Preparing Standard Solutions of Cyanmethemoglobin

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In 1956 this laboratory was asked to prepare a large quantity of a dilute solution of cyanmethemoglobin to be distributed as a "National Standard" to clinical laboratories for the standardization of hemoglobinometry.[1, 2, 3] Earlier experience on a smaller scale had demonstrated that such solutions of cyanmethemoglobin provided a useful standard of reference,[4] and repeated examination of these first standard solutions indicated that after three years they had faded about one per cent if stored at ice box temperatures and about four per cent if stored at room temperature.

Preparation of large lots of cyanmethemoglobin presented difficulties. However, a satisfactory material was prepared and distributed. The purpose of this communication is to describe the method whereby it was done.

The specifications for the standard solution were these:

1. A solution of cyanmethemoglobin prepared from the hemoglobin of human red blood cells, the concentration to be approximately 60 mg. per 100 ml. as determined by optical density. The formula for this calculation:

$$\text{Hb(mg./100 ml.)} = \frac{\text{MW} \times 0.1 \times \text{D}}{\text{C}}$$

Where MW = molecular weight of Hb, 16,667
D = optical density of the solution determined in the Beckman DU spectrophotometer at a wave length of 540 mμ.
C = Coefficient of extinction, 11.5
"A solution containing one milliatom of hemoglobin iron per liter shall have a millimolar extinction coefficient of 11.5 at a wave length of 540 mμ." (1)
(1) The iron content of Hb was accepted to be 0.335 per cent.*

2. Free of particulate matter (clumps of cellular stroma, etc.) so that centrifugation does not alter the optical density.
3. Free of turbidity.
4. Free of bacterial and other growth.
5. The optical density should remain constant for at least six months.
6. The solution should be dispensed for use in quantities of 25 ml. in amber glass.

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* It has recently been agreed by the International Union of Pure and Applied Chemistry to assign the value 0.338 per cent as the concentration of iron in Hb. The Panel on the Establishment of a Hemoglobin Standard, National Research Council, has recommended the acceptance of this value. The molecular weight assigned to Hb would therefore be 16,520. So far as the Hb standard is concerned, this change of less than one per cent is of no significance.

METHOD

1. For starting material a unit of outdated blood from the blood bank was obtained. The plasma was removed and the red cells were washed three times with equal volume of 0.9 per cent solution of NaCl.

2. When the last of the saline had been discarded an equal volume of cold, distilled water was added to the packed red cells and the mixture was shaken for one minute. Toluol was then added in a quantity equal to 0.4 volume of the washed red cells. The mixture was vigorously shaken for 5 minutes and then centrifuged at 2,000 r.p.m. for 20 minutes at 4 C.

3. A long, slender needle was inserted through the top toluol-stroma layer and the clear solution of hemoglobin was aspirated into another flask. If any sediment was aspirated with the solution it was removed by filtration through paper of medium porosity in a Buchner funnel.

4. The concentration of hemoglobin in the solution was established by the cyanmethemoglobin method,[4] accurately diluting a measured sample with Drabkin's solution in a volumetric flask. The concentration of hemoglobin in the several lots was found to be 10 to 15 Gm. per 100 ml.

5. The standard solution was prepared in 20-liter carboys. The carboys were acid cleaned

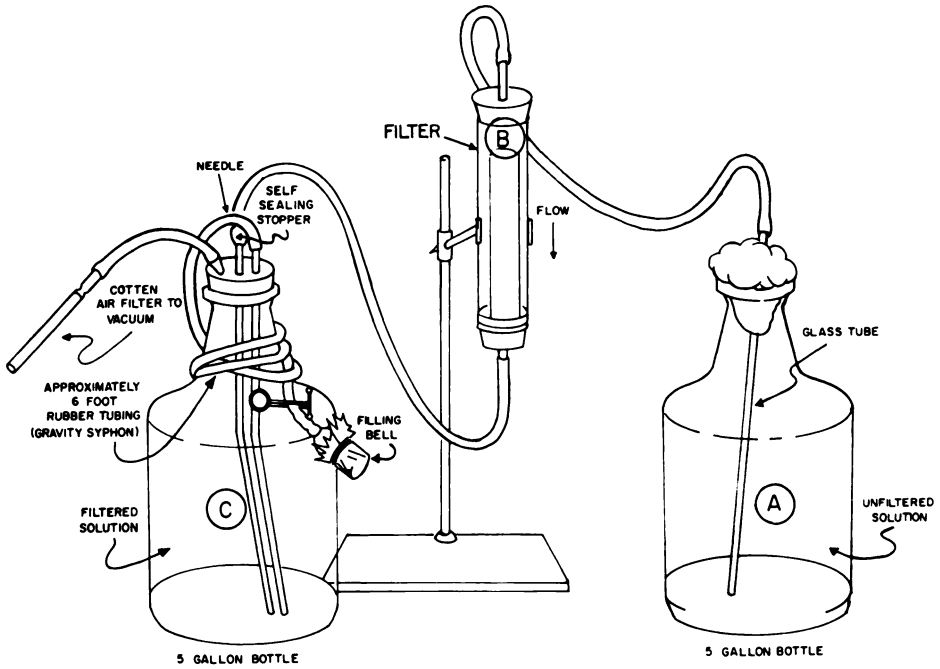

FIG. 1.—The filtration assembly used in the preparation of the Cyanmethemoglobin Standard Solutions.

Filter assembly B consisting of a glass aspirator tube, porcelain filter, and outlet tubing with needle is sterilized in the autoclave as a separate unit from bottles C and A.

Bottle C is sterilized as a unit. The assembly consists of a three hole stopper (size 13) with outlets for a syphon tube for dispensing solution, inlet tube for introducing filtered solution to bottle, and an open filter tube (cotton plugged) which is utilized as an attachment for vacuum during filtration and as a sterile airway during the dispensing operation.

A self-sealing vaccine-bottle stopper is placed on one outlet as a means of introducing filtered solution and withdrawing samples for assay and bacteriological study. Evacuated sleeve-stoppered bottles are utilized for withdrawing pilot samples for studies and assay prior to the dispensing operation. Closed system technic is employed.

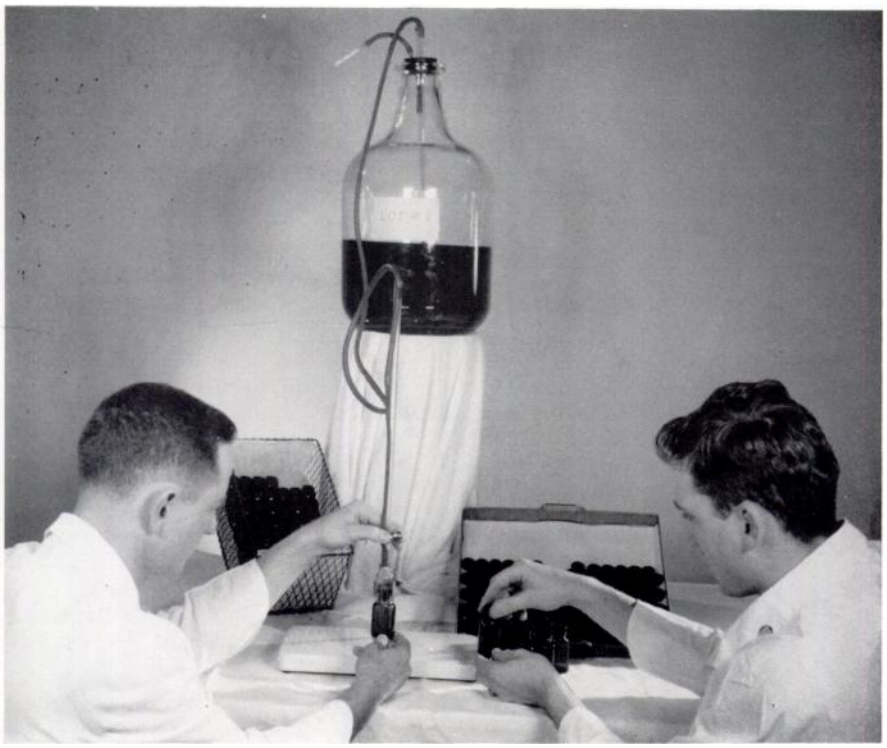

FIG. 2.—Dispensing the Cyanmethemoglobin Standard Solution. The amber glass bottles were autoclaved upside down in the wire basket; their caps were autoclaved in the tray. The bottles are filled under the filling bell and immediately capped.

and thoroughly rinsed. Eighteen liters of distilled water were placed in each. They were then capped with gauze and paper, autoclaved and allowed to cool overnight. The volume had been marked on the side of the carboy and any loss due to autoclaving was made up by adding boiled distilled water. The chemical ingredients of Drabkin's solution were added: 18 Gm. of $NaH\,CO_3$; 0.9 Gm. of KCN; 5.5 Gm. of $K_3Fe(CN)_6$. These were added when the water had cooled to avoid effervescence of the carbonate.

6. When the salts had dissolved, a portion of the hemoglobin solution was added to obtain the desired concentration of cyanmethemoglobin, i.e., 60 mg. per 100 ml. The amount of Hb needed for 18 liters was 10.8 Gm. If, for example, the concentration of the solution (par. 4) proved to be 12 Gm. per 100 ml. it was necessary to add 90 ml. of the Hb solution to the carboy.

7. To avoid the possibility of bacterial or mycotic contamination the cyanmethemoglobin solution was transferred into a second sterile carboy through a closed system (fig. 1) containing a porcelain candletype line filter (Type VFA-88 filter, pore size 0.6 μ. Scientific Glass Co., Bloomfield, N. J.). After 24 hours at room temperature a small amount of the solution was withdrawn for cultures and for measurement of the concentration of cyanmethemoglobin.

8. The standard solution was dispensed into one-ounce, brown-glass prescription bottles that had been autoclaved. As a precaution against contamination a filling bell was used. The sterilized bottles and caps remained upside down until the moment of filling and capping (fig. 2).

9. Each carboy was designated with a lot number. Three lots were prepared for distribution as a "National Standard" and three additional lots were prepared for distribution at scientific meetings. The hemoglobin concentration was established by averaging the spec-

trophotometric results from four laboratories. (D. L. Drabkin, University of Pennsylvania Graduate School of Medicine; A. T. Ness, National Institutes of Health; J. H. Gould, National Bureau of Standards; J. I. Munn, Walter Reed Army Institute of Research).

Lot Number	1	2	3	4	5	6
Drabkin	0.407	0.408	0.422	0.405	0.408	0.412
Ness	0.404	0.402	0.415	0.399	0.402	0.405
		0.403	0.414			
Gould	0.409	0.401	0.418	0.405	0.405	0.410
Munn	0.412	0.410	0.423	0.404	0.404	0.410
		0.415	0.420			
Average	0.408	0.405	0.419	0.403	0.405	0.409
Hemoglobin value mg./ 100 ml.	59.2	58.7	60.8	58.5	58.7	59.4

10. The following controls were carried out:

a. Material from each lot was inoculated into thyoglycolate medium and incubated at 37 C. for nine days before it was said to be free of contamination.

b. Samples of cyanmethemoglobin solution were placed in flame-sealed cuvettes and tumbled end over end 30 r.p.m. for 24 hours at room temperature in order to determine whether there was surface denaturation of the protein. The optical density of the solution was unaffected. Similar cuvettes were shipped to Korea and to Panama and back. The optical density was unaffected. This was not done on material from the six lots mentioned above.

c. Material from each lot was found to be free of turbidity, and centrifugation did not alter the optical density.

d. Material from each lot was stored in amber glass containers at room temperature and at 4C. Its optical density was measured thereafter at intervals.

Date	Lot No.					
	1	2	3	4	5	6
Initial Readings						
11 June 1956	0.412	0.405	0.420	—	—	—
20 July 1956	—	—	—	0.404	0.404	0.410
Stored at Room Temperature						
14 Aug. 1956	0.408	0.399	0.420	—	—	—
6 Dec. 1956	0.410	0.403	0.418	0.404	0.408	0.410
13 Feb. 1956	0.418	0.390	0.390	0.407	0.407	0.407
Stored at 4C.						
14 Aug. 1956	0.413	—	0.420	—	—	—
6 Dec. 1956	0.417	0.409	0.420	0.402	0.408	0.410
13 Feb. 1956	0.422	0.410	0.421	0.415	0.410	0.420

Summary

The method is described whereby solutions of cyanmethemoglobin were prepared for distribution as a National Standard for the calibration of methods for clinical hemoglobinometry. The characteristics of the solutions are given.

Summario in Interlingua

Es describite le methodo usate in preparar le solutiones de cyanmethemoglobina destinate al distribution como Standard National in le calibration del methodos de hemoglobinometria clinic. Le characteristicas del solutiones es date.

REFERENCES

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[4] Crosby, W. H., Munn, J. L., and Furth, F. W.: Standardizing a method for clinical hemoglobinometry. U. S. Armed Forces M. J. *5:* 693, 1954.