Survival of ^{125}Iodine-Labeled Factor VIII in Normals and Patients with Classic Hemophilia

OBSERVATIONS ON THE HETEROGENEITY OF HUMAN FACTOR VIII

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ABSTRACT Radiolabeled human Factor VIII was used to study its survival in normals and patients with classic hemophilia, and to study the heterogeneity of Factor VIII. Purified Factor VIII was radiolabeled with ^{125}iodine (^{125}I-VIII) without loss of its structural integrity. The survival of ^{125}I-VIII was studied in six normals and six hemophiliacs of whom four of the hemophiliacs had received transfusions with normal cryoprecipitate before the ^{125}I-VIII infusion. No significant difference was observed between the disappearance of Factor VIII coagulant activity and radioactivity in these hemophiliacs. ^{125}I-VIII in plasma showed a biphasic disappearance with an average $t_{1/2}$ of 2.9±0.4 h (SEM) for the first phase and 18.6±0.7 h (SEM) for the second phase, respectively. The survival of ^{125}I-VIII was similar comparing normals and hemophiliacs.

The highest molecular weight forms of Factor VIII disappear more rapidly than the lower molecular weight ones. This was established by analysis of the fractions obtained by gel chromatography of plasma collected at several times after infusion and by analysis of the in vivo disappearance of three subfractions of Factor VIII. The fraction of ^{125}I-VIII binding to platelets in the presence of ristocetin (containing the highest molecular weight forms of Factor VIII including the ristocetin cofactor) represented about 50% of the radioactivity present in plasma after infusion and showed a $t_{1/2}$ of 11.7±0.9 h (SEM) for the second phase. The fraction, which was recovered in cryoprecipitate of the recipient's plasma, represented about 90% of the initial radioactivity and showed a $t_{1/2}$ of 16.3±0.8 h (SEM) for the second phase. The fraction of ^{125}I-VIII remaining in the cryosupernatant plasma (containing low molecular weight forms of Factor VIII) showed a $t_{1/2}$ of 27.2±1.1 h (SEM). The first phase of the disappearance of ^{125}I-VIII is caused in part by the disappearance of the highest molecular weight forms, which are possibly removed by the reticuloendothelial system.

INTRODUCTION

Abnormalities of human Factor VIII (antihemophilic factor) are primarily associated with two congenital hemorrhagic disorders: classic hemophilia and von Willebrand's disease. Factor VIII was originally defined as the factor correcting the clotting defect in blood of patients with classic hemophilia (1). This coagulant activity (VIII:C)[1] is now recognized to be associated with antigen (Factor VIII-related antigen, VIIIR:Ag), as detected by heterologous antisera (2) and with a factor correcting the prolonged bleeding time (3), the abnormal glass bead platelet-retention test (4, 5) and the defective ristocetin-induced platelet aggregation (6, 7) in von Willebrand's disease (von Willebrand factor, VIIIR:WF).[2]

This work was presented in part at the 54th General Meeting of the British Society for Haematology and the Netherlands Haematology Society, Amsterdam, The Netherlands, October 1976 (1977. *Br. J. Haematol.* 35: 680) and at the VIth Congress of the International Society on Thrombosis and Haemostasis, Philadelphia, June 1977 (1977. *Thromb. Haemostasis.* 38: 51).

Received for publication 19 December 1977 and in revised form 27 February 1978.

[1] *Abbreviations used in this paper*: cryo-VIII, Factor VIII present in cryoprecipitate; DTT, dithiothreitol; ^{125}I-VIII, ^{125}iodine-labeled Factor VIII; PRA-VIII, platelet ristocetin-associated Factor VIII; SDS, sodium dodecyl sulfate; sup-VIII, Factor VIII present in cryosupernate; TCA, trichloroacetic acid; VIII: C,[2] Factor VIII coagulant activity; VIIIR:Ag,[2] Factor VIII-related antigenic activity as measured with a rabbit antiserum; VIIIR:WF,[2] ristocetin cofactor, vonWillebrand factor as measured in an assay using ristocetin.

[2] Abbreviations according to the final report of the Task Force on Nomenclature of Factor VIII-Related Activities, VIth Congress of the International Society on Thrombosis and Haemostasis, Philadelphia, June 1977.

Apart from the complexity of these Factor VIII-related activities, Factor VIII in plasma appears to be heterogeneous. This is manifested by the presence of multiple molecular forms showing differences in Factor VIII-related activities as well as in molecular weight (8–11).[3,4] Factor VIII binding to platelets in the presence of ristocetin (platelet ristocetin-associated Factor VIII: PRA-VIII) has been shown to represent the most highly aggregated forms of Factor VIII, which contain VIIIR:WF.[3] The Factor VIII that is recovered in cryoprecipitate of normal plasma (cryo-VIII) also contains high molecular weight forms of Factor VIII and all VIIIR:WF, whereas the Factor VIII remaining in the cryosupernatant plasma (sup-VIII) consists of the forms of relatively low molecular weight, which have no VIIIR:WF (11).

Studies concerning the survival of Factor VIII in humans have been based initially on the disappearance of VIII:C administered to classic hemophilia patients (12–14). In this way the biphasic nature of the disappearance curve was discovered. The early data showed some discordance, but more recent studies have shown that the $t_{1/2}$s are in the range of about 12–18 h. The introduction of the quantitative assay for VIIIR:Ag using heterologous antisera (2) allowed the analysis of the disappearance of Factor VIII on the basis of VIIIR:Ag. It was then reported that this VIIIR:Ag decreased more slowly than VIII:C after transfusion of normal cryoprecipitate into hemophiliacs (15–17).

Infusion of a trace amount of radiolabeled Factor VIII might allow an accurate analysis of this phenomenon in a different way. Because the VIIIR:Ag represents the main protein constituent of the molecule, one may assume that the disappearance of protein-associated radiolabel will reflect the catabolism of VIIIR:Ag. To our knowledge no data in humans obtained with well-characterized, purified, and radiolabeled Factor VIII have been reported. In this paper we describe the disappearance of ^{125}iodine-labeled Factor VIII (^{125}I-VIII) both in normals and in hemophiliacs. No significant difference was observed in the survival of VIII:C and ^{125}I-VIII in transfused hemophiliacs. Basic catabolism of ^{125}I-VIII was not significantly different in normals and hemophiliacs either. Further analysis made it possible to study the heterogeneity of Factor VIII by following the disappearance of various molecular forms in ^{125}I-VIII: (*a*) the fraction of ^{125}I-VIII that binds to platelets in the presence of ristocetin (^{125}I-PRA-VIII), (*b*) the fraction that is cryoprecipitable when added to plasma (^{125}I-cryo-VIII), and (*c*) the fraction that remains in cryosupernatant plasma (^{125}I-sup-VIII). After infusion, the highest molecular weight forms of Factor VIII as present in PRA-VIII and cryo-VIII disappear more rapidly than the Factor VIII forms of lower molecular weight like those in sup-VIII.

[3] Doucet-de Bruïne, M. H. M., J. J. Sixma, J. Over, and N. H. Beeser-Visser. Heterogeneity of human Factor VIII. II. Characterization of Factor VIII binding to platelets in the presence of ristocetin. Manuscript submitted for publication.

[4] Van Mourik, J. A., and P. A. Bolhuis. Dispersity of human Factor VIII. Manuscript submitted for publication.

METHODS

VIII:C was assayed in a one-stage clotting time assay using the kaolin-activated partial thromboplastin time as described by Veltkamp et al. (18) and modified by Bouma and Starkenburg (19). Frozen (−70°C) reference plasma pooled from four normal donors was standardized against fresh pooled plasma of 40 normal donors (mean age 31.5 yr, ratio women/men = 1, nonpregnant women not taking oral contraceptives). 1 U of VIII:C is defined as the amount of VIII:C in 1 ml of the fresh normal plasma pool of 40 donors. The coefficient of variation was 8.2% (n = 10) (19).

The VIIIR:Ag assay was carried out by the method of Laurell (20) with rabbit antifactor VIII (21) according to Over et al. (11, 22). The dilution of antibody in the gel was chosen to produce precipitation peaks of 3–4 mm height for the eightfold diluted reference plasma. 1 U of VIIIR:Ag is defined as the amount present in 1 ml of a normal plasma pool of 40 healthy donors, frozen and stored at −70°C. The coefficient of variation was 8.0% (n = 36) for a normal plasma (22).

VIIIR:WF was assayed as ristocetin cofactor activity using formalin-fixed platelets according to Macfarlane et al. (23). Ristocetin was obtained from H. Lundbeck & Co. A/S (Copenhagen, Denmark).

Crossed immunoelectrophoretic analysis (24) of VIIIR:Ag was performed according to Over et al. (11).

Radioactivity of ^{125}iodine was counted in a gamma-counter (type 4222, Nuclear-Chicago Corp., Des Plaines, Ill., or type Trigamma 600, Baird Atomic, Inc., Bedford, Mass.) designed to count samples (2–4 ml) in polystyrene tubes (1.4 × 11 cm).

Protein concentration was determined according to Lowry et al. (25) using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a reference.

All chemicals, obtained from commercial sources (E. Merck AG, Darmstadt, West Germany or J. T. Baker Chemicals B. V., Deventer, The Netherlands), were of the highest purity available.

Preparation of ^{125}I-VIII. Purification of Factor VIII was performed essentially as described by Van Mourik and Mochtar (26) with minor modifications. Frozen blood bank cryoprecipitates obtained from four normal donors were dissolved in the residual amount of cryosupernatant plasma, frozen, and cryoprecipitated again by thawing in melting ice overnight. After centrifugation, the pellets were dissolved in saline (pH 7.0) and centrifuged three times. Floating lipids and sedimented insoluble material were removed. The solution was applied to gel chromatography on Sepharose 6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in the presence of dextran (Rheomacrodex Dextran Poviet 10%, mol wt 40,000; Povite Products B. V., Oss, The Netherlands) (26). Freshly packed columns were treated with cryoprecipitate of four donors or 100 ml of normal plasma before use to prevent excessive adsorption of Factor VIII to the gel material. The void volume fractions were pooled and dialyzed against a mixture of 6.5 vol of distilled water and 3.5 vol of a saturated (at 4°C) ammonium sulfate solution, ad-

justed to pH 7.0 with ammonia. The precipitate was spun down (3,500 *g*, 10 min, 4°C) and the supernate was removed except for the last 2 ml. The specific activities of the Factor VIII per milligram protein were 24.7±8.6 U (SD, $n = 14$) of VIII:C, 65.4±17.3 U ($n = 9$) of VIIIR:WF, and 70.9±20.0 U ($n = 11$) of VIIIR:Ag. Recoveries calculated from cryoprecipitate as starting material were 25.3±7.5% (SD, $n = 8$), 24.4±12.1% ($n = 9$), and 24.3±11.8% ($n = 10$), respectively. In most instances the precipitate was radiolabeled immediately after preparation.

Radiolabeling of Factor VIII was achieved with ^{125}iodine by means of the lactoperoxidase method derived from Marchalonis (27). 1 ml of the ammonium sulfate suspension of Factor VIII was centrifuged (10,000 *g*, 2 min, 20°C) in a swing-out centrifuge (type 3200, Eppendorf Gerätebau, Netheler and Hinz GmbH, Hamburg, West Germany). The supernate was removed carefully and the tube was dried with filter paper. The Factor VIII precipitate was dissolved in 0.35 ml of Michaelis (28) buffer (0.0285 M sodium acetate, 0.0285 M sodium barbital, 0.116 M NaCl, pH 7.35). An amount of 100 μl was kept apart for analysis of Factor VIII-related activities and protein content. To the remaining part (±200 μg of Factor VIII protein) 2.5 μl of a lactoperoxidase solution (10 mg/ml, final protein content 25 μg; 67 U/mg, Sigma Chemical Co.), 10 μl of a D-glucose solution (90 mg/ml, final concentration 3.3 mg/ml), and 10 μl of a glucose-oxidase solution (0.1 mg/ml, final protein content 1 μg; 210 U/mg, C. F. Boehringer-Mannheim GmbH, Mannheim, West Germany) were added. All reagents were in Michaelis buffer (pH 7.35). After gentle mixing of the reaction mixture, 1 μl of carrier-free ^{125}iodine (100 μCi; sodium iodide in dilute NaOH, sp act 11–17 mCi/μg iodide, The Radiochemical Centre, Amersham, Eng.) was added. The whole mixture was incubated for 1 h at 20°C with repeated mixing. The solution was centrifuged once (10,000 *g*, 2 min, 20°C), and the supernate was applied to a column (1.6 × 40 cm, Pharmacia Fine Chemicals) packed to a height of 35 cm with Sephadex G 200 (Pharmacia Fine Chemicals; bed vol 70 ml) equilibrated in Michaelis buffer (pH 7.35). Chromatography was carried out at 4°C with a flow rate of 12 ml/h. Radiolabeled Factor VIII was eluted in the void volume, separated from the enzymes and the bulk of free ^{125}iodine. The three void volume fractions (1.8 ml each) were pooled. Specific radioactivity was 3.0±1.4, μCi/U VIIIR:Ag (SD, $n = 7$).

Sterilization of ^{125}I-VIII was achieved by means of filtration through a 0.45-μm filter (type Swinnex-13, Millipore Corp., Bedford, Mass.). The pooled void volume eluate containing ^{125}I-VIII was mixed with a human albumin solution (GPO plasma substitute, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) in a volume ratio of 3:2 before filtration, which was found to prevent retention of ^{125}I-VIII by the sterilization filter. The sterilized material was infused within 15 min after this procedure.

Characterization of ^{125}I-VIII. VIII:C, VIIIR:Ag, and VIIIR:WF were determined at all stages of the preparation procedure. The ratios of VIII:C over VIIIR:Ag and VIIIR:WF over VIIIR:Ag were calculated and used as indicators for the denaturation of Factor VIII. The ratio VIII:C/VIIIR:Ag (0.35 ±0.07 [SD, $n = 10$] in the starting cryoprecipitate) had decreased by about 40% before the radiolabeling of Factor VIII (0.20±0.09), which suggests some denaturation, but in the subsequent radiolabeling and sterilization procedure the ratio remained constant (0.19±0.08). The recovery of the ^{125}I-VIII preparation in vivo (about 72%, see Results) also suggested that some denatured Factor VIII was present. In four preparation procedures, the ratio VIIIR:WF/VIIIR:Ag was determined. No evidence for inactivation of VIIIR:WF was obtained. VIIIR:Ag was completely recovered after radiolabeling and sterilization. The specific antigenic activity (71 U VIIIR:Ag/mg protein) did not change in the radiolabeling procedure.

Crossed immunoelectrophoretic analysis of Factor VIII at various stages of the procedure and of the sterilized ^{125}I-VIII revealed no change in mobility. After autoradiography (Cronex, Lo'dose mammography film, E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.; 2-wk exposure), the radiolabel showed a homogeneous distribution over the immunoprecipitate (Fig. 1).

Gel chromatography of ^{125}I-VIII on Sepharose 2B (Pharmacia Fine Chemicals) was performed as described in the gel chromatography section (see below). No differences were observed between the elution behavior of VIII:C and VIIIR:Ag of the starting cryoprecipitate and that of the radiolabel of the sterilized ^{125}I-VIII.

The distribution of the Factor VIII molecular forms in the ^{125}I-VIII preparation was examined by cryoprecipitation and

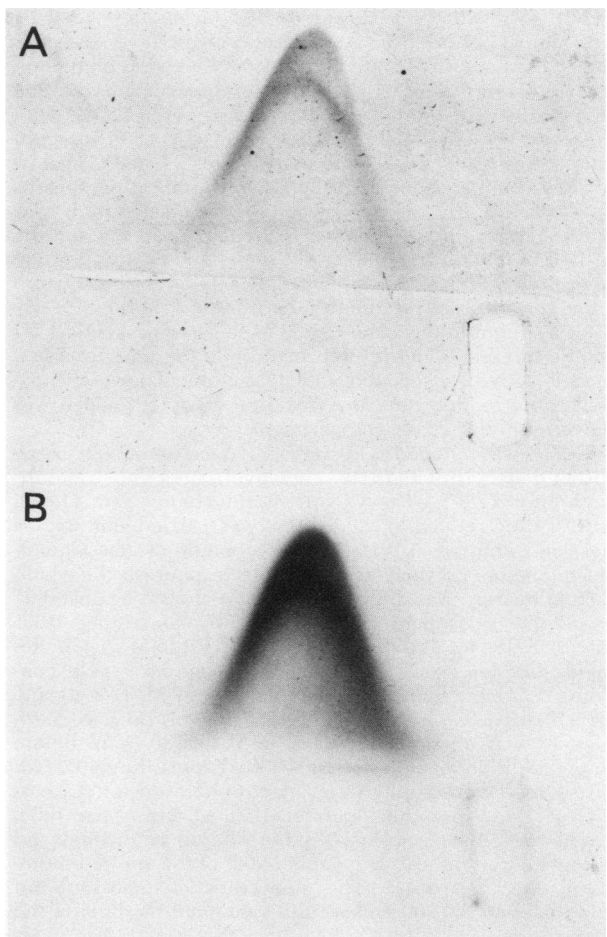

FIGURE 1 Crossed, immunoelectrophoretic analysis of sterilized ^{125}I-VIII. The 50-μl sample contained 0.47 U/ml of VIIIR:Ag with a sp act of 4.07 μCi/U of VIIIR:Ag. Nonlabeled Factor VIII had not been added to the sample. Electrophoresis conditions were according to Over et al. (11). After washing and staining of the gel, an autoradiographic film was superimposed for 2 wk and developed thereafter. (A) Stained immunoprecipitation pattern; (B) autoradiographic pattern.

assay of PRA-VIII (see below). Although the Factor VIII was originally purified from cryoprecipitate and is therefore not entirely representative for the Factor VIII normally present in plasma, cryoprecipitation of normal plasma to which sterilized ^{125}I-VIII was added showed that 12.4±4.6% (SD, n = 9) of the initial radioactivity in plasma (corrected for the presence of free ^{125}iodine, see below) remained in the cryosupernatant plasma. This is not necessarily caused by the presence of a contaminant, because this percentage does not deviate from results obtained after a second cryoprecipitation of normal cryo-VIII, when analyzed for VIII:C.[5]

The PRA-VIII assay showed that 54.8±8.9% (SD, n = 7) of the initial radioactivity (corrected for the presence of free ^{125}iodine) was bound to the platelets. When the platelet-bound fraction (^{125}I-PRA-VIII) and the remaining supernatant fraction were analyzed by gel chromatography on 2% agarose, patterns of radioactivity were observed identical to the patterns of VIII:C and VIIIR:Ag when normal PRA-VIII and its remaining supernatant fraction were analyzed.

Large-pore polyacrylamide gel electrophoresis of nonreduced, sterilized ^{125}I-VIII was carried out as described by Van Mourik et al. (8). Acrylamide, methylene bisacrylamide, ammonium persulfate, and N,N,N^1,N^1,-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Richmond, Calif.). Gels were 4.35% with respect to acrylamide and 0.029% with respect to bisacrylamide. Sterilized ^{125}I-VIII was dialyzed for 2 h at 4°C against 0.01 M sodium phosphate (pH 7.0) before application. Results are shown in Fig. 2. A typical polydisperse pattern was obtained indicating the heterogeneity that exists even in the purified product. After slicing of the gel and counting of the radioactivity, 85% of the applied radioactivity was recovered in the gel, the rest presumably being free ^{125}iodine. Nearly 80% was found in the same region as the stained Factor VIII protein, the rest being present mainly on the top of the gel. No radioactivity associated with low molecular weight material could be detected, free ^{125}iodine excepted, which was recovered in the anode compartment.

^{125}I-VIII was reduced as described by Van Mourik (29) using sodium dodecylsulfate (SDS, 1% wt/vol), urea (8 M), and dithiothreitol (DTT, 1% wt/vol; Calbiochem, San Diego, Calif.). After reduction, alkylation was carried out with a final concentration of 0.11 M iodoacetamide (Sigma Chemical Co.). Before polyacrylamide gel electrophoresis the whole reaction mixture was dialyzed overnight at 20°C against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% (wt/vol) SDS, 0.1% (wt/vol) DTT, and 0.125 M NaCl. Reduced samples were electrophoresed on 6.5% gels containing 1% methylenebisacrylamide in 0.37 M Tris-glycine (pH 9.5), 0.1% (wt/vol) SDS, and 0.1% (wt/vol) DTT. Electrophoresis was carried out at 2 mA for 30 min before sample addition. Samples of 100–350 μl containing 40% (wt/vol) sucrose were applied and electrophoresed for 0.5 h at 20°C at 1 mA per tube and for 1.5 h at 2 mA per tube. Results are shown in Fig. 3. After slicing of the gel and counting of the radioactivity, about 90% of the applied radioactivity was recovered. The characteristic single subunit band of about 270,000 mol wt (29) contained the bulk of the recovered radioactivity.

Solid phase immunoadsorption. The possible presence of a contaminant was further studied by submitting the preparation to solid phase immunoadsorption utilizing a highly specific antibody. The antiserum was raised in rabbits against the slow-moving component (21) obtained by dialysis

[5] Over, J., B. N. Bouma, J. J. Sixma, P. A. Bolhuis, and R. A. A. Vlooswijk. Manuscript submitted for publication.

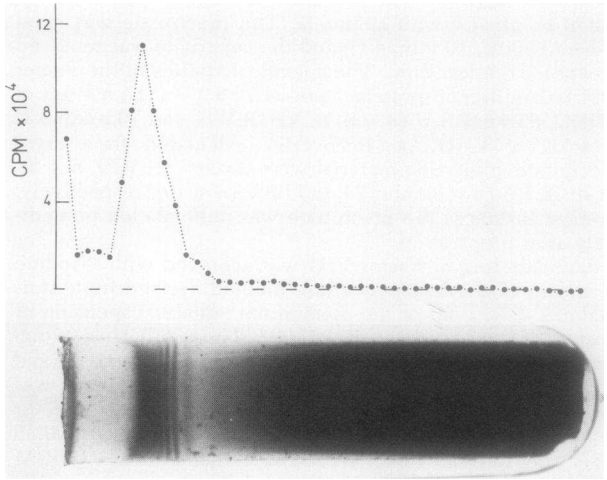

FIGURE 2 Large-pore polyacrylamide gel electrophoresis of nonreduced ^{125}I-VIII. Gel was 4.35 and 0.029% with respect to acrylamide and bisacrylamide, respectively (8). The 350-μl sample contained about 40 μg of radiolabeled Factor VIII protein and about 5 mg of protein of plasma substitute (albumin mainly). Nonlabeled Factor VIII had not been added to the sample. The heavy staining on the right is caused by proteins of the added plasma substitute, which was necessary in the sterilization procedure. After destaining, the gel was immersed in distilled water for 6 h, frozen at −70°C, and cut in 1-mm slices. These were counted for radioactivity (●).

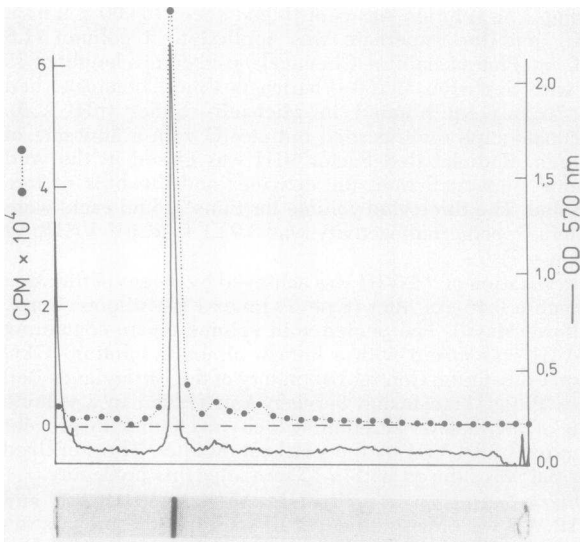

FIGURE 3 Polyacrylamide gel electrophoresis of reduced ^{125}I-VIII. The gel was 6.5 and 1% with respect to acrylamide and bisacrylamide, respectively. Nonsterilized ^{125}I-VIII was reduced with DTT in the presence of urea and SDS (29). The 300-μl sample contained about 15 μg of radiolabeled Factor VIII protein. Nonlabeled Factor VIII had not been added to the sample. The gel was scanned for stained protein bands, sliced (in 2-mm cuts), and counted for radioactivity. The extinction profile (solid line) is depicted together with the radioactivity profile (●).

of highly purified Factor VIII at low ionic strength (8). This particular component of Factor VIII had previously been electrophoresed on polyacrylamide gel (8) making it unlikely that the antiserum was directed also to substances other than Factor VIII, appearing in the void volume of the Sepharose 6B column used for the purification of Factor VIII. The γ-globulins of the antiserum were partially purified and immobilized to Sepharose beads (21). For the control, γ-globulins of normal rabbit serum were used. 1 ml of test sample was added to about 0.5 ml of antibody-coupled Sepharose (in 0.02 M Tris-HCl, pH 7.5, 0.10 M NaCl plus 5.1 mM trisodium citrate). The mixture was gently stirred for 6 h at 20°C and centrifuged (1,000 *g*, 5 min, 20°C) thereafter. The supernate was removed from the Sepharose pellet and assayed for VIII:C, VIIIR:Ag, and radioactivity. The Sepharose was washed five times with normal cryosupernatant plasma to remove nonspecifically adsorbed radioactivity and finally it was washed with buffer. The washed pellet and the supernates were counted for radioactivity. The total amount of radioactivity present in the washings was added to that of the first supernate.

Binding of radioactivity was never complete. This was caused by the presence of free ^{125}iodine (±1.5–12%) as quantified by protein precipitation with trichloroacetic acid (TCA) (see below). Additionally, Factor VIII was never bound completely, as established by measuring residual VIII:C and VIIIR:Ag in the supernate (±10–15%). Some radioactivity bound nonspecifically (±1.5–3%), which was shown by counting the washed control Sepharose. After correction for these values, plasma samples before, and at various times after, infusion (obtained from five infusion experiments) contained between 90 and 108% of radioactivity associated with VIIIR:Ag (mean±SD: 99.4±5.2%, n = 12). No indication was found for a smaller percentage in later (23-h) postinfusion samples as compared to preinfusion or 15-min samples.

Normals and Patients

Normals were six male volunteers, physicians, or biochemists of the Department of Hematology. Patients were four severely affected hemophiliacs and two moderately severe hemophiliacs (W. K.: VIII:C 3%; R. M.: VIII:C 6%). All patients had been treated by us for a long time. No trace of an inhibitor had been demonstrated on any of several occasions. Ages of the volunteers ranged from 25 to 40 yr. The investigation was submitted to the Committee on Medical Ethics of our hospital and approved. The nature, purpose, and possible risks of the study were fully explained to each subject before obtaining his voluntary consent. All persons received an intravenous injection of 200 mg NaI 2–4 h before the injection of ^{125}I-VIII. Lugol's solution, 10 drops three times daily, was used during 1 wk after administration. Four of the patients with hemophilia received cryoprecipitate from 20 donors from the same source as used for purification of Factor VIII. This material was infused during ¾–1 h. ^{125}I-VIII was injected within 15 min after the end of the cryoprecipitate infusion. In general, 15 μl of the infused ^{125}I-VIII solution was mixed with 15 ml of fresh plasma of the volunteers collected before infusion in order to make it comparable to the postinfusion samples. This preinfusion plasma was characterized similarly to the plasma samples obtained after infusion.

^{125}I-VIII was injected intravenously. The quantity administered was determined by weighing the syringe before and after injection. The injected quantities ranged from 0.2 to 3.2 U of VIII:C (mean 1.1 U, n = 11), 3.7 to 16.4 U of VIIIR:WF (mean 8.2 U, n = 4), and 1.2 to 12.3 U of VIIIR:Ag (mean 5.8 U, n = 12). A mean of 14.5 μCi (range 4.1 to 44.7 μCi, n = 12) of ^{125}iodine was infused.

Methods after infusion

Blood collection was performed by separate venipuncture from the cubital vein. Nine parts of blood were drawn into one part of 0.129 M trisodium citrate. From eight individuals, samples were collected at 15 min, 1, 2, 3½, 9, 19, 27, 42, 51, and 66 h after infusion of ^{125}I-VIII. From four persons (K. S., R. M., M. H., and A. M.) blood was also collected at 30 min, 6, 23, and 32 h after infusion. Whole plasma samples (2 ml) were counted for radioactivity immediately after the last blood collection. Platelet-poor plasma was obtained by centrifuging whole blood at 3,500 *g* for 10 min at 4°C.

Correction for free ^{125}iodine. As a few percent of radioactivity appeared to be unbound in spite of the Sephadex G 200 chromatography, fresh platelet-poor plasma (2 ml) at all times of blood collection was treated with 2 ml of 20% (wt/vol) TCA in four infusion studies (K. S., R. M., M. H., and A. M.). In three experiments (C. S., J. M., and A. V.) TCA precipitation was carried out on plasma stored at −70°C. Precipitated proteins were spun down after 10 min (1,800 *g*, 5 min, 20°C). The precipitate and supernate were counted for radioactivity. The radioactivity present in the supernate is referred to as free ^{125}iodine. The percentage was 0.9–4.8% at 15 min postinfusion and maximally 8–12% at late (27–32 h) postinfusion times. This percentage was used for correction of the whole plasma value. Estimation of free ^{125}iodine by means of dialysis against Michaelis buffer (pH 7.35, 5.1 mM trisodium citrate added) confirmed the results obtained by protein precipitation with TCA. The presence of free ^{125}iodine was also reported for bovine Factor VIII (30) and fibrinogen (31).

Assay of ^{125}I-PRA-VIII was performed according to Doucet-de Bruïne et al.[3] An 8-μl aliquot of ristocetin solution was added to 0.5 ml of test sample containing ^{125}I-VIII and thoroughly mixed. Then 0.5 ml of formalin-fixed platelet suspension was added (final concentration of ristocetin 1.6 mg/ml). The mixture was gently tilted, incubated for 10 min at 37°C without stirring, and then quickly centrifuged (10,000 *g*, 2 min, 20°C). The supernate was carefully removed and counted for radioactivity as was the platelet pellet. Two control experiments were carried out. These consisted of mixing test plasma with platelet suspension without adding ristocetin and of mixing test plasma and ristocetin without adding the platelet suspension. The value obtained in the test assay was corrected for this. In preceding experiments the platelet concentration of the suspension had been chosen by titration as just sufficient to remove all VIIIR:WF.[3]

^{125}I-cryo-VIII and ^{125}I-sup-VIII were assayed by means of cryoprecipitation of 2 ml of whole plasma (11) obtained at 15 min, 1, 2, 3½, 9, 19, 27, 42, 51, and 66 h after infusion, and counting of the radioactivity in the precipitate and supernatant plasma, respectively.

Gel chromatography was carried out using Sepharose 2B. When whole plasma or ^{125}I-VIII preparations were chromatographed, the gel was equilibrated in deaerated Michaelis buffer (pH 7.35) containing 1.7 mM trisodium citrate and 38.8 mM ε-aminocaproic acid. In the experiments concerning ^{125}I-PRA-VIII, the gel was in Tris-buffered saline (0.005 M Tris-HCl, 0.15 M NaCl, pH 7.4). Fractions of about 3.4 ml each were collected and counted for radioactivity. Other conditions have been dealt with in detail (11).

Recovery determination. The blood volume for the recovery determination was calculated on the basis of body surface area (using the formula: blood volume [in liters] = 2.7

× [body surface area in m^2] − 0.14 [32]). The formula used for the body surface area calculation was that of Boyd (33). The recovery was calculated from the radioactivity present in the first whole blood sample obtained.

Calculation of $t_{1/2}$. The best fitting curves were constructed by means of a calculator (type 31, Tektronix, Inc., Beaverton, Ore.) programmed for analysis of complex logarithmic functions using an iterative "peeling-off" method (34). At most, two phases of disappearance were assumed to be present.

Statistical analysis of the data was performed with Student's *t* test (for two means as well as for paired data) and Mann-Whitney statistic as described by Snedecor and Cochran (35).

RESULTS

Survival of ^{125}*I-VIII.* Radiolabeled Factor VIII was infused in six normals and six hemophiliacs. After infusion radioactivity was counted in plasma samples collected at several times. A typical example of such an experiment is shown in Fig. 4. The survival of ^{125}I-VIII can be described as a double exponential curve. In the first hours after infusion a rapid disappearance of radioactivity is observed, which is followed by a second phase characterized by a slower decrease. The $t_{1/2}$s of these phases (from now on called first and second phase) were calculated. The results were similar in normals and hemophiliacs. The $t_{1/2}$s of the first and second phase were 2.9±1.3 and 18.6±2.5 h (SD, n = 12; Table I). Because a small percentage of radioactivity in the infused material was present as free ^{125}iodine, the results obtained in three normals and four hemophiliacs were corrected for this. The $t_{1/2}$s did not differ significantly from those without correction (not shown).

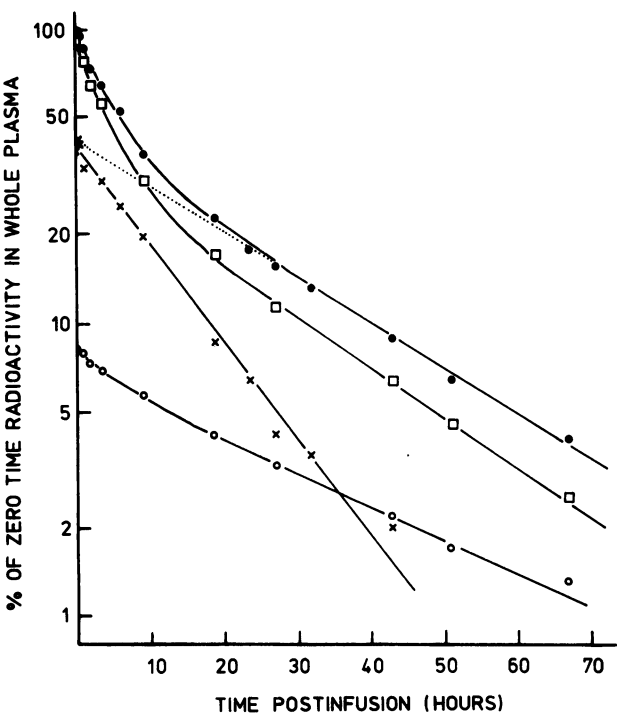

FIGURE 4 Disappearance of ^{125}I-VIII and subfractions of ^{125}I-VIII in vivo. 14 samples of blood were collected at different times after infusion of ^{125}I-VIII (patient M. H.). Radioactivity was measured in whole plasma (^{125}I-VIII, ●), in the platelet pellet after centrifugation of an incubation mixture of whole plasma, platelets, and ristocetin (^{125}I-PRA-VIII, ×), in cryoprecipitate of the plasma sample (^{125}I-cryo-VIII, □), and in the corresponding cryosupernatant plasma after correction for free ^{125}iodine (^{125}I-sup-VIII, ○). The values are expressed as percentage of the intercept on the *y*-axis, obtained by extrapolation of the whole plasma curve (●).

The recovery of ^{125}I-VIII (71.6±12.7% [SD, n = 12], Table I) was lower than is usually reported for VIII:C (14, 36, 37, and Table VI). This may be due to rapid clearance of Factor VIII denatured during the preparation procedure. Although the recovery did not correlate with the VIII:C/VIIIR:Ag ratio, a low recovery was found in the experiments with a low ratio (W. K. and J. W., Table I).

The percentage of ^{125}I-VIII that disappeared in the initial phase (Table I) was computed by extrapolation of both phases to the *y*-axis (see Fig. 4). The values for the normals (41.7±9.0% [SD, n = 6]) seemed to be different from those of the four hemophiliacs, who were transfused with cryoprecipitate from 20 donors (27.8 ±11.7% [SD, n = 4]), but this difference was not significant (*t* test, P = ±0.07).

Survival of ^{125}*I-PRA-VIII.* The survival of ^{125}I-PRA-VIII, i.e. the fraction of ^{125}I-VIII binding to platelets in the presence of ristocetin, was studied. A rapid disappearance of the radioactivity in this fraction was observed. This limited the number of time-points available for computation to 5–8 in most instances. The kinetic data are therefore less reliable than those obtained for whole plasma.

The recovery of ^{125}I-PRA-VIII (mean 69.7%, n = 4) tended to be lower, but was not significantly different from that of total ^{125}I-VIII. The percentage of ^{125}I-VIII that was present as ^{125}I-PRA-VIII was 50.2 ±18.4% (SD, n = 8) at zero disappearance time as established by computed extrapolation of the disappearance curves to the *y*-axis and 46.3±14.5% (SD, n = 8) at 15 min post-infusion. These values were corrected for the presence of free ^{125}iodine.

The disappearance curve of ^{125}I-PRA-VIII was double exponential in most cases with a very short $t_{1/2}$ for the first phase (mean 1.2 h [n = 10] for the first phase and 11.7±3.1 h [SD, n = 12] for the second phase). The survival was shorter than that of total ^{125}I-VIII present in plasma (Fig. 4 and Table II; paired *t* test, $P < 0.01$ for the first $t_{1/2}$ and $P < 0.001$ for the second $t_{1/2}$).

The $t_{1/2}$s of both phases of ^{125}I-PRA-VIII were not different in the normals and the hemophiliacs. Yet, the difference of the second $t_{1/2}$ of ^{125}I-PRA-VIII from that of total ^{125}I-VIII seemed to be smaller in transfused hemophiliacs. This phenomenon was studied by determin-

TABLE I
Recovery and Survival of Total ^{125}I-VIII

	Ratio* VIII:C/VIIIR:Ag	Recovery	$t_{1/2}$ First phase‡	$t_{1/2}$ Second phase‡	In first phase§
		%	*h*	*h*	%
Normals					
J. S.	0.18	71	2.2	15.0	40
C. S.	—	78	1.6	16.1	37
J. O.	0.27	72	4.4	19.7	43
J. M.	0.24	87	2.1	19.0	28
J. W.	0.11	58	4.6	20.2	48
K. S.	0.12	70	2.7	21.1	54
Mean±SD	0.18±0.07	72.7±9.6	2.9±1.3	18.5±2.4	41.7±9.0
Hemophiliacs					
A. V.	0.23	67	1.1	14.0	16
W. K.	0.08	50	1.1	18.8	22
J. Ma.	0.19	60	3.1	17.6	30
R. M.	0.10	86	3.6	21.0	43
M. H.‖	0.18	93	3.6	19.1	58
A. M.‖	0.28	67	4.5	21.8	29
Mean±SD	0.18±0.08	70.5±16.1	2.8±1.4¶	18.7±2.8	33.0±15.2¶

* Ratio of the ^{125}I-VIII preparation infused.

‡ The $t_{1/2}$s were computed from the amount of radioactivity present in 10–14 samples of whole plasma (see Methods). The measured values had a mean relative deviation from the computed curve ranging from 3.6 to 10.8% (mean 5.6%).

§ The percentage removed in the first phase was calculated from the intercept of the computed exponential lines on the *y*-axis (see Fig. 4).

‖ These patients did not receive cryoprecipitate from 20 normal donors before the ^{125}I-VIII infusion.

¶ The values probably are not in a normal distribution.

ing the time needed till only 10% of the initial ^{125}I-PRA-VIII value was left (Table III). This overall disappearance of ^{125}I-PRA-VIII was faster in the normals than in the transfused hemophiliacs (Mann-Whitney, $P < 0.01$). This shorter survival of ^{125}I-PRA-VIII in nontransfused subjects was also apparent when analyzed in a different way. Calculation of the ratio of the second $t_{1/2}$ of ^{125}I-PRA-VIII over that of total ^{125}I-VIII showed that a significant smaller ratio was obtained in the nontransfused subjects (*t* test, $P < 0.005$).

The short survival of ^{125}I-PRA-VIII suggested that the disappearance of this material might be responsible for the rapid first phase of total ^{125}I-VIII (Fig. 4). When the data of ^{125}I-PRA-VIII were subtracted from the ^{125}I-VIII values in total plasma, single exponential curves in all but three experiments (J. S., J. W., and M. H.) were obtained. This suggests that the disappearance of radioactivity in the first phase of the survival curve of ^{125}I-VIII is mainly due to the disappearance of ^{125}I-PRA-VIII.

Survival of ^{125}I-cryo-VIII. The amount of ^{125}I-VIII present as cryo-VIII and as sup-VIII at different times after injection was determined as described in Methods. The recovery of ^{125}I-cryo-VIII was slightly (about 2%) but significantly (paired *t* test, $P < 0.025$) higher than found for total ^{125}I-VIII. The amount of ^{125}I-VIII recovered in cryoprecipitate of the samples at zero disappearance time was 88.8±5.3 (SD, $n = 7$) and 89.6±4.7% (SD, $n = 7$) of 15-min postinfusion samples. These values were corrected for the presence of free ^{125}iodine.

The disappearance curve of ^{125}I-cryo-VIII was double exponential (Fig. 4). The $t_{1/2}$s of both phases were similar in the normals and in the hemophiliacs (mean 2.5 h for the first $t_{1/2}$ and 16.3±2.6 [SD, $n = 12$] for the second $t_{1/2}$, Table IV), but they are shorter than those of total ^{125}I-VIII (paired *t* test, $P < 0.025$ for the first $t_{1/2}$ and $P < 0.001$ for the second $t_{1/2}$).

The percentage of ^{125}I-cryo-VIII removed in the first phase was different between nontransfused and transfused subjects (46.4±9.6% [SD, $n = 8$] and 29.8±14.6% [SD, $n = 4$], respectively; *t* test, $P < 0.05$). The percentage of ^{125}I-cryo-VIII disappearing in the first phase was larger than that of total ^{125}I-VIII (paired *t* test, $P < 0.01$), but equal to that of ^{125}I-PRA-VIII. Also the $t_{1/2}$s of the first phase of ^{125}I-cryo-VIII and ^{125}I-PRA-VIII did not differ. The $t_{1/2}$ of the second phase was larger for ^{125}I-cryo-VIII, however (paired *t* test, $P < 0.001$).

Survival of ^{125}I-sup-VIII. The recovery of ^{125}I-sup-VIII was significantly lower (about 14%; paired *t* test,

TABLE II
Survival of ^{125}I-PRA-VIII

	$t_{1/2}$ First phase*	$t_{1/2}$ Second phase*	In first phase‡
	h	*h*	%
Normals			
J. S.	—	5.0	—
C. S.	1.2	8.2	31
J. O.	1.3	12.8	58
J. M.	0.3	9.6	65
J. W.	0.2	12.7	80
K. S.	0.5	13.1	43
Mean±SD	0.7±0.5§	10.2±3.3	55.4±19.1
Hemophiliacs			
A. V.	1.4	12.3	26
W. K.	1.6	15.3	28
J. Ma.	0.7	12.4	29
R. M.	3.5	15.4	45
M. H.‖	—	9.4	—
A. M.‖	1.7	14.3	28
Mean±SD	1.8±1.0§	13.2±2.3	31.2±7.8

* The measured values of radioactivity had a mean relative deviation from the computed curve ranging from 6.2 to 32.7% (mean 14.5%).
‡ Determined by extrapolation (see Table I).
§ The values are probably not in a normal distribution.
‖ Not transfused (see Table I).

$P < 0.025$) than found for total ^{125}I-VIII. The percentage of ^{125}I-VIII remaining in cryosupernatant plasma was 11.2±5.3% (SD, $n = 7$) at zero disappearance time and 10.4±4.7% (SD, $n = 7$) at 15 min postinfusion. Because of the small percentage these values were corrected for the presence of free ^{125}iodine. This correction was carried out for seven disappearance curves. These were single exponential in four cases. The $t_{1/2}$s of the monophasic curves or of the second phase of the biphasic ones (27.2±2.9 h; SD, $n = 7$) were longer than those of ^{125}I-VIII in whole plasma (Fig. 4 and Table V; paired t test, $P < 0.001$). This suggests that of total ^{125}I-VIII in plasma the higher molecular weight forms as present in cryo-VIII are disappearing more rapidly.

TABLE III
Disappearance of ^{125}I-PRA-VIII

Normals	90% Disappearance time*	Hemophiliacs	90% Disappearance time*
	h		*h*
J. S.	17	A. V.	36
C. S.	23	W. K.	43
J. O.	26	J. Ma.	35
J. M.	18	R. M.	38
J. W.	13	M. H.‡	31
K. S.	33	A. M. ‡	41

* Disappearance is expressed as time required till only 10% of the initial ^{125}I-PRA-VIII remained. The initial value (determined by extrapolation to zero disappearance time on the y-axis) and the 10% point were determined from the computed curves.
‡ Not transfused (see Table I).

TABLE IV
Survival of ^{125}I-Cryo-VIII

	$t_{1/2}$ First phase*	$t_{1/2}$ Second phase*	In first phase‡
	h	*h*	%
Normals			
J. S.	2.3	12.7	43
C. S.	1.3	13.6	41
J. O.	4.3	17.1	43
J. M.	2.1	18.2	35
J. W.	4.3	18.5	55
K. S.	2.3	18.2	53
Mean±SD	2.8±1.2	16.4±2.6	45.0±7.6
Hemophiliacs			
A. V.	1.0	12.0	18
W. K.	0.8	16.3	23
J. Ma.	2.0	13.5	27
R. M.	2.2	17.9	51
M. H.§	2.8	17.7	63
A. M.§	4.5	19.8	38
Mean±SD	2.2±1.4‖	16.2±2.9	36.7±17.5‖

* The $t_{1/2}$s were computed from the data obtained by cryoprecipitation of 10 plasma samples. The measured values of radioactivity had a mean relative deviation from the computed curve ranging from 2.5 to 13.1% (mean 6.1%).
‡ Determined by extrapolation (see Table I).
§ Not transfused (see Table I).
‖ The values are probably not in a normal distribution.

TABLE V
Survival of ^{125}I-Sup-VIII

	$t_{1/2}$ First phase*	$t_{1/2}$ Second phase*	In first phase‡
	h	*h*	%
Normals			
C. S.	1.5	24.6	26
J. M.	—	25.0	—
K. S.	1.2	26.9	55
Hemophiliacs			
A. V.	—	26.7	—
R. M.	—	32.8	—
M. H.§	3.0	25.3	14
A. M.§	—	29.0	—

* The $t_{1/2}$s were computed from the data obtained by cryoprecipitation of 10 plasma samples after correction for free ^{125}iodine. The measured values of radioactivity had a mean deviation from the computed curve ranging from 1.0 to 14.2% (mean 6.5%).
‡ Determined by extrapolation (see Table I).
§ Not transfused (see Table I).

TABLE VI
Recovery and Survival of VIII:C in Four Transfused Hemophiliacs

Hemophiliacs	Recovery	$t_{1/2}$ First phase*	$t_{1/2}$ Second phase*	In first phase*
	%	*h*	*h*	%
A. V.	102 (67)‡	0.5 (1.1)‡	10.8 (14.0)‡	31 (16)‡
W. K.	115 (50)	3.0 (1.1)	14.4 (18.8)	47 (22)
J. Ma.	88 (60)	1.7 (3.1)	17.4 (17.6)	31 (30)
R. M.	87 (86)	4.5 (3.6)	17.9 (21.0)	46 (43)

* See Table I. The measured values of VIII:C had a mean relative deviation from the computed curve ranging from 8.3 to 19.3% (mean 12.0%).

‡ The corresponding values for ^{125}I-VIII in whole plasma (Table I) are given in parentheses.

This should then lead to a relative increase of the percentage of ^{125}I-sup-VIII of total ^{125}I-VIII. Indeed this percentage increased from 10.4 ±4.7% at 15 min to 19.1±5.2% at 19 h and 35.2±11.1% (SD, n = 7) at 66 h after infusion. The $t_{1/2}$s of the second phase for the hemophiliacs were in the same range as in the normals.

Disappearance of VIII:C and ^{125}I-VIII in hemophiliacs. To compare the disappearance of VIII:C with that of ^{125}I-VIII, the VIII:C levels obtained after transfusion of normal cryoprecipitate prepared from plasma of 20 donors (approximately 950 U of VIII:C) into hemophiliacs were determined at the same times as the total radioactivity. Recovery, $t_{1/2}$s, and percentage removed in the first phase are shown in Table VI. The recovery of VIII:C (98.0±13.2%; SD, n = 4) was higher than that of ^{125}I-VIII, although not significantly (paired t test, P = ±0.06). The $t_{1/2}$s of both phases of VIII:C were also not significantly different from those of ^{125}I-VIII (paired t test, P = ±0.06). The disappearance of VIII:C in cryo-VIII and sup-VIII was similar to that of the radioactivity in these fractions in that cryo-VIII:C disappeared faster than plasma-VIII:C and sup-VIII:C showed a longer $t_{1/2}$.

Gel chromatography of ^{125}I-VIII after infusion. For further characterization of ^{125}I-VIII after infusion, plasma samples of each individual collected at several times after infusion were chromatographed on 2% agarose. Elution profiles of ^{125}I-VIII are shown in Fig. 5. Immediately after infusion most of the radioactivity was eluted at or near the void volume. At later times a larger proportion of ^{125}I-VIII was detected in later eluting fractions, suggesting again that the higher molecular weight forms of Factor VIII are disappearing more rapidly.

Crossed immunoelectrophoretic analysis of ^{125}I-VIII after infusion. Autoradiography of the precipitation peaks obtained by crossed immunoelectrophoresis of postinfusion plasma samples was carried out in six individuals (J. M., K. S., A. V., R. M., M. H., and A. M.). The autoradiographic pattern at early times (15 min, 1, and 3½ h after infusion) coincided with the protein

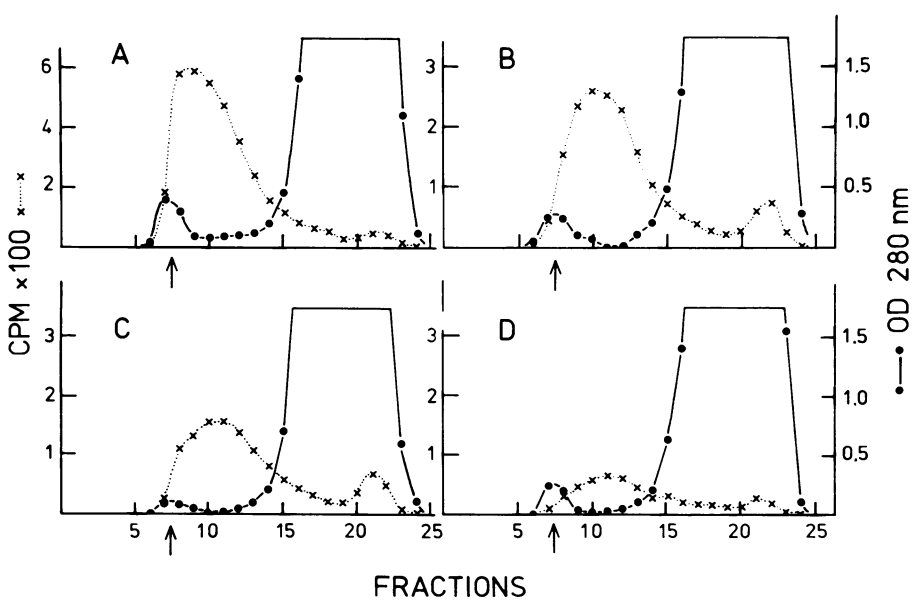

FIGURE 5 Gel chromatographic analysis of ^{125}I-VIII in plasma. Whole plasma (normal person J. W.) was chromatographed on 2% agarose, and the fractions were counted for radioactivity. (A) Elution pattern of ^{125}I-VIII (×) obtained for plasma collected at 1 h after infusion (965 cpm/ml plasma); (B) at 9 h (525 cpm/ml); (C) at 19 h (320 cpm/ml); and (D) at 42 h (120 cpm/ml) after infusion. Recoveries of radioactivity after gel chromatography were 98, 91, 101, and 106%, respectively. Optical density at 280 nm is also shown (●). For details see Methods and reference 11.

staining. In general, the amount of radioactivity in the immunoprecipitate was too small to detect the expected shift of radioactivity toward more anodally migrating forms of Factor VIII at later times after infusion.

Excretion of ^{125}iodine in urine. The urine excretion of ^{125}iodine was followed in five individuals (J. O., J. W., K. S., R. M., and A. M.). A very consistent picture was obtained. The sum of radioactivity in plasma and the radioactivity excreted decreased for 6 to 10 h, but returned to about 70% of the radioactivity infused (71.8±7.1% [SD, n = 5]) after 66 h.

DISCUSSION

Transfusion studies of ^{125}I-VIII into normals and hemophiliacs were undertaken to compare the disappearance of the radiolabel with that of VIII:C and to study the heterogeneity of Factor VIII. Our results indicate that no significant difference was present in the disappearance of ^{125}I-VIII and VIII:C in transfused hemophiliacs nor in the catabolism of ^{125}I-VIII comparing normals and hemophiliacs. Evidence was obtained that the highest molecular weight forms of Factor VIII as present in PRA-VIII and cryo-VIII disappear more rapidly than the lower molecular weight forms, as present in sup-VIII.

The overall shape of the disappearance curve of ^{125}I-VIII (Fig. 4) in hemophiliacs as well as in normals conformed to the disappearance of VIII:C in the hemophiliacs and to the data reported in the literature (12–14, 36, 37). In the first hours after infusion a rapid disappearance of radioactivity was observed, followed by a second phase characterized by a slower decrease. The $t_{1/2}$ of this phase was slightly longer than usually reported for VIII:C in transfused hemophiliacs (12, 13, 36, 37). In this study also the $t_{1/2}$ of the second phase of VIII:C seemed to be shorter (Table VI). The curves were so similar, however, that we feel that this difference is of no importance in comparison to the data reported by others for the half-disappearance time of VIII:C and VIIIR:Ag in transfused hemophiliacs (15–17). Analysis of the survival of VIIIR:Ag in the four transfused hemophiliacs in this study showed that in A. V. and R. M. the disappearance was similar to that of ^{125}I-VIII (second $t_{1/2}$: 15 and 17 h, respectively). In W. K. and J. Ma., a $t_{1/2}$ of 45 and 54 h, respectively, was found. These discrepancies were probably caused by the low accuracy of the measurements, which was presumably due to the high baseline level of VIIIR:Ag. Therefore, the data do not permit a conclusion concerning the survival of VIIIR:Ag.

The first phase is at least in part due to the disappearance of the highest molecular weight forms of Factor VIII. Evidence hereof was obtained from the observation that ^{125}I-PRA-VIII disappeared faster than ^{125}I-VIII (Fig. 4, Tables I and II), and subtraction of ^{125}I-PRA-VIII values from ^{125}I-VIII values produced in most cases a single exponential curve that conformed largely to the second phase of ^{125}I-VIII. Previous studies had shown that PRA-VIII consists of the most highly aggregated forms of Factor VIII, possessing all VIIIR:WF present in plasma.[3]

In addition, by means of cryoprecipitation of plasma of the recipient at different times, it was shown that ^{125}I-cryo-VIII (Table IV) had a shorter $t_{1/2}$ than total ^{125}I-VIII (Table I), and ^{125}I-sup-VIII lived longer (Fig. 4, Table V). Furthermore, survival curves of ^{125}I-sup-VIII tended to be monophasic. Previous studies from our laboratory have shown that sup-VIII represents forms with lower apparent molecular weight without VIIIR:WF (11). This suggested again that the higher molecular weight forms disappear faster. This was further substantiated by gel chromatographic analysis, which showed a preponderance of smaller forms at later times (Fig. 5). In addition, the observation that VIII:C in cryosupernatant plasma of the transfused hemophiliacs also had a longer $t_{1/2}$ than VIII:C in whole plasma or cryoprecipitate supported the conclusion of a longer survival of sup-VIII.

An alternative explanation for the biphasic nature and the longer survival of lower molecular weight forms of Factor VIII might be a continuous conversion of higher molecular weight forms into forms of lower molecular weight. Some indication hereof was obtained in the hemophiliacs A. V., R. M., and A. M., who showed a slight absolute increase (A. V. and R. M.) or a constant amount (A. M.) of ^{125}I-sup-VIII during 19, 9, and 3½ h after infusion, respectively. Comparison of the ratio of the $t_{1/2}$ of ^{125}I-sup-VIII over that of total ^{125}I-VIII showed that this ratio was higher for the transfused hemophiliacs A. V. and R. M. as compared to the other five persons (three normals and two nontransfused hemophiliacs, Tables I and V). This observation was supported by results obtained in similar studies with von Willebrand's disease patients.[6] This higher ratio would suggest that transfusion decreased the removal of ^{125}I-sup-VIII. However, the absolute increase in ^{125}I-sup-VIII, in combination with the longer overall survival of ^{125}I-PRA-VIII (Table III), supported an alternative explanation. A previous transfusion with cryoprecipitate may decrease the removal of high molecular weight forms, like PRA-VIII. Thus, the concentration of these forms thereby decreases more slowly. This will then lead to an absolute or relative increase of lower molecular weight Factor VIII forms, like sup-VIII.

The conversion process, however, if really existing, will most certainly not be the only one responsible for removal of high molecular weight forms, as the overall disappearance of ^{125}I-VIII showed that a large amount

[6] Over, J., J. J. Sixma, B. N. Bouma, R. A. A. Vlooswijk, and N. H. Beeser-Visser. Manuscript in preparation.

of radioactivity was rapidly eliminated from the circulation (Fig. 4). The urine values also indicated that a certain amount of radioactivity was sequestered in the body. A possible location of the compartment into which high molecular weight forms may disappear is the reticuloendothelial system. This concept was supported by the more rapid removal of the highest molecular weight forms and by the observation that a preceding transfusion of cryoprecipitate decreased the amount disappearing in the first phase for ^{125}I-VIII and ^{125}I-cryo-VIII and that it increased the overall survival of ^{125}I-PRA-VIII. However, the latter effects were not highly significant ($P = \pm 0.07$, $P < 0.05$, $P < 0.05$, respectively), but results obtained in von Willebrand's disease patients[5] supported this concept. This effect of a previous transfusion suggests that the compartment was saturable. In this respect, it is of particular interest that the disappearance of VIII:C is monophasic in multiply transfused (13) or continuously transfused (38) hemophiliacs.

The concept of the reticuloendothelial system being responsible for the first phase is supported by a recent study of Sodetz et al. (39), who infused ^{125}iodine-labeled human Factor VIII into rabbits. Blocking of the reticuloendothelial system diminished the percentage removed in the first phase. It was also found that a plasma sample containing ^{125}I-VIII, obtained from a rabbit at a time after which the first rapid removal (of the highest molecular weight forms?) had occurred, did not show a significant first phase again, when reinfused into a second rabbit. In addition, this experiment excluded the possibility of an equilibrium-distribution phenomenon toward extravascular compartments. The high molecular weight nature of Factor VIII is probably a serious impediment for passage to the extravascular space. Although it was found that Factor VIII may be bound by platelets to some extent (40–42),[7] no Factor VIII was taken up by the platelets of transfused, severe von Willebrand's disease patients (43–45). Similarly, no Factor VIII was detected in the endothelium after transfusion of such patients (46). As a consequence, the extravascular compartment must be very small for Factor VIII.

The loss of about 30% of radioactivity within the first 15 min after infusion could not be accounted for merely by removal of the highest molecular weight forms, as was established by extrapolation of the survival curves to zero disappearance time. This may be caused by rapid clearance of some denatured Factor VIII, present in the ^{125}I-VIII preparation. The presence of free ^{125}iodine also contributes to a low recovery, due to its large distribution volume.

In conclusion, no basic difference in the survival of ^{125}I-VIII existed between normals and hemophiliacs. Our data show a preferential removal of high molecular weight forms of Factor VIII, as can also be inferred from studies in transfused swine with von Willebrand's disease (47). The long survival of the low molecular weight forms as present in sup-VIII may be a real phenomenon, but it may also be caused by continuous generation of low molecular weight forms like sup-VIII, from higher molecular weight forms, like PRA-VIII and cryo-VIII. The data do not allow, however, a choice between the two alternatives. Direct estimation of the $t_{1/2}$ of sup-VIII by infusion of this material will be needed to solve this question, which is of importance for the relation between the various Factor VIII forms present in plasma.

[7] Doucet-de Bruïne, M. H. M. Unpublished observation.

ACKNOWLEDGMENTS

Appreciation is expressed to the volunteers for their enthusiastic participation in the study. The authors also wish to thank Doctor J. M. M. Roelofs, Department of Medical Physics, University Hospital, Utrecht, for his help in the computer analysis of the data.

The Foundation for Medical Research FUNGO, which is subsidized by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.), is acknowledged for financial support (grant 13-30-02A).

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