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Radioenzymic Determination of Homocysteine in Plasma and Urine

Helga Refsum, Svein Helland, and Per M. Ueland

Using a modification of the radioenzymic assay described previously (*J Biol Chem* **259**: 2360–2364, 1984) we measured homocysteine in freshly prepared plasma and urine from volunteers. The concentration of free homocysteine—i.e., the amount measurable in plasma after deproteinization by strong acid—was 2.27 (SEM 0.11) μmol/L for 18 men and 1.95 (SEM 0.13) μmol/L for 16 women ($p > 0.05$, not significant). About 70% of the total homocysteine in human plasma was associated with plasma proteins, and was precipitated with strong acid. The concentration of protein-bound homocysteine in plasma was 6.51 (SEM 0.32) μmol/L for men and 7.29 (SEM 0.65) μmol/L for women, a significantly ($p < 0.01$) different spread. Homocysteine was rapidly released from plasma proteins in the presence of a reducing agent, dithioerythritol. By gel filtration of plasma on a "high-performance" liquid-chromatographic column, albumin was shown to be the sole carrier of homocysteine in plasma. Because the fraction bound to protein as determined by this procedure equaled that obtained by precipitation of plasma proteins with acid, we conclude that homocysteine is bound to albumin in vivo. The concentration of homocysteine in urine ranged from 3.5 to 9.5 μmol/L, about 6 μmol of homocysteine being excreted per 24 h.

Additional Keyphrases: *reference interval · sex-related differences · free and protein-bound amino acid · heritable disorders*

Homocysteine is a metabolite of the endogenous transmethylase inhibitor, *S*-adenosylhomocysteine (*1, 2*). This amino acid is further metabolized to methionine via a pathway that in most tissues requires 5-methyltetrahydrofolate as the methyl donor (*3*). Homocysteine metabolism may thus be affected by treatment with an anti-folate drug, such as methotrexate, and this has been suggested to play a role in the hepatotoxic effect observed with this drug (*4*). Exploitation of homocysteine metabolism in the management of malignant diseases has been considered, because certain malignant cells do not grow in a culture medium in which methionine has been replaced with homocysteine (*5*).

The inherited disease homocystinuria has been ascribed to deficiencies of enzymes involved in homocysteine metabolism (*6*). Patients with this disease suffer from thromboembolism, and homocysteine is a possible atherogenic agent (*7*). Higher concentrations of homocysteine in plasma of post-menopausal women than in that of pre-menopausal women have been related to the increasing incidence of coronary artery disease in women at old age (*7, 8*).

The possible clinical importance of homocysteine makes it pertinent to determine the amount of this amino acid in plasma and urine. Recent reports (*8–13*) describe low concentrations of homocysteine in plasma under physiological conditions, but the published values vary, and little attention has been paid to the possibility that homocysteine might be associated with plasma proteins in vivo (*12*). Here we report our findings on the amount of homocysteine in human plasma and urine, and we demonstrate that most of the homocysteine in plasma forms an albumin–homocysteine mixed disulfide.

Materials and Methods

Materials

L-Homocysteine thiolactone, DL-homocysteine, and dithioerythritol were from Sigma Chemical Co., St. Louis, MO. [8-^{14}C]Adenosine (0.59 kCi/mol) was from Amersham International, Amersham, Bucks, U.K. 2′-Deoxycoformycin was a gift from Parke-Davis Research Laboratories, Ann Arbor, MI. Columns (0.46 × 10 cm) for reversed-phase liquid chromatography were packed with 3-μm ODS Hypersil (Shandon Southern Products, Cheshire, U.K.), as described previously (*14*). *S*-Adenosylhomocysteine hydrolase (EC 3.3.1.1) was purified from mouse liver as described (*15*).

Procedures

Preparation of plasma. Blood was collected by venipuncture in the morning after overnight fasting from 18 men and 16 women volunteers, ages 25 to 55 years. Whole blood was collected in Vacutainer Tubes containing EDTA, cooled on ice, and immediately centrifuged at 2000 × *g* for 5 min at 0–2 °C. One part of the EDTA-treated plasma was frozen in liquid nitrogen and stored at −20 °C until assay. Another part was immediately diluted fourfold with ice-cold perchloric acid, 0.8 mol/L, containing 10 mmol of EDTA per liter. The precipitated protein was removed by centrifugation, and the supernate was neutralized to pH 7.5 with a solution containing 1.44 mol of KOH and 1.2 mol of $KHCO_3$ per liter, then centrifuged to remove the insoluble potassium perchlorate.

Determination of total homocysteine in plasma. When not otherwise stated, we routinely diluted plasma fourfold with potassium phosphate buffer (100 mmol/L, pH 7.4), added dithioerythritol and 2′-deoxycoformycin (final concentrations 10 mmol/L and 1 μmol/L, respectively), and incubated for 10 min at 37 °C. Then homocysteine in plasma was condensed with [^{14}C]adenosine to form *S*-[^{14}C]adenosylhomocysteine in the presence of *S*-adenosylhomocysteine hydrolase (9 U/L), as described in detail previously (*16*). *S*-[^{14}C]Adenosylhomocysteine was isolated and quantified by "high-performance" liquid chromatography (HPLC) (see below) after precipitation of plasma proteins with perchloric acid.

Determination of free homocysteine in plasma. The neutralized supernate from plasma deproteinized with perchloric acid was incubated with dithioerythritol, 10 mmol/L final concentration, for 10 min at 37 °C. Then homocysteine was converted to *S*-[^{14}C]adenosylhomocysteine and quantified as described above and detailed in ref. *16*.

Determination of homocysteine in urine. Urine was also diluted fourfold with potassium phosphate buffer and processed as described for free homocysteine in plasma.

Quantification of S-*[^{14}C]adenosylhomocysteine by re-*

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versed-phase liquid chromatography. Samples of 90 µL were injected into the ODS Hypersil column equipped with a 2.5-cm guard column. The columns were eluted with a 3 to 90 mL/L gradient of methanol in acetate buffer (15 mmol/L, pH 4.5) at a flow rate of 3 mL/min, as detailed previously (*16*). The *S*-[^{14}C]adenosylhomocysteine eluted from the column was collected by a programmable fraction collector equipped with a peak separator (*16*). *S*-[^{14}C]Adenosylhomocysteine was determined by liquid scintillation counting.

Gel filtration of human plasma. We injected 25 µL of plasma, prepared at 0–2 °C, into an I-250 HPLC protein column (Waters Associates, Milford, MA), equilibrated and eluted at a flow rate of 0.8 mL/min with Tris HCl (50 mmol/L, pH 7.4) containing 100 mmol of KCl per liter. The protein standards (bovine serum albumin and myoglobin) were chromatographed under the same conditions. To determine the total volume of the column, we injected a low-M_r compound (e.g., *S*-adenosylhomocysteine). The absorbance of the effluent was recorded at 280 nm with a variable-wavelength detector (Kratos, Model Spectroflow 773).

Sephadex G-100 chromatography. We applied 5 mL of human plasma to a 100 × 2 cm column of Sephadex G-100, equilibrated and eluted with Tris HCl (50 mmol/L, pH 7.5).

Statistical Methods

We used Student's *t*-test for unpaired observations when comparing two sets of parameters showing a normal distribution (as judged by a Q–Q plot) (*17*) and having a variance not significantly different between the two groups, as determined by analysis of variance. If the values did not show a normal distribution, we used the Wilcoxon test for relative spread in unpaired samples (*18*), followed by an ordinary Wilcoxon two-sample rank test if the variances were not found to be different. Significant levels were always expressed as two-tailed.

Results

Optimization of the assay procedure. This method for determining homocysteine in plasma and urine is a modification of a procedure developed for use with tissue extracts. It is based on the enzymic conversion of homocysteine to *S*-[^{14}C]adenosylhomocysteine in the presence of [^{14}C]adenosine and *S*-adenosylhomocysteine hydrolase (*16*). In that procedure, interfering compounds in tissues—adenosine and *S*-adenosylhomocysteine—were removed by adsorption to charcoal, leaving homocysteine in solution (*16*). However, there are no interfering compounds such as adenosine or *S*-adenosylhomocysteine in human plasma (cooled on ice) or in urine, so charcoal treatment is not required in the method reported here.

The amount of endogenous homocysteine and the analytical recovery of exogenous homocysteine were determined at various dilutions of plasma and urine. The amount of endogenous homocysteine determined both in plasma and urine varied linearly with the dilution factor for dilutions of two- to 16-fold. More than 90% of added homocysteine was accounted for (data not shown). Plasma and urine were routinely diluted fourfold with phosphate buffer, or plasma was deproteinized with three volumes of the perchloric acid reagent. Under these conditions less than 20% of [^{14}C]adenosine was consumed during the enzymic condensation with homocysteine. Preincubation for 10 min in the presence of dithioerythritol and further incubation for 40 min with [^{14}C]adenosine and enzyme sufficed for nearly complete conversion of homocysteine in plasma and urine to *S*-[^{14}C]adenosylhomocysteine (data not shown).

To isolate *S*-[^{14}C]adenosylhomocysteine formed from homocysteine in tissue extracts (*16*), plasma, and urine, we used the same chromatographic system (see *Materials and Methods*). The chromatograms of urine were complex, with peaks close to that for *S*-adenosylhomocysteine. For urine analysis, we used a narrow time window in combination with a peak separator to collect *S*-[^{14}C]adenosylhomocysteine in a single tube during unattended analysis.

We constructed a standard curve for the amount of homocysteine vs the amount of radioactive *S*-adenosylhomocysteine formed (*16*).

Free and protein-bound homocysteine in human plasma. The concentration of free homocysteine in plasma was 2.27 (SEM 0.11) µmol/L for the 18 men and 1.95 (SEM 0.13) µmol/L for the 16 women. The difference was statistically insignificant ($p > 0.05$, Student's *t*-test). The concentration of protein-bound homocysteine, calculated by subtracting free homocysteine from total homocysteine, was 6.50 (SEM 0.32) µmol/L for men and 7.29 (SEM 0.65) µmol/L for women. The variances for the values for men and women differed significantly ($p < 0.01$, analysis of variance), as was confirmed by the Wilcoxon test for relative spread (*18*). The ratio between free and protein-bound homocysteine was significantly ($p < 0.01$) higher for men (0.35, SEM 0.014) than for women (0.28, SEM 0.015) by Student's *t*-test. These data are summarized in Table 1.

When plasma diluted fourfold with phosphate buffer was incubated at 37 °C, with or without dithioerythritol, we observed that dithioerythritol increased the amount of free homocysteine, which reached the total amount of homocysteine. In plasma not supplemented with dithioerythritol, the value for free homocysteine remained constant for 20 min, then declined (Figure 1). These data suggest that, in the presence of a reducing agent at least, bound homocysteine is present in plasma as a fast-exchange protein–homocysteine mixed disulfide.

Linear regression analysis showed a positive correlation ($r = 0.58$, $p < 0.001$) between the concentration of free and protein-bound homocysteine in plasma from men and women (Figure 2).

Association of homocysteine with plasma proteins. To investigate whether homocysteine is associated with plasma proteins in vivo, we subjected freshly separated plasma to gel filtration on an HPLC protein column (Figure 3). Homocysteine was eluted from the column as two distinct peaks. About 75% co-chromatographed with serum albumin, and another peak appeared in the total volume. These data were confirmed by conventional gel chromatography on Sephadex G-100 (data not shown).

About 75% of homocysteine was bound to proteins in plasma, as determined by gel filtration (Figure 3). The proportion bound to proteins as measured by this method equaled that determined in a parallel experiment as the difference between total homocysteine and (free) homocysteine in plasma deproteinized by perchloric acid (data not

Table 1. Homocysteine in Plasma and Urine

Homocysteine, µmol/L		Ratio	Homocysteine excreted in
Protein bound	Free	[free]/[bound]	urine, µmol/24 h
Men (n = 18)			
6.51 ± 0.32[a]	2.27 ± 0.11	0.35 ± 0.014	6.92 ± 0.64[b]
(4.22–9.12)	(1.52–3.17)		(4.81–9.77)
Women (n = 16)			
7.29 ± 0.65[c]	1.95 ± 0.13	0.28 ± 0.015[d]	5.12 ± 0.68[e]
(3.55–12.97)	(1.09–2.98)		(3.52–7.28)

[a]Mean ± SEM (and range). [b]n = 10. [c]Values show significantly greater spread than those for men: $p < 0.01$, analysis of variance and Wilcoxon test for relative spread in unpaired samples (*18*). [d]Significantly different from the ratio for men ($p < 0.01$, Student's *t*-test). [e]n = 7.

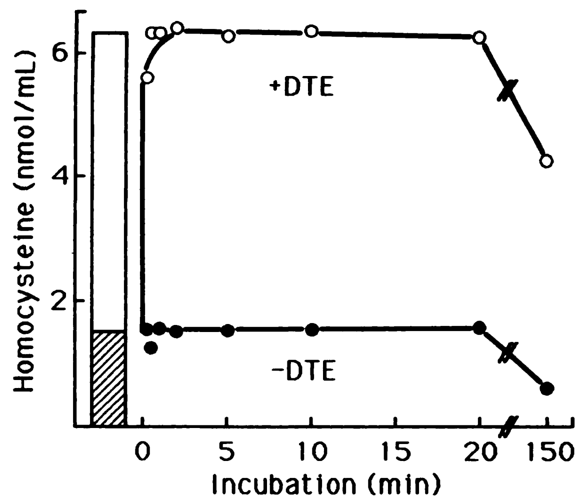

Fig. 1. Effect of dithioerythritol (DTE) on free and protein-bound homocysteine in plasma

Freshly prepared human plasma was diluted fourfold with potassium phosphate buffer (100 mmol/L, pH 7.4), then divided into two portions, one to be supplemented with dithioerythritol, 10 mmol/L; both were incubated at 37 °C for various periods before addition of perchloric acid (final concentration 0.6 mol/L), removal of the precipitated protein by centrifugation, and measurement of the homocysteine remaining in the supernate. The *bar* to the left shows the total amount of homocysteine determined by running the *S*-adenosylhomocysteine hydrolase reaction in whole plasma in the presence of dithioerythritol (see *Materials and Methods*); the *cross-hatched part* shows the amount of free homocysteine in the same plasma sample

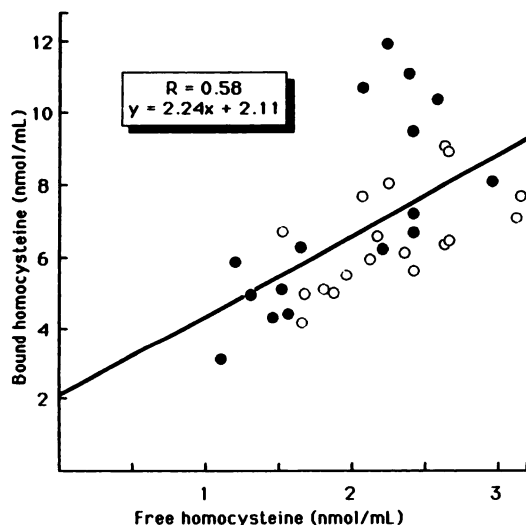

Fig. 2. Relation between free and protein-bound homocysteine in plasma from men (○) and women (●)

shown). Evidently the protein binding of homocysteine measured by the latter procedure occurs in vivo and is not an artifact related to precipitation of plasma proteins with strong acid.

Storage of whole blood and plasma. When whole blood supplemented with EDTA was left at room temperature or on ice for 2 h, there was no change in free and protein-bound homocysteine (data not shown). Incubation of plasma for 1.5 h at 37 °C decreased the concentration of free homocysteine by about half (*cf.* Figure 1). When plasma was frozen and stored at −20 °C for a week, the concentration of free homocysteine decreased to about 40%, but the total concentration of homocysteine was unaffected. Repeated freezing and thawing diminished the concentration of free homocysteine to traces.

Homocysteine in urine. We measured homocysteine in urine of normal men and women who were on their usual diet. The concentration ranged from 3.5 to 9.5 µmol/L. The

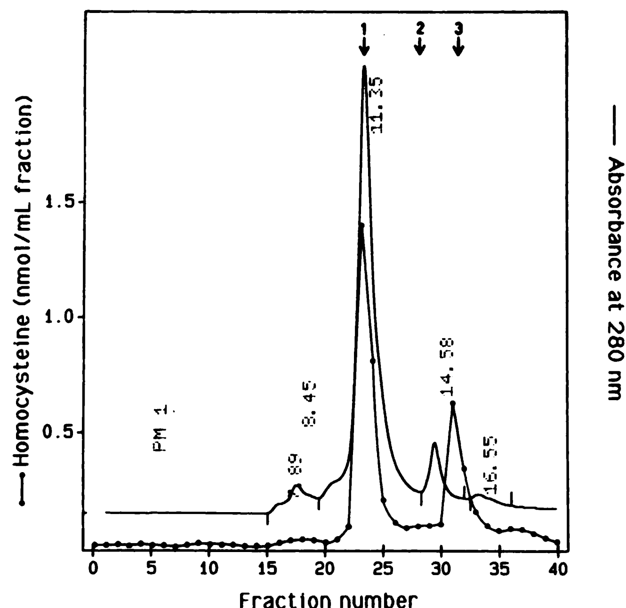

Fig. 3. HPLC gel filtration of human plasma

A 25-µL sample of freshly prepared plasma was subjected to gel filtration on an HPLC column as described in the text. (——) elution profile of whole plasma; (●—●) elution profile of endogenous homocysteine in plasma after fractionation of the effluent in 400-µL fractions and determination of homocysteine in each fraction. *Arrows* indicate where protein standard eluted: (*1*) bovine serum albumin (M_r = 68 000), (*2*) myoglobin (M_r = 16 900), and (*3*) *S*-adenosylhomocysteine (indicating the total volume of the column)

amount of homocysteine excreted during 24 h was 6.92 (SEM 0.64) µmol for men and 5.12 (SEM 0.68) µmol for women. The sex-related difference (Table 1) was not significant (p >0.05, Wilcoxon two-sample test).

Discussion

The present method is characterized by high specificity because compounds, to interfere, must serve both as a substrate for *S*-adenosylhomocysteine hydrolase and the reaction product must co-chromatograph with *S*-adenosylhomocysteine on a reversed-phase column. The sensitivity of the method is about 1 pmol [about 100 counts/min (*16*)], and less than 25 µL is required for analysis, which facilitates multiple blood sampling. The present method is far more sensitive than determination of homocysteine with an amino acid analyzer (*8, 10, 19*). Methods based on HPLC and electrochemical detection (*9, 20, 21*) are about as sensitive as the present method, but the former procedures are subject to interference from reducing agents such as dithioerythritol, which are required for homocysteine to be liberated from plasma proteins (Figure 1).

Recent data on homocysteine in human plasma under physiological conditions have been somewhat inconsistent. Reported values range from 1 to 20 mmol/L (*8–13, 22*). The possibility that a major portion of homocysteine in human plasma is bound to plasma proteins has been largely overlooked. Kang et al. (*12*) found that protein-bound homocysteine concentrations ranged from 0.5 to 2.2 µmol/L, far below the values we found, about 7 µmol/L (Table 1). This discrepancy may be related to the different procedures used to liberate homocysteine from plasma proteins.

We were able to demonstrate homocysteine bound to native plasma protein by gel filtration of plasma on an HPLC protein column (Figure 3) and on a Sephadex G-100 column. With the former procedure, free and bound homocysteine are separated within 15 min (Figure 3). Because the ratio between free and bound homocysteine (in the absence of dithioerythritol) was stable for 20 min (Figure 1),

we assume that the bound fraction obtained with gel filtration reflects the binding of homocysteine to plasma proteins in vivo. The protein binding of homocysteine as determined by gel filtration equaled that obtained by deproteinization of plasma with strong acid. Because deproteinization is less laborious, we prefer it for routine analysis.

Co-chromatography of bound homocysteine with albumin (Figure 3) suggests that this protein is the main carrier of homocysteine in plasma.

Homocysteine bound to plasma proteins was recovered as free homocysteine within 1 min in the presence of dithioerythritol (Figure 1). This suggests that the homocysteine associated with plasma proteins is a fast-exchange homocysteine–albumin mixed disulfide, at least in the presence of dithioerythritol. Others have reported similar observations for the interaction of other thiols and disulfides with albumin (*9, 23, 24*).

Cysteine bound to plasma proteins represents a large portion of total plasma cyst(e)ine (*25*), as was originally suggested by Stein and Moore (*26*). Smolin and Benevenga (*27*) have recently reported that homocysteine decreased the plasma protein binding of cysteine in rats, whereas displacement of homocysteine binding by cysteine could not be demonstrated. Homocysteine may thus have a higher affinity than cysteine for the binding sites on plasma proteins (*27*).

Our finding that the free homocysteine in plasma from men was slightly but not significantly higher than in women (2.27 vs 1.95 μmol/L) agrees with the data of Wilcken and Gupta (*11*), although they reported that the difference was significant: cysteine–homocysteine mixed disulfide in men was 3.3 μmol/L, 2.4 μmol/L in women. Note that the present radioenzymic method does not distinguish between homocysteine and its (mixed) disulfides.

We found that the amount of protein-bound homocysteine was slightly but not significantly higher in women than in men; however, the ratio between free and bound homocysteine was significantly ($p < 0.01$, Student's *t*-test) higher in men than in women (Table 1). This suggests that men and women have different characteristics for the binding of homocysteine to plasma proteins. Whether this is related to properties of the homocysteine binding sites or to the presence of endogenous compounds in plasma competing for homocysteine remains to be established.

Frimpter (*28*) demonstrated the presence of homocysteine–cysteine mixed disulfide in urine from patients with cystinuria and, in trace amounts, in urine from one of two normal individuals after administration of [^{35}S]methionine. We here report, for the first time, the values for urinary excretion of homocysteine (or its disulfides) in normal men and women. The amount of homocysteine excreted in the urine was slightly higher for men than for women (Table 1). The fact that the concentration of homocysteine in the urine (3.5–9.5 μmol/L) exceeds the concentration of free homocysteine in plasma (1–3 μmol/L) suggests that renal clearance of homocysteine may contribute significantly to the total clearance of this compound from plasma.

The concentration of homocysteine in extracellular fluids such as urine (3.5–9.5 μmol/L) and plasma (about 10 μmol/L) was higher than that in several tissues of mouse and rat, e.g., about 1 nmol of homocysteine per gram, except for liver, which contained about 3.5 nmol/g (*16*). The intracellular concentration of homocysteine is extremely low in isolated cells; thus, homocysteine apparently is excreted against a concentration gradient into the extracellular fluids (unpublished results). Therefore, determination of homocysteine in urine and plasma may provide information on perturbations of homocysteine metabolism in humans during diseases or pharmacological interventions that affect metabolism of one-carbon compounds.

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Immunoenzymometric Assay for Insulin Involving Column Chromatography and Insulin Immobilized on Sepharose

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This practical assay for measuring insulin involves use of a 4 × 8 mm chromatographic column. Serum samples are incubated at 30 °C for 1 h with β-D-galactosidase-labeled antibody to insulin, then passed through the 0.1-mL column containing insulin immobilized on Sepharose 4B. After the column is washed to remove the bound label, the buffer in the column is replaced with a solution of o-nitrophenyl-β-D-galactoside. The column is then incubated at 30 °C for 1 h, the enzyme reaction is stopped by washing the column with sodium carbonate solution, and the absorbance of the eluate is measured at 420 nm. Results obtained by this method were compared with those by a radioimmunoassay and a solid-phase enzyme immunoassay.

Our recently developed enzyme immunoassay, which includes a covalent chromatographic-separation method (*1*), is useful for competitive immunoassays (*1–3*) and immunoassays for antibodies (*1, 4*) without interference from serum components (*5*). Now we have improved the column-separation method, making it more practicable and simple, and have applied this technique to assays for thyroxin (*6*), triiodothyronine (*6*), and secretory immunoglobulin A (*7*).

We describe here an enzyme immunoassay for insulin, in which we use the improved column-separation method, antibody labeled with enzyme, and antigen immobilized on Sepharose 4B.

Materials and Methods

Materials

We use de-ionized water throughout.

Buffer G. This was sodium phosphate buffer (10 mmol/L, pH 7) containing 0.3 mol of NaCl, 1 mmol of $MgCl_2$, 1 g of NaN_3, 1 g of bovine serum albumin (Cohn Fraction V; Armour Pharmaceutical Co., Chicago, IL), and 5 g of digested gelatin per liter. The digested gelatin was prepared by treating gelatin (Difco Laboratories, Detroit, MI) with a protease (Protease T1; Amano Pharmaceutical Co., Nagoya, Japan) (*8*).

Antibody. Guinea pig antiserum to porcine insulin was obtained from Medical and Biological Laboratories, Nagoya, Japan. The IgG fractions were isolated from antiserum by precipitation with $(NH_4)_2SO_4$, dialysis, and chromatography on diethylaminoethyl-cellulose (*5*).

Antibody–β-D-galactosidase conjugate. F(ab')$_2$ fragments of anti-insulin antibody, prepared by digesting (anti-insulin) IgG fractions with pepsin (EC 3.4.23.1), were reduced with 2-mercaptoethylamine and coupled to β-D-galactosidase (EC 3.2.1.23, from *Escherichia coli;* Boehringer Mannheim, Mannheim, F.R.G.) by use of *N,N'*-o-phenylenedimaleimide (*9*). The amounts of the conjugate were expressed in terms of units of enzyme activity, and 1 unit (U) of activity was defined as that which hydrolyzed 1 μmol of o-nitrophenyl-β-D-galactoside per minute under the conditions described below.

Insulin immobilized on Sepharose. We mixed 1 mg of crystalline insulin (Sigma Chemical Co., St. Louis, MO), dissolved in 80 mL of sodium carbonate buffer (0.1 mol/L, pH 9), with 20 mL of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), and stirred this at 4 °C overnight. The insulin immobilized on Sepharose was then washed with Tris HCl buffer (0.1 mol/L, pH 8) and stored at 4 °C.

Standard serum. A porcine insulin ("Actrapid"; Novo Industri A/S, Copenhagen, Denmark) was diluted with human serum that had been freed of insulin by passage through a column containing anti-insulin antibody immobilized on Sepharose (100 mL of serum was passed through a 25-mL column). The anti-insulin immobilized on Sepharose was prepared as described previously (*6*).

Comparison method. Using our enzyme immunoassay method, we measured our standard serum and that in a radioimmunoassay kit (Insulin "Eiken" RIA Kit from Eiken Chemical Co., Tokyo, Japan), then calculated the value of our standard serum from the standard curve obtained with the kit's standard serum. The value for our standard serum was 97.3% as great as that for the standard serum specified with the radioimmunoassay kit.

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