, 25 \(\mu\)g/ml bovine serum albumin, 0.5 mM diithothreitol, 2 mM CaCl\(_2\) and 2 \(\mu\)l nuclear protein extract. The samples were incubated at 37°C for 1 hour and the reaction stopped by the addition of 0.2 vol. of 1% SDS and 0.2 M EDTA. The relative digestion of plasmid DNA by the nuclear protein extracts was visualized by agarose gel electrophoresis and ethidium bromide staining. One unit of endonuclease activity was defined as the conversion of 1 \(\mu\)g covalently closed circular (form I) plasmid DNA to open circular (form 2) or linear (form 3) DNA after 1 hour digestion. Scanning densitometer (Bio-Rad GS-70) was utilized to quantify the relative amount of form I plasmid DNA converted to forms 2 and 3 after endonuclease exposure.

Poly(ADP)ribosepolymerase activity in nuclei
Nuclei (50 \(\mu\)g DNA) were isolated from livers of rats fed the control and deficient diets as previously described (17) and resuspended in 500 \(\mu\)l of reaction buffer (25 mM Tris, pH 8.0, 10 mM MgCl\(_2\), 0.5 mM diithothreitol, 0.1 M NaCl). Enzymatic ribosylation of DNA in the permeabilized nuclei was initiated by the addition of 20 \(\mu\)l of 0.5 \(\mu\)Ci [\(^{3}H\)]NAD (ICN Radiochemicals, Irvine, CA) (18). The reactions were stopped after a 10 min incubation at 37°C by the addition of 200 \(\mu\)l of ice-cold 50% trichloroacetic acid (TCA). After centrifugation, the pellets were washed until no radioactivity could be detected in the supernatants. The TCA precipitates were solubilized by overnight incubation at 37°C in 0.1 M NaOH and 2% SDS. The incorporation of radiolabel into DNA was quantified in a Packard 1900TR scintillation counter using Ultima Gold scintillant.

**Results**

**Body weights, liver weights, total liver protein and DNA content**
This data is summarized in Table I. Three weeks after randomization to the semi-purified folate/methyl deficient or the supplemented control diet, the mean body weight of the deficient rats was 92% that of the control-fed rats; after 9 weeks, the body weight of the deficient rats was 80% of control. At 3 weeks, the liver weights of the deficient rats were almost twofold that of control livers reflecting the massive lipid accumulation associated with lipotrope deficiency (20). By 9 weeks, however, the difference in liver weights between groups was considerably less (20%), consistent with significant regression of lipid accumulation as previously described (21). Associated with the peak in liver weight at 3 weeks was an increase in total protein, a decrease in total DNA content and a significant increase in protein–DNA ratio (\(P < 0.01\)). By 9 weeks, total protein and DNA were not different from control values. An increase in protein–DNA ratio at 3 weeks is consistent with a delay or arrest in DNA synthesis (S-phase) occurring in a significant proportion of hepatocytes.

**Apoptosis and proliferation**
Figure 1 is a representative example of in situ labeling of apoptotic bodies in a liver section from a folate/methyl deficient rat 3 weeks after diet intervention. The end-labeling method was used as a means to facilitate identification of morphologically distinct ABs. It should be noted that positive nuclear staining with in situ labeling is not unique to apoptotic cells and can be observed in necrotic cells (22) as well as in viable cells with excessive DNA strand breakage (23); thus, accurate identification and enumeration of ABs requires careful attention to morphology (22). The identification of stained ABs was confirmed by specific morphological criteria including nuclear condensation, cytoplasmic compaction and detachment from neighboring cells (24). In the present evaluation, hepatocytes with necrotic morphology were a rare occurrence and foci of inflammatory cells were absent. As shown in Figure 2, the apoptotic index (ABs/1000 hepatocytes) was significantly increased in deficient rats (\(P < 0.001\)) 2 days after diet initiation and remained significantly elevated at all subsequent sampling intervals. The apoptotic index in liver of control-fed rats did not change at any sampling interval. Two days after diet initiation, the initial increase in ABs was paralleled by an acute increase in mitotic figures, which appeared to undergo transient homeostatic adjustment on day 5, followed by a
Table I. Effect of folate/methyl deficiency on body weight, liver weight, total liver protein and DNA content

<table>
<thead>
<tr>
<th>Time on diet</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
<th>Total protein (mg)</th>
<th>Total DNA (mg)</th>
<th>Protein/DNA (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Days</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>49.4 ± 5.2</td>
<td>2.8 ± 0.3</td>
<td>232.2 ± 74</td>
<td>11.2 ± 1.6</td>
<td>18.1 ± 2.0</td>
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<tr>
<td>Folate/methyl def.</td>
<td>49.6 ± 4.4</td>
<td>3.1 ± 0.3</td>
<td>198.6 ± 33</td>
<td>8.3 ± 1.1</td>
<td>23.3 ± 3.0</td>
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<tr>
<td>1 week</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>83.2 ± 6.7</td>
<td>4.1 ± 0.3</td>
<td>322.1 ± 38</td>
<td>15.7 ± 0.4</td>
<td>21.8 ± 2.5</td>
</tr>
<tr>
<td>Folate/methyl def.</td>
<td>80.8 ± 12.4</td>
<td>5.8 ± 1.3</td>
<td>312.2 ± 14</td>
<td>12.0 ± 0.3</td>
<td>26.3 ± 8.0</td>
</tr>
<tr>
<td>3 weeks</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>157.2 ± 8.9</td>
<td>7.3 ± 0.9</td>
<td>642.9 ± 124</td>
<td>34.3 ± 3.6</td>
<td>19.1 ± 2.3</td>
</tr>
<tr>
<td>Folate/methyl def.</td>
<td>145.2 ± 2.6</td>
<td>13.3 ± 0.3</td>
<td>804.9 ± 129</td>
<td>27.9 ± 0.9</td>
<td>31.3 ± 1.6</td>
</tr>
<tr>
<td>9 weeks</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>337.3 ± 14.3</td>
<td>12.9 ± 0.9</td>
<td>1167.3 ± 115.4</td>
<td>40.0 ± 1.64</td>
<td>30.7 ± 1.8</td>
</tr>
<tr>
<td>Folate/methyl def.</td>
<td>267.2 ± 7.0</td>
<td>16.8 ± 0.9</td>
<td>1280.9 ± 141.6</td>
<td>45.1 ± 1.62</td>
<td>29.7 ± 1.6</td>
</tr>
</tbody>
</table>

aP < 0.05; bP < 0.01.

Table I. Effect of folate/methyl deficiency on body weight, liver weight, total liver protein and DNA content

Means ± SD; n = 3–6 rats/group.

Folate/methyl deficiency in rats

sustained elevation that paralleled apoptosis. A direct comparison between the relative increases in apoptotic bodies and mitotic figures is not possible because these indices have different durations in vivo.

Endonuclease activity

In order to confirm biochemically the mechanism of cell death, the activity of nuclear Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease was measured in protein extracts from nuclei isolated from folate/methyl deficient and control-fed rats. This nuclear enzyme is responsible for the internucleosomal fragmentation during late stage apoptosis (25) and is characterized by cation and pH dependency that is distinct from enzymes involved in necrotic DNA fragmentation (26). Using the plasmid incision assay, the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease activity in protein extracts from nuclei of 2 day and 9 week samples from folate/methyl deficient livers was compared. As shown in Figure 3, an early peak of activity was apparent 2 days after initiation of the deficient diet. After a transient decline at 5 and 7 days, endonuclease activity again increased and was maintained at elevated levels after 3 and 9 weeks on the deficient diet.

Poly(ADP-ribose) polymerase activity and NAD levels

Poly(ADP-ribose)polymerase (PARP) is a nuclear enzyme that is activated by DNA strand breaks and is part of a rapid cellular stress response to DNA damage that is involved in the initiation of DNA base excision repair (27). PARP physically binds to the DNA break site and undergoes rapid auto-ribosylation with newly synthesized negatively charged ADP-ribose polymers. Nicotinamide adenine dinucleotide (NAD) is the sole substrate for PARP and provides the source of ADP-ribose subunits that are degraded within minutes of production (28). The activity of PARP in liver nuclei from the folate/methyl deficient rats was significantly elevated 2 days after diet initiation, remained elevated at 5 and 7 days, and exhibited further increases in activity at 3 weeks and 9 weeks (Figure 4). This pattern of activity, with an acute increase at day 2 followed by sustained elevations at 3 and 9 weeks is very similar to that observed with the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent nuclear endonuclease (Figure 3) and also similar to progressive changes in apoptotic rate (Figure 2). In a separate experiment, hepatic levels of NAD were measured by HPLC as described.
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The parallel changes in apoptotic rate and endonuclease activity in the present study suggest that DNA strand break accumulation may be due, in part, to endonuclease-mediated DNA cleavage during apoptotic cell death. In this report, we provide evidence that apoptosis appears to be the primary mechanism of cell death during early phases of methyl deficiency induced with semi-purified diets. This is an important clarification since the initial observations of cell death with methyl deficiency referred to ‘necrosis’ as the mechanism of cell death (13, 41). However, in the early literature, necrosis was used as a more general pathological term encompassing all forms of cell death without regard to mechanism (42).

Whether apoptosis is the primary form of cell death with the more severe deficiency induced by AA defined methyl deficient diets remains to be determined.

Fig. 4. Changes in poly(ADPribose) polymerase (PARP) activity and nicotinamide adenine dinucleotide (NAD) levels in folate/methyl deficient rats over the 9-week feeding period expressed as percentage change from the control values (means ± SE; n = 4–6 rats/interval).

for intracellular nucleotides. NAD levels were significantly reduced after 7 days on the deficient diet (P < 0.05) and remained depressed through 9 weeks at which point NAD levels fell to 55% that of control samples (Figure 4).

Deoxynucleotide (dNTP) pools

A representative example of the HPLC elution pattern of hepatic nucleotides and deoxynucleotides is presented in Figure 5. To our knowledge, this is the first attempt to quantify dNTP levels in liver tissue obtained in vivo. Most previous nucleotide analyses have been performed on extracts from proliferating cell lines in vitro (29, 30). The NTP and dNTP pools were quantified using appropriate calibration standards and Beckman System Gold software and were expressed per mg protein in each sample. None of the hepatic nucleotide pools (CTP, UTP, GTP or ATP) were significantly different from control samples over the 9-week feeding period. No significant differences in dNTP pools were found in liver samples at sampling intervals before 3 weeks due to wide variation between samples at the early time points (data not shown). After 3 weeks and 9 weeks of folate/methyl deficiency, however, significant increases in the dUTP pools were associated with significant decreases in the dTTP pools relative to control values as shown in Figure 6. As a result, the ratio of dUTP/dTTP was elevated ~3-fold over control at 3 and 9 weeks. At 9 weeks, significant decreases in both dCTP and dATP were observed consistent with a compromise in folate-dependent de novo purine synthesis.

Discussion

Increased interest in the methyl deficient model of endogenous hepatocarcinogenesis has generated new mechanistic information that underscores the multifactorial nature of multistage carcinogenesis. Several non-exclusive hypotheses have been proposed for this model that implicate: (i) DNA hypomethylation (31, 32); (ii) oxidative free radical damage (33, 34); (iii) aberrant membrane phospholipid metabolism, (35); (iv) abnormal deoxynucleotide metabolism (36, 37); and (v) aberrant oncogene and tumor suppressor gene expression (38–40). In a previous study, we demonstrated that DNA hypomethylation was associated with increased DNA strand breaks and proposed that hypomethylation with folate/methylation deficiency may alter chromatin conformation and accessibility of the DNA to oxidant and enzyme-induced DNA breaks (17). The parallel changes in apoptotic rate and endonuclease activity in the present study suggest that DNA strand break accumulation may be due, in part, to endonuclease-mediated DNA cleavage during apoptotic cell death. This is an important clarification since the initial observations of cell death with methyl deficiency referred to ‘necrosis’ as the mechanism of cell death (13, 41). However, in the early literature, necrosis was used as a more general pathological term encompassing all forms of cell death without regard to mechanism (42). Whether apoptosis is the primary form of cell death with the more severe deficiency induced by AA defined methyl deficient diets remains to be determined.

The balance between proliferation and apoptosis determines cell number homeostasis and tissue growth under normal physiologic conditions. Under pathologic conditions, apoptosis provides a protective mechanism by the selective elimination of single DNA damaged or potentially neoplastic cells (24). Within hepatic preneoplastic foci, an increase in proliferation rate is accompanied by a parallel increase in apoptotic rate that may account for the slow growth phenotype in these lesions (43). To assess proliferation rates under conditions of folate deficiency, the mitotic index (MI) may provide the most accurate method. Methods based on BrdU or [3H]thyidine incorporation may overestimate actual cell division rates because these analogs are incorporated via the salvage pathway for thymidine synthesis which is up-regulated in folate deficient, but not control cells (36). It is highly likely that the sustained increase in apoptosis observed in the methyl deficient liver represents an ongoing attempt to counteract the chronic diet-induced biochemical stress and DNA damage. Nonetheless, the implicit assumption in this model of chronic apoptosis, regenerative proliferation and carcinogenesis is that the rare initiated cell must eventually escape the apoptotic pathway to attain the transformed, immortalized phenotype with proliferative advantage. Supporting this assumption, folate deprivation of CHO cells in vitro leads to endonuclease activation and apoptosis in the majority of cells; however, a minority of cells fail to undergo apoptosis and survive with increased mutation frequency (44).

The acute and chronic stimulation of PARP in liver of the folate/methyl deficient rats is consistent with our previous observations of DNA strand break accumulation (17) and confirms earlier observations by Henning et al. (45). Stimulation of hepatic PARP activity with folate/methyl deficiency may result from strand breaks induced by endonuclease cleavage (46), oxidative stress (47) and/or dNTP imbalance (48). After 1 week, the sustained increase in PARP was associated with a sustained decrease in intracellular NAD levels. The combination of unrepaired DNA strand breaks, stimulation of PARP and depletion of NAD has been shown to initiate apoptosis in several cell types (27, 49) and may contribute to the sustained increase in apoptotic rate observed during the later phases of chronic folate/methyl deficiency.

A final consideration is the genetic consequences of increased proliferation (DNA synthesis) under conditions of dNTP pool imbalance. Because the dNTP are the substrate for the DNA polymerases, the fidelity of DNA replication and repair synthesis is critically dependent on the correct balance
of dNTP (50). The size of the nuclear dNTP pools is extremely small and can sustain DNA synthesis for only 30 s to 3 min (51). Most previous evaluations of dNTP imbalance have been done in proliferating cell lines in vitro (29,30). The nucleotide distribution pattern in liver is unique in that the NTP/dNTP ratios are much smaller compared with cell lines or lymphocyte models in which NTP are generally several fold higher than the dNTP. This may reflect the greater nucleotide turnover rate and increased requirement for protein synthesis in the liver. In addition, the dUTP levels are generally negligible in rapidly proliferating cell lines; however, in the liver, the dUTP levels were higher than dTTP levels in control samples. Again, this may be due to the unique biochemistry and requirements in liver with relatively low cell proliferation rate. Consistent with this possibility, cytoplasmic UTPase has been shown to vary with proliferation status and is negligible in non-dividing cells (52).

The most notable change in hepatic dNTP pools with folate/methyl deficiency was a threefold increase in the dUTP/dTTP ratio after 3 weeks, which was maintained through 9 weeks of deficiency. The feeding of orotic acid, a precursor for uridine biosynthesis, similarly elevates uridine nucleotides and is also a potent liver tumor promoter in rats (53,54). An increase in dUTP/dTTP in vitro promotes folate fragile site expression (55), DNA strand breakage (56) and error prone DNA repair (57,58). Several investigators have proposed that an increase in dUTP/dTTP ratio causes a delay in DNA replication fork progression (59), cell cycle arrest (60) and genomic instability (44,61). Recent reports of uracil in the DNA of folate deprived HL-60 cells and human lymphocytes (62–64) confirm earlier reports of uracil misincorporation associated with increased intracellular dUTP/dTTP ratios (65).

Misincorporation of uracil, however, does not constitute a base mispair mutation since the DNA polymerase does not distinguish uracil from thymine and will insert an adenine during DNA replication or repair. Rather, the genetic consequences of uracil misincorporation are secondary to the efficient uracil DNA glycosylase DNA repair activity and the induction of abasic sites and DNA strand breaks, which can be significant premutagenic lesions (66). Finally, although not generally appreciated, the misincorporation of uracil for thymine may constitute an important secondary mechanism of DNA hypomethylation (loss of the thymine methyl group) as originally proposed by Krumdieck (67). Supporting this possibility, uracil substitution for thymine has been recently shown to alter the binding of sequence-specific transcription factors (68).

As a final comment, it is interesting to note that the magnitudes of biochemical and molecular changes observed with dietary manipulation in vivo are generally much less than those observed with acute nutrient deprivation in vitro. The long latency period in this nutritional model of multistage carcinogenesis may reflect the significant impact of small changes chronically imposed over long intervals.

**Acknowledgements**

This work was supported by a research grant from the American Cancer Society (no. CN-73E, SJH) and by appointments to the Postgraduate Research Program administered by the Oak Ridge Institute from Science and Education

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Received on March 19, 1996; revised on September 20, 1996; accepted on October 17, 1996