Biochemical Pharmacology, Vol. 37, No. 11, pp. 2259–2266, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

pH-RELATED EFFECTS OF SODIUM CYANATE ON MACROMOLECULAR SYNTHESIS AND TUMOR CELL DIVISION

JENNIFER J. HU, ALEYKUTTY LUKE, MOHAN CHELLANI, KARIMULLAH A. ZIRVI and MICHAEL A. LEA*

Departments of Biochemistry and Surgery, UMDNJ-New Jersey Medical School, Newark, NJ 07103, U.S.A.

(*Received* 29 *July* 1987; *accepted* 28 *October* 1987)

Abstract—In past work, the selective effects of sodium cyanate on macromolecular synthesis in tumors have not been seen with cells in culture. We have explored the possibility that differences in the response of tumor cells to cyanate *in vivo* and *in vitro* may be related to the pH in the environment to which cells are exposed. When rat hepatoma (HTC) cells were incubated with sodium cyanate (0.25 mg/ml), there was a greater inhibition of precursor incorporation into RNA and DNA with a decrease in pH from 7.4 to 6.6. At pH 7.4 there was no significant effect of sodium cyanate on the incorporation of [^{3}H]leucine into protein of rat hepatocytes and HTC cells, but at pH 6.6 there were decreases of 50% or greater. The time of response and the reversibility of the inhibitory effects of sodium cyanate were not those anticipated from carbamoylation of amino groups but were compatible with modification of sulfhydryl groups. The uptake of [^{14}C]sodium cyanate in HTC cells and human colon cancer (HT29) cells was greater at pH 6.6 than at 7.4. Over a period of 4 days there was a slower rate of cell division by HTC and HT29 at pH 6.6 than at pH 7.4. The addition of sodium cyanate caused a further reduction in the rate of proliferation, and at a concentration of 0.25 mg sodium cyanate/ml there were decreases in cell numbers. The data suggested that a lower interstitial pH in tumors than normal tissues would result in greater sensitivity to inhibitory effects of sodium cyanate on macromolecular synthesis.

Sodium cyanate has been shown to exert inhibitory effects on protein synthesis in tumors under conditions in which there is little or no inhibition in normal tissues [1–3]. In agreement with Allfrey *et al.* [2], we found that cyanate has a greater effect on protein synthesis in Novikoff hepatoma cells growing in the peritoneal cavity of rats rather than in culture, and we have shown further that the inhibitory effect of cyanate on amino acid incorporation is greater in solid tumors than in ascites cells (unpublished observations). While it appears that tumor cells in culture or in ascites form are less responsive to the action of sodium cyanate than are solid tumors [2], it is less clear why there should be such a distinction. Two hypotheses have been presented in the literature. One proposes hepatic metabolism to an active compound [4] and the other suggests an action on circulation in tumors [5]. Allfrey *et al.* [2] found that protein synthesis in primary colon tumors was inhibited by cyanate but the sensitivity of tumor cells to cyanate was greatly diminished in culture. They then provided evidence that cyanate can be activated by a liver S9 fraction and NADPH to yield an unidentified compound toxic to neoplastic cells [4]. On the other hand, the magnitude of the inhibition of protein synthesis was not as great as that which can be observed *in vivo*. In addition, our studies on potential metabolism of sodium cyanate by liver S9 or microsomal fractions gave little support but did not exclude such a mechanism [6]. However, the data also indicate that inhibitory effects of cyanate on protein synthesis in hepatomas are greater and more prolonged than would be expected from the action on tumor circulation.

We would like to propose a third hypothesis which is not mutually incompatible with the two above. This takes into consideration the lower pH in hepatomas than in liver [7]. A lower pH favors the formation of un-ionized isocyanic acid and would thereby enhance uptake by cells. It would, in addition, increase carbamoylation and the stability of certain carbamoylation products, notably of sulfhydryl groups [8, 9]. An examination of the influence of pH on the inhibitory effects of sodium cyanate on macromolecular synthesis forms the subject of this report. Preliminary communications of this work have been presented [10, 11].

MATERIALS AND METHODS

Cells. Rat hepatoma (HTC) cells and human colon cancer (HT29) cells were maintained in Chee's essential medium (B & B/Scott Laboratories, Fiskeville, RI) containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY). Hepatocytes were prepared by collagenase digestion from Sprague–Dawley rats. Where indicated, HTC cells were also

* Address correspondence to: Dr. M. A. Lea, Department of Biochemistry, UMDNJ-New Jersey Medical School, 185 South Orange Ave., Newark, NJ 07103.

obtained by collagenase digestion of tumors transplanted subcutaneously in Buffalo-strain rats. In studies on the incorporation of isotope-labeled precursors into macromolecules, the cells were incubated in Eagle's minimal essential medium which was obtained from GIBCO Laboratories.

Reagents. Sodium cyanate was obtained from ICN Pharmaceuticals, Plainview, NY. L-[4,5-^{3}H]Leucine (44 Ci/mmol), [*methyl*-^{3}H]thymidine (62 Ci/mmol) and [5-^{3}H]uridine (16 Ci/mmol) were purchased from ICN Radiochemicals, Irvine, CA. [^{14}C]Potassium cyanate was purchased from NEN Products, Boston, MA, and was diluted with unlabeled sodium cyanate to give a concentration of 20 mg/ml and a radioactivity of 20 μCi/ml.

Assay methods. The procedures for measurement of the incorporation of ^{3}H-labeled precursors into RNA, DNA and protein were described previously [12]. In measurements of the stability of cyanate in media at different pH values, the colorimetric assay of Guilloton and Karst [13] was used. In studies on the uptake of ^{14}C-labeled cyanate, the medium containing cells was layered over 0.8 ml of a silicone–mineral oil mixture (4.85:1.0). Incubations at 37° were terminated by centrifugation in an Eppendorf microfuge. The microfuge tubes were frozen, and the pellets were obtained by cutting the tubes. Radioactivity in the pellets was measured by liquid scintillation counting.

Statistical analysis of data was done by Student's *t*-test with a probability of less than 5% being considered significant on a two-tailed test.

RESULTS

The mean incorporation of [^{3}H]uridine into RNA of rat hepatoma (HTC) cells at pH 6.6 was 73% of the incorporation at pH 7.4 (Table 1). When the cells were incubated with 3.85 mM sodium thiocyanate, there was no significant effect on incorporation of [^{3}H]uridine at either pH relative to controls. In contrast, in the presence of 3.85 mM sodium cyanate at pH 6.6, the incorporation was 41–60% of control values, whereas at pH 7.4 there was no significant effect of cyanate. In addition to the effect of sodium cyanate at pH 6.6 shown in Table 1 for cultured cells, a similar effect was seen with cells freshly isolated by collagenase digestion of solid tumors in which there was a 40% inhibition of [^{3}H]uridine incorporation at pH 6.6, while no effect was seen at pH 7.4. An inhibitory effect of sodium cyanate on the incorporation of [^{3}H]thymidine into DNA was also found to be affected by pH (Fig. 1). The data for the HTC cells indicate a progressively greater inhibition as the pH of the medium fell from 7.4 to 6.6. Studies with human colon cancer (HT29) cells showed a marked inhibition of incorporation of [^{3}H]thymidine into DNA by sodium cyanate at pH 6.6, and at this pH there was a concentration-dependent effect (Fig. 2).

A pH-dependent inhibitory effect on the incorporation of [^{3}H]leucine into protein was observed when either HTC cells (Fig. 3) or normal hepatocytes (Fig. 4) were incubated with sodium cyanate (0.25 mg/ml). The dose-dependent nature of the inhibition at pH 6.6 is illustrated for hepatocytes and HT29 cells in Fig. 5. When HT29 cells were incubated with sodium cyanate (0.25 mg/ml), there was a 10% inhibition of [^{3}H]leucine incorporation at pH 7.4 (data not shown) but at pH 6.6 there was a 77% inhibition relative to controls (Fig. 5). The influence of sodium cyanate concentration was similar for rat hepatocytes and human colon cancer cells (Fig. 5).

Since differences in uptake might be one factor mediating the increased response to sodium cyanate at a lower pH, we examined the uptake of ^{14}C-labeled sodium cyanate in HTC cells (Fig. 6) and HT29 cells (Fig. 7). For both cell lines the uptake was greater at pH 6.6 than at pH 7.4. When the cells were washed after a 10-min incubation with the ^{14}C-labeled sodium cyanate, it was found that the uptake was largely reversible. Cells were washed once with 1 ml medium at the pH corresponding to the original incubation and were resuspended in 1 ml medium. The percent-retained radioactivity after the wash was 7% at pH 7.4 for both HTC and HT29 cells. At pH 6.6 the retained radioactivity was 15% for HTC cells and 10% for HT29 cells. The question of reversibility

Table 1. Effects of pH and sodium cyanate on the incorporation of [^{3}H]uridine into RNA of HTC cells

pH	Incorporation (cpm/10^4 cells)	Incorporation as % control	
		3.85 mM NaOCN	3.85 mM NaSCN
7.4	118 ± 28	92 ± 12	99, 103
6.6	87 ± 22*	49 ± 8†	105, 123

HTC cells (2×10^5) were preincubated for 10 min at 37° in 1 ml of Eagle's minimal essential medium containing 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 7.4 or 6.6. There was a further incubation for 30 min after the addition of 2.5 μCi [^{3}H]uridine in 10 μl water. The incorporation of isotope into RNA in the presence of either 3.85 mM sodium cyanate (0.25 mg/ml) or 3.85 mM sodium thiocyanate is expressed relative to incorporation by cells in the medium alone. The data for incorporation of isotope into RNA are means ± SD for four experiments with sodium cyanate and for individual experiments with sodium thiocyanate. In each experiment there were triplicate incubations.

* $P < 0.05$, relative to paired controls at pH 7.4.

† $P < 0.05$, relative to paired controls at pH 6.6.

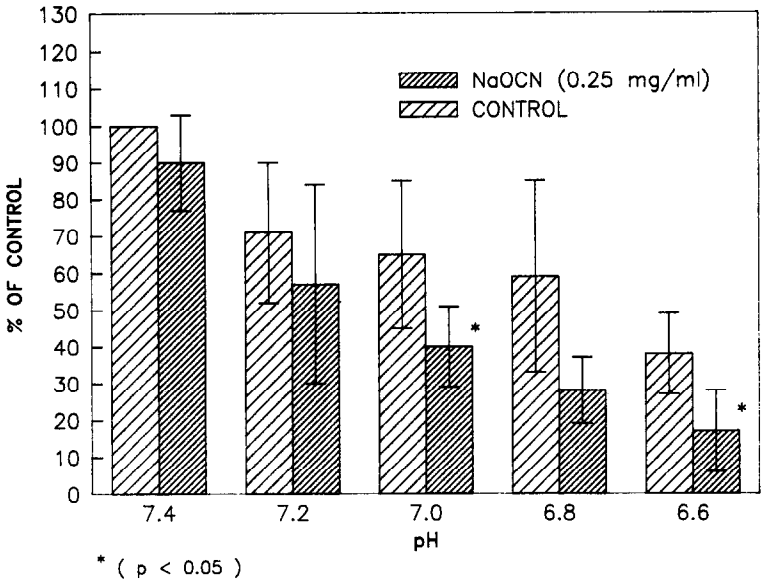

Fig. 1. Effect of pH on the inhibition of thymidine incorporation into DNA by sodium cyanate. Freshly isolated HTC cells (2×10^5) were preincubated for 10 min with sodium cyanate (0.25 mg/ml) in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES before addition of 2.5 μCi [^{3}H]thymidine in 10 μl water and a further 30 min incubation. Incorporation of isotope into DNA is expressed as a percentage of the incorporation in control cells at pH 7.4 (499 ± 188 cpm/10^4 cells). Each bar represents the mean ± SD of three separate experiments in triplicate.

was also examined with respect to effects on macromolecular synthesis. Although preincubation of HTC cells with sodium cyanate for 40 min did not have a significant effect on the incorporation of [^{3}H]thymidine into DNA in fresh medium at pH 7.4, an inhibitory effect of low pH in the preincubation was retained in the subsequent incubation for 30 min at pH 7.4 (Table 2). The data in Table 3 confirm the inhibitory effect of sodium cyanate on the incorporation of [^{3}H]leucine into protein of HTC cells but

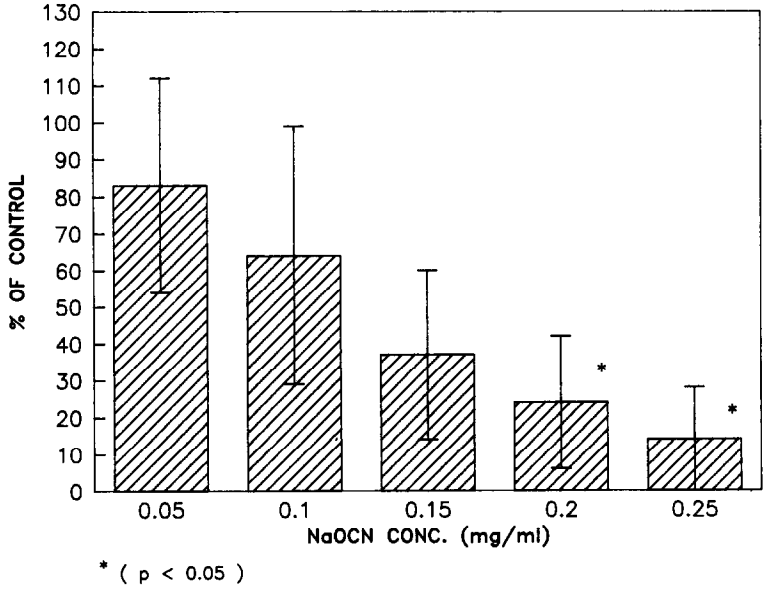

Fig. 2. Effect of sodium cyanate concentration on the incorporation of [^{3}H]thymidine into DNA of HT29 cells at pH 6.6. The cells (4×10^5) were preincubated for 10 min with sodium cyanate at the stated concentration in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES before addition of 2.5 μCi [^{3}H]thymidine in 10 μl water and a further 30-min incubation. Incorporation of isotope into DNA is expressed as a percentage of the incorporation in control cells incubated without cyanate (249 ± 243 cpm/10^4 cells). Each bar represents the mean ± SD of three separate experiments in triplicate.

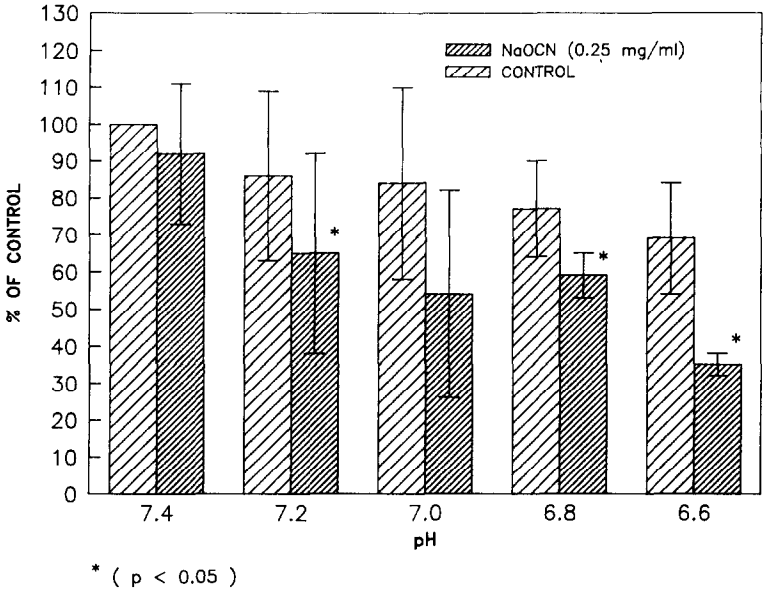

Fig. 3. Effect of pH on the inhibition of leucine incorporation into protein of HTC cells by sodium cyanate. Freshly isolated HTC cells (2×10^5) were preincubated for 10 min with sodium cyanate (0.25 mg/ml) in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES before addition of 5.0 μCi [^{3}H]leucine in 10 μl water and a further 30-min incubation. Incorporation of isotope into protein is expressed as a percentage of the incorporation in control cells at pH 7.4 (609 ± 387 cpm/10^5 cells). Each bar represents the mean ± SD of three separate experiments in triplicate.

show that this effect was essentially lost if cyanate was removed by resuspending the cells in fresh medium before addition of the isotope-labeled precursor.

Before examining the effect of sodium cyanate on tumor cell division we investigated the stability of sodium cyanate at 37° at either pH 6.6 or 7.4. Over a 20-hr period at 37° there was less than 5% hydrolysis of sodium cyanate at pH 7.4, but when incubated in medium at pH 6.6 there was a 69% decrease in

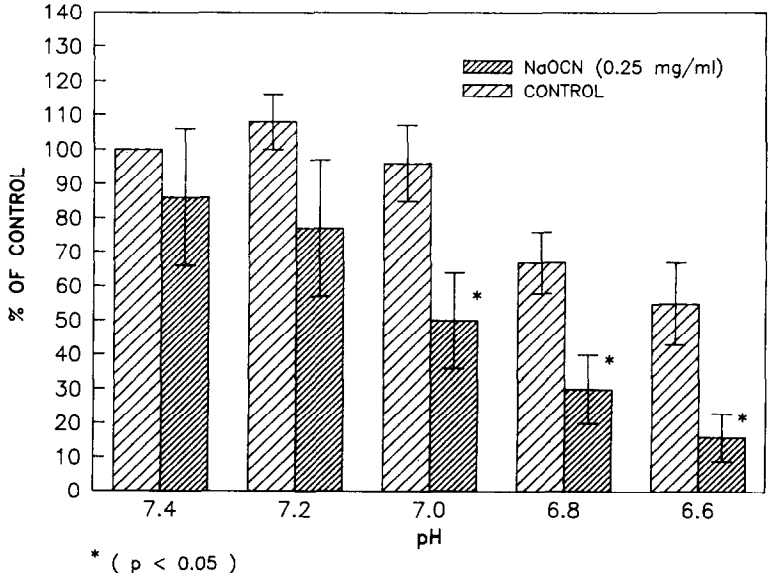

Fig. 4. Effect of pH on the inhibition of leucine incorporation into protein of hepatocytes by sodium cyanate. Freshly isolated hepatocytes (2×10^5) were preincubated for 10 min with sodium cyanate (0.25 mg/ml) in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES before addition of 5.0 μCi [^{3}H]leucine in 10 μl water and a further 30-min incubation. Incorporation of isotope into protein is expressed as a percentage of the incorporation in control cells at pH 7.4 (164 ± 45 cpm/10^4 cells). Each bar represents the mean ± SD of three separate experiments in triplicate.

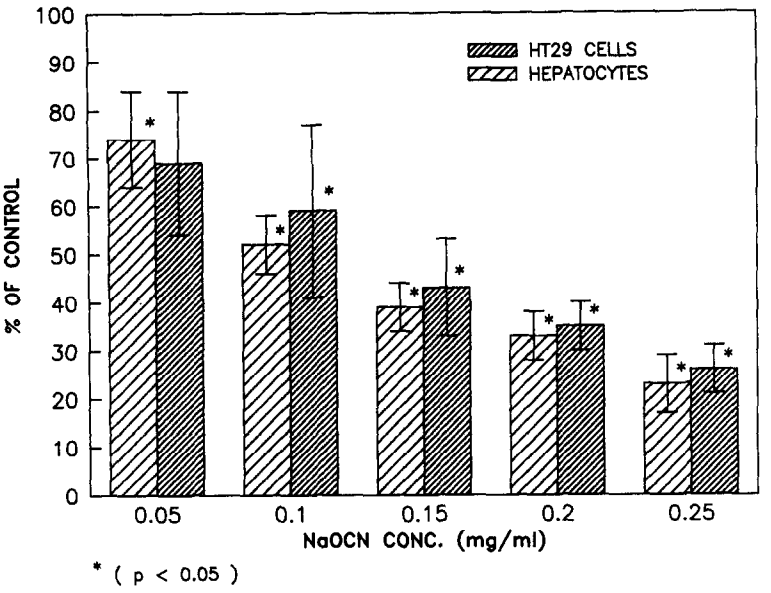

Fig. 5. Effect of sodium cyanate concentration on the incorporation of [^{3}H]leucine into protein of HT29 cells and rat hepatocytes at pH 6.6. The cells (4×10^5) were preincubated for 10 min with sodium cyanate at the stated concentration in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES before addition of 5.0 μCi [^{3}H]leucine in 10 μl water and a further 30-min incubation. Incorporation of isotope into protein is expressed as a percentage of the incorporation in control cells incubated without cyanate (338 ± 147 cpm/10^5 HT29 cells and 664 ± 303 cpm/10^5 hepatocytes). Each bar represents the mean ± SD of three separate experiments in triplicate.

cyanate concentration over this time period. On the basis of these results, we chose to change the medium every 24 hr in experiments in which cells were incubated with control medium or medium containing sodium cyanate. The data in Fig. 8 indicate that proliferation of HTC cells was less rapid at pH 6.6 than at pH 7.4. Sodium cyanate at a concentration of 0.1 or 0.25 mg per ml caused an inhibition of cell division at both pH values, but only at pH 6.6 was there a large decrease in the number of viable cells.

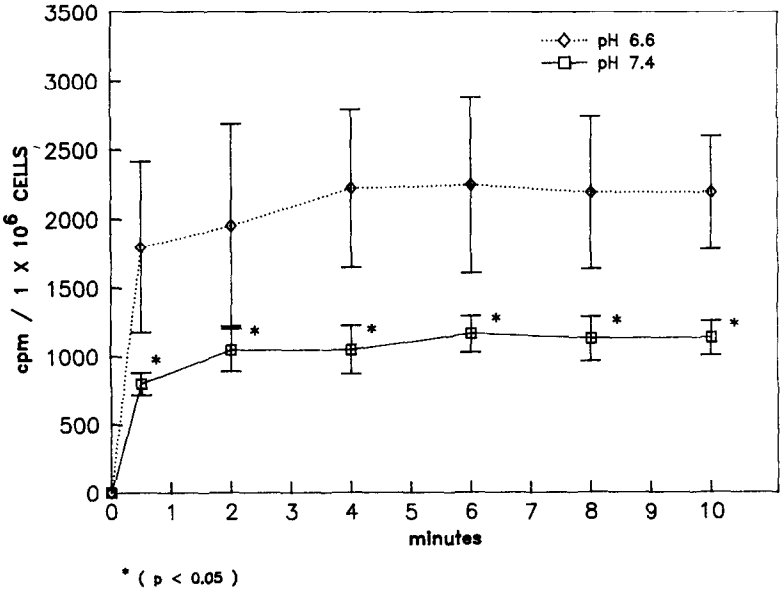

Fig. 6. Uptake of [^{14}C]sodium cyanate in HTC cells. Cultured HTC cells (10^6) were incubated with [^{14}C]sodium cyanate (0.25 mg and 0.25 μCi per ml of Eagle's minimal essential medium containing 50 mM PIPES buffer, pH 7.4 or 6.6). The uptake of isotope at different time points is expressed as cpm per 10^6 cells. Each point represents the mean ± SD of three separate experiments in duplicate. Statistical significance is indicated for the incubations at pH 6.6 relative to the corresponding observations at pH 7.4.

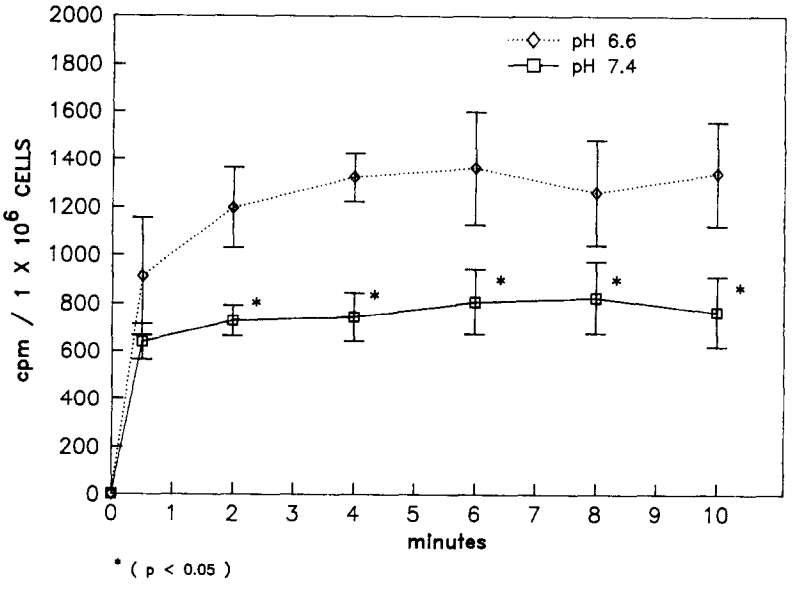

Fig. 7. Uptake of [^{14}C]sodium cyanate in HT29 cells. The experimental conditions and presentation of data were as described for Fig. 6.

Similar responses were seen with HT29 cells under the same conditions (Fig. 9).

DISCUSSION

Sodium cyanate has greater inhibitory effects on macromolecular synthesis in hepatomas than in the livers of tumor-bearing rats [1, 14]. In previous work little inhibitory effect of sodium cyanate has been observed with cultured cells at concentrations which might be attained *in vivo*. Such studies were performed with cells incubated at pH 7.4. In solid tumors, however, the interstitial pH is generally less than pH 7.4 [7, 15, 16]. Our work shows that tumor cells can proliferate in a medium with a pH as low as 6.6 albeit more slowly than at pH 7.4. At pH 6.6 there were marked inhibitory effects of sodium cyanate on macromolecular synthesis in tumor cells and on cell division. The inhibitory effect on [^{3}H]leucine incorporation into protein was not restricted to neoplastic cells but was also seen with rat hepatocytes. This suggests that the greater sensitivity of tumor cells to cyanate *in vivo* may be related more to differences in the microenvironment of the cells than to inherent differences in sensitivity.

Table 2. Incorporation of [^{3}H]thymidine into DNA of HTC cells at pH 7.4 after preincubation with sodium cyanate in media of different pH

Preincubation pH	NaOCN (mg/ml)	Incorporation as % control
7.4	0	100
	0.25	91 ± 17
7.2	0	61 ± 13*
	0.25	48 ± 18*
7.0	0	46 ± 12*
	0.25	52 ± 17*
6.8	0	58 ± 15*
	0.25	48 ± 8*
6.6	0	40 ± 14*
	0.25	42 ± 10*

HTC cells (2×10^5) were preincubated for 40 min with or without sodium cyanate at 37° in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES buffer at the stated pH. The cells were centrifuged and resuspended in fresh medium at pH 7.4 without sodium cyanate and were incubated for 30 min after addition of 2.5 μCi [^{3}H]thymidine in 10 μl water. Incorporation of isotope into DNA is expressed as a percentage of the incorporation in control cells at pH 7.4 (206 ± 33 cpm/10^4 cells). The results are means ± SD for three experiments. In each experiment there were triplicate incubations.

* $P < 0.05$, relative to controls at pH 7.4. Data for cells preincubated with sodium cyanate were not significantly different from controls at the same pH.

Table 3. Reversibility of the effect of sodium cyanate on the incorporation of [^{3}H]leucine into protein of HTC cells

pH of medium	NaOCN (mg/ml)	Incorporation as % control	
		Not resuspended	Resuspended
7.4	0	100	100
7.4	0.25	85 ± 10	97 ± 5
7.4	0.50	75 ± 10	103 ± 14
6.6	0	92 ± 9	93 ± 6
6.6	0.25	54 ± 10*	92 ± 4

Cultured HTC cells (0.5 to 2 × 10^6) were preincubated for 10 min with or without sodium cyanate at 37° in 1 ml of Eagle's minimal essential medium containing 50 mM PIPES buffer at pH 7.4 or 6.6. With those cells designated as "Resuspended", the cells were centrifuged and washed once with medium and resuspended in medium of the original pH but without sodium cyanate. All cells were then incubated for 30 min after addition of 5 μCi [^{3}H]leucine in 10 μl water. Data are expressed as a percentage of the incorporation of isotope into protein of control cells incubated at pH 7.4 (112 ± 59 cpm/10^5 cells). Each value represents the mean ± SD of three separate experiments in triplicate.

* $P < 0.05$, relative to control at pH 6.6.

Even at pH 7.4 we found that there was inhibition of cell division, and it may be noted that inhibition of normal tissue growth was an early observation in studies on the pharmacology of sodium cyanate [17, 18].

Carbamoylation of macromolecules occurs primarily on proteins with very little modification of DNA [19]. Reactions have been observed with amino, hydroxyl, carboxyl, imidazole, phosphate and sulfhydryl groups [20, 21], but only the reaction with amino groups is an irreversible process under physiological conditions. It is not known what reaction of cyanate is critical for the inhibition of macromolecular synthesis or even if this involves a carbamoylation reaction. However, the data are more compatible with carbamoylation of sulfhydryl groups than of amino groups. Carbamoylation of amino groups is a slow process occurring over several hours [22], whereas reaction with sulfhydryl groups occurs in a few minutes [9]. The reversibility in the

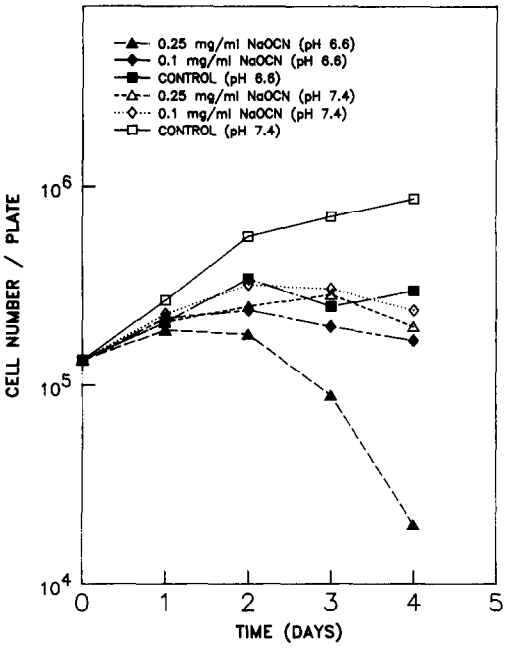

Fig. 8. Effects of pH and sodium cyanate on growth of HTC cells. The cells were cultured in Chee's essential medium with 10% fetal calf serum at pH 7.4 or 6.6. Control cells or cells incubated with sodium cyanate (0.1 or 0.25 mg/ml) were incubated at 37° in 35 mm petri dishes. The cell number per plate is given over the 4-day culture period. Each point represents the mean of two separate experiments in duplicate.

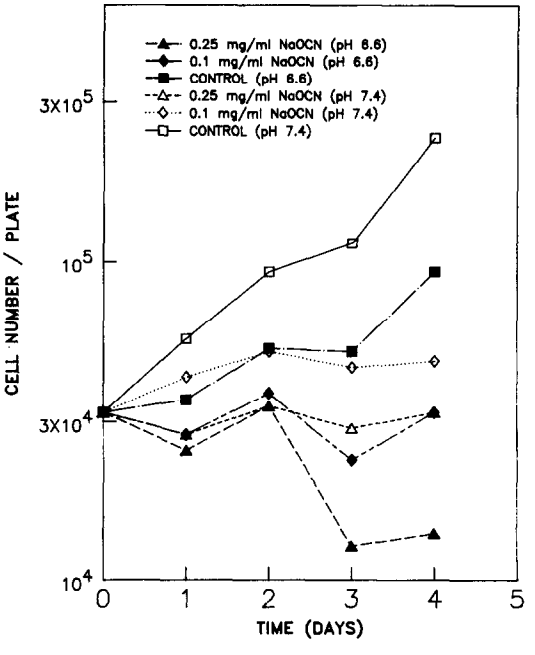

Fig. 9. Effects of pH and sodium cyanate on growth of HT29 cells. The experimental conditions and presentation of data were as described for Fig. 8.

effect of cyanate would not be anticipated if amino group carbamoylation was involved but would be expected if there was carbamoylation of sulfhydryl groups.

A decrease in pH stabilizes the carbamoylation of sulfhydryl groups and is a possible factor in the enhanced inhibitory effects we have seen at a lower pH. Stark [9] observed that the carbamoyl derivative of cysteine is stable below pH 6 but between pH 6 and pH 8 the position of the equilibrium is dependent upon pH. We found that carbamoylation of glutathione in cells by cyanate was greater when the pH of the incubation medium was decreased from pH 7.4 to 6.6 (J. J. Hu, M. J. Dimaira and M. A. Lea, unpublished observations). Glutathione is a potential target for such carbamoylation. Depletion of glutathione has been seen with cyanate in erythrocytes [23] and reticulocytes [24]. On the other hand, Freedman *et al.* [24] concluded that the decrease in the level of glutathione was not the sole factor in the inhibition by cyanate of protein synthesis in rabbit reticulocytes.

In view of the increased uptake of ^{14}C-labeled cyanate by tumor cells at pH 6.6 relative to uptake in medium at pH 7.4, the degree of ionization of isocyanic acid may be an important factor in the pH dependence of cyanate action. The uncharged isocyanic acid, which exists in equilibrium with the cyanate ion, would be expected to enter cells more readily than the cyanate ion. Isocyanic acid is the carbamoylating species, and one cannot exclude effects on the cell membrane independent of reactions within the cell. Growth inhibition of *Escherichia coli* by potassium cyanate was observed to be enhanced at higher H^+ concentrations and, from this evidence, Guilloton and Karst [25] concluded that the inhibition was likely to be mediated by isocyanic acid. In the case of organic isocyanates, which like inorganic cyanate can exert preferential effects on macromolecular synthesis in tumors *in vivo* [26], there would not be a pH effect related to ionization. For organic isocyanates some other mechanism for tissue specificity must be identified. The present data suggest that a lower interstitial pH in tumors will enhance the inhibitory effects of sodium cyanate on macromolecular synthesis, but a mechanism for this response requires further characterization.

Acknowledgements—This work was supported by NIH Grants CA-35315 and CA-37138. We are grateful to Mr. George Dikdan for the preparation of hepatocytes.

REFERENCES

1. M. A. Lea, M. R. Koch and H. P. Morris, *Cancer Res.* **35**, 2321 (1975).
2. V. G. Allfrey, L. C. Boffa and G. Vidali, *Cancer, N.Y.* **40**, 2692 (1977).
3. M. A. Lea and M. R. Koch, *J. natn. Cancer Inst.* **63**, 1279 (1979).
4. L. C. Boffa, S. Kozak and V. G. Allfrey, *Cancer Res.* **41**, 60 (1981).
5. M. A. Lea and J. Parsons, *Cancer Res.* **41**, 4988 (1981).
6. J. Hu, A. Luke, O. Velazquez, C. F. Martinson, J. S. H. Yoo, C. S. Yang and M. A. Lea, *Fedn Proc.* **45**, 447 (1986).
7. P. M. Gullino, *Progr. exp. Tumor Res.* **8**, 1 (1966).
8. G. R. Stark, W. H. Stein and S. Moore, *J. biol. Chem.* **235**, 3177 (1960).
9. G. R. Stark, *J. biol. Chem.* **239**, 1411 (1964).
10. J. J. Hu, A. Luke and M. A. Lea, *J. Cell Biol.* **103**, 29a (1986).
11. J. J. Hu, A. Luke, V. Oliphant, M. Chellani, K. Zirvi and M. A. Lea, *Proc. Am. Ass. Cancer Res.* **28**, 416 (1987).
12. M. A. Lea, *J. natn. Cancer Inst.* **71**, 1073 (1983).
13. M. Guilloton and F. Karst, *Analyt. Biochem.* **149**, 291 (1985).
14. M. A. Lea, M. R. Koch, V. G. Allfrey and H. P. Morris, *Cancer Biochem. Biophys.* **1**, 129 (1975).
15. S. Harguindey, *Med. pediat. Oncol.* **10**, 217 (1982).
16. J. L. Wike-Hooley, J. Haveman and H. S. Reinhold, *Radiother. Oncol.* **2**, 343 (1984).
17. K. M. Birch and F. Schutz, *Br. J. Pharmac. Chemother.* **1**, 186 (1946).
18. F. Schutz, *Experientia* **5**, 133 (1949).
19. M. A. Lea, *CRC Crit. Rev. Oncol. Hematol.*, **7**, 329 (1987).
20. G. R. Stark, in *Methods in Enzymology* (Ed. C. H. W. Hirs), Vol. 2, p. 590. Academic Press, New York (1967).
21. J. Carreras, A. Chabas and D. Diederich, in *The Urea Cycle* (Eds. S. Grisolia, R. Baguena and F. Mayor), p. 501. John Wiley, New York (1976).
22. M. A. Lea, A. Luke, C. Martinson and O. Velazquez, *Int. J. Pept. Protein Res.* **27**, 251 (1986).
23. B. E. Glader and M. E. Conrad, *Nature, Lond.* **237**, 336 (1972).
24. M. L. Freedman, F. J. Schiffman and M. Geraghty, *Br. J. Haemat.* **27**, 303 (1974).
25. M. Guilloton and F. Karst, *J. gen. Microbiol.* **133**, 645 (1987).
26. M. A. Lea, A. Luke, J. J. Hu and O. Velazquez, *Biochem. Pharmac.* **36**, 2775 (1987).