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# Influence of pH on the modification of thiols by carbamoylating agents and effects on glutathione levels in normal and neoplastic cells

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**Summary.** In previous studies, we have suggested that the selective inhibitory effect of sodium cyanate (NaOCN) on hepatoma metabolism may be due to the lower pH observed in tumors relative to normal tissues. Lower pH might enhance the action of NaOCN by increasing the formation of isocyanic acid and carbamoylation of sulfhydryl groups. In the present work, studies were conducted on the effect of pH on the carbamoylation of sulfhydryl groups. The data indicated that carbamoylation of the sulfhydryl group of glutathione by NaOCN was enhanced by decreasing the pH from 7.4 to 6.6. A less pH-dependent response was observed with organic isocyanates. However, all reactions were reversible after the pH was increased by the addition of base. Kinetic studies showed that the rate of the reaction is very rapid, a maximal effect occurring within the first 10 min. Dose-dependent modifications of cellular glutathione by NaOCN and organic isocyanates were observed in human HT29 colon tumor cells, rat HTC hepatoma cells, and rat hepatocytes. The rate of carbamoylation of the glutathione sulfhydryl group in cells was similar to that of pure glutathione (GSH). The effect of buthionine sulfoximine on GSH levels in cells was at least as great as that of sodium cyanate, but only the latter showed inhibitory effects on macromolecular synthesis; these were very rapid, pH-dependent, and reversible in tumor cells. Our results suggest that cellular sulfhydryl group(s) other than that of GSH might be involved in the effect of NaOCN on macromolecular synthesis.

## Introduction

It has been demonstrated that cyanate can carbamoylate a variety of functional groups of proteins, such as the amino, sulfhydryl, carboxyl, phenolic, hydroxyl, imidazole, and phosphate groups [6, 21]. We have postulated [13] that the selective inhibitory effect of sodium cyanate (NaOCN) on macromolecular synthesis in tumors might be due to the lower pH observed in many tumors. In the present study we showed that low pH can enhance the uptake of NaOCN into cells and, hence the inhibitory effect on macromolecular synthesis and cell proliferation. The action of NaOCN in cells is very rapid, pH-dependent, and reversible; these features are compatible with the carbamoylation of sulfhydryl groups. Support for this hypothesis may be derived from the study of Stark [20], who observed rapid carbamoylation by cyanate of cysteine sulfhydryl groups to form S-carbamoylcysteine, which is stable below pH 5 but decomposes at a pH higher than 8.

Glutathione (GSH) is the major nonprotein thiol in cells that might be a critical target for cyanate in modulating some important biological functions. A correlation has been reported between cellular GSH content and cell growth in 3T3 fibroblasts [19]. In DU145 human prostatic carcinoma cells, treatment with estramustine resulted in the decrease of intracellular GSH and glutathione-S-transferase activity, and these effects might enhance the anticytoskeletal and antimitotic properties of the drug [23].

The cellular concentration of GSH has been found to influence the cytotoxicity of several chemotherapeutic drugs [2, 18, 25]. Babson et al. [4] have shown that the isocyanate derived from 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) can inhibit glutathione reductase activity. This resulted in a 70% decrease of cellular GSH, and BCNU enhanced the cytotoxicity of Adriamycin. However, a treatment with diethyl maleate that decreased GSH to 20% of control levels in hepatocytes did not affect the toxicity of Adriamycin [4]. In a study by Begleiter et al. [5], the melphalan-resistant mutants of Chinese hamster ovary cells were compared with sensitive cells, and the results suggest that elevated sulfhydryl groups might be at least partially responsible for the resistance to melphalan.

However, other mechanisms such as drug uptake and efflux are also involved in the development of drug resistance. In addition, resistance to melphalan has been shown to be related to critical membrane -SH groups [7]. Moreover, the work by Russo et al. [18] has clearly shown the close relationship between GSH levels and drug response as well as drug-induced resistance to chemotherapeutic drugs such as melphalan, cyclophosphamide, BCNU, Adriamycin, bleomycin, and neocarzinostatin. These authors concluded that the modulation of GSH levels, might be advantageous in chemotherapy [18]. Further attention was focused on the possibility of selective depletion of GSH in tumors, since this would be useful in enhancing the therapeutic effect of many antineoplastic drugs. Babson and Reed [3] have shown that the carbamoylating activity of some nitrosoureas can inhibit glutathione reductase and decrease the GSH level. Direct bindings between cyanate and reduced glutathione have been observed in erythrocytes [9] and reticulocytes [8].

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The present study was undertaken to determine the influence of pH on the carbamoylation of sulfhydryl groups and to examine the correlation between the inhibitory effect of NaOCN on macromolecular synthesis and the decrease in cellular GSH. Both normal rat hepatocytes and neoplastic cells were used in these studies.

## Materials and methods

*Chemicals.* Sodium cyanate (NaOCN) was obtained from ICN-K & K Laboratory (Plainview, NY). Cyclohexyl isocyanate, ethyl isocyanate, 2-chloroethyl isocyanate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and reduced GSH were purchased from Aldrich Chemical Co. (Milwaukee, Wis). DL-buthionine-[S. R.]-sulfoximine (BSO), glutathione reductase, and NADPH were obtained from Sigma Chemical Co. (St. Louis, Mo). L-[4,5-$^3$H]-leucine (44 Ci/mmol), and [methyl-$^3$H]-thymidine (62 Ci/mmol) were obtained from ICN Radiochemicals. (Irvine, Calif).

*Cells.* Rat hepatoma (HTC) and human colon cancer (HT29) cells were maintained in Chee's essential medium (CEM; B & B/Scott Laboratories, Fiskeville, RI) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37° C in a humidified atmosphere (95% saturation) of 5% carbon dioxide. Rat hepatocytes were freshly isolated from Sprague-Dawley rats according to the method established by Machiedo et al. [16].

*Carbamoylation of sulfhydryl groups.* The -SH groups were measured as previously described by Alexander [1]. This method was based on the change of absorbance of *N*-ethylmaleimide at 300 nm, and the amount of -SH was calculated by using the molar extinction coefficient of 620.

*GSH determination.* The intracellular reduced and oxidized GSH levels were measured by the method described by Griffith [10], who used an enzymatic recycling assay based on the oxidation of GSH by DTNB and the reduction by NADPH catalyzed by glutathione reductase. First, the cells were harvested, counted, and resuspended in Eagle's minimum essential medium (MEM) at pH 7.4 or 6.6. After pH and drug treatments, the cells were collected by centrifugation and protein was precipitated with a final concentration of 3% sulfosalicylic acid. This treatment results in the hydrolysis of cyanate and isocyanates and prevents the inhibition of glutathione reductase in the assay medium. Aliquots of the supernatant were taken for total GSH determination. The assay for total GSH was started by mixing 0.7 ml 0.3 m*M* NADPH, 0.1 ml 6 m*M* DTNB, and 0.1 ml phosphate buffer (all solutions were prepared in buffer containing 125 m*M* sodium phosphate and 6.3 m*M* sodium-EDTA, pH 7.5), with 0.1 ml GSH sample or buffer as reference. The solutions were mixed in a cuvette at room temperature and the absorbance was continuously monitored at 412 nm after the addition of 5 µl glutathione reductase (50 units/ml). The GSH content was determined by reference to a standard curve. Glutathione disulfide was selectively measured when reduced glutathione was masked by treatment with 2-vinylpyridine. Aliquots of sample (100 µl) were placed in plastic vials and mixed with 2 µl 2-vinylpyridine. Pure triethanolamine (6 µl, 45 µmol) was added and vigorously mixed. The final pH was about 7–7.5. Reduced glutathione was completely derivatized after 20–60 min at 25° C, depending on the final pH; the glutathione disulfide content was then assayed by the method described above.

*Incorporation of precursors into DNA and protein.* The incorporation of precursors into DNA and protein was determined following previously described procedures [14].

*Statistical evaluation.* Statistical significance of the results was determined by Student's paired *t*-test; a probability of $< 5\%$ was considered significant.

## Results

Our first studies were carried out with solutions of GSH; the effects of NaOCN on the sulfhydryl group of cellular GSH was later investigated. The data in Fig. 1 indicate that with incubation times up to 40 min, the effect of NaOCN on thiol levels was greater at pH 6.6 than at pH 7.4. In the presence of cyanate, the percentage of initial sulfhydryl group concentration fell 61% at pH 7.4, whereas at pH 6.6 there was an 85% decrease. The addition of acid to solutions at pH 7.4 decreased the pH to 2.7, causing a further decline in the sulfhydryl level. In contrast, the addition of base to give a pH of 12.2 essentially caused a complete reversal of the effect of the cyanate. The pH dependence of sulfhydryl-group carbamoylation was less obvious with organic isocyanates (Fig. 2) than with NaOCN (Fig. 1). The lower pH enhanced the carbamoylation of the sulfhydryl group of GSH by ethyl isocyanate to some degree. However, no significant pH-dependent response was ob-

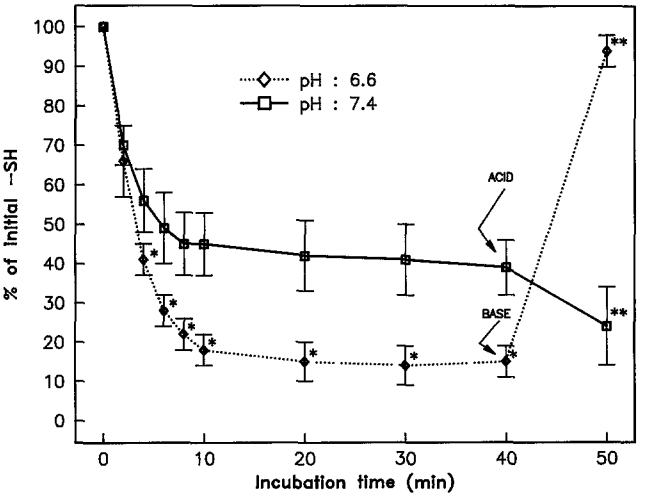


**Fig. 1.** Effect of pH on carbamoylation of the sulfhydryl groups of GSH by NaOCN. The reactions were started by mixing equal volumes (2 ml) of GSH and NaOCN solutions at concentrations of 20 m*M* and 0.2 *M*, respectively (in 0.1 *M* phosphate buffer, pH 7.4 or 6.6). The incubation took place at 37° C. At the indicated time points, 0.1-ml aliquots were taken and mixed with 1.9 ml N-ethylmaleimide solution (1 mM) at the corresponding pH. At 40 min, 0.4 ml HCl (2*N*) and KOH (3*N*) was added to the reaction mixture at pH 7.4 and pH 6.6, respectively, Aliquots were taken after 10 min and mixed with *N*-ethylmaleimide solution as described above. The -SH content was determined and data were expressed as a percentage of the initial total -SH groups. Each data point represents the mean ± SD of three separate experiments carried out in duplicate. * ($P < 0.05$) pH 7.4 vs pH 6.6; ** ($P < 0.05$) 40 min vs 50 min

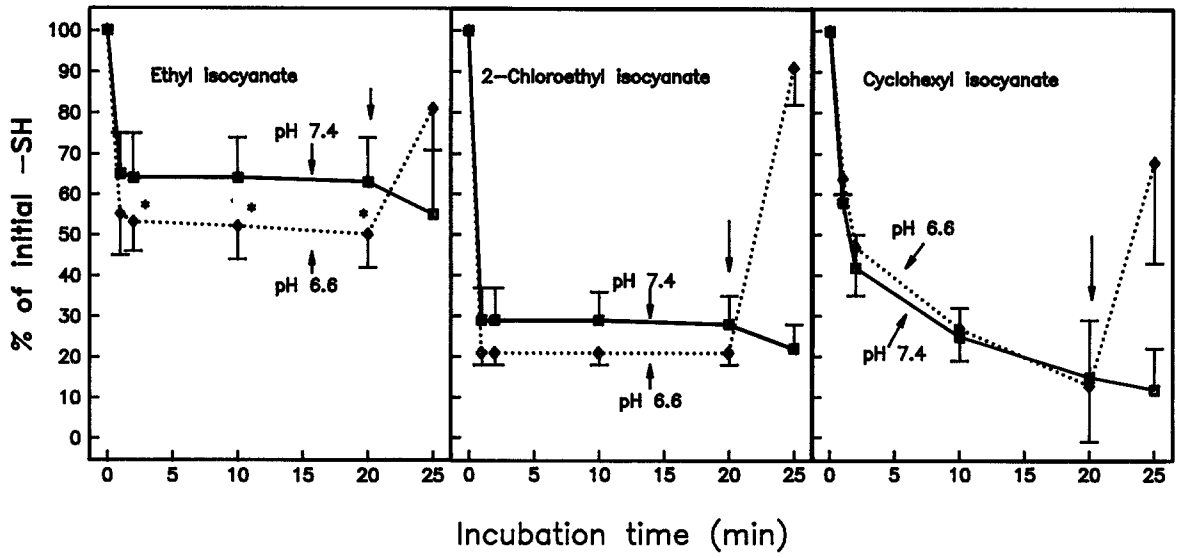


**Fig. 2.** Effect of pH on carbamoylation of the sulfhydryl groups of GSH by organic isocyanates. The reactions were started by adding the isocyanates GSH solution to give final concentrations of 0.01 *M* for both isocyanates and GSH (in 0.1 *M* phosphate buffer, ph 7.4 or 6.6). The procedures and presentation of data are the same as described for Fig. 1. Each data point represents the mean ± SD of at least three separate experiments carried out in duplicate. * ($P < 0.05$) pH 7.4 vs pH 6.6

served between pH 6.6 and pH 7.4 with 2-chloroethyl isocyanate or cyclohexyl isocyanate. Nevertheless, the more drastic change in pH resulting from the addition of base did reverse the effects of the isocyanates.

The effect of carbamoylating agents on cellular GSH levels was studied. Preliminary data revealed that oxidized glutathione (GSSG) represented <4% of the total cellular GSH in both rat hepatocytes and HT29 cells and indicated that GSSG was not affected by carbamoylation. Since GSSG did not substantially contribute to total GSH levels, the latter could be used as a measure of reduced glutathione; total GSH contents measured in subsequent studies are reported in this paper. The cellular GSH level was decreased by NaOCN treatment (Fig. 3) in a dose- and pH-dependent manner. A greater effect was observed at both lower pH and higher concentration of NaOCN. The carbamoylation of GSH was also studied with isocyanates at pH 7.4 and pH 6.6. Both 2-chloroethyl isocyanate and ethyl isocyanate affected the cellular GSH in a dose-dependent manner; a pH-dependent effect was observed with 2-chloroethyl isocyanate but not with ethyl isocyanate (Fig. 4).

The time course of the carbamoylation of cellular GSH by NaOCN was studied (Fig. 5). The rates of reaction were very rapid in both rat hepatocytes and human HT29 cells; a maximal effect could be achieved within the first 10 min.

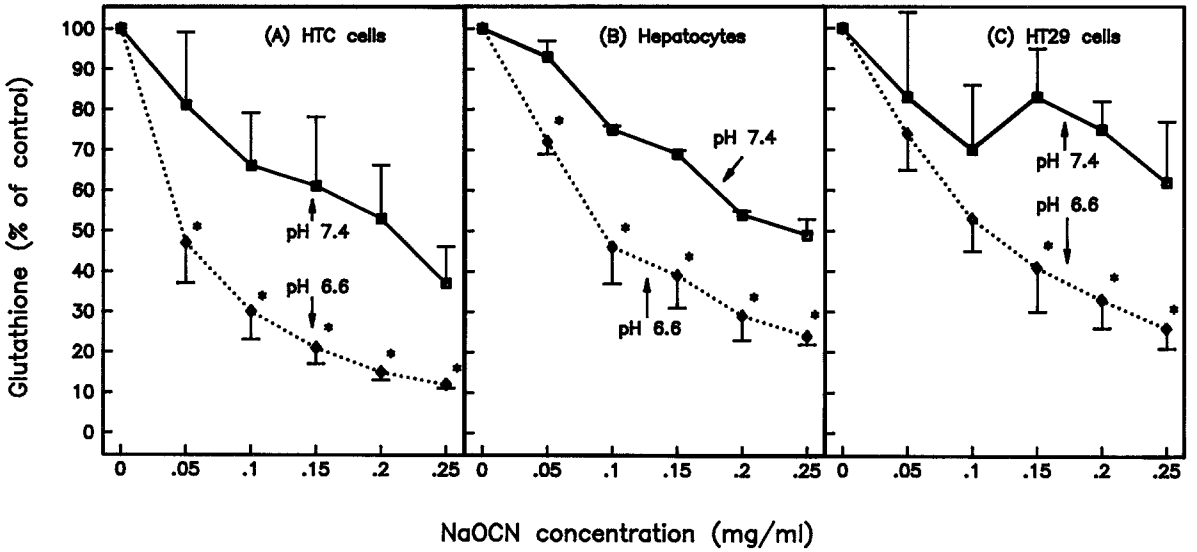


**Fig. 3.** Dose-dependent depletion of cellular GSH by NaOCN. The cells ($1.5-3 \times 10^6$ cells) were incubated with NaOCN at the stated concentrations in 1 ml Eagle's MEM containing 50 m*M* PIPES (pH 7.4 or 6.6) at 37° C for 30 min. Cells were collected and mixed with 0.3 ml 3% sulfosalicyclic acid and the total cellular GSH was determined. Data are expressed as a percentage of the value of control cells (μg/$10^6$ cells) at pH 7.4 (HTC: 1.84±0.16; rat hepatocyte: 5.9±0.1; HT29, 1.5±0.1). Each data point represents the mean ± SD of three separate experiments carried out in duplicate. * ($P < 0.05$) pH 7.4 vs pH 6.6

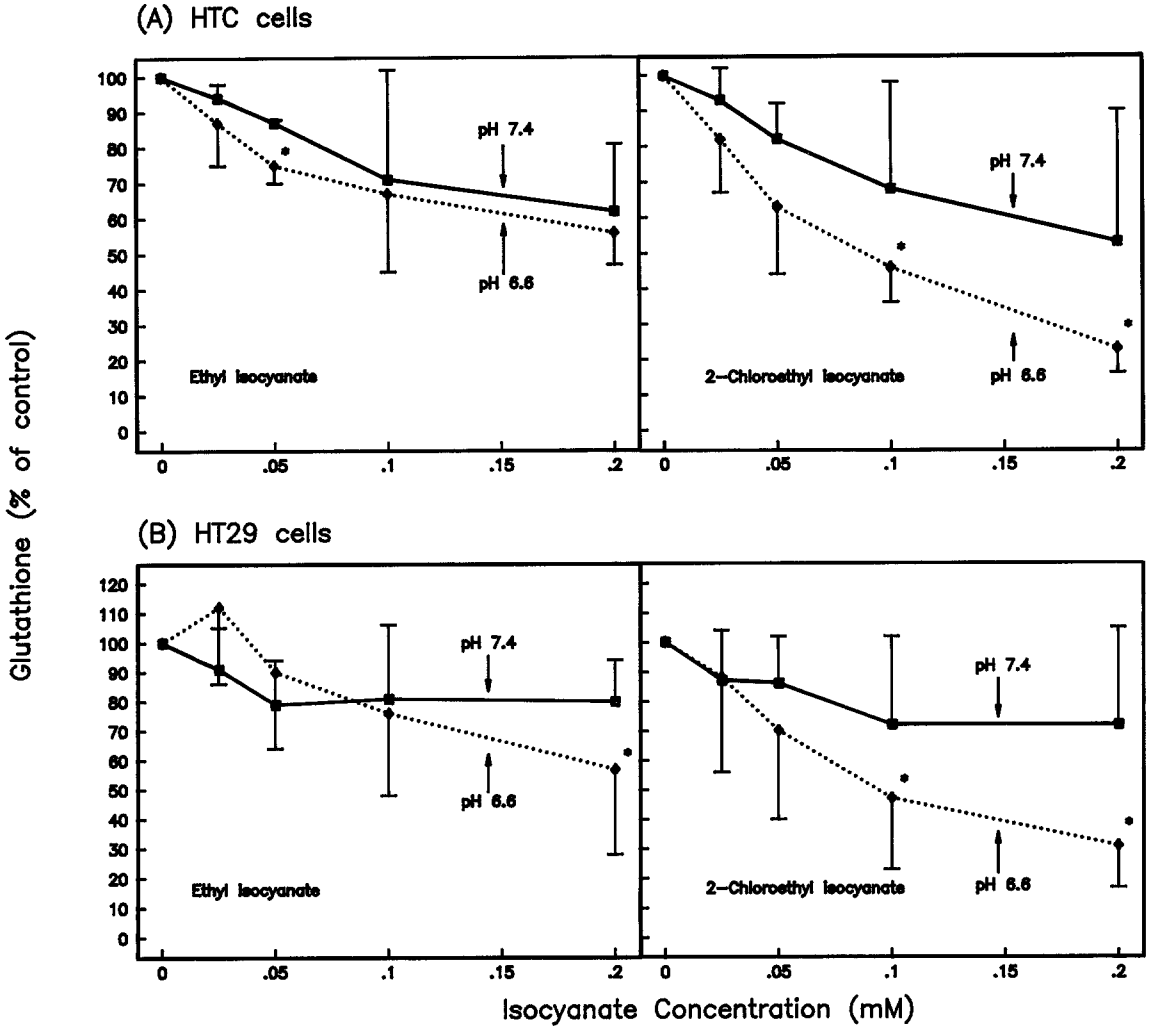


**Fig. 4.** Dose-dependent depletion of cellular GSH by organic isocyanates. Procedures of experiments were the same as described in Fig. 3. Data are expressed as a percentage of the value of control cells at pH 7.4 as described for Fig. 3. Each data point represents the mean ± SD of three separate experiments carried out in duplicate. * ($P < 0.05$) pH 7.4 vs pH 6.6

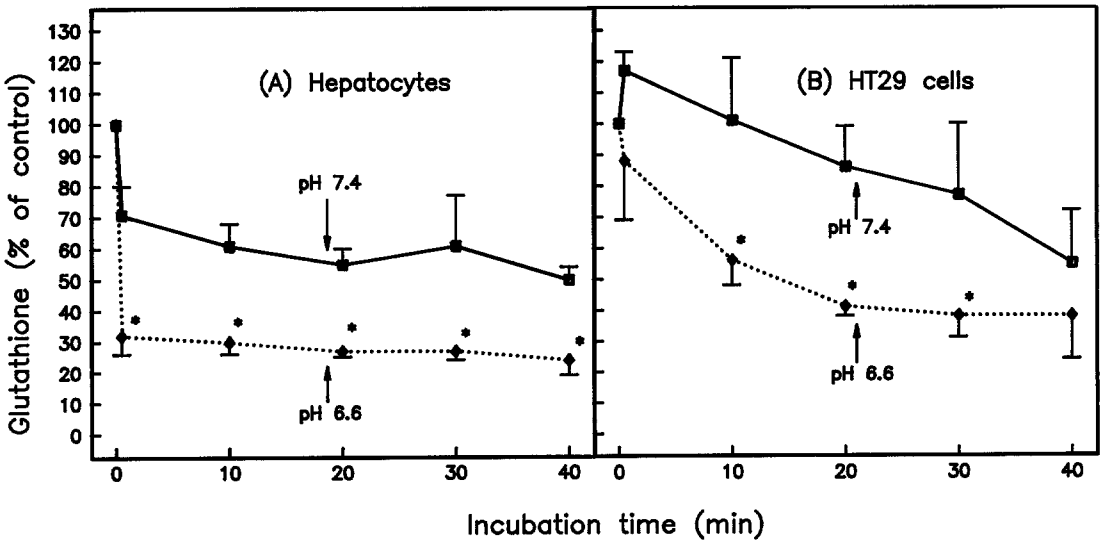


**Fig. 5.** Time course of the carbamoylation of cellular GSH by NaOCN. Rat hepatocytes or human colon cancer cells ($1.5-3 \times 10^6$) were incubated with NaOCN (0.25 mg/ml) at 37° C. Samples were collected at the indicated time points and the GSH content was determined. Data are expressed as a percentage of the value of control cells at pH 7.4. Each data point represents the mean ± SD of three separate experiments carried out in duplicate. * ($P < 0.05$) pH 7.4 vs pH 6.6

**Table 1.** Reversibility of the effect of NaOCN on cellular glutathione level

| Cell | pH | NaOCN (mg/ml) | % of control | |
|---|---|---|---|---|
| | | | Non-resuspended | Resuspended |
| HTC | 7.4 | 0 | 100 | 100 |
| | 7.4 | 0.25 | 37±9 | 80±19* |
| | 6.6 | 0.25 | 12±1 | 63±20* |
| HT29 | 7.4 | 0 | 100 | 100 |
| | 7.4 | 0.25 | 62±15 | 87±16* |
| | 6.6 | 0.25 | 26± 5 | 79±15* |

Cultured HTC and HT29 cells ($1.5-3\times10^6$) were preincubated with NaOCN at 37°C for 10 min in Eagle's MEM containing 50 m*M* PIPES buffer at pH 7.4 and 6.6. For cells designated as *Resuspended,* the cells were centrifuged, washed once with medium, and resuspended in medium of the original pH but without NaOCN. All cells were then incubated, for 30 min at 37°C. The cells were centrifuged and mixed with 0.15–0.3 ml 3% sulfosalicylic acid. After centrifugation, the supernatant was stored at −20°C before assay of the glutathione content. Data are expressed as a percentage of the value of control cells (HTC, 1.84±0.16 μg/$10^6$ cells; HT29, 1.5±0.1 μg/$10^6$ cells). Each data point represents the mean ± SD of three separate experiments carried out in duplicate
* $P < 0.05$; Non-resuspended vs resuspended with Student's two-tailed *t*-test

**Table 2.** Effect of BSO, low pH, and NaOCN on cellular glutathione level in HT29 cells

| BSO | pH | NaOCN (mg/ml) | % of control (m*M*) | | |
|---|---|---|---|---|---|
| | | | 0 min | 10 min | 40 min |
| 0 | 7.4 | 0 | 100 | – | – |
| | 7.4 | 0.25 | – | 67±47 | 62±21* |
| | 6.6 | 0.25 | – | 41±20 | 31±14* |
| 0.2 | 7.4 | 0 | 19±14* | – | – |
| | 7.4 | 0.25 | – | 11± 6* | 6± 4* |
| | 6.6 | 0.25 | – | 8± 5* | 5± 3* |
| 1 | 7.4 | 0 | 14±14* | – | – |
| | 7.4 | 0.25 | – | 7± 3* | 6± 4* |
| | 6.6 | 0.25 | – | 6± 3* | 4± 2* |
| 2 | 7.4 | 0 | 14± 8* | – | – |
| | 7.4 | 0.25 | – | 7± 3* | 4± 4* |
| | 6.6 | 0.25 | – | 5± 2* | 4± 2* |

HT29 cells were cultured in CEM with BSO (0.2–2 m*M*) at 37°C for 24 h. The cells were then incubated with NaOCN (0.25 mg/ml) in Eagle's MEM containing 50 m*M* PIPES buffer at pH 7.4 or 6.6. The procedures and presentation of data were as described for Table 1. Data are expressed as a percentage of the value of control cells (1.5±0.1 μg/$10^6$ cells). The results represent the mean ± SD of three separate experiments with duplicate incubations
* $P < 0.05$; Treated vs control with Student's two-tailed *t*-test

However, the carbamoylation of cellular GSH was largely reversible in both HTC and HT29 cells (Table 1). The carbamoylating moiety dissociated from GSH after washing with fresh medium; nevertheless, the reaction was not completely reversible at pH 6.6. The results provided evidence that S-carbamoylcysteine is more stable at lower pH.

Further investigations were concentrated on evaluating the correlation between the cellular GSH level and macromolecular synthesis. After HT29 cells were cultured in the presence of the inhibitor of glutathione synthesis, buthionine sulfoximine (BSO), cellular GSH decreased significantly. Expressed as a percentage of control levels, the values dropped to 19%, 14%, and 14% at BSO concentrations of 0.2, 1, and 2 m*M*, respectively. Further small decreases were observed in the presence of NaOCN, but they were not statistically significant (Table 2). A similar response was also observed in HTC cells: only 9% of control GSH was present after the cells were treated with BSO (1 m*M*) for 24 h. A slight, nonsignificant decrease in GSH was observed following treatment with NaOCN (Fig. 6). Although cellular GSH was significantly decreased by BSO in HT29 and HTC cells, the incorporation of precursors into DNA and protein was not affected in our systems (Table 3). The inhibition of macromolecular synthesis was observed only after NaOCN treatment.

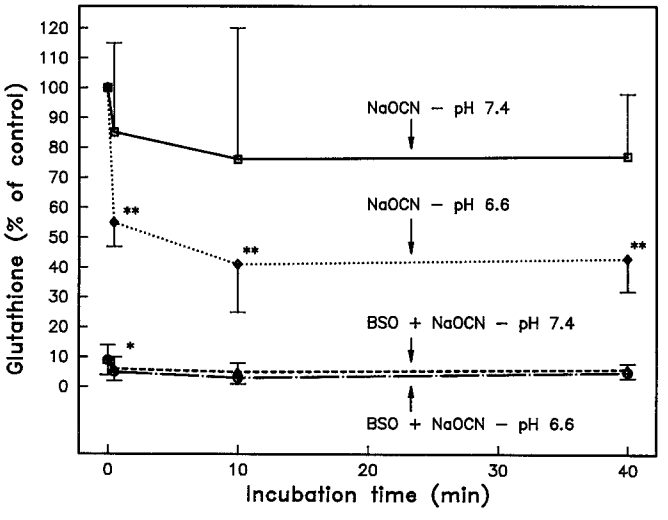


**Fig. 6.** Effect of BSO, low pH, and NaOCN on the cellular GSH level in HTC cells. HTC cells were treated following the procedures described in Table 2. Data are expressed as a percentage of the value of control cells. The results represent the mean ± SD for three separate experiments carried out in duplicate. * ($P < 0.05$) BSO treated vs control; ** ($P < 0.05$) NaOCN treated vs control

## Discussion

The present studies show that the carbamoylation of sulfhydryl groups by NaOCN is enhanced at lower pH, the reaction being reversible after the addition of base to change the pH from 6.6 to 12.2. A further decrease in -SH groups could be observed after the addition of acid to change the pH from 7.4 to 2.7 (Fig. 1). However, less pH-dependent responses were observed with the three organic isocyanates studied (Fig. 2). This difference in responses at different pH might be attributed to the equilibrium of NaOCN with isocyanic acid ($pKa = 3.8$). The ratio of the ionic and nonionic form is dependent on the pH of the medium. The difference in ionization affects the carbamoylation of -SH groups since the nonionic form is the carbamoylating moiety. Such a factor does not apply to organic isocyanates, which might explain the less pH-dependent responses of the three organic isocyanates compared with NaOCN in the carbamoylation of -SH groups.

We have reported [13, 15] that a lower pH could enhance the inhibitory effect of NaOCN on macromolecular

**Table 3.** Effect of BSO, low pH and NaOCN on macromolecular synthesis

| Cell | NaOCN 0.25 (mg/ml) | pH | Incorporation (% of control) | | | |
|---|---|---|---|---|---|---|
| | | | Protein | | DNA | |
| | | | Control | BSO | Control | BSO |
| HT29 | – | 7.4 | 100 | 117±23 | 100 | 121± 1 |
| | – | 6.6 | 93±12 | 98±22 | 75±16 | 86±25 |
| | + | 7.4 | 74± 6 | 90±24 | 107±38 | 108±64 |
| | + | 6.6 | 29± 6* | 41± 5* | 6± 5* | 3± 4* |
| HTC | – | 7.4 | 100 | 93±24 | 100 | 94±23 |
| | – | 6.6 | 84± 9* | 89±13 | 71±14* | 69±20* |
| | + | 7.4 | 77±13* | 77±32 | 63±18* | 55±21* |
| | + | 6.6 | 21± 2* | 12± 7* | 2± 1* | 2± 1* |

HTC and HT29 cells were cultured in CEM supplemented with 10% fetal bovine serum. Some dishes were treated with BSO (1 m*M*) for 24 h. The cells were then collected, washed several times, and resuspended in Eagle's MEM containing 50 m*M* PIPES buffer at pH 7.4 and 6.6. The cells were preincubated for 10 min with NaOCN (0.25 mg/ml) in 1 ml MEM at the pH indicated before the addition of 5 μCi [$^3$H]-leucine or 2.5 μCi [$^3$H]-thymidine in 10 μl and a further 30-min incubation. Incorporation of precursors into protein or DNA is expressed as a percentage of that in control cells at pH 7.4 (HT29 cells: protein, 122±45 cpm/$10^4$ cells; DNA, 116±60 cpm/$10^4$ cells; HTC cells: protein, 168±71 cpm/$10^4$ cells; DNA, 147±43 cpm/$10^4$ cells). Each data point represents the mean ± SD of three separate experiments carried out in triplicate

* $P < 0.05$; Treated vs control with Student's two-tailed *t*-test

synthesis and tumor cell proliferation. Two possible mechanisms were proposed: first, a low pH favors the formation of nonionic isocyanic acid, which can preferentially enter the cells; second, the carbamoylation of critical cellular -SH groups may be enhanced at a lower pH [13]. Evidence was obtained that the uptake of NaOCN was higher in cells incubated at pH 6.6 than in those incubated at pH 7.4.

Initially, we considered that the inhibitory effect of NaOCN on macromolecular synthesis might be mediated through -SH groups, since the response is very rapid, pH-dependent, and reversible. The cellular GSH might play an important role, as previous studies [19] have suggested that GSH might be involved in DNA synthesis in various systems. The results presented in this paper clearly demonstrate that the carbamoylation of -SH groups is enhanced by low pH and that the reaction is reversible. However, the depletion of cellular GSH by BSO did not affect the macromolecular synthesis in either HT29 or HTC cells. Further treatment with NaOCN significantly inhibited the macromolecular synthesis at pH 6.6. The data suggest that, at least in our system, GSH may not be responsible for the inhibitory effect of NaOCN on macromolecular synthesis. This inhibitory effect might be mediated through cellular sulfhydryl groups other than that of glutathione or by a mechanism not involving sulfhydryl groups. Further characterization of the target molecules of NaOCN will be necessary for the determination of the mechanism involved.

Modulation of cellular GSH levels may play an important role in drug metabolism and resistance to some chemotherapeutic agents [2]. Inhibition of GSH reductase and a decrease in GSH levels have been observed in carcinoma cells treated with a carbamoylating nitrosourea [22]. If a reagent could preferentially affect the GSH level in tumors but not in normal tissues, the use of such a compound would be advantageous in solving some of the problems of drug resistance in cancer therapy. Therefore, we believe that NaOCN might play an important role in combination cancer therapy. The concentrations of cyanate used in the present work might be achieved after a single injection but would not be tolerated on a chronic basis. The interstitial pH of tumors is generally more acidic than that of normal tissues [11, 24], which should enhance the uptake of NaOCN and the carbamoylation of -SH groups. This selective effect might potentiate the action of chemotherapeutic drugs that are inactivated by reaction with GSH. Furthermore, we could demonstrate that NaOCN can affect intracellular pH regulation in tumor cells (submitted for publication).

The therapeutic effects of some cancer treatments have been reported to be enhanced by acidic pH [24]. In addition, the combination of several compounds that interfere with the regulation of intracellular pH has shown greater cytotoxicity to tumor cells than that of each agent alone, and these compounds were suggested to be potentially useful in anticancer therapy [17]. Although a previous phase I clinical trial indicated that treatment with NaOCN alone had no therapeutic effect on patients with advanced colorectal carcinoma [12], we believe that the use of carbamoylating agents in combination cancer chemotherapy is worthy of further exploration.

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