Role of Thiocyanate Ion in Detoxification of the Anticancer Agent Chlorambucil

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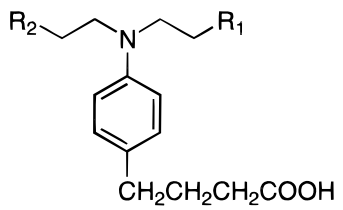
N,*N*-Bis(2-chloroethyl)-*p*-aminophenylbutyric acid (chlorambucil, **1**) is an orally administrated drug widely used in the chemotherapy of chronic lymphocytic leukemia. We have recently described a new metabolic path for the decomposition of **1** in human gastric juice based on its reactions with saliva-derived thiocyanate ion. We report here our quantitative data on the reactions of thiocyanate ion with CLB in various fluid matrixes at 37 °C. The rate of decomposition of **1** is zero-order with respect to SCN^- concentration up to 100 mM. However, thiocyanate ion reacts ca. 18 300 times faster than water with the aziridinium ion derived from **1** at neutral and acidic pH. When the SCN^- concentration was greater than 10 mM, practically no *N*,*N*-bis(2-hydroxyethyl)-*p*-aminophenylbutyric acid, **4**, the product of chlorambucil hydrolysis, could be detected. Thiocyanate ion also effectively overcompensates for the rate retardation caused by Cl^-; 10 mM SCN^- is enough to decrease the effect of 0.5 M chloride ion to one-half. This is an important factor in human gastric juice where the chloride ion concentration is normally high.

Introduction

Chronic lymphocytic leukemia is the most common form of leukemia in Western countries. Its chemotherapy has been based on the use of *N*,*N*-bis(2-chloroethyl)-*p*-aminophenylbutyric acid (chlorambucil, **1**; Chart 1) for more than 30 years (*1*). Chlorambucil is an alkylating agent that is known to bind covalently to many types of cellular molecules, such as DNA, RNA, and proteins (*2*). Its binding to DNA is thought to be the mechanism of its cytotoxicity as well as of its mutagenicity and carcinogenicity. The covalent binding of the drug to DNA leads to misreading of the DNA code, cross-linking of DNA, and single- and double-stranded breaks of DNA. Since the alkylating agents preferentially bind at sites of DNA that are actively transcribed, they are more toxic to cancer cells than to normal cells. The N7 position of guanine is thought to be the principal site of DNA alkylation. Despite the central role of chlorambucil in clinical chemotherapy, very little attention has been paid to it during the past two decades (*3*).

CLB[1] is given by oral administration, the daily dose being usually 4−10 mg. It is absorbed from the gastrointestinal track, presumably mainly from the stomach, and the peak of plasma concentration is achieved ca. 1 h after administration (*4*). We started our investigations of **1** by determining its kinetic parameters in different matrixes, such as human blood, red cells, plasma, plasma ultrafiltrate, gastric juice, and phosphate-buffered saline (*5*). In our recent communication, we reported a new metabolic pathway for **1** based on its reactions in human gastric juice with saliva-derived thiocyanate ion (*6*). We report here our quantitative data on the reactions of thiocyanate ion with chlorambucil in various fluid matrixes in vitro. Also, the role of thiocyanate ion as a pharmacokinetic modulator of CLB is discussed.

Chart 1



1: $R_1 = R_2 = Cl$
2: $R_1 = SCN$, $R_2 = Cl$
3: $R_1 = OH$, $R_2 = Cl$
4: $R_1 = R_2 = OH$
5: $R_1 = OH$, $R_2 = SCN$
6: $R_1 = R_2 = SCN$

Experimental Section

General. *N*,*N*-Bis(2-chloroethyl)-*p*-aminophenylbutyric acid was purchased from Sigma. All inorganic reagents were of ACS grade or better. *N*,*N*-Bis(2-hydroxyethyl)-*p*-aminophenylbutyric acid (**4**), *N*,*N*-bis[(2-thiocyano)ethyl]-*p*-aminophenylbutyric acid (**5**), and *N*-(2-hydroxyethyl)-*N*-[(2-thiocyano)ethyl]-*p*-aminophenylbutyric acid (**6**) were synthesized as described previously (*5*, *6*). *N*-(2-Chloroethyl)-*N*-(2-hydroxyethyl)-*p*-aminophenylbutyric acid (**3**) was synthesized according to a literature procedure (*7*). Human gastric juice and saliva samples were obtained as described previously (*6*). Protein-depleted leukemia cell ultrafiltrate was prepared from 2×10^{10} leukemia cells. The cells were obtained, after informed consent, from a leukapheresis sample of a patient suffering from T-cell prolymphocytic leukemia. Cells were disrupted with a Teflon homogenizer, and protein-depleted ultrafiltrate (ca. 1 mL) was prepared (1000*g*) using Amicon's "Centrifree" micropartition system for separation of free from protein-bound microsolutes. The thiocyanate ion

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[1] Abbreviations: CLB, chlorambucil; CLL, chronic lymphocytic leukemia.

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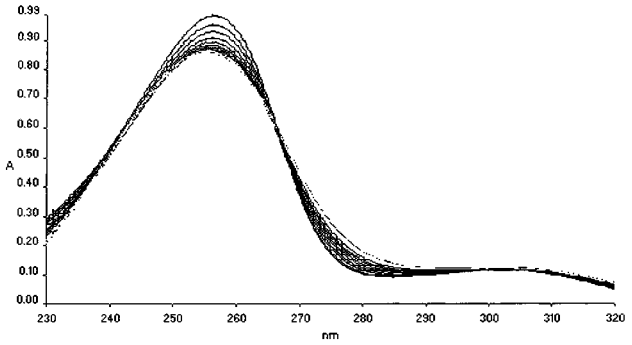


Figure 1. Decomposition of chlorambucil (**1**) in cacodylic acid buffer (0.2 M, 50% base) in the presence of 1 mM thiocyanate ion followed by UV spectrophotometry over 10 half-lives at 37 °C. The ionic strength was adjusted to 1.0 M with $NaClO_4$.

concentrations of human fluid matrixes were determined VIS spectrophotometrically as an $FeSCN^{2+}$ complex (*8*).

UV spectra were recorded on a Perkin-Elmer Lambda 2 spectrophotometer equipped with a thermostated cell holder. HPLC analyses were performed on a Waters 2000 or a Merck-Hitachi 6000 instrument consisting a UV detector ($\lambda = 267$ nm) and a reversed phase column (Hypersil C18, 4.6 mm × 240 mm, particle size of 6 μm). The mobile phase consisted of buffer A (0.1 M ammonium acetate) and buffer B [0.1 M ammonium acetate in 50% (v/v) aqueous acetonitrile]. The gradient was 100% A from 0 to 10 min and a linear gradient from 100% A to 100% B from 10 to 40 min. The flow rate was 1.0 mL min^{-1}.

Kinetic measurements were performed as described previously (*6*).

Results

We have already shown that chlorambucil (**1**) reacts with thiocyanate ion present in gastric juice and saliva samples in vitro (*6*). Even at rather low thiocyanate ion concentrations (<2 mM), considerable amounts of thiocyanate adducts were formed. To find a simple method for quantifying the product distribution of the decomposition of **1** in various fluid matrixes, chlorambucil was allowed to react with thiocyanate ion (1.0 mM) in a non-nucleophilic buffer (cacodylic acid, 50% base; ionic strength adjusted to 1.0 M with $NaClO_4$), and the reaction was followed on UV spectrophotometry over 10 half-lives at 37 °C. The UV spectra have an isosbestic point at 267 nm (Figure 1). This wavelength was chosen when the reactions of **1** were followed by HPLC techniques; at this wavelength, the sum of the areas of all the peaks did not change as a function of time. Accordingly, **1** and all the reaction intermediates and end products have the same extinction coefficients at 267 nm even in our HPLC system, and the areas of their peaks are proportional to the concentrations of each reaction component in the solution. Although the chosen wavelength is almost 10 nm off from the absorption maximum, each reaction component absorbed enough to warrant reliable product analysis.

Decomposition of chlorambucil was investigated in the presence and absence of thiocyanate ion at acidic and neutral pH. In the presence of thiocyanate ion, the drug is converted into two reaction intermediates (**2** and **3**) and three stable metabolites (**4**−**6**). The stable products are *N*,*N*-bis(2-hydroxyethyl)-*p*-aminophenylbutyric acid (**4**), *N*-(2-hydroxyethyl)-*N*-[(2-thiocyano)ethyl]-*p*-aminophenylbutyric acid (**5**), and *N*,*N*-bis[(2-thiocyano)ethyl]-*p*-aminophenylbutyric acid (**6**) (Chart 1). The reaction intermediates are characterized as *N*-(2-chloroethyl)-*N*-(2-hydroxyethyl)-*p*-aminophenylbutyric acid (**3**) and *N*-(2-chloroethyl)-*N*-(2-thiocyano)-*p*-aminophenylbutyric acid (**2**). Identification of the peaks of **3**−**6** was confirmed on HPLC by spiking with authentic samples synthesized via an independent route (*5*−*7*). Characterization of **2** is based on the following observations. (i) According to the mole fractions of each reaction component as a function of time, **2** is produced directly from CLB. (ii) At high SCN^- concentrations when no CLB hydrolysis was observed, **2** was the only reaction intermediate. (iii) It was not produced in the absence of SCN^-. (iv) When **3** was allowed to decompose in the presence of thiocyanate ion, no **2** was formed. Mole fractions of each reaction component of CLB decomposition in the presence of 10 mM thiocyanate ion at neutral pH as a function of time are shown in Figure 2.

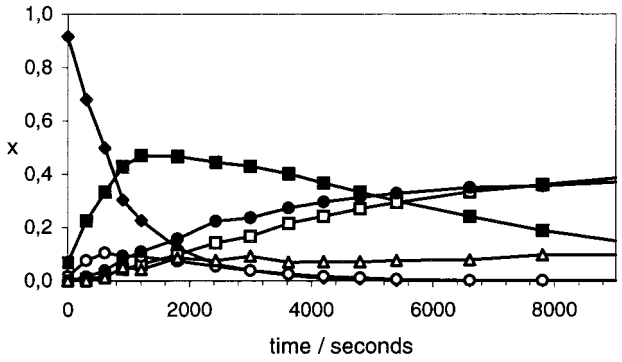


Figure 2. Product distribution of CLB disintegration as a function of time at 37 °C (pH 6.8, $[SCN^-] = 10$ mM, $I = 1.0$ M with $NaClO_4$): **1** (◆), **2** (■), **3** (○), **4** (△), **5** (●), and **6** (□).

Table 1. Effect of pH and $[SCN^-]$ on the Rate of Decomposition and Product Distribution of 1 at 37 °C[a]

pH	$[SCN^-]$ (M)	k_{obs} ($\times 10^{-4}$ s^{-1})	product distribution		
			4	**5**	**6**
6.8	0	9.91 ± 0.04	1.00	0.00	0.00
6.8	0.010	10.71 ± 0.12	0.03	0.34	0.63
6.8	0.100	10.00 ± 0.16	0.00	0.00	1.00
2.0	0	1.40 ± 0.20	1.00	0.00	0.00
2.0	0.010	1.31 ± 0.17	0.02	0.34	0.64
2.0	0.100	1.39 ± 0.10	0.00	0.00	1.00

[a] Ionic strength adjusted to 1 M with $NaClO_4$.

The effect of thiocyanate ion concentration on the rate of decomposition of chlorambucil was investigated at two different pHs at a constant ionic strength (Table 1). At physiological pH where the drug can be assumed to be completely deprotonated [pK_a of **1** is 2.5 (*9*)], no rate enhancement was observed when the SCN^- concentration was increased from 0 to 100 mM. Analogously, at pH 2.0, where more than 50% of **1** is protonated, no rate acceleration was detected within experimental error.

The addition of chloride ion to the reaction medium is known to suppress the rate of decomposition of nitrogen mustards significantly (*9*, *10*). We reinvestigated the effect of chloride ion on the rate of decomposition of **1** at a constant ionic strength. The increase in Cl^- concentration from 0 to 0.5 M decreased the rate of hydrolysis of **1** in the absence of SCN^- by a factor of 5.7. The observed rate retardation is somewhat larger than that reported by Chatterji et al. (*9*) and Culls et al. (*10*) in phosphate-buffered media. The difference arises, in all likelihood, from the reaction of **1** with phosphate dianion which partially cancels the effect of Cl^-. When thiocyanate ion was added to the reaction medium, the chloride effect

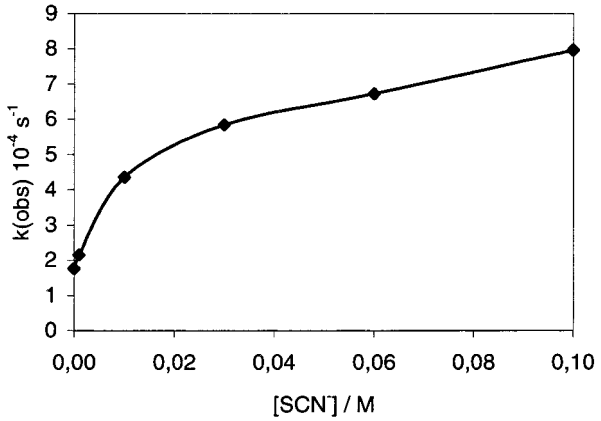


Figure 3. Effect of SCN^- concentration on the rate of CLB decomposition in the presence of 0.5 M Cl^-. t = 37 °C, and I = 1.0 M ($NaClO_4$).

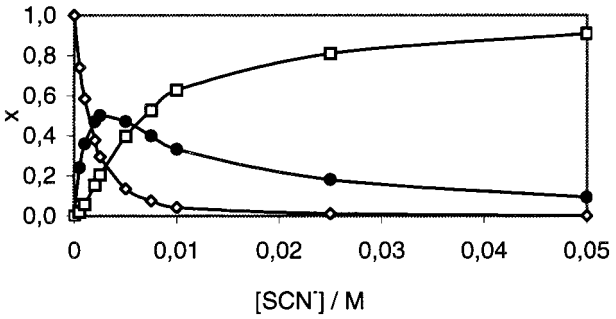


Figure 4. End product distribution of CLB decomposition as a function of thiocyanate ion concentration at pH 6.8 and I = 1.0 M ($NaClO_4$): **4** (◇), **5** (●), and **6** (□).

was suppressed considerably (Figure 3). For example, 0.01 M SCN^- decreased the rate retardation of 0.5 M Cl^- to a factor of 2.3.

We studied also the end product distribution of chlorambucil (0.6 mM) decomposition as a function of thiocyanate ion concentration at acidic and neutral pH at a constant ionic strength (Table 1 and Figure 4). Similar amounts of products of chlorambucil hydrolysis and thiocyanate alkylation were formed at 3 mM SCN^-. When the SCN^- concentration was greater than 0.01 M, practically no *N*,*N*-bis(2-hydroxyethyl)-*p*-aminophenylbutyric acid, **4**, the product of CLB hydrolysis, could be detected. At acidic pH, the mole fractions of thiocyanate adducts were slightly larger than at neutral pH at a constant thiocyanate ion concentration.

Reaction of Chlorambucil in Human Fluid Matrixes in Vitro. As shown previously (*6*), chlorambucil is converted in human gastric juice and saliva into two reaction intermediates (**2** and **3**) and three stable end products (**4**−**6**). The product distribution is dependent on the thiocyanate ion concentration of the fluid matrix. The thiocyanate ion concentration in various gastric juice and saliva samples varied from 0.2 to 2.0 and from 0.4 to 5.6 mM, respectively, when it was analyzed VIS spectrophotometrically as an $FeSCN^{2+}$ complex. The mole fractions of each reaction component in a gastric juice and saliva sample as a function of time are shown in Figures 5 and 6. We measured spectrophotometrically the SCN^- concentration also in the ultrafiltration of CLL cells, but it was under the detection level of the method of analysis. Hence, CLB decomposition in that matrix was not investigated in this study.

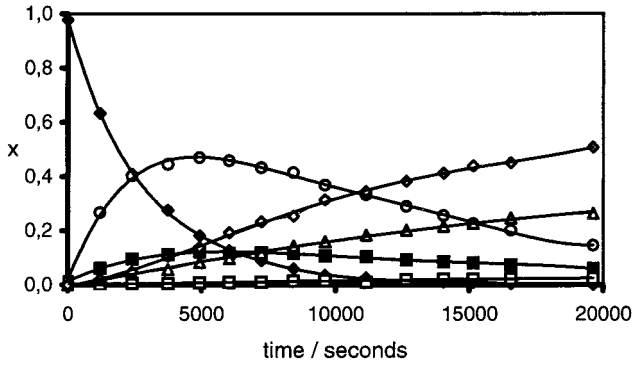


Figure 5. Product distribution of CLB disintegration in a human gastric juice sample (37 °C, pH 2.5, [SCN^-] = 0.7 mM, [Cl^-] = 150 mM) as a function of time: **1** (◆), **2** (■), **3** (○), **4** (◇), **5** (●), and **6** (□).

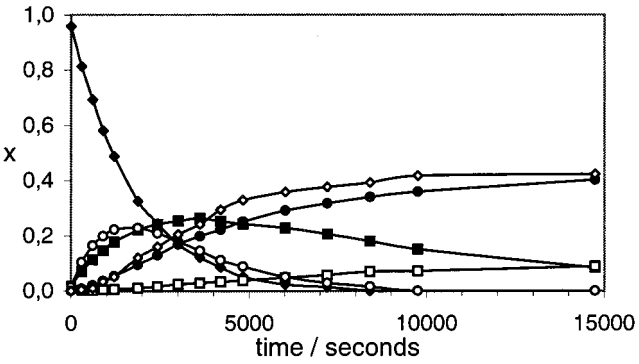


Figure 6. Product distribution of CLB disintegration in a human saliva sample (37 °C, pH 7.4, [SCN^-] = 1.7 mM, [Cl^-] = 150 mM) as a function of time: **1** (◆), **2** (■), **3** (○), **4** (◇), **5** (●), and **6** (□).

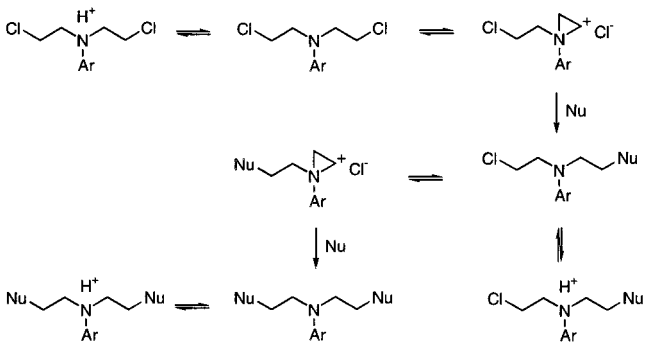
Table 2. Half-Lives and Product Distributions of the Decomposition of Chlorambucil (1) in Various Human Fluid Matrices at 37 °C

medium	pH	[SCN^-] (mM)	[Cl^-] (mM)	$t_{1/2}$ (min)	product distribution		
					4	**5**	**6**
gastric	0.9	0.09	113	413	nd	nd	nd
	2.5	0.2	97	39	0.60	0.36	0.04
	7.0	2.0	85	25	0.58	0.37	0.06
saliva	7.5	1.4	14	17	0.73	0.20	0.05
	7.6	2.1	nd[a]	19	0.40	0.45	0.15

[a] nd, not determined.

The observed pseudo-first-order rate constants and the end product distribution for the decomposition of **1** in various human fluid matrixes are collected in Table 2. At low gastric pH, the product distribution could not be determined due to the extreme stability of the reaction intermediates **2** and **3**. It is clearly demonstrated that high gastric pH induced by a protonic pump inhibitor Losec has a dramatic destabilizing effect on **1**. However, the observed rate constants for the decomposition in human fluid matrixes were somewhat smaller than would be predicted according to their H^+, Cl^-, and SCN^- concentrations. This can be tentatively attributed to noncovalent binding of **1** to biomolecules present in gastric juice and saliva. This would not be surprising, since the binding of **1** to albumin in plasma has already been demonstrated to have a stabilizing effect on CLB (*5*). Also, the mole fractions of thiocyanate adducts formed in gastric juices and saliva were smaller than the measured SCN^- concentration would predict. The difference results, at least partially, from the heterogeneity of gastric juice and saliva samples. Furthermore, the thio-

Scheme 1



cyanate ion concentrations in physiological solutions were determined spectrophotometrically as an $FeSCN^{2+}$ complex in 1 M nitric acid (*8*). In gastric juice and saliva, a part of SCN^- may also be bound to biomolecules (*11*), and the concentration of free SCN^- capable of reacting with CLB might be lower than the one determined in strong acid. Also, thiocyanate ion chlorambucil concentrations were very close to each other. In some experiments, the concentration of free thiocyanate ion may be even smaller than that of CLB (chlorambucil is capable of consuming 2 equiv of thiocyanate ion). This could explain the somewhat anomalous product distributions.

Discussion

It is widely accepted that chlorambucil like other aromatic and aliphatic nitrogen mustards decomposes in aqueous media by a mechanism outlined in Scheme 1 (*9*, *10*, *12*, *13*). Accordingly, an intramolecular, rate-determining attack of the unprotonated nitrogen to form an aziridinium ion intermediate is followed by attack of an external nucleophile. The mechanism is supported by the following observations. (i) The rate of decomposition is proportional to the mole fraction of the deprotonated **1** in solution. Since only deprotonated nitrogen can form the aziridinium ion, the reactivity of protonated **1** is negligible. (ii) Chloride ion retards the rate of decomposition. (iii) The reaction is of zero-order with respect to other nucleophiles. An exception to the above reaction is the alkylation of a thiol nucleophile by **1** in *tert*-butyl alcohol which occurs by direct S_N2 displacement (*14*). In this case, direct S_N2 displacement by SCN^- ion to the deprotonated or protonated **1** can be ruled out as an alternative reaction pathway, since the increase in the SCN^- concentration does not accelerate the reaction in neutral or acidic media. Thus, the reaction proceeds via an aziridinium ion intermediate in the presence or absence of SCN^- ions. However, thiocyanate ion reacts very fast with the aziridinium ion derived from **1**; similar amounts of products of chlorambucil hydrolysis and thiocyanate alkylation were formed at 3 mM SCN^-. Since the H_2O concentration is 55 M, SCN^- reacts 18 300 times faster than water with the aziridinium ion derived from **1**.

Although thiocyanate ion does not promote the decomposition of **1** by changing the reaction mechanism, it acts as a pharmacokinetic modulator by decreasing the common ion effect. SCN^- at 0.01 M is enough to decrease the effect of 0.5 M Cl^- to less than one-half. Unlike the biologically relevant thiol nucleophile, glutathione, thiocyanate ion is nucleophilic even in highly acidic media (pK_as of HSCN and glutathione are −1.8 and 8.8, respectively); the chloride effect is suppressed at acidic pH as well. This is an important factor in gastric juices, where oxonium and chloride ion concentrations are normally high.

Although the comparison of the results obtained in strictly controlled media and human fluid matrixes is not straightforward, it is clear that high gastric SCN^- concentrations decrease the bioavailability of CLB by suppressing the chloride ion effect. This, however, assumes intensive chewing and saliva swallowing. The most dramatic diminishing of CLB bioavailability is caused by high gastric pH. Prednisolone, which may cause gastric irritation, is commonly prescribed together with **1**. The irritation may then be treated with proton pump inhibitors, decreasing the secretion of HCl by the stomach wall and hence accelerating the chlorambucil detoxification.

There are several indications that SH groups containing biomolecules (predominantly glutathione) play an important role in the intracellular resistance to alkylating agents (*15*). GSH is known to prevent **1** as well as other alkylators from forming DNA cross-links, either by reacting directly with them or by quenching their DNA monoadducts (*16*). Glutathione, on other hand, is also known to increase the toxicity of some rather harmless compounds such as halogenated hydrocarbons (e.g., dichloromethane and 1,2-dibromoethane) by converting them to derivatives of sulfur half-mustards (*17*). Naturally, thiocyanate ion does not have the latter effect.

GSH is the most abudant cellular low-molecular mass thiol. Its concentration in human erythrocytes is 2 mM and in hepatocytes greater than 10 mM (*15*). By contrast, the GSH concentration in blood is less than 1 μM (*18*). Thiocyanate ion, in turn, is derived endogenously as a detoxification product of the reaction between cyanide and thiosulfates in the liver. It is abundantly present in blood (*19*, *20*), and especially in saliva (*21*, *22*). Its concentration varies widely depending on diet and smoking habits. Exposure to tobacco smoke, metabolism of vitamin B_{12}, certain foods containing cyanide or cyanogenic glycosides (such as almonds, nuts, cabbage, cauliflower, and broccoli), and some medications (e.g., nitroprussine) are known increase SCN^- concentration. Normal nonsmokers have a saliva SCN^- concentration of 0.5−2 mM, while some smokers may have a SCN^- concentration of up to 6 mM (*20*). We confirmed that the saliva thiocyanate ion concentration of 147 subjects varied from 0.4 to 5.6 mM when analyzed spectrophotometrically as an $FeSCN^{2+}$ complex (*23*). The thiocyanate concentration in serum ranges from 0.02 mM for nonsmokers to 0.08 mM for smokers (*18*). Its binding to albumin has also been described (*11*, *24*). There is no information available on the presence of SCN^- in the cells. We confirmed that the thiocyanate concentration in the ultrafiltration of CLL cells was negligible.

In summary, thiocyanate ion might be regarded as "an inorganic glutathione" in CLB detoxification in saliva, gastric juice, and blood, where the GSH concentration is small or negligible. Although the role of SCN^- in detoxifying ingested alkylators other than CLB in vivo is not yet clear, the ion undoubtedly deserves more attention.

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