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Stability and Spontaneous Production of Blood Cyanide During Heating

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ABSTRACT: To investigate the effects of heat on blood cyanide concentrations, *in vitro* experiments were performed using a headspace gas chromatographic method. Cyanide concentrations were determined for solutions of human hemoglobin (Hb) at neutrality, and for blood which was sealed in a vial and incubated at 25, 50, 63, 75 and 90°C for 1 h. Spontaneous cyanide production was also measured. Nearly all of the added cyanide was recovered in both the Hb and for blood samples which were heated below 63°C. Cyanide recovery in Hb decreased in a temperature-dependent manner at temperatures above 75°C, and more than half of the recovered cyanide was found to be in the free form. In contrast, cyanide in blood disappeared more rapidly, and a major portion of it existed in the bound form. Cyanide concentrations in Hb solutions which were heated at 90°C dropped in the two phases; a rapid initial phase, followed by a slower process. Spontaneous cyanide production was observed at temperatures above 50°C for Hb and above 63°C for blood. Under optimal conditions (75°C heating), about 0.2 mmol of cyanide was produced per mol heme of Hb.

KEYWORDS: forensic science, cyanide, blood, hemoglobin, postmortem alteration, heating

Cyanide is a potent toxic agent that prevents tissue use of oxygen by inhibition of the terminal cellular respiratory enzyme cytochrome oxidase (1). Besides widespread usage in industry, hydrogen cyanide (HCN) is produced when nitrogen-containing materials pyrolize or burn (2). Recently, it has been reported that significant amounts of cyanide are detectable in the bloods of fire victims (3), suggesting that cyanide may be involved as a lethal agent in fires (4). Therefore, cyanide determinations in blood should be conducted not only as evidence of acute cyanide poisoning in unnatural death (5), but also as a diagnostic parameter of inhalation of HCN gas in order to elucidate the cause of death in fires (6). The blood samples drawn from heavily burned fire victims often show signs of heating as evidenced by blood coagulation. Therefore, cyanide alterations caused as a result of a fire must be taken into account when such unusual blood samples are examined. Although changes in blood cyanide concentrations during storage have been investigated extensively (7), postmortem cyanide alteration by heating has not been examined extensively. In this paper, using headspace gas chromatography (HS-GC) as a cyanide assay method (8), the effects of heating on the alteration of cyanide concentrations in human hemoglobin (Hb) and in blood samples is examined.

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Materials and Methods

Cyanide Determination by Head-Space Gas Chromatography

Cyanide concentrations were determined by the HS-GC method as described previously (8,9). A 5890A gas chromatograph (Yokogawa Hewlett-Packard, Tokyo, Japan), equipped with a nitrogen phosphorus detector and split injector was used for the study. Stationary phase was a GS-Q column (30 m × 0.53 mm i.d., J& W Scientific, Folson, CA) and helium was used as the carrier-gas at a flow-rate of 4.7 mL/min. The injection port, detector and column oven were maintained at 200°C, 250°C and 130°C, respectively. The split ratio was adjusted to 3.5. HS equilibrium was attained using a screw cap septum vial (7 mL, Pierce Co., Rockford, IL). Samples were added into the vial, which was sealed with a Tuf-Bond Disc (Pierce Co.), and 0.2 mL of 50% phosphoric acid was introduced through the disc using a glass tip syringe (0.5 mL, Top Co., Tokyo, Japan) and needle (25 gauge × 1″, 0.50 × 25 mm, Terumo Co., Tokyo, Japan). The mixture was allowed to stand at 50°C for 30 min, and 0.5 mL of gaseous phase in the sealed vial was injected into the GC using a glass tip syringe (1.0 mL) and needle (25 gauge × 1″). The cyanide concentration was then normalized to the pressure value in the HS vial. The retention time of HCN was 2.7 min, and the quantitation limit was roughly 1 ng (S/N = 3.5) cyanide.

Heating Experiments

Human Hb was prepared from out-dated transfusion blood and its heme concentration was measured as described previously (9). A standard cyanide solution was prepared from potassium cyanide as described previously (8). Heating of blood or Hb solutions were performed in a HS vial. Into a vial, 0.82 μmol (heme) of Hb or 0.2 mL of blood, 20 μmol of sodium phosphate (pH 7) in 0.8 mL aqueous solution with or without cyanide (200 ng for Hb, 100 ng for blood) was added, sealed as described above, and incubated at constant temperature (25, 50, 63, 75, 90°C) for 1 h. At the end of the heating period, 0.5 mL of HS gas was injected into the GC (for temperatures above 63°C, HS gas was applied after an additional 50°C incubation for 30 min). Next, 0.2 mL of 50% phosphoric acid was introduced into the remaining vial, which was allowed to stand at 50°C for 30 min, and 0.5 mL of HS gas was injected into the GC. The free cyanide is defined as cyanide form, which is detected in blood or Hb sample solution without phosphoric acid treatment, and the bound one is as that which is only detected with phosphoric acid treatment. Standard samples

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for a calibration curve were performed as follows, in consideration of the matrix effect (10), for the partition coefficient of HCN between the gas phase and the liquid phase could be changed according to the sample matrix alteration produced by heat treatment. Heat-treated Hb or blood samples devoid of cyanide were fortified with known amounts of cyanide, phosphoric acid was introduced into the HS vial, and the cyanide concentration was determined by HS-GC method. The matrix effect of the heat-treated samples gave values nearly identical to the non-treated samples.

Results

Cyanide Disappearance During Heating

The Hb solution that was fortified with cyanide in phosphate buffer (pH 7) was incubated at an appropriate temperature for 1 h. The free and the total (free plus bound) cyanide concentrations, determined by the HS-GC before and after phosphoric acid addition, are shown in Fig. 1. Below 63°C, almost all of the fortified cyanide was recovered as the bound form. Above 75°C, the total cyanide concentration was lower with increasing temperature, and more than half of the recovered cyanide was found as the free form.

Figure 2 shows the recovery results for heated blood. Below 63°C, almost all of the added cyanide was recovered as the bound form. Above 75°C, the recovery was considerably lower with increasing temperature, and greater portions of the recovered cyanide was found as the bound form.

Figure 3 shows the curve for the disappearance of cyanide in Hb solution incubated at 90°C. The total cyanide concentrations decreased rapidly within 10 min to about a 60% level (diminution rate 8%/min), and then gradually decreased (0.4%/min). Except for the initial rapid phase, the free cyanide consisted of nearly half of the total cyanide recovered.

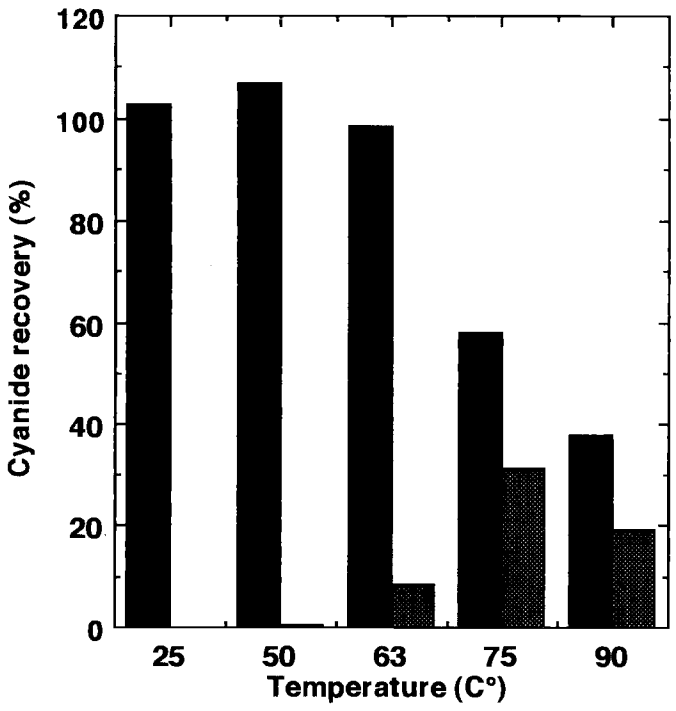

FIG. 1—*Cyanide recovery in heated hemoglobin solutions. Cyanide concentrations before* (shaded boxes) *and after* (drawn boxes) *phosphoric acid addition were expressed as the percentage of those for untreated samples.*

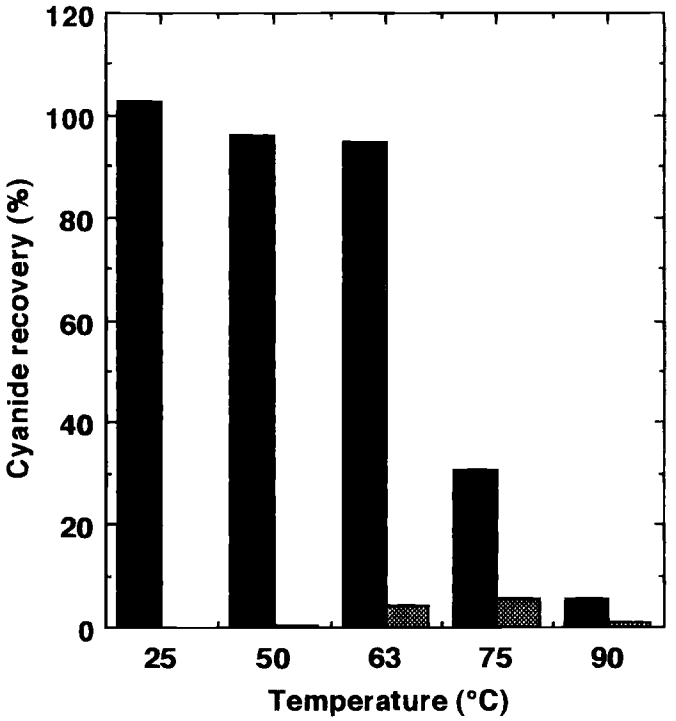

FIG. 2—*Cyanide recovery in heated blood solution. Cyanide concentrations before* (shaded boxes) *and after* (drawn boxes) *phosphoric acid addition were expressed as the percentage of those for untreated samples.*

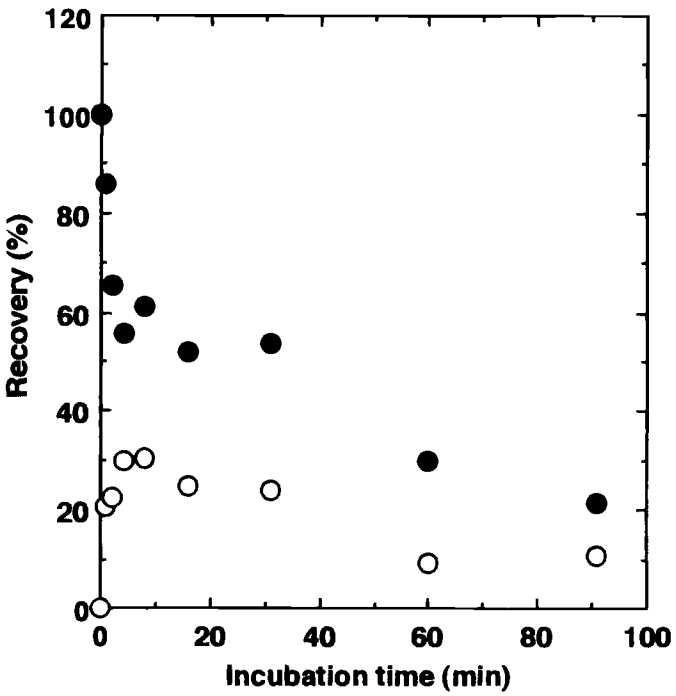

FIG. 3—*Cyanide disappearance in hemoglobin solution during heating. Cyanide (7.7 nmol) was incubated in 0.8 mL solution containing 0.82 μmol heme of Hb and 20 μmol of sodium phosphate (pH 7) at 90°C for a designated time, then quickly cooled to 0°C. After 50°C incubation for 30 min, 0.5 mL of HS gas was injected into the GC. Next, the remaining HS vial was introduced with 0.2 mL of 50% phosphoric acid, incubated at 50°C for 30 min, and 0.5 mL of HS gas was applied to GC. Cyanide concentrations determined by HS-GC before (○) and after (●) phosphoric acid addition were expressed as the percentage of those for untreated samples.*

Similar recovery experiments were performed for the cyanide solutions (200 ng) in buffers with and without bovine serum albumin (15 mg). At 75°C and 90°C heating for 1 h, 90% and 75% of the cyanide were recovered in the buffer solution, respectively. At 75°C and 90°C heating for 1 h in albumin solution, 43% and 9.7% of the cyanide were recovered as the free form, and 49% and 8.9% as the total form, respectively.

Spontaneous Cyanide Production During Heating

A Hb solutions (830 nmol heme) in phosphate buffer (pH 7) in a sealed vial (which was not spiked with cyanide) was heated for 1 h. The cyanide concentrations determined by HS-GC before and after phosphoric acid addition are shown in Fig. 4. Cyanide production in Hb was only observed above 50°C. At 50°C heating, all of the produced cyanide was recovered as the bound form. At 63°C heating, about half was found as the free form, and above 75°C, a considerably larger portion was found as the free form. Under optimal conditions, 4.7 ng (0.18 nmol) of cyanide was produced by heating Hb at 75°C.

As shown in Fig. 5, spontaneous cyanide production in blood (0.2 mL) was also found above 63°C. Almost all of the cyanide was recovered as the bound form. Under optimal conditions, 9.6 ng (0.37 nmol) of cyanide was produced by heating blood at 75°C.

Discussion

The following three postmortem alterations of toxic substances (10) should be considered in forensic toxicology: phase I, a change that occurs between the time of death and the time of sampling; phase II, a change which occurs between the time of sampling and the time of analysis, that is, alteration during sample storage and transfer; and phase III, a change which occurs during analysis.

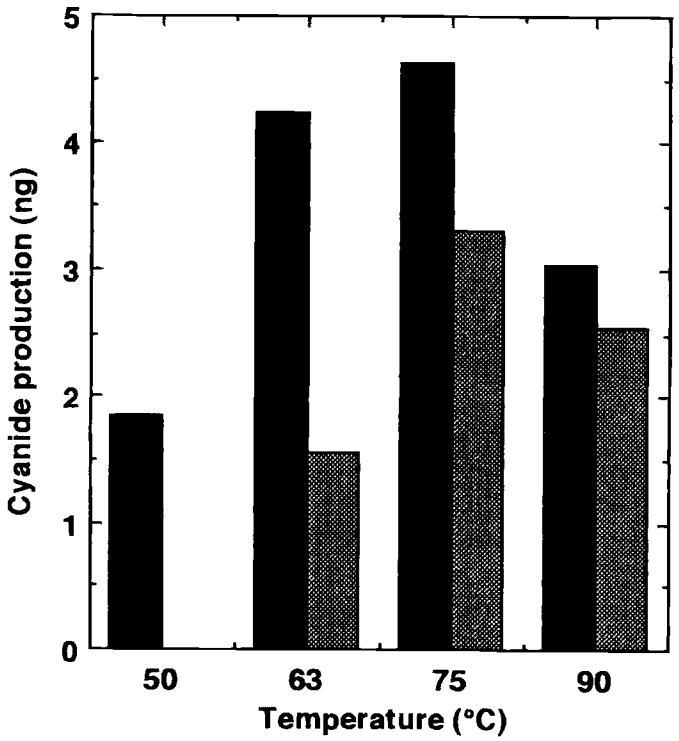

FIG. 4—*Spontaneous cyanide production in hemoglobin solution during heating. Cyanide concentrations were determined by HS-GC before* (shaded boxes) *and after* (drawn boxes) *phosphoric acid addition.*

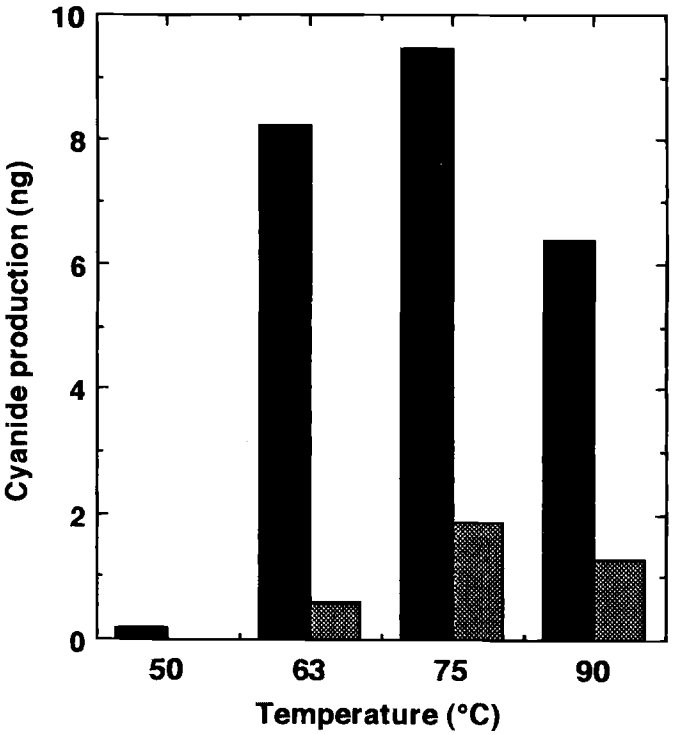

FIG. 5—*Spontaneous cyanide production in blood during heating. Cyanide concentrations were determined by HS-GC before* (shaded boxes) *and after* (drawn boxes) *phosphoric acid addition.*

In contrast, as *in vivo* cyanide alterations, the following molecular events should be considered: metabolism to thiocyanate by mitochondrial rhodanase (11), covalent binding with proteins containing cystine (12) and ligand interactions with cobalamine or heme proteins (1). These changes can be expected to occur, in part, as postmortem alterations. Existence of erythrocytic thiocyanate oxidase activity has also been suggested (13), but this may be an artifact (9). There are also reports alluding to the metabolic conversion of organic nitrile or organic thiocyanate to cyanide in animal tissues (14), but human blood does not appear to support these conversions.

There are many reports about cyanide phase II alterations such as Ballantyne's extensive works (7). Concerning the phase I changes, some laboratories have investigated the effects of heating on blood carbon monoxide concentrations (15,16) and toluene concentrations in eggs (17). However, there appears to be no published reports about cyanide alteration during heating. Most of the cyanide added to whole blood is localized in erythrocytes, and methemoglobin (Met-Hb) is the main component, which traps cyanide in blood (18). When blood is subject to heating, the first observable event is protein denaturation. Oxyhemoglobin is converted to Met-Hb (19), and Met-Hb is readily denatured and precipitates via hemichrome (20), in contrast to stable Met-Hb cyanide complex. This bound form of cyanide can be released by acid treatment (9) or by heating. The liquid phase conditions determine whether released HCN is stable or decomposes. In this paper, HCN was found to be relatively stable in buffer solution. Even after a 90°C incubation, 75% of the added cyanide was recovered. The portion corresponding to HCN loss (25%) may be due to leakage from HS vial or the spontaneous breakdown of HCN. Considerable amounts of cyanide disappeared from both blood and BSA solution heated above 75°C. Cystine found in albumin could scavenge

HCN by covalent binding (12), and this may explain some of the disappearance. In contrast, almost half of the cyanide was recovered in Hb solution heated above 75°C, and the released HCN fraction was found as the free form. The loss in heated Hb solution does not appear to be due to the specific scavenging by cystine, but may be derived from the nonspecific reaction of HCN with peptide chains or hemes. The recovery results in blood can be explained from a combination of ligand binding by Met-Hb and scavenging by albumin. Therefore, it should be concluded that both Hb and albumin are involved in the *in vitro* kinetics of cyanide in blood.

Postmortem cyanide production could give improper estimation of cyanide poisoning from blood analysis. Considerable cyanide production has been reported during storage (phase II change) and the extent depends upon the storage conditions (7). Cyanide is also produced as an artifact from thiocyanate under acidic condition in the presence of erythrocytes (phase III change) (8,21). Superoxide anion radical is produced through oxyhemoglobin acidification and oxidizes thiocyanate to form cyanide (9), and about 15 nmol per 1 μmol thiocyanate can be expected to be produced by 0.2 mL blood. It is possible to prevent thiocyanate oxidation by ascorbic acid (9). It is also thought that, during the phase I change, superoxide anion radical, which is produced through Hb heat denaturation, oxidizes proteins to liberate cyanide. However, the extent of cyanide production via this pathway is quite small. The observable heat-induced production levels (less than 50 ng/mL blood) is far less than the toxic blood cyanide level (1 μg/mL) (22). This suggests that there is negligible influence of spontaneous production on the confirmation of cyanide poisoning for blood samples from burned victims.

It can be speculated, for *in vivo* cyanide behavior during burning, that HCN is released from Met-Hb cyanide complex by heat denaturation and the released HCN diffuses out of blood vessels via plasma during which it encounters albumin. Therefore, there is a possibility that the determined cyanide concentrations for the heat-denatured blood are underestimated due to the postmortem cyanide disappearance.

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