Similar incubation studies have been conducted on bone marrow aspirations. As might be expected from the spectrophotometric studies of Thorell relating to hemoglobin synthesis (*6*), the uptake of radioiron by normoblasts is much greater than that observed in reticulocytes. Again, radioactive heme could be demonstrated after incubation of radioiron with these marrow cells.

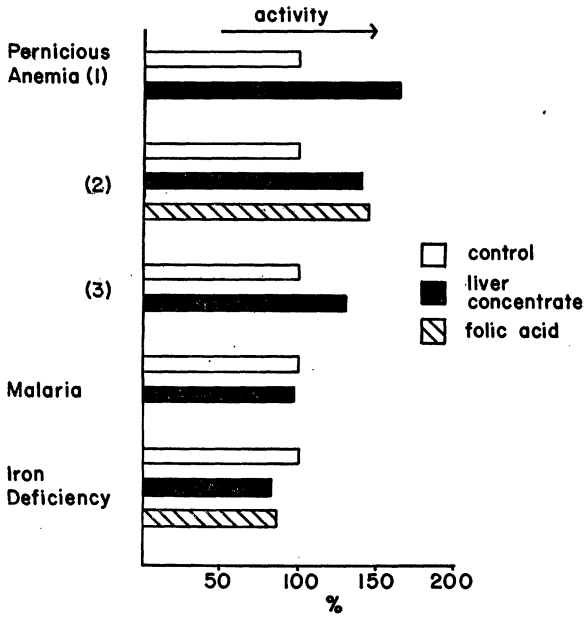

FIG. 2. Marrow incubation. Effect of folic acid and liver. Marrow cells obtained by sternal aspiration from patients with untreated pernicious anemia showed an increase in radioiron uptake of 66%, 44%, and 28% after addition of liver (1×10^{-5} unit) or folic acid (5 μg). This was in contrast to slight decreases found in other conditions not involving a deficiency in these substances.

It is possible to use radioiron as an indicator of altered hematopoiesis, as shown in Fig. 2. Suspensions of marrow from patients with untreated pernicious anemia have repeatedly shown an acceleration of the rate of iron uptake after the addition of liver or folic acid as compared with control studies. This has not occurred in conditions other than those characterized by specific deficiencies of the substances used. These observations on the marrow cells in untreated pernicious anemia indicate that these effective therapeutic agents act directly on the immature erythrocytes.

References

1. FINCH, C. A. *et al.* *J. Lab. Clin. Med.* In press.
2. GEIMAN, Q. M. *et al.* *J. exp. Med.*, 1946, **84**, 583.
3. GIBSON, J. G. II *et al.* *J. clin. Invest.*, 1946, **25**, 616.
4. HAHN, P. F. *et al.* *Science*, 1940, **92**, 131.
5. SURGENOR, D. M., KOECHLIN, B. A., and STRONG, L. E. *J. clin. Invest.*, 1949, **28**, 73.
6. THORELL, B. *Studies on the formation of cellular substances during blood cell production.* London: Henry Kimpton, 1937–1947.
7. VALLEE, B. L., HUGHES, W. L., and GIBSON, J. G. II. *Blood*, 1947, Special Issue No. 1, 82.

Freezing of Whole Blood

Max M. Strumia[1]

John S. Sharpe Research Laboratory
and the Laboratory of Bryn Mawr Hospital,
Bryn Mawr, Pennsylvania

It is a generally accepted hypothesis that freezing and thawing of red cells results in hemolysis. Alternate freezing and thawing is a procedure commonly employed in the laboratory for the purpose of obtaining hemolysis. Preliminary experiments carried out in 1942 showed that the hypothesis is not correct under all conditions, i.e., it is possible to freeze whole blood in a solid mass and thaw it without appreciable hemolysis. "Freezing of blood" is here intended to mean solidification of blood by means of temperatures well below the freezing point of blood.

Early in our experiments, evidence showed that the type of water crystallization resulting from freezing was not an essential factor in the behavior of the red cells. Thus, equally good preservation of red cells was obtained by slow freezing at −3° C or by very rapid freezing at −60° C. In the case of slow freezing, the mass of frozen blood showed formation of large crystals; whereas with fast freezing, the mass of frozen blood appeared very uniform. Fast freezing was carried out as follows: 1 ml of whole human blood collected in an acid-citrate-dextrose mixture was placed in a glass test tube. The blood was frozen by manually rotating the tube in cracked CO_2 ice. Freezing occurred in a few seconds, and the tube containing the solid blood was removed instantly upon solidification and placed in the water bath at 37° C to thaw with the aid of agitation. The hematocrit before freezing was 39.37; after freezing and thawing, 39.07; the supernatant fluid showed no appreciable discoloration from hemoglobin. However, if frozen blood was allowed to remain in contact with CO_2 ice for even a few seconds after freezing, massive hemolysis resulted upon thawing.

Experiments on freezing of whole blood were resumed about a year ago. More than 150 specimens of blood have been frozen and thawed under varying conditions of temperature, heat dissipation (affecting the time of freezing), concentration of electrolytes, pH, concentration of diffusible and nondiffusible sugars (affecting the size of the erythrocytes), etc. In a series of a little over 100 specimens of blood, freezing and thawing were accomplished with a resulting hemolysis of less than 1% of the cells. Most of these specimens were collected in an acid citrate solution, with and without glucose. In these experiments the hematocrit was determined with the use of an air turbine (*2*) and the osmotic fragility by the method proposed by Parpart (*3*). The concentration of hemoglobin in the supernatant plasma was measured by the method of Karr and Chornock (*1*).

It is of practical importance to note that even when freezing and thawing of red cells at −3° C results in

[1] With the technical assistance of Miss Margaret M. Dolan and Miss Louise Colwell.

some hemolysis, the remaining red cells appear to be undamaged.

Experiments have shown that regardless of the mode of freezing of blood, rapid thawing at +37° C in the water bath with agitation is the best method to avoid hemolysis. Results similar to those reported for whole citrated blood may be expected, and have been obtained, with red cells suspended in various media. Much better results have been obtained when red cells are frozen after crenation produced by a hypertonic solution of sucrose, than when red cells are swollen by the addition of a hypotonic solution of glucose.

The preservation of blood in the frozen state is of particular interest for the obvious practical advantages which it offers. In all, about 100 specimens of blood have been satisfactorily preserved for varying periods of time up to 1 month.

The importance of temperature control for proper preservation of blood in the frozen state is best emphasized by the following experiment: 500 ml of whole blood were mixed with 75 ml of chilled anticoagulant solution.[2] Blood was maintained at room temperature for the first 3 hr. It was then distributed in 10-ml aliquots in rubber-stoppered, thin-walled test tubes. A tube (#8) was placed in a rapidly circulating water-alcohol mixture cooled to −14° C. The blood in the tube was agitated and the thermometric readings were as follows:

Time (min)	Temperature (° C)
0	+21
1	+ 6
2	+ 1
3	± 0
4	− 1
5	− 2.4
6	− 0.455

Initial freezing occurred at this point, and the tube was allowed to remain in the cooled bath for an additional 14 sec. While the thermometer still registered about −0.455° C, the tube of semifrozen blood was removed and placed at −3° C in an air cabinet. Within a few minutes at this temperature the blood became completely solid, and was maintained solid at −3° C for 1 hr.

Tube #3 was treated in a similar manner, but after initial freezing at about −0.5° C, the tube was maintained at −14° C for a little over 3 min; this tube then was also placed at −3° C and maintained in the solid state for 1 hr.

A control tube (C) contained whole blood preserved in A.C.D. solution and maintained at +4° C for about 4 days.

Results of hematocrit determinations, and of determinations of hemoglobin in the supernatant fluid, and of osmotic fragility in hypotonic salt solution after thawing are shown in the table and figure.

The results may be summarized as follows: When whole citrated blood is frozen to a solid mass in a cooled circulation bath, removed as soon as freezing has started and placed at −3° C (+0.5) for periods of several hours, minimal hemolysis and minimal changes in the hematocrit and osmotic resistance are noted comparable to that obtained with whole blood preserved in A.C.D. solution at +4° C for 4 days. When frozen blood is allowed to cool for even a very short period of time at temperatures below −3° C, rapid and severe hemolysis occurs.

When whole citrated blood is placed in an air cabinet, cooled at −3° C and allowed to remain undisturbed, it will remain liquid for an indefinite period of time. This has allowed comparative study of blood preserved at −3° C in the solid and in the liquid states.

[2] Standard A.C.D.

TABLE 1

HEMATOCRIT READING AND HEMOGLOBIN OF SUPERNATANT PLASMA OF WHOLE BLOOD FROZEN AND PRESERVED FOR 1 HR AT −3° C IN THE FROZEN STATE

	Hematocrit readings	Hemoglobin in supernatant plasma
Control	36.61	3 mg %
Tube #8*	36.84	6 mg %
Tube #3†	30.40	240 mg %

* Tube #8 was properly frozen and thawed.

† Tube #3 was allowed to remain too long after initiation of freezing in the bath at −14° C. The discrepancy between the hemoglobin in the supernatant plasma and the hematocrit reading is due to the fact that high centrifugal force, developed in the air turbine used for hematocrit determinations, breaks damaged red cells. This discrepancy is always an indication of poor preservation of red cells. The hematocrit of damaged cells is generally difficult to determine and not too reliable.

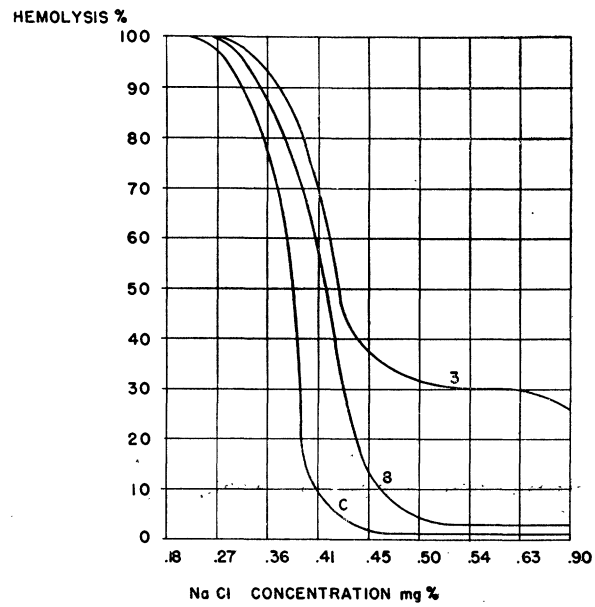

FIG. 1. Fragility of frozen whole blood stored for 1 hr at 3° C in the frozen state.

The results obtained so far in the preservation of frozen and liquid blood at −3° C are sufficiently encouraging to justify further studies, which are now under way.

References

1. KARR, W. G. and CHORNOCK, F. W. *J. clin. Invest.*, 1947, **26**, 685.
2. PARPART, A. K. and BALLENTINE, R. *Science*, 1943, **98**, 545.
3. PARPART, A. K. *et al.* *J. clin. Invest.*, 1947, **26**, 636.

A "Free Manometer" Method of Using the Standard Warburg Apparatus[1]

Avram Goldstein

Department of Pharmacology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole, Massachusetts

In the Warburg apparatus, as commonly employed, a gas reaction occurs within a closed system of constant volume. The temperature being held constant, pressure changes (read on the open manometer arm) are related by a linear function to the volume of gas evolved or taken up. This function is known as the "vessel constant" and depends upon gas-space volume, fluid volume, temperature, solubility of the reacting gas, atmospheric pressure, and manometer fluid density (*1*).

It can be shown that within the limits of accuracy generally accepted in the Warburg technique (±1%), a constant volume is not in fact required; that if both pressure and volume are permitted to vary, a linear function (vessel constant) is still obtained; and that consequently the repeated leveling of manometer fluid, whereby a constant volume is maintained, can be eliminated.

The free manometer technique presents the following unique features: automatic recording, by a time-controlled camera, becomes feasible; certain small errors inherent in the process of leveling the manometer fluid are eliminated; readings can be made more rapidly, more frequently, and with greater ease, and arithmetical steps are greatly simplified; the capacity of the standard manometer is substantially increased, while its sensitivity is correspondingly reduced. A total gas change three to four times as great as by the constant volume method can be measured without resetting the manometer. This increased capacity has been found desirable in at least two applications: in measuring substrate oxidation by cells or tissues in the face of a high endogenous rate, and in studying the protracted time course of enzyme-substrate reactions.

The fluid on the vessel side of the manometer is set initially to the manometer midpoint and subsequent readings are made on this same arm of the manometer. The fluid adjustment is not touched again after the initial setting. A reciprocal and equal change occurs in the fluid levels of the two arms as a reaction proceeds, but the fluid in the open arm is ignored. Thermobarometer corrections, provided they are not exceedingly large (< 5 mm), are made in the usual fashion, but the thermobarometer vessel must contain the same volume of fluid as the other vessels.

If the vessel constants are determined empirically no special problems arise. If they are calculated from mercury calibration of the gas space an additional factor M is required. M is the linear volume of the manometer (cu mm/mm) and is obtained automatically if one follows the calibration method suggested by Burris (*3*, p. 50), filling the manometer first to a point above, and then to a point below the midpoint mark. The full vessel constant equation is:[2]

$$k = \left[\frac{V P_o + V_f R T \alpha_x}{P_o R T}\right]\left[\frac{p_x P_o M}{V P_o + V_f R T \alpha_x} + \frac{p_z P_o M}{V P_o + V_f R T \alpha_z} + 2\right]$$

It should be noted that the only variables requiring calibration are V and M. The observed change in level (mm) on one arm of the manometer multiplied by the vessel constant gives moles of gas reacting at NTP.

The full equation must be used for CO_2, but O_2 and other gases of low solubility can be determined with the following simplified vessel constant:

$$k = \frac{2V + M\,(P_o - p_w)}{RT} + V_f\,\alpha_x\left[\frac{2V + M\,(P_o - p_w)}{V P_o}\right]$$

where p_w is the vapor pressure of water (mm manometer fluid) at temperature T.

The method described here has been in use in this laboratory for some time. Replicate determinations of CO_2 and O_2 by free manometer and constant volume techniques under diverse conditions agree satisfactorily. It has proved convenient to calculate vessel constants by both methods and to use the free manometer technique routinely, reserving the constant volume technique for those occasions when high sensitivity is desired. Full theoretical and practical details of the method will be published elsewhere (*2*).

References

1. DIXON, M. *Manometric methods, as applied to the measurement of cell respiration and other processes.* 2nd Ed. New York: Macmillan, 1943.
2. GOLDSTEIN, A. *Proc. Amer. Acad. Arts Sci.*, 1949, **77**, No. 7.
3. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. *Manometric techniques and related methods for the study of tissue metabolism.* Minneapolis: Burgess, 1945.

[1] The work reported in this paper was done during tenure of a Lalor Fellowship and was also supported by a grant from the Abbott Laboratories, North Chicago, Illinois.

[2] V = gas space (cu mm)
M = manometer factor (cu mm/mm)
$R = 8.21 \times 10^4 \times P_o$
T = absolute temperature
p_x = initial partial pressure of reacting gas (mm manometer fluid)
p_z = initial partial pressure of inert gas (mm manometer fluid)
P_o = atmospheric pressure (mm manometer fluid)
V_f = fluid volume (cu mm)
α_x, α_z = solubility coefficients of the gases (moles/cu mm at P_o, T)