ABSTRACT

Sodium cyanate is a selective in vivo inhibitor of protein synthesis in a variety of mammalian tumor cells without a corresponding effect on the normal tissues of tumor-bearing animals. The in vivo decrease of protein synthesis observed 4 h post-NaOCN i.p. administration in the murine P388 leukemia cell cannot be explained by decreased amino acid pools in the mouse peritoneal cavity. In addition, the decrease in protein synthesis observed with NaOCN in isolated P388 cells was shown not to be secondary to (a) alterations in the kinetics of amino acid transport or (b) effects on total nucleotide pools. The incorporation of [14C]phenylalanine in P388 cell-free lysates from NaOCN-pretreated mice was significantly decreased to approximately 55% of control lysates in the presence of exogenous amino acids. The addition of exogenous calf liver tRNA to the lysates did not alter this result. However, no difference was observed in polyuridylic acid-directed [14C]phenylalanine incorporation into polypeptides in micrococcal nuclease-treated P388 lysates from NaOCN-pretreated or control mice. Quaternary initiation complex (48S) formation and mRNA synthesis were found to be significantly decreased by 35 and 38%, respectively, in P388 cells from NaOCN-pretreated mice. DNA synthesis was decreased by 66% of control at 1 h and 62% at 4 h post-NaOCN i.p. administration. No apparent effect with NaOCN was observed on total RNA synthesis in P388 cells. These results suggest that the decrease in P388 cell protein synthesis observed with NaOCN in vivo appears to be due to alterations manifested in the synthesis of cellular mRNA and protein synthesis initiation processes. NaOCN does not appear to affect the P388 cell ribosomal machinery, tRNA, or protein synthesis elongation processes.

INTRODUCTION

Sodium cyanate has been described as having a variety of effects on various tissues. Of particular interest is the selective in vivo inhibition of protein synthesis by NaOCN in mammalian tumor cells, including various transplantable hepatomas, the MK3 kidney tumor, the Ehrlich ascites cell, the Novikoff ascites hepatoma cell, the LK1 and T36 colon cancer, and a 1,2-dimethylhydrazine-induced colon adenocarcinoma, since no corresponding effect is observed on the nonmalignant tissue of the tumor-bearing animal (1–4). The selective sensitivity of tumor cells to NaOCN was also observed with normally insensitive chick fibroblasts transformed with Rous sarcoma virus. In addition, NaOCN failed to cause a decrease in protein synthesis in normally sensitive HeLa tumor cells after the cells were exposed to sodium butyrate, a substance which suppresses the malignant phenotype (2). The only normal tissue yet reported to show a decrease in protein synthesis with NaOCN was the normal tissue of dimethylhydrazine-induced colon adenocarcinoma, since no effects on various tissues. Of particular interest is the selective inhibition requires the activation of NaOCN by the cytochrome P-450 fraction of the liver and that NADPH is a necessary cofactor for this reaction (2, 9). In addition, it was found that this selective inhibition was not secondary to differences in cyanate uptake into tumor versus normal tissues (10). However, tumor-selective decreases in amino acid and amino acid analogue uptake (1–3), phosphate uptake (3), and ATP levels (3) have been observed with NaOCN.

We have extended these studies on the selective inhibition by NaOCN of protein synthesis in tumor cells. We had previously reported that NaOCN caused a decrease in protein synthesis in the murine P388 leukemia cell without a corresponding inhibition in various nonmalignant murine tissues (11). In the present study, we have examined the possible sites of action of NaOCN in the P388 tumor cell. Since NaOCN acts selectively to inhibit protein synthesis in tumor cells, determining the mechanism of NaOCN action may eventually provide some information on the differences between normal and transformed cells.

MATERIALS AND METHODS

Materials. NaOCN was supplied by ICN Pharmaceuticals Inc. McCoy’s 5A modified medium was purchased from Gibco, and bovine serum albumin (Fraction V, powder, low-salt, and salt-free fractions) was supplied by Miles laboratories. Hemin, unlabeled amino acids, unlabeled AIB, and baker’s yeast RNA were obtained from Sigma Chemicals. Unlabeled BCH isomer was purchased from Boehringer-Mannheim. l-[U-14C]leucine, α-amino[1,14C]isobutyrlic acid, l-[2,2,2-13C]threonine, l-[1-14C]glutamine, l-[1-14C]glycine, [carboxyl-14C]cholin, [methyl-14C]thymidine, initiated water, and l-[3H]methionine were all purchased from Amersham. [S-35]H, uridine was obtained from ICN Biochemicals while l-[U-14C]phenylalanine was supplied by both ICN Biochemicals and Amersham. d1-β-aminobicyclo[2,2,1]heptane-2-carboxylic acid (carboxyl-14C) was purchased from New England Nuclear. Sparsomycin was a kind gift from Dr. M. Sussman of the National Cancer Institute, Bethesda, MD (NCS No. 59729-6/10) while salmon sperm DNA was kindly supplied by Dr. J. Hiscott of the Lady Davis Institute for Medical Research, Montreal, Quebec, Canada.

Harvesting of Murine P388 Leukemia Cells and NaOCN Administration.

Received 3/11/87; revised 6/15/87; accepted 7/1/87.

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tion. Male BALB/c × DBA/2 F1 mice (hereafter called CD2F, mice) weighing 17-25 g and maintained on laboratory mouse chow pellets and water ad libitum were used throughout this study. CD2F, mice were given implants i.p. of 2 x 10^6 murine P388 leukemia cells. Four-five days after implantation, groups of 3-6 mice received 0.1 ml/10 g body weight of either NaOCl or saline (pH 7.4). NaOCl was dissolved in phosphate-buffered saline (pH 6.0) at a concentration of 25 mg/ml. Unless otherwise indicated, the mice were sacrificed by cervical traction at 1 or 4 h post-NaOCl administration, and the P388 cells were aspirated from their abdomens and suspended in the appropriate media.

The cells were then washed twice with centrifugations at 200 x g for 5 min at 23°C and suspended at a final concentration of 4 x 10^6 P388 cells/ml.

Cell viability was typically 95% by trypan blue exclusion. The percentage of RBC in P388 cell suspensions was always ≤10%.

Inhibition of Protein Synthesis. P388 cells (4 x 10^6 cells/ml) were preincubated for 15 min at 37°C immediately after cell suspension in McCoy's 5A modified medium. To each ml of cell suspension was added 0.025 μCi of [U-14C]leucine (3,700 mCi/mmol) in 20 μl 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and incubated for 1 h at 37°C. The cells were homogenized in 5% trichloroacetic acid and washed twice in the same solution at 4°C. The final pellets were solubilized overnight in 1 N NaOH and assayed for radioactivity as described previously (12).

Amino Acid Transport Studies. The labeled amino acid or amino acid analogues used in this study were α-aminoc[1-14C]isobutyrlic acid (58 mCi/mmol), L-α-[14C]glutamine (280 mCi/mmol), L-α-[14C]lysine (334 mCi/mmol), and D,L-α-2-amino-bicyclo-[2,2,1]heptane-2-carboxylic acid (carboxyl-14C) (4.95 mCi/mmol). The P388 cells were washed and suspended in either a Na+-rich medium containing phosphate-buffered saline (pH 7.4), 0.25% dextrose, and 0.1 mM low-salt bovine serum albumin, or a ν-t'nv choline-substituted medium containing 0.1% dextrose and 0.1 mM salt-free bovine serum albumin, or a ν-t'nv choline-substituted medium containing 0.1% dextrose and 0.1 mM salt-free bovine serum albumin as described previously (12, 13). Prior to the initiation of transport experiments, all cell suspensions and amino acid solutions were preincubated for 15 min at 37°C. Transport was initiated by the addition of labeled amino acid in the appropriate medium to an equivalent volume of suspended P388 cells (4 x 10^6 cells/ml) and incubated at 37°C. Four hundred-μl aliquots were removed from the amino acid-P388 cell incubation mixture and assayed for radioactivity as previously described (12, 13). The intracellular water space was determined with tritiated water plus [carboxyl-14C]ulinin for all transport studies by centrifugation through Versilube F-50 silicone oil to separate medium from cells as previously described (12, 14).

The uptakes were linear for up to at least 1 min for all amino acids or amino acid analogues. The concentrations utilized were always between 0.005 and 1.0 mM and were incubated for 30 s or 1 min, as indicated in the text. Kinetic parameters for both BCH and lysine uptakes were measured in Na+-free media; the observed velocities in Na+-free media were subtracted for AIB, threonine, and glutamine measurements so that only the Na+-dependent velocities were measured. Kinetic parameters for all amino acids or analogues were determined by linear regression analysis and were equivalent when plotted on either Lineweaver-Burk or Edie-Hofstee plots.

ATP Measurements. P388 cells were washed in cold (4°C) Tris-saline solution (146 mM NaCl, 35 mM Tris-HCl, pH 7.5) and were then lysed by the addition of cold hypotonic buffer (1.5 mM magnesium acetate, 10 mM potassium acetate, 0.5 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5; 0.5 g cell weight/ml buffer). After 10-15 min at 0°C, the cells were disrupted by 30-40 strokes in a Dounce homogenizer, and an equal volume of cold 12% trichloroacetic acid was added. The homogenate was centrifuged at 2000 x g for 10 min at 3°C, and the ATP in the protein-free supernatant was assayed using a coupled enzyme system with phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase as described previously (15, 16).

[14C]Phenylalanine Incorporation into Protein in P388 Cell-free Ly- sates. Cell lysates were prepared fresh the day of each experiment, and all operations were performed at 0-4°C unless otherwise indicated. One vol of 1 mM hemin dissolved in 75% ethylene glycol, 0.2 mM Tris-HCl (pH 7.6), and 0.05 N KOH was added to 9 vol of lysed P388 cells and centrifuged at 15,000 x g for 15 min. Protein synthesis in the supernatant (S-15) was monitored by adding 0.5 vol of extract to 0.5 vol of a solution containing the following components: 20 mM Tris-HCl (pH 7.6); 0.6 M potassium acetate; 12 mM magnesium acetate; 10 mM dithiothreitol; 2.4 mM ATP; 0.6 mM GTP; 20 mM creatine phosphate; 0.4 mg/ml creatine kinase; 0.8 mM spermidine; 0.08 mM each of 19 unlabeled amino acids (except phenylalanine); and 5 μCi/ml L-[U-14C]phenylalanine (450 or 522 mCi/mmol). Incubations were at 37°C and were allowed to proceed for up to 30 min. Protein synthesis was measured as described previously (17).

When exogenous polyuridylic acid was added to the cell-free lysate, the endogenous mRNA was degraded by a 15-min incubation of lysate samples with 150 units/ml micrococcal nuclease plus 1 mM CaCl2 at 37°C, as described previously (18, 19). One μM cycloheximide, 0.1 μM puromycin, 2 mg/ml polyuridylic acid, and 0.1 mg/ml calf liver tRNA (coding for all 20 amino acids) were added to the incubation mixture, as indicated in the text.

Initiation Experiments. The methods utilized were similar to those of Cohen and Glazer (20) with a few modifications. P388 cells were incubated at 5 x 10^6 cells/ml in Na+ -rich medium and preincubated for 20 min at 37°C with 0.1 mM sparsomycin [an inhibitor of elongation of protein synthesis (21)] prior to the addition of 0.9 μCi/ml L-4-[14C]methionine (1.19 or 1.32 Ci/μmol). After a 10-min incubation at 37°C, the cells were rinsed 4 times in cold saline (pH 7.4), and homogenized with 30-40 strokes of a Dounce homogenizer in cold buffer containing 0.5% Nonidet P-40, 2.5 mM magnesium acetate, 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, and 75 mM KCl. Formaldehyde was added to a final concentration of 0.2% and the homogenate was centrifuged at 10,000 x g for 10 min at 3°C. Four hundred-μl samples of the supernatant (S-10), equivalent to the extract from 5 x 10^6 P388 cells, were layered onto 12 ml of 15-40% glycerol gradients and spun for 16.5 h at 18,000 x g in an SW41 rotor at 3°C. Fractions (0.4 ml) were spectrophotometrically analyzed at 260 nm and then collected on Whatman GF/A glass fiber filters with 10% trichloroacetic acid. Radioactivity was assayed as described previously (12). P388 cell protein synthesis initiation was measured in NaOCl versus control samples utilizing the ratio of radioactivity (cpm)/sum of absorbance (absorbance units), under the 40S peak of their respective RNA profiles. Each experiment was performed with duplicate NaOCl and control samples.

DNA and RNA Synthesis Measurements. P388-containing CD2F, mice were given injections of 0.2 μCi [methyl-14C]thymidine (61 mCi/ mmol) and 20 μCi [5-3H]uridine (17 Ci/mmol) or 1 h post-NaOCl administration. After 1 h, the mice were sacrificed and the P388 cells were collected and washed 3 times with Tris-saline prior to lysis in H2O (0.5 g cell weight/ml H2O) with 30 strokes of a Dounce homogenizer. An alkaline extraction of RNA followed by an acid extraction of DNA was then performed using a modification of the Schmidt Thannhauser procedure as previously described (22, 23). DNA- and RNA-rich extracts were collected, analyzed spectrophotometrically at 260 nm, and assayed for radioactivity (3H or 14C, respectively) as previously described (12). Estimates of RNA and DNA content as a function of spectrophotometric readings at 260 nm were determined from standard curves made from baker's yeast RNA or low molecular weight salmon sperm DNA.

[3H]Uridine Incorporation into P388 Cell Poly(A) RNA. Mice were given i.p. injections of 10 μCi [5-3H]uridine (17 Ci/mmol) or 1 h post-NaOCl administration. After 1 h, the mice were sacrificed and the P388 cells were collected and washed 3 times with Tris-saline prior to lysis in H2O (0.5 g cell weight/ml H2O) with 30 strokes of a Dounce homogenizer. An alkaline extraction of RNA followed by an acid extraction of DNA was then performed using a modification of the Schmidt Thannhauser procedure as previously described (22, 23). DNA- and RNA-rich extracts were collected, analyzed spectrophotometrically at 260 nm, and assayed for radioactivity (3H or 14C, respectively) as previously described (12). Estimates of RNA and DNA content as a function of spectrophotometric readings at 260 nm were determined from standard curves made from baker's yeast RNA or low molecular weight salmon sperm DNA.

Isoamyl alcohol (50:49:1) extraction. The procedure was performed in triplicate prior to the addition of 0.2 N NaCl followed by 2.5 vol absolute ethanol. The RNA was precipitated overnight at ~70°C, spun down, and washed twice in ~70°C ethanol. The poly(A)+ RNA was then isolated using oligodeoxynucleotidyl acid cellulose as described previously (24). Samples were analyzed spectrophotometrically at 260
Am of lysine transport but significantly (P < 0.025) increased NaOCN on the integrity of the protein synthetic machinery. but this difference was not significant.pared with 0.289 ± 0.053 µmol ATP x g^-1 cells from controls), P388 cells. The levels of total ATP in the protein-free super
the Vra".affected. In addition, NaOCN had no significant effect on the
decreased by NaOCN while the Vm,x was not significantly
The Km of glutamine transport was significantly (P < 0.025) system A,5 and (c) threonine, shown to enter P388 cells specif
system L, (b) AIB, an amino acid analogue shown to enter
transport kinetics of (a) BCH, an amino acid analogue known
to enter cells specifically by the neutral amino acid transport
Menton kinetics of in vitro amino acid transport in P388 cells
NaOCN on amino acid transport was studied. The Michaelis-

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<th>BCH</th>
<th>AIB</th>
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<th>Glutamine</th>
<th>Lysine</th>
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<tr>
<td>Km (µM)</td>
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<td>770</td>
<td>119</td>
<td>222</td>
<td>112</td>
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<tr>
<td>Vmax (nmol x µl^-1 intracellular water space x min^-1)</td>
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<td>4.48 ± 0.67</td>
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<td>Control</td>
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<td>1.37 ± 0.28</td>
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nm and assayed for radioactivity as previously described (12).
Student's t test (one tail) was utilized for statistical analysis of all experiments.

RESULTS

The incorporation of [3H]leucine into protein P388 cells from NaOCN-pretreated mice was previously shown to be decreased by 75% as compared to P388 cells from control mice following the i.p. administration of [3H]leucine 4 h after the i.p. administration of NaOCN (250 mg/kg) or 1 n saline (11). In the present work, a 1-h in vitro incubation of [14C]leucine with P388 cells from NaOCN-pretreated and control mice at 37°C was performed. P388 cells had been aspirated from mice which had been sacrificed 4 h after the i.p. administration of NaOCN (250 mg/kg) or 1 n saline. It was found that the incorporation of [14C]leucine into protein in cells from NaOCN-pretreated mice was significantly (P < 0.01) decreased by 46% as compared to cells from control mice [i.e., 392 ± 12 (SE) dpm/2 x 10^6 cells from NaOCN-pretreated mice versus 736 ± 54 dpm/2 x 10^6 cells from control mice]. To determine the site of action of NaOCN, the effect of NaOCN on amino acid transport was studied. The Michaelis-Menton kinetics of in vitro amino acid transport in P388 cells obtained from NaOCN-pretreated and control mice are shown in Table 1. NaOCN had no significant effect on the amino acid transport kinetics of (a) BCH, an amino acid analogue known to enter cells specifically by the neutral amino acid transport system L, (b) AIB, an amino acid analogue shown to enter P388 cells specifically by the neutral amino acid transport system ASC (12). The Km of glutamine transport was significantly (P < 0.025) decreased by NaOCN while the Vmax was not significantly affected. In addition, NaOCN had no significant effect on the Km of lysine transport but significantly (P < 0.025) increased the Vmax.

It was also possible that NaOCN altered the ATP pools in P388 cells. The levels of total ATP in the protein-free supernatant of P388 cells from NaOCN-pretreated and control mice were determined. Cells from NaOCN-pretreated mice contained slightly less ATP than cells from control mice (0.221 ± 0.030 µmol ATP x g^-1 cells from NaOCN-pretreated mice as compared with 0.289 ± 0.053 µmol ATP x g^-1 cells from controls), but this difference was not significant.
P388 cell-free lysates were developed from cells from both NaOCN-pretreated and control mice to examine the effects of NaOCN on the integrity of the protein synthetic machinery.

Table 1 Effect of NaOCN on Michaelis-Menton kinetics of amino acid transport in P388 cells

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Fig. 1 shows the incorporation of [14C]phenylalanine into protein directed by the endogenous mRNA of P388 cell-free lysates from NaOCN-pretreated and control mice. The levels of incorporated [14C]phenylalanine were significantly decreased over a 30-min incubation period for all points tested in NaOCN lysate samples as compared to controls (P < 0.005 for all points). The incorporation in NaOCN lysate samples was 55% of control at 4 min, but rose to 69% of control by 30 min. The addition of 1 mM cycloheximide and 0.1 mM puromycin significantly inhibited protein synthesis in both lysates as shown in Fig. 1 (P < 0.005 for all points). The observed decrease in [14C]phenylalanine incorporation into protein in NaOCN lysate samples was not affected by the addition of exogenous calf liver tRNA, as seen in Fig. 2. The incorporation in tRNA-rich NaOCN lysate samples was approximately 55% of control from 4–15 min and 66% of control at 30 min. The addition of 1 mM cycloheximide and 0.1 mM puromycin to the lysate produced a similar decrease in [14C]phenylalanine incorporation as that described above (results not shown).

Fig. 3 represents the incorporation of [14C]phenylalanine into polypeptides as directed by exogenous polyuridylic acid in micrococcal nuclease-treated P388 lysates (i.e., no endogenous mRNA). The levels of polyuridylic acid-directed incorporation were similar in both NaOCN and control lysate samples, because approximately 2.2 pmol phenylalanine x µg^-1 RNA were incorporated by 30 min in both lysates. In the absence of added

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Unpublished results.

Fig. 1. Effect of NaOCN on protein synthesis in P388 cell-free lysates. P388 cell-free lysates were prepared from cells from both NaOCN-pretreated and control mice. Protein synthesis proceeded at 37°C for up to 30 min with incubation mixtures containing 5 µCi l-[1-14C]phenylalanine (Phe) as described under "Materials and Methods." Radioactivity was assayed as described in the text. A, control lysate; O, NaOCN lysate; △, control lysate plus 1 mM cycloheximide plus 0.1 mM puromycin; O, NaOCN lysate plus 1 mM cycloheximide plus 0.1 mM puromycin. Data are the mean ± SE of at least 5 determinations.
was 35% (significant at P < 0.025). In addition, peaks were found in both samples in the monosome, or 80S region (fraction 23-26) of the gradient. The data in Table 2 record the inhibition of \(^{14}C\)thymidine incorporation into DNA in P388 cells from NaOCN-pretreated rats, in the present study no such decrease in protein synthesis in HeLa (26) and Ehrlich ascites tumor cells (27). Although a decrease in the uptake of amino acids and AIB had been observed by others in hepatoma cells and tumor cells (27). It has been previously reported that the in vivo NaOCN inhibition of protein synthesis in transplantable hepatoma and colonic tumors of the rat may be secondary to alterations in the tissue distribution of labeled substrates into tumors versus normal cells (25). The observed decrease in P388 cell protein synthesis by NaOCN cannot, however, be totally explained by alterations in absorption in the mouse peritoneal cavity. The observed decrease with NaOCN in incorporation of labeled uridine into RNA was not significantly affected by NaOCN when labeled uridine was injected at either 1 or 4 h post-NaOCN administration.

DISCUSSION

It has been previously reported that the in vivo NaOCN inhibition of protein synthesis in transplantable hepatoma and colonic tumors of the rat may be secondary to alterations in the tissue distribution of labeled substrates into tumors versus normal cells (25). The observed decrease in P388 cell protein synthesis by NaOCN cannot, however, be totally explained by alterations in absorption in the mouse peritoneal cavity. The observed decrease with NaOCN in incorporation of labeled uridine into P388 cell mRNA was significantly (P < 0.01) reduced by 38% in NaOCN samples from NaOCN-pretreated and control mice. In contrast to the data observed for total RNA synthesis, \(^{3}H\)uridine incorporation into P388 cell mRNA was significantly (P < 0.005) reduced by 66% 4 h post-NaOCN administration. Table 2 is also representative of the incorporation of \(^{3}H\)uridine into poly(A)+ RNA or mRNA in P388 cells from NaOCN-pretreated and control mice. In contrast to the data observed for total RNA synthesis, \(^{3}H\)uridine incorporation into P388 cell mRNA was significantly (P < 0.01) reduced by 38% in NaOCN samples from NaOCN-pretreated and control mice. When \(^{14}C\)thymidine was injected i.p. 1 h post-NaOCN administration, a 66% inhibition in incorporation into DNA was observed (significant at P < 0.01). This inhibition was maintained when labeled thymidine was injected 4 h post-NaOCN administration (62%; significant at P < 0.005). In contrast, the inhibition was not observed at the level of total RNA synthesis. As seen in Table 2, the incorporation of \(^{3}H\)uridine into RNA was not significantly affected by NaOCN when labeled uridine was injected at either 1 or 4 h post-NaOCN administration.

Radioactivity:absorbance ratios at the 40S peak for 6 experiments were 1482 and 2246 cpm/absorbance unit \(^{-2}\) for the NaOCN and control samples, respectively. The overall increase in radioactivity:absorbance ratios at the 40S peak in NaOCN samples as compared to controls for 6 experiments was 35% (significant at P < 0.025). In addition, peaks were found in both samples in the monosome, or 80S region (fractions 23-26) of the gradient. The data in Table 2 record the inhibition of \(^{14}C\)thymidine incorporation into DNA in P388 cells from NaOCN-pretreated cells and control mice. When \(^{14}C\)thymidine was injected i.p. 1 h post-NaOCN administration, a 66% inhibition in incorporation into DNA was observed (significant at P < 0.01). This inhibition was maintained when labeled thymidine was injected 4 h post-NaOCN administration (62%; significant at P < 0.005). In contrast, the inhibition was not observed at the level of total RNA synthesis. As seen in Table 2, the incorporation of \(^{3}H\)uridine into RNA was not significantly affected by NaOCN when labeled uridine was injected at either 1 or 4 h post-NaOCN administration.

Fig. 2. Effect of NaOCN on protein synthesis in P388 cell-free lysates containing exogenous calf liver tRNA. P388 cell-free lysates were prepared from cells from both NaOCN-pretreated and control mice as described under "Materials and Methods." Protein synthesis was monitored at 37°C for up to 30 min with incubation mixtures containing 5 μCi/ml L-\(^{14}C\)phenylalanine (Phe) and 0.1 mg/ml exogenous calf liver tRNA (coding for all 20 amino acids) as described under "Materials and Methods." • control lysate; O NaOCN lysate. Data are the mean ± SE (bars) of 2 determinations.

Fig. 3. Effect of NaOCN on polyuridylic acid-directed protein synthesis in P388 cell-free lysates. P388 cell-free lysates were prepared from both NaOCN-pretreated and control mice as described under "Materials and Methods." Endogenous mRNA was degraded utilizing 150 units/ml micrococcal nuclease. Protein synthesis was monitored at 37°C for up to 30 min with incubation mixtures containing 5 μCi/ml L-\(^{14}C\)phenylalanine (Phe) and 2 mg/ml exogenous polyuridylic acid as described under "Materials and Methods." • control lysate; O NaOCN lysate. Data are the mean ± SE (bars) of 3 determinations.

Fig. 4. Effect of NaOCN on quaternary initiation complex formation in P388 cell-free lysates. P388 cells were isolated from mouse abdomens 4 h post-NaOCN administration and preincubated for 20 min at 37°C with 0.1 msiu sparsomycin as described under "Materials and Methods." Cells were then incubated with 0.9 mCi/ml L-\(^{14}C\) methionine for 10 min at 37°C prior to cell lysis as described in the text. S-10 samples were layered onto 15-40% glycerol gradients and spun for 16.5 h at 18,000 rpm in an SW41 rotor at 3°C. Fractions (0.4 ml) were obtained prior to spectrophotometric analysis and assaying for radioactivity as described in the text. Experiments were performed with duplicate NaOCN and control samples. Data are representative of one of 6 experiments.

polyuridylic acid, \(^{14}C\)phenylalanine incorporation was reduced in both lysates to 8% of uninhibited values at 30 min (results not shown).

The formation of intermediary initiation complexes containing radioactive methionine was examined by glycerol gradient analysis of sparsomycin-treated P388 cell lysates (see "Materials and Methods"). Fig. 4 shows the gradient distribution of radioactive methionine relative to absorbance in incubation samples prepared from lysed P388 cells from NaOCN-pretreated and control mice. In the control sample, a major peak of radioactivity was obtained at the top of the gradient in fractions 1-6, representing an approximate S value of 5, and another in fractions 16-19, being associated with 40S ribosomal subunits or 48S initiation complexes. Similar peaks were found in the NaOCN sample, but the peak associated with the 40S subunits was less pronounced than that of the control samples. The radioactivity:absorbance ratios for the 40S peak for 6 experiments were 1482 and 2246 cpm/absorbance unit \(^{-2}\) for the NaOCN and control samples, respectively. The overall decrease in radioactivity:absorbance ratios at the 40S peak in NaOCN samples as compared to controls for 6 experiments was 35% (significant at P < 0.025). In addition, peaks were found in both samples in the monosome, or 80S region (fractions 23-26) of the gradient.

The data in Table 2 record the inhibition of \(^{14}C\)thymidine incorporation into DNA in P388 cells from NaOCN-pretreated cells and control mice. When \(^{14}C\)thymidine was injected i.p. 1 h post-NaOCN administration, a 66% inhibition in incorporation into DNA was observed (significant at P < 0.01). This inhibition was maintained when labeled thymidine was injected 4 h post-NaOCN administration (62%; significant at P < 0.005). In contrast, the inhibition was not observed at the level of total RNA synthesis. As seen in Table 2, the incorporation of \(^{3}H\)uridine into RNA was not significantly affected by NaOCN when labeled uridine was injected at either 1 or 4 h post-NaOCN administration.

Table 2 is also representative of the incorporation of \(^{3}H\)uridine into poly(A)+ RNA or mRNA in P388 cells from NaOCN-pretreated and control mice. In contrast to the data observed for total RNA synthesis, \(^{3}H\)uridine incorporation into P388 cell mRNA was significantly (P < 0.01) reduced by 38% in NaOCN samples from NaOCN-pretreated and control mice. When \(^{14}C\)thymidine was injected i.p. 1 h post-NaOCN administration, a 66% inhibition in incorporation into DNA was observed (significant at P < 0.01). This inhibition was maintained when labeled thymidine was injected 4 h post-NaOCN administration (62%; significant at P < 0.005). In contrast, the inhibition was not observed at the level of total RNA synthesis. As seen in Table 2, the incorporation of \(^{3}H\)uridine into RNA was not significantly affected by NaOCN when labeled uridine was injected at either 1 or 4 h post-NaOCN administration.

DISCUSSION

It has been previously reported that the in vivo NaOCN inhibition of protein synthesis in transplantable hepatoma and colonic tumors of the rat may be secondary to alterations in the tissue distribution of labeled substrates into tumors versus normal cells (25). The observed decrease in P388 cell protein synthesis by NaOCN cannot, however, be totally explained by alterations in absorption in the mouse peritoneal cavity. The observed decrease with NaOCN in incorporation of labeled leucine into P388 cell protein after a 1-h in vitro incubation suggests that the inhibition of P388 cell protein synthesis is caused by a direct effect of NaOCN on the P388 cell.
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NaOCN. The relationship between these alterations in amino acid transport and the inhibition by NaOCN of protein synthesis remains unclear.

ATP levels were previously shown to be significantly decreased by NaOCN in the HTC hepatoma but not in hepatoma 7777 (29). Although a 23% decrease in ATP was observed in P388 samples from NaOCN-pretreated mice as compared to controls, it was not significant at P < 0.05. However, the usefulness of these data may be limited by the fact that P388 cell ATP levels may decrease during the preparation of cell extracts at 0–4°C as performed in this study. In addition, NaOCN caused significant decreases in labeled phenylalanine incorporation into protein in P388 cell-free lysates despite the presence of excess exogenous ATP and GTP. These results suggest that NaOCN inhibition of P388 cell protein synthesis cannot be explained by reductions in nucleotide pools.

The NaOCN inhibition of P388 cell protein synthesis appears to be secondary to alterations in the P388 cell protein synthetic machinery. The incorporation of labeled phenylalanine into protein was decreased at all time points tested in untreated P388 cell-free NaOCN lysates as compared to controls. The presence of 40 μM excess exogenous amino acids in the lysate mixtures excluded NaOCN alterations of P388 cell amino acid pools as an apparent cause for the decreased phenylalanine incorporation into protein. In addition, this decrease was maintained when excess exogenous calf liver tRNA, coding for all amino acids, was added to the lysates. However, no decrease in phenylalanine incorporation into polypeptides was observed in NaOCN micrococcal nuclease-treated lysates containing polyuridylic acid as compared to controls. These results suggest that NaOCN is not affecting the protein synthetic machinery of the P388 cell through changes manifested in the ribosomal machinery, tRNA, or protein synthesis elongation processes.

The initiation of protein synthesis in eukaryotic cells is a process known to involve many steps requiring various factors and cofactors. The formation of a ternary complex of GTP, Met-tRNA<sup>Met</sup>, and eukaryotic initiation factor 2 and the subsequent joining of the 43S ribosomal complex (40S ribosomal subunit plus eukaryotic initiation factor 3 protein complex) appear to be the vital steps in protein synthesis initiation (30–33). Our results suggest that the formation of the quaternary 48S initiation complex (43S-GTP-Met-tRNA<sup>Met</sup>-eukaryotic initiation factor 2) is significantly decreased by NaOCN in P388 cells. The levels of labeled methionine bound at the 40S peak in glycerol gradients were significantly decreased in NaOCN samples as compared to control. The fact that no difference was observed in polyuridylic acid-directed phenylalanine incorporation into polypeptides in NaOCN versus control lysates was not inconsistent with these observations since normal initiation processes do not appear to be necessary for polyuridylic acid translation (34).

NaOCN was also observed to decrease labeled thymidine incorporation into DNA of P388 cells. This is similar to results obtained with NaOCN in various transplantable hepatomas and the murine L1210 leukemia cell (10). In addition, NaOCN was observed to cause significant decreases in labeled uridine incorporation into P388 cell mRNA with no significant effect on incorporation into total cellular RNA. These results suggest that NaOCN is a specific inhibitor of a variety of synthetic pathways in the P388 cell. The observed decreases by NaOCN in the levels of formation of initiation intermediates and in mRNA synthesis could result in corresponding decreases in protein synthesis. Since a decrease in protein synthesis is not observed with NaOCN in most normal tissues, the alterations observed with NaOCN on P388 tumor cell mRNA synthesis and protein synthesis initiation could represent significant differences in these processes between malignant and normal tissues. Whether these effects are manifested in the normal tissues of the P388-containing mice remains to be determined.

The relationship between the NaOCN effect on DNA synthesis and the decrease in protein synthesis in P388 cells remains unclear. It was previously reported that NaOCN caused decreases in DNA synthesis in nonmalignant rat intestinal mucosa and regenerating liver without a corresponding decrease in protein synthesis, suggesting that they are independently occurring events (10). However, the general pattern of inhibition observed with NaOCN in P388 cells is similar to that of other agents. Phyllanthoside is an antitumor agent found to inhibit both P388 cell protein and DNA synthesis without a corresponding effect on RNA synthesis (35). In addition, the sesquiterpene lactones, of which helalamin is the most stereotypic agent, are antitumor agents known to selectively alkylate thiol-bearing enzymes, suppressing both DNA and protein synthesis in P388 cells (36) and interfering with the formation of the 48S initiation complex in the rabbit reticulocyte (37).

It remains to be determined how NaOCN interferes with DNA and mRNA synthesis and the formation of the 48S initiation complex in P388 cells. It is probable that NaOCN is altering one or more of the enzymes or factors necessary for the normal progression of these events. The observed carbamylation of NaOCN to basophilic sites of proteins in vitro may be the chemical basis for the NaOCN actions in vivo. However, this question can only be resolved when the nature of the in vivo NaOCN metabolite is elucidated.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Michael Laughrea for his advice and helpful suggestions and Barry Posner for critical reading of the
manuscript. The authors are also thankful to Andrea Dorato-Lazarus, Angela McQuillan-Lemieux, Lea Brakier-Gingras, and Robert L. Glazer for their valuable help and discussions. The authors thank Sandy Fraiberg for the preparation of the manuscript.

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Mechanism of Decrease of Protein Synthesis by Sodium Cyanate in Murine P388 Leukemia Cells

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