

LIQUID-SCINTILLATION RADIOASSAY OF ¹⁴C-LABELED HEMOGLOBIN IN A THIXOTROPIC GEL

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The use of ¹⁴C-labeled amino acids, particularly of 2-¹⁴C-glycine, to study the metabolism of hemoglobin and its components has been well established (1-4). The small percentage of utilization of this amino acid in hemoglobin synthesis results in low specific activities, a method with high efficiency is desirable to assay blood samples for this isotope.

The use of liquid-scintillation-counting techniques in the radioassay of labeled hemoglobin has had limited value because of the quenching effect of heme and the organic-protein content of globin. Planchette counting with gas-flow systems (2) and liquid-scintillation counting using Van Slyke combustion (5) or a modified Schoniger combustion technique (6,7) have been used. The efficiencies of these systems range from 20 to 80%.

A high-efficiency system is particularly useful for *in vivo* studies of hemoglobin metabolism. Such a system would permit a smaller dose of isotope to be administered and would reduce the amount of blood to be withdrawn from a patient who may already be severely anemic. To carry out a study concerned with the incorporation rate of ¹⁴C-glycine into hemoglobin in a variety of congenital anemias, we have developed a reproducible, simple method for radioassaying ¹⁴C-labeled hemoglobin using a thixotropic gel scintillator. The optimum efficiency of the system is 87.8%.

MATERIALS AND METHODS

The scintillation mixture used contained 7 gm of 2,5-diphenyl-oxazole (PPO), 0.3 gm of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP) and 100 gm of naphthalene dissolved in 1 liter of spectro grade dioxane. Each counting vial contained 12-15 ml of a 5% mixture of the scintillator in the thixotropic gel (Cab-O-Sil, thixotropic gel, Packard Instrument Co.).

Radioassay was performed in a Packard Tri-Carb Model 3000 Series Liquid Scintillation Counter. Settings for this instrument were: Channel A, red, 45-1,000 divisions; Channel B, green, 300-800 divisions. Gain was set at 15% on both channels. Background was 25 cpm for Channel A and 5 cpm

for Channel B. Quenching was determined by the channels ratio system (8). The ratio of the green channel (300-800 divisions) to the red channel (45-100 divisions) was used.

Each subject was injected intravenously with 150-180 μ Ci of sterile 2-¹⁴C glycine dissolved in normal sterile saline. Venous blood samples were then withdrawn at selected intervals.

The erythrocytes were washed in physiological saline and lysed with distilled water; the stromal lipids were separated by adding toluene. The hemolysate was converted to carbomonoxy hemoglobin or cyanmethemoglobin and adjusted to a concentration of 6 gm % with distilled water. The hemoglobin sample was then dialyzed against distilled water for 48 hr to remove any trace of free glycine. A 0.1-ml aliquot of the hemoglobin solution was transferred volumetrically in a calibrated pipette to a glass scintillation vial. Then 0.2 ml of 0.1 normal sodium hydroxide was added to form the alkaline hemochromogen. The pH of the solution was 12.7. The sample was allowed to stand at room temperature for 30 min to insure that the conversion to the hemochromogen was complete. Decolorization was then achieved by adding 0.2 ml of 30% hydrogen peroxide to the mixture. This reaction required a minimum of 12 hr for completion. The caps of the vials were loosely fastened during this time to prevent the accumulation of gas. Before the scintillator was added, the resultant solution was a faint yellow color. Twelve to 15 ml of a 5% mixture of Cab-O-Sil scintillator were added to each vial and thoroughly shaken. The samples were then placed in the liquid-scintillation counter, and counts were recorded with the samples kept at 6°C.

The change in counting efficiency with increasing amount of hemoglobin solution is shown in Table 1. The hemoglobin solution was made as follows: 5 ml of hemolysate (6 gm % concentration) was

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TABLE 1. EFFECT OF THE AMOUNT OF HEMOGLOBIN SOLUTION ON COUNTING EFFICIENCY

Sample No.	Hemoglobin solution (ml)	Hemoglobin (mg)	Counting rate (cpm)	Efficiency*
1	0	0	13,061	88.7
2	0.2	6	12,925	87.8
3	0.4	12	12,727	86.5
4	0.6	18	12,600	85.6
5	0.8	24	12,461	84.7
6	1.0	30	12,240	83.0
7	1.5	45	11,627	79.0
8	2.0	60	11,084	75.3
9	2.5	75	10,517	71.4

* Efficiency = dpm ¹⁴C benzoic acid added/cpm ¹⁴C benzoic acid recorded.
Standard: dioxane gel + 14,720 dpm ¹⁴C benzoic acid.

added to 10 ml of 0.1 N sodium hydroxide and converted to the alkaline hemochromogen. The 10 ml of 30% hydrogen peroxide was added for decolorization. This solution was made in the same proportion as the hemoglobin solution which was used in the experiments described below. Increasing amounts of the hemoglobin solution were added to vials containing the scintillator and a standard of 14,720 dpm ¹⁴C benzoic acid in dioxane gel (Table 1). The counting efficiency was determined by comparing the counts recorded in each vial to the control which contained no hemoglobin solution.

The decrease in counting efficiency that was noted may be attributed to a quenching effect of hemoglobin, sodium hydroxide, hydrogen peroxide and water. The counting efficiency fell from 88.7% when no hemoglobin solution was present to 71.4% when 2.5 ml of hemoglobin solution or 75 mg of hemoglobin was added.

DISCUSSION

In the patients studied, peak specific activities varied from 14 cpm/mg to 55 cpm/mg depending on the isotope dose injected, the blood volume and the erythropoietic status.

Hemoglobin must be maintained in the alkaline state throughout decolorization to prevent loss of radioactive CO₂ from the sample. This was achieved by adding 0.1 N sodium hydroxide to the mixture. The initial pH before decolorization was 12.7; the pH after decolorization was 9.5.

Quenching at the higher hemoglobin concentrations was caused by protein, alkali and water in the reaction mixture and the color of heme- or hemo-

proteins. Dioxane scintillator was used rather than toluene for hemoglobin radioassay because of its greater capacity to maintain counting efficiency in the presence of water. Precipitation does occur when the hemoglobin solution is added to liquid dioxane alone without the thixotropic gel. Therefore the gel was used to suspend the precipitate. The efficiency of the system was 86.5% when not more than 12 mg of hemoglobin was used for each sample.

Pyridine and hydroxide of hyamine, which are basic, were tested in place of sodium hydroxide in the system. They were unsatisfactory because decolorization was less effective after the addition of hydrogen peroxide and thus higher quenching values were found when compared to sodium hydroxide.

SUMMARY

A simple method of liquid-scintillation radioassay of ¹⁴C-labeled hemoglobin in a thixotropic gel is described. The optimal counting efficiency for this system is 86–88%. This method can be used to study hemoglobin metabolism in patients in whom hemoglobin has been labeled *in vivo*.

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