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BINDING OF ^{99m}Tc ION TO HEMOGLOBIN

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The mechanism and preferential site of binding of ^{99m}Tc ion to hemoglobin had been determined by the separation of ^{99m}Tc-hemoglobin from ^{99m}Tc-citrate and ^{99m}Tc-pertechnetate ion with a Sephadex G25 column. This purified fraction was analyzed by the HCl/acetone mixture to determine the ^{99m}Tc activity distribution with heme and globin. Most of the ^{99m}Tc activity is associated with globin fraction. The preferred chain for ^{99m}Tc ion binding was determined by the splitting of ^{99m}Tc-hemoglobin with parachloromercuribenzoate solution followed by separation with a diethylaminoethyl cellulose column equilibrated with phosphate buffer. The ^{99m}Tc ion, like Cr^{3+} ion, tends to bind preferentially with the beta chain of hemoglobin.

Several investigators (*1–5*) reported the labeling of red blood cells with ^{99m}Tc isotope and subsequent uses in determination of red-cell mass and volume and imaging spleen with altered labeled cells. Several peptides and proteins had been labeled with reduced technetium ion. We recently modified the labeling procedure (*6*). The development of the kit method of preparation of ^{99m}Tc-labeled red blood cells will be described elsewhere. In this investigation on ^{99m}Tc-hemoglobin, methods used for the separation of heme from globin, the splitting of the hemoglobin chain with parachloromercuribenzoate and the activity distribution with the hemoglobin units after separating them with the diethylaminoethyl cellulose column (*7,8*) will be described. The site of ^{99m}Tc binding in terms of specific activity in heme and globin and hemoglobin subunit chain had been determined. A degree of similarity was observed in the binding of ^{99m}Tc and ^{51}Cr with the hemoglobin (*9–13*).

MATERIALS AND METHODS

The author's blood collected in ACD solution was centrifuged at 200 G value for 5 min, the supernatant removed, and the red cells were washed free of plasma with isotonic saline solution. The cells were then incubated with a small volume of ^{99m}Tc-pertechnetate in saline solution and subsequently treated with the content of a kit containing mainly stannous citrate and glucose. The method of labeling (*6*) is described below.

Twenty milligrams of $SnCl_2$ were dissolved in 20 ml of ACD solution (Abbott). The solution was filtered with 0.22-micron Millipore filter paper. A 1-ml aliquot transferred to a serum vial was freeze-dried and preserved under nitrogen atmosphere to prevent hydrolysis and oxidation of Sn(II) citrate. The kit was reconstituted with 1 ml of isotonic saline solution, and the content was transferred to a washed red-cell pellet containing suitable amount of $^{99m}TcO_4^-$ ion and incubated for 15 min at 37°C. The free $^{99m}TcO_4^-$ ion was removed by washing with isotonic saline solution. The labeled cells were lysed with water-toluene mixture. The residual insoluble material was removed by centrifugation and filtration. The aliquots of these hemolysates were used for the following analyses.

TCA precipitation. A small fraction of the ^{99m}Tc-labeled hemolysate was precipitated with freshly prepared 10% TCA solution before and after gel filtration. The precipitate was further washed with an equal volume of 10% TCA solution and the activity in the supernatant before and after gel filtration was determined.

Separation of heme from globin before and after gel filtration with Sephadex G25 column. To a 1% hydrochloric acid solution in acetone (v/v) a few drops of mercaptoethanol were added and the solution was stored at −20°C. One milliliter of tagged hemoglobin was mixed with 20 ml of HCl-acetone

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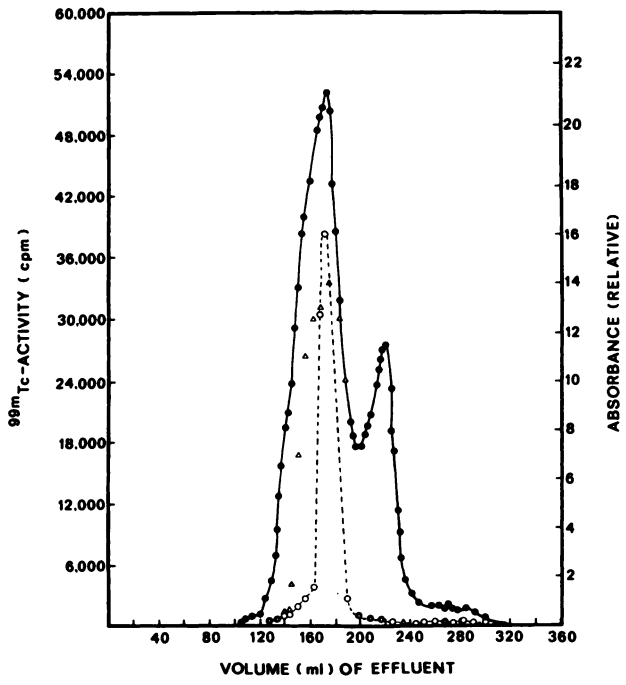

FIG. 1. Elution curve of ^{99m}Tc-hemoglobin on Sephadex G200 column (2.5 cm × 38 cm) and measurement of absorbance at wavelengths of 280 nm (—○—) and 400 nm (—△—) with spectrophotometer. ^{99m}Tc activity —●—.

solution, vortexed for 5 min, and centrifuged for 10 min at 1,120 G value with the Sorvall RC-3 refrigerated centrifuge. The sediment was washed four to five times with 5 ml fractions of cold acetone until a white globin precipitate was obtained. The pellet was dissolved in 8 *M* urea for a constant geometry. The ^{99m}Tc activities in the supernatant, washings, and globin were determined in a well-type NaI(Tl) detector. Hemin was also separated from ^{99m}Tc-hemoglobin with the modified method of Labbe and Nishida (*9*) where a 2% strontium chloride solution in glacial acetic acid mixed with acetone was used for hemin extraction.

Method of separation of alpha and beta hemoglobin chains. Technetium-99m activity associated with hemoglobin was separated by the modified method of Bucci and Fronticelli (*7*) and Jensen, et al (*8*). The ^{99m}Tc-hemoglobin was eluted from G25 column with 0.05 *M* phosphate buffer (pH 5.8). The hemoglobin fraction was equilibrated for 5 min with carbon monoxide. To a 1-ml aliquot were added 0.1 ml of 1.0 *M* sodium chloride solution and 0.1 ml of 1% chloromercuribenzoate in 0.07 sodium hydroxide solution. The solution was stored at 4°C for 24–36 hr. The mixture was centrifuged at 1,120 G value for 15 min. The supernatant was eluted through a Sephadex G25 column and equilibrated with 0.01 *M* phosphate buffer at pH 8.0. A 0.5–1-ml aliquot was placed on DE-52 column (0.9 cm × 12 cm) and the alpha chain was eluted with the same buffer. Subsequently the beta chain was separated with the same buffer containing 0.2 *M* sodium chloride solution. The activities in different fractions were determined in a well counter. A few drops of Drabkin's solution were added to the fractions to convert them in cyanomethemoglobin and the absorbances were measured at 280, 400, and 540 nm with a Carl Zeiss uv spectrophotometer.

RESULTS

A labeling efficiency of 65–85% was obtained in the labeling procedure. The method is independent of collection of blood in ACD or heparin solution and the order of incubation with pertechnetate and stannous citrate solution does not affect the labeling efficiency. Figure 1 indicates that 25–35% of $^{99m}TcO_4^-$ associated with red blood cells could not be removed easily by saline washing although the bulk of the ^{99m}Tc activity was associated with the hemoglobin as observed by spectrophotometric measurements. This observation was further corroborated by the TCA precipitation experiment as well as by the results of HCl/acetone separation method where a higher amount of ^{99m}Tc activity was retained with the heme fraction before the sample was purified by gel filtration. Similar value of globin activity (87%) was obtained by the modified method of Labbe and Nishida (*9*). (10 ± 2) % of the ^{99m}Tc activity was associated with intact hemin. In the TCA precipitation experiment, pre- and post-gel filtration, (60 ±) % and (90 ± 5) % of ^{99m}Tc activity were retained with the hemoglobin. Table 1 also

TABLE 1. ACTIVITY DISTRIBUTION OF HEME AND GLOBIN PRE- AND POST-GEL FILTRATION OF ^{99m}Tc-HEMOGLOBIN WITH SEPHADEX G25 COLUMN

	^{99m}Tc-hemoglobin (cpm)	^{99m}Tc-hemoglobin and impurities (cpm)
HCl acid/acetone (heme)	2,735	9,319
First washing	1,833	4,113
Second washing	193	1,131
Third washing	15	413
Globin pellet-dissolved in cone Urea solution (%) of activity	16,076	21,590
with globin	78 ± 2	60 ± 3
Yield (%)	95 ± 2	94 ± 2

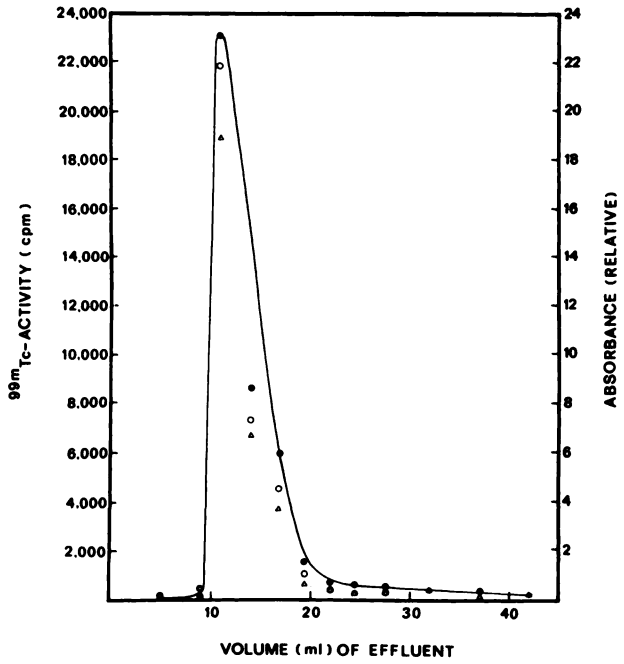

FIG. 2. Elution curve of split ^{99m}Tc-labeled hemoglobin treated with parachloromercuribenzoate on DE-52 column (0.9 cm × 12 cm). Elution was made with 0.2 *M* saline solution; relative absorbances were measured at wavelengths of 280 (—○—) and 400 nm (—△—); ^{99m}Tc activity (—●—).

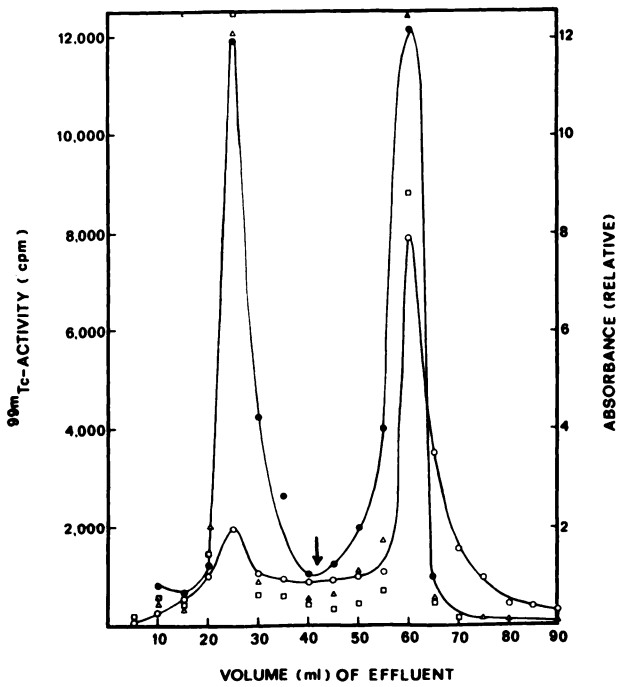

FIG. 3. Elution curve of split ^{99m}Tc-labeled hemoglobin treated with parachloromercuribenzoate on DE-52 column (0.9 cm × 12 cm). Arrow indicates onset of elution with 0.2 *M* saline solution. Relative absorbances were measured at wavelengths of 280 (—□—), 400 (—△—), and 540 mm (—●—), respectively; ^{99m}Tc activity (—○—).

indicates that (78 ± 2) % of ^{99m}Tc activity is associated with globin. Figure 2 indicates that in the absence of suitable Na+ ion gradient, both the alpha and beta chain elute simultaneously. On the other hand, in Fig. 3, the alpha chain is eluted at low Na+ ion concentration followed by the beta chain at higher Na^+ ion concentration. The ^{99m}Tc activity distribution indicates that only a very small fraction of ^{99m}Tc activity is associated with the alpha chain although the concentrations of protein and hemoglobin as measured by spectrophotometric measurements at wavelengths of 280, 400, and 540 nm, respectively, in the two subunit chains are not very different. In the separation procedure with the DEAE column about 40–60% of the ^{99m}Tc activity is eluted and 5–8% of the hemoglobin is not split which appears sometimes as a second band after the separation of the alpha chain.

DISCUSSION

These results indicate that there is a similarity in the nature of binding of technetium and chromium ion to hemoglobin. In the RBC labeling procedures with ^{99m}Tc and ^{51}Cr isotopes, the cells are first incubated with pertechnetate and chromate ion followed by reduction with stannous citrate or ascorbic acid. The nature of binding of Cr^{3+} to hemoglobin had been studied by several investigators. Some aspects of the results are controversial. The reduced Cr^{3+} ion has a preferential tendency to bind with S-atom (*11–13*). Apparently in the incubation period of red blood cells with TcO_4^- ion in saline, TcO_4^- ion readily diffuses through the cell membrane but this activity generally washes out. As stannous citrate in ACD solution is added in the latter phase of labeling, a fraction of stannous ion reduces TcO_4^- ion in the lower valence state, probably Tc(IV), which binds irreversibly with globin. Technetium-99m ion is bound to the beta chain most probably by coordinate covalent bond formation. Although reduced ^{99m}Tc ion is used for binding albumin (*14*) and polypeptide (*15*), not much information is available regarding the nature and site of binding. Though it is known that ^{99m}Tc-HSA is metabolized faster with respect to ^{131}I-IHSA, it is not known whether this higher metabolic rate is due to denaturing of albumin or to ^{99m}Tc-chelation or impurities in human serum albumin. Further investigation by tryptic digestion of long-lived Tc-labeled hemoglobin will determine the exact site of ^{99m}Tc binding. Since not much information is available regarding the binding of ^{111}In, ^{67}Ga, and ^{203}Pb with hemoglobin, these separation methods will prove equally useful for studying the binding of these isotopes with hemoglobin.

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