Preservation of Blood Ammonia by Rapid Freezing—A Method for Delayed Determinations

Marshall J. Orloff* and Clarence O. Stevens†

A method is described for preservation of blood for delayed determinations of ammonia by rapid freezing of the samples in a dry ice and acetone solution followed by storage of the specimens in the frozen state. The method was evaluated by first establishing the reproducibility of the chemical method used for blood ammonia analysis in 714 consecutive samples of dog blood which were analyzed in duplicate, and then determining whether freezing caused greater changes in ammonia concentration than one would expect knowing the reproducibility of the chemical method. Studies in 200 samples of dog blood with ammonia levels of 16 to 2904 μg./100 ml., each of which was analyzed immediately and after 24 hr. of freezing, showed that freezing and storage for 24 hr. did not significantly affect the blood ammonia content. Studies of 22 samples of frozen dog blood with ammonia levels of 11 to 2624 μg./100 ml., parts of each of which were analyzed daily for 5 consecutive days, showed that storage in the frozen state for up to 72 hr. had no significant effect on the ammonia concentration, but that storage for 96 hr. or longer resulted in increases in blood ammonia beyond acceptable limits. It is concluded that accurate delayed measurements of blood ammonia may be accomplished by rapid freezing of the blood immediately after shedding, and storage of the frozen samples for up to 72 hr.

The determination of blood ammonia has wide applicability in clinical medicine and research. Nevertheless, many hospital laboratories do not perform ammonia determinations because of difficulties encountered in the collection and analysis of specimens. A major reason

From the Departments of Surgery, Harbor General Hospital, and the University of California, Los Angeles School of Medicine, Torrance, Calif.
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*Markle Scholar in Academic Medicine.
†Postdoctoral Fellow of the United States Public Health Service in the Department of Chemistry, University of Colorado, Boulder, Colo.
for excluding this procedure has been the necessity to perform the analysis within 10 to 30 min. of obtaining the sample, according to presently recommended technics, which imposes too great a burden on both laboratory and clinical personnel and interferes with the routine of a busy laboratory.

In a previous report (17) reference was made to a method developed in our laboratory for the preservation of blood ammonia by rapid freezing of the blood sample immediately after shedding. The method consists simply of immersing the Pyrex glass test tube containing the specimen of blood in a metal container of acetone, the temperature of which has been lowered by prior introduction of dry ice. An ordinary commercial can may be used as the container. In preparing the acetone solution, violent bubbling occurs on contact of the dry ice with the acetone. Chips of dry ice are added until the further addition of ice produces only mild bubbling. At this point, the temperature of the solution is \(-50^\circ\) to \(-70^\circ\). Complete freezing of the blood occurs within 2 min., following which the specimen may be stored in the freezer compartment of an ordinary refrigerator until later analysis. Just prior to the ammonia determination, the sample is removed from the refrigerator and thawed in a water bath at 37\(^\circ\), a process which requires 1 to 3 min.

The technic of rapid freezing has now been used in more than 2000 blood ammonia determinations over a period of 3 years and has proved to be of value in both the hospital and the research laboratory. It is the purpose of this communication to report the findings of a study aimed at comparing the results of delayed determinations of blood ammonia after rapid freezing with those of immediate determinations.

### Materials and Methods

**Sources of Material**

In order to ascertain whether or not freezing and delayed determinations resulted in significant changes in blood ammonia content, it was necessary to determine the range of variation associated under ordinary circumstances with multiple simultaneous determinations on the same sample, i.e., the reproducibility of the chemical method of blood ammonia analysis. This was accomplished by analyzing the differences between duplicate determinations performed on each of 714 consecutive specimens of dog blood taken from records of experiments. These samples of blood had been obtained from normal dogs and from dogs with experimental ammonia intoxication induced by the
intravenous administration of glycine and by the oral administration of ammonium salts to animals with Eck fistulas and portacaval transpositions. Ammonium sulfate was added to a few of the specimens to produce high levels of ammonia. The 714 samples of blood contained levels of ammonia ranging from 0 to 2894 µg./100 ml.

Initial studies were directed at determining the influence on the blood ammonia level of rapid freezing and storage for 24 hr. Two hundred samples of dog blood were obtained by venipuncture and each was immediately divided into two parts. One part was analyzed for ammonia content immediately, and the other part was frozen and analyzed for ammonia content after 24 hr. of storage. The specimens were obtained from normal dogs and from dogs with ammonia intoxication induced by the administration of glycine intravenously. The ammonia levels ranged from 0 to 2904 µg./100 ml.

Additional studies were undertaken to determine the length of time for which blood specimens could be stored in the frozen state without a significant change in ammonia concentration. Twenty-two samples of dog blood were obtained by venipuncture and each was immediately divided into six parts. One part was analyzed for ammonia content immediately and the other five were rapidly frozen and stored. At the end of each 24-hr. period for 5 consecutive days, a part of the sample was thawed and analyzed for ammonia. The blood specimens were obtained from normal dogs and from dogs with ammonia intoxication induced by intravenous glycine, and the ammonia levels ranged from 11 to 2624 µg./100 ml.

Method of Ammonia Determination

The method employed for measuring blood ammonia nitrogen was based on the microdiffusion technic of Conway (8) combined with colorimetric analysis of the diffused ammonia as described by Russell (21). Russell's method represents a modification of a technic originally reported by Van Slyke and Hiller (23) and improved by Borsook (2). It is based on the reaction of phenol with ammonia in the presence of sodium hypochlorite to give a blue color, which can be quantitatively analyzed in a photoelectric colorimeter or spectrophotometer. The method is sufficiently sensitive to allow detection of 0.1 µg. ammonia nitrogen in 1.0 ml. blood.

All blood samples were collected by venipuncture in syringes lightly coated with a fresh heparin* solution, and were immediately trans-

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*Heparin manufactured by Abbott Laboratories was used in all experiments.
ferred to Pyrex glass tubes and sealed with rubber stoppers. Determinations of blood ammonia were initiated within 5 min. of shedding. Similarly, samples subjected to freezing were frozen within 5 min. of shedding and were stored in the freezer compartment of an ordinary refrigerator.

The ammonia was liberated from the blood by treatment in a Conway dish with an alcohol-borate buffer mixture and was collected in 0.02N H₄SO₄ in the center well of the dish. Diffusion was allowed to continue for 1 hr. at room temperature, after which the solution in the center well was treated with manganous sulfate, cold alkaline phenol reagent, and sodium hypochlorite. Maximum blue color was developed by heating in a boiling water bath for 5 min. Analysis of the color was accomplished at 628 mμ in a Klett-Summerson photoelectric colorimeter with filter No. 66. Analysis of an ammonium sulfate standard solution and of a distilled water blank solution subjected to the same treatment as the blood were performed simultaneously with each blood sample.

Since all of the studies were concerned with comparing the results of repeated determinations on the same sample, no correction was made for nonspecific liberation of ammonia owing to the action of alkali on the blood. The method of ammonia analysis described above has no clear advantages over several other methods presently available and the rapid freezing technic may be used with any of the commonly employed analytical procedures.

Method of Statistical Analysis

The reproducibility of the chemical method employed for determination of blood ammonia was established by calculating the differences between duplicate determinations performed on 714 samples and plotting the 714 differences on a distribution curve (Fig. 1). Similarly, the change in blood ammonia after freezing was determined by calculating the difference between the ammonia determination performed immediately, and the ammonia determination performed on the same blood specimen after 24, 48, 72, 96, and 120 hr. of freezing, and these differences were plotted on a distribution curve for each of the 5 freezing periods (Figs. 2 and 3). The curves depicting the distribution of differences in the frozen specimens were then compared with the curve depicting the distribution of differences in the duplicates in order to determine whether freezing caused greater changes in ammonia concentration than one would expect, knowing the reproducibility of
the method used to make the ammonia analysis. This comparison was made according to the relation described by Fisz (10).

Results

Analysis of the results of duplicate determinations of ammonia performed on 714 samples of dog blood showed that the difference in ammonia concentrations between members of a duplicate pair was 30 \( \mu g./100 \text{ ml.} \) or less in 91% of the samples and 35 \( \mu g./100 \text{ ml.} \) or less in 97% of the samples (Fig. 1). The differences between duplicate determinations in the specimens containing high concentrations of ammonia (2001 to 2894 \( \mu g./100 \text{ ml.} \)) were neither greater nor smaller than the differences in the samples with low ammonia levels (0 to 400 \( \mu g./100 \text{ ml.} \)).

Studies of the effects on the blood ammonia level of rapid freezing

![Graph](image-url)
and storage for 24 hr., involving 200 samples of dog blood with ammonia levels ranging from 16 to 2904 μg./100 ml., showed a mean difference between the portions of the specimens analyzed immediately and those analyzed after 24 hr. of freezing of 9.9 μg./100 ml. The differences ranged from 0 to 24 μg./100 ml. Comparison of the 24-hr. difference curve with the duplicate difference curve (or "reducibility of the chemical method" curve) by the Fisz test led to the conclusion that freezing for 24 hr. did not affect the blood ammonia content (Fig. 2 and Table 1).

Studies involving 22 samples of dog blood with ammonia levels

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**Fig. 2.** Differences between immediate determinations of blood ammonia and determinations after 24 hr. of freezing on 200 samples of dog blood. The differences between immediate determinations of blood ammonia and determinations on the same sample after 24 hr. of freezing are plotted on the broken line, and for comparison the duplicate difference curve from Fig. 1, indicating the reproducibility of the chemical method of ammonia analysis, is shown on a solid line.
ranging from 11 to 2624 μg./100 ml., aimed at determining how long blood could be stored in the frozen state without a significant change in ammonia concentration, are summarized in Figures 3 and 4 and Table 1. Regarding the first 24 hr. of freezing, the findings of the previous study were confirmed. Determinations at the end of 24 hr. showed a mean difference from the immediate determinations of 8.4 μg./100 ml. with a range of 0 to 22 μg./100 ml. A comparison of curves indicated that the ammonia level was unaltered during this period of freezing.

The findings were similar at the end of 48 hr. of preservation in the frozen state, when the mean difference between the immediate and delayed determinations was 13 μg./100 ml. with a range of 0 to 27 μg./100 ml.

After 72 hr. of freezing, the mean difference between the immediate

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Immediate determination (μg./100 ml.)</th>
<th>Difference in delayed determination after freezing (μg./100 ml.)</th>
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<td>48 hr.</td>
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<td>77</td>
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and delayed determinations was 19 $\mu$g./100 ml. and 21 of the 22 samples showed a difference of 29 $\mu$g./100 ml. or less. One sample, however, was found to have an ammonia level which had increased 79 $\mu$g./100 ml. after 72 hr. Application of the Fisz test to the 72 hr. group

indicated that freezing for this length of time would not be expected to alter the blood ammonia content.

Following 96 hr. of freezing, 8 of the 22 samples were found to have ammonia concentrations which had increased more than 35 $\mu$g./100 ml. above the levels found upon immediate determination. The mean increase in this group was 43.2 $\mu$g./100 ml. and the range of increase was 1 to 144 $\mu$g./100 ml. It is of interest that 7 of the 8 samples showing a significant increase had blood ammonia levels greater than 500 $\mu$g./100 ml., which suggests that blood containing large quantities of ammonia is not as stable in the frozen state as is blood having the usual small ammonia content. Application of the Fisz test to the 96-hr. group showed that freezing for this length of time failed to preserve the ammonia concentration of blood within the acceptable limit.

After 120 hr. of freezing, 14 of the 22 samples, including 7 in the 11 to 400 $\mu$g./100 ml. range, showed a significant increase in the level of ammonia. The mean increase was 78.7 $\mu$g./100 ml. and the range was 5 to 227 $\mu$g./100 ml. which was clearly beyond the limits of acceptability.
Discussion

Medwedew (13), in 1911, was the first to show the progressive increase in the ammonia levels of shed blood which occurs on standing. Using blood from normal dogs, he demonstrated an increase of ammonia content after 3.5 hr. of standing at room temperature of as much as 540 μg./100 ml., and after 24 hr. the rise was as great as 1400 μg./100 ml. Medwedew attributed the elevation of blood ammonia to "deamidization resulting from ferment action." Subsequently, numerous authors confirmed his observations on blood from a variety of species, including man (1, 3, 4, 9, 14, 18–20).
Conway (4) and Conway and Cooke (5–7) have described the formation of ammonia in shed blood as occurring in three stages. The first stage, which they called “alpha ammonia,” appeared during the first 5 min. after shedding and amounted to approximately 40 μg./100 ml. They believed that this stage resulted from the action on adenosine of a specific deaminase in blood. Several authors have disputed the existence of “alpha ammonia” as well as the proposed underlying mechanism (3,11,19,24). The second stage, which the authors termed “beta ammonia,” began after 5 min. and continued for 3 to 5 hr. after shedding. In the rabbit, “beta ammonia” amounted to 1000 μg./100 ml., and Conway and Cooke proposed that the breakdown of adenylpyrophosphoric acid (ATP) was responsible for its formation. The third stage, called “gamma ammonia,” formed after 4 to 5 hr. of standing and amounted to approximately 350 μg./100 ml. in human blood. The authors suggested that the source of “gamma ammonia” was vegetable adenyllic acid or adenyldeoxyribonucleotide, which underwent a preliminary dephosphorylation and then was deaminated.

The rate of ammonia formation in shed blood has been found to vary greatly from one species to the next (5, 7). In human blood, the rate has been reported as 0.01 μg./ml./min. by Conway and Cooke (7), 0.007 μg./ml./min. by Kirk (12), and 0.003 μg./ml./min. by Seligson and Hirahara (22).

Little attention has been given to the effect of cooling on the formation of ammonia in shed blood, although through the years occasional mention has been made of the retarding influence of reduced temperature upon the processes which lead to evolution of ammonia. Discrepant reports have appeared regarding the influence on blood ammonia of lowering the temperature short of freezing. Rhode (20) and Merchant et al. (14) reported that cooling short of freezing was without effect on ammonia formation, while Nash and Benedict (15), Nathan and Rodkey (16), Jacquez et al. (11), and Reif (19) found that cooling had a definite retarding influence for as much as 2 to 48 hr. Freezing of the blood, on the other hand, recently has been reported to halt ammonia formation for 24 hr. (14).

The results of the present study clearly demonstrate that the ammonia content of blood is preserved without significant change for as long as 72 hr. by rapid freezing and storage of the blood in the frozen state. After 96 hr. or more of storage there is a significant rise in the blood ammonia level. It is recommended, therefore, that analysis of
quick-frozen samples be accomplished within 72 hr. of shedding in order to assure valid measurement of blood ammonia concentration.

Rapid freezing and storage of blood specimens for ammonia analysis overcomes the major objection to presently used methods, namely, the urgency to perform the determination within several minutes of obtaining the blood sample. The equipment required for freezing the specimens is simple and can readily be obtained in most hospitals. A small quantity of acetone, an ordinary commercial can, a bit of dry ice, and a refrigerator are all that is necessary. With the use of the freezing technic, almost any hospital or research laboratory can leisurely perform ammonia determinations at a convenient time most appropriate to the schedule of the laboratory. When used in research studies involving many ammonia determinations, a large number of samples may be collected over several days and analyzed at one sitting.

References