Isolation of Human Complex-forming Glycoprotein, Heterogeneous in Charge (Protein HC), and Its IgA Complex from Plasma

PHYSICOCHEMICAL AND IMMUNOCHEMICAL PROPERTIES, NORMAL PLASMA CONCENTRATION

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Human complex-forming glycoprotein, heterogeneous in charge (protein HC) has previously been isolated from urine and immunochemically shown to be present in low and high molecular weight forms in blood plasma (Tejler, L., and Grubb, A. O. (1976) Biochim. Biophys. Acta 439, 82–94). In the present work, the major low and high molecular weight forms of the protein were isolated from plasma by immunosorption followed by gel chromatography. The plasma low molecular weight protein HC and the urinary protein had similar, if not identical, molecular weight, amino acid composition, NH₂-terminal and carboxyl-terminal amino acid sequences and electrophoretic mobility. The low molecular weight plasma protein HC carried a yellow chromophore like the urinary protein, but its molar extinction coefficient at 280 nm was lower and its charge heterogeneity less pronounced than that of urinary protein HC.

The plasma high molecular weight protein HC had a hydrodynamic volume which was greater than that of monomeric IgA but smaller than that of dimeric IgA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the isolated high molecular weight protein HC followed by electrophoretic blotting and immunochromical analysis demonstrated that the protein contained four polypeptide chains: two light immunoglobulin chains (M₀ = 23,000), one IgA α-chain (M₀ = 54,000), and one chain with M₀ ~ 90,000 which carried both α-chain and protein HC antigenic determinants. Whether the protein HC IgA complex is a functionally significant part of the human immune system cannot be decided without further experimentation, but the complex was found to be completely absent from the blood plasma of patients with a selective deficiency of IgA-secreting immunocytes.

The isolated low and high molecular weight plasma protein HC components were used as standard proteins in the construction of a quantitative crossed immunoelectrophoretic assay for the simultaneous quantitation of the two major protein HC components in blood plasma. The plasma concentrations of the low and high molecular weight protein HC components were measured by this method in 13 healthy Caucasians. The results for the low molecular weight protein HC were:

- Mean, 20.3 mg/liter, S.D., 3.2 mg/liter, range, 13.6–26.0 mg/liter;
- For the protein HC-IgA complex: mean, 293 mg/liter, S.D., 176 mg/liter, range, 36–620 mg/liter.

Human complex-forming glycoprotein, heterogeneous in charge (protein HC) is a recently described LMW glycoprotein originally isolated from normal human urine (1). It shows a considerable charge heterogeneity, carries an unidentified yellow-brown chromophore and has been immunochemically demonstrated to occur in normal human plasma, both as a low and high molecular weight component (1). The HMW component displays, in addition to its protein HC immunoreactivity, IgA immunoreactivity (1).

The protein is closely related to two other recently described glycoproteins, α₁-microglobulin and α₂-microglycoprotein, both of which were isolated from the urine of patients with renal tubular dysfunction (2–5). Recent publications of the complete amino acid sequences of human urinary protein HC (6) and α₁-microglobulin (7) demonstrated nearly identical sequences of the single polypeptide chains of the two molecules, although the one of α₁-microglobulin was reported to be 14 residues shorter than that of protein HC.

Although protein HC and α₁-microglobulin have been shown to be produced by hepatocytes (8, 9) and to possess immunoregulatory properties (10), the plasma components carrying the same immunoreactivity as the pure urinary proteins have not so far been isolated and characterized.

Several attempts have been made to measure the plasma and urinary concentration of these proteins by immunochemical procedures, but with greatly varying results. For example, the reported mean plasma concentration of protein HC or α₁-microglobulin in healthy adults has varied from 1.8 to 98 mg/liter (1, 11).

The present work describes the isolation from human plasma of both the low and high molecular weight components carrying protein HC immunoreactivity and some of their physico- and immunochemical properties. The isolated components are also used to elucidate the difficulties in the immunochemical quantitation of protein HC or α₁-microglobulin and a procedure for the simultaneous correct quantitation of both the low and high molecular weight plasma protein HC component is proposed.

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1 The abbreviations used are: protein HC, human complex-forming glycoprotein, heterogeneous in charge; BSA, bovine serum albumin; HMW, high molecular weight; LMW, low molecular weight; PBS, 0.05 M phosphate buffer, pH 7.4, with 0.10 M NaCl; SDS, sodium dodecyl sulfate.
EXPERIMENTAL PROCEDURES

Materials

Sephadex G-25, Sephadex G-200, and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and prepared according to the instructions supplied. Ultrigel AcA 22 was from LKB-Produkt AB (Bromma, Sweden). DEAE-cellulose (DE50) was from Whatman Ltd. (Springfield Mill, Maidstone, Kent, England), nitrocellulose membrane filters (0.2 µm) were from Schleicher and Schüll GmbH (Dassel, Germany), cellulose thin layer plastic plates were from Merck AG (Darmstadt, Germany), and nitrocellulose membrane filters (0.2 µm) were from Schleicher and Schüll GmbH (Dassel, Germany), cellulose thin layer plastic plates were from Merck AG (Darmstadt, Germany), and nitrocellulose membrane filters (0.2 µm) were from Schleicher and Schüll GmbH (Dassel, Germany). Cellulose thin layer plastic plates (DE52) were from Whatman Ltd. (Springfield Mill, Maidstone, Kent, England), nitrilotriacetic acid (NTA) and agarse (Sea Kem, ME) was from Marine Colloids Inc. (Rockland, ME). Trypsin with 1-tosylamido-2-phenyl-ethyl chloromethyl ketone and carboxypeptidase A and carboxypeptidase Y were from Merck AG, BSA (Fraction V) was from Miles Laboratories Ltd. (Stoke Poges, Slough, England), protein A was from Pharmacia Fine Chemicals, iod[1-14C]acetic acid (54 mCi/mmol) was from the Radiochemical Centre (Amersham, England), Coomassie brilliant blue R-250 was from Schwarz/Mann (Orangeburg, New York), polyethylene glycol 6000 was from KEBO AB (Stockholm, Sweden), and all biochemicals were of highest available quality.

Methods

Isolation of Urinary Protein HC—Protein HC was isolated from the urine of a single individual (J. L.) with tubular proteinuria by ion-exchange chromatography followed by gel filtration and immunoprecipitation (17) or of highest quality.

Isolation of an IgA(κ) M Component—Plasma obtained by plasmapheresis from a patient (M. E.) suffering from multiple myeloma was used as starting material for the isolation of an IgA(κ) M component. Solid ammonium sulfate was added to 1.0 liter of plasma to a final saturation of 55%, and the mixture was incubated at 6°C overnight and centrifuged at 2000 × g for 15 min. The pellet was dissolved in water and dialyzed against 0.02 M sodium phosphate buffer, pH 8.2. The solution was then chromatographed on a column (5 x 80 cm) of DEAE-cellulose (Whatman DE52) equilibrated with the dialysis buffer. The column was stepwise eluted with the dialysis buffer containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.075, 0.1, and 0.125 M NaCl and the eluted fractions were tested for the presence of the IgA M component by agarose gel electrophoresis as described by Johansson (12). Suitable fractions were pooled and concentrated by pressure ultrafiltration (Amicon Diaflo cell with a UM-10 membrane). The concentrate was applied to a column (5 x 90 cm) of Sephadex G-200 in 0.05 M Tris-HCl buffer, pH 7.4, with 0.1 M NaCl and 0.002 M EDTA. The eluted fractions were monitored for the presence of the IgA M component by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis. Suitable fractions were pooled and tested for contamination by other plasma proteins, including IgG and IgM by agarose gel electrophoresis.

Isolation from Plasma of the Low and High Molecular Weight Components Carrying Protein HC Immunoreactivity—Plasma obtained by plasmapheresis from a patient (M. E.) with multiple myeloma was used as starting material for the isolation. This plasma contained an IgA(κ) M component and had a comparatively high final saturation of 55% and after incubation at 22°C for 16 h the mixture was divided in 25 parts which were successively run on a column (1.6 x 90 cm) of Sephadex G-200 and lyophilized and used directly for automatic amino acid analysis.

The same material of native protein as described above was used for fractionation with carboxypeptidase Y. The digestion was carried out in 150 µl of pyridine acetate buffer, pH 5.5, at 37°C for 1-3 min with an enzyme-substrate ratio of 1:200 (w/w). The digests were frozen and lyophilized and used directly for automatic amino acid analysis.

Proteolytic Digestion—120-150 µg of native LMW plasma protein HC or 5 nmol of its COOH-terminal CNBr fragment were digested with carboxypeptidase B. The digestion was carried out in 150 µl of 0.2 M N-methylmorpholine buffer, pH 8.2, for 16 h at 37°C with an enzyme-substrate ratio of 1:200 (w/w). The digests were frozen and lyophilized and used directly for automatic amino acid analysis.

Amino Acid Analysis—Salt-free samples were hydrolyzed with 200 µl of 5.7 M HCl containing 0.05% (v/v) 2-mercaptoethanol in evacuated and sealed tubes at 110°C for 24, 48, and 72 h. The analyses were performed in a Beckman 121-M analyzer equipped with a Beckman 126 data system. Half-cystine was determined either as carboxymethylcysteine or as cysteic acid after performic acid oxidation (17). Tryptophan was determined by hydrolysis with 3 ml of 6 M hydrochloric acid at 110°C for 2, 4, 6, and 20 h. Sialic acid was determined after hydrolysis with 0.05 M sulfuric acid at 80°C for 1 h using the thiobarbituric acid assay (20).

Protein HC Complex from Human Plasma—One mg of native purified LMW plasma protein HC was dissolved in 30 µl of pyridine acetate buffer, pH 5.5, and lyophilized and used directly for automatic amino acid analysis. The oxidized protein (500 µg) was applied on a thin layer cellulose plate (20 x 20 cm) and subjected to electrophoresis in pyridine/acetic acid/water (1:2:4: 40 (v/v), pH 4.4) at 20 V/cm for 2 h. After electrophoresis the plate was stained with a 1% ninhydrin solution in acetic acid.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—
Polyacrylamide gel slab electrophoresis in the presence of SDS was carried out at pH 8.9 as described by Laemmli (15). The separation gel contained 15% (w/v) acrylamide and 0.4% (w/v) bisacrylamide. Samples were sometimes reduced by boiling for 5 min in 1% (v/v) SDS and 1% (v/v) β-mercaptoethanol prior to application to the gel.

Proteins and polypeptide chains were sometimes transferred from the SDS-polyacrylamide gels to unmodified nitrocellulose sheets by the electrophoretic procedure described by Burnette (21) which removes all SDS from the separated molecules and allows immunochromical analysis of them. The molecules reacting with a specific rabbit antiserum were visualized by overnight incubation of the nitrocellulose sheets at 37 °C in a PBS buffer, pH 7.4, with 50 mg/ml BSA, incubation for 1 h at 37 °C in the PBS buffer with 25 mg/ml BSA supplemented with rabbit antiserum, washing for 30 min in PBS, incubation for 1 h in PBS containing 125I-labeled protein A and, finally, by washing for 1 h in PBS before autoradiography.

**Densitometric Scanning of SDS-Polyacrylamide Slab Gels—** Isolated IgA(λ) M component, urinary protein HC, and HMW plasma protein HC component were completely reduced by boiling for 5 min in 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS and then applied in increasing amounts to the same SDS-polyacrylamide slab gel. After the electrophoresis, the gel was stained with 0.5% (w/v) Coomassie brilliant blue R-250 in acetic acid/methanol/water (1:8:10, v/v) and then destained in the same solution without Coomassie blue until no background staining of the gel could be observed. The wet gel was placed on a glass plate and scanned in a Zeineh soft laser scanning densitometer (Bionized Instruments, Inc., Chicago, IL). Each electrophoretic track was scanned three times and the content of heavy and light IgA polypeptide chains and of the protein HC component were completely reduced by boiling for 1 h in PBS before autoradiography.

**Crossed Immunoelectrophoresis—** For qualitative purposes, the system of Laurell (22) as described by Grubb (14) was used. The intermediate gel technique of Axelsen (23) was used to identify complex-constituents.

For quantitative purposes, the system described by Grubb et al. (24) was used. Pure bovine coagulation factor X (kindly provided by Dr. J. Stenflo, Allmänna Sjukhuset, Malmö) and a monospecific rabbit antiserum against this protein were used to produce the internal standard precipitation arc in the system. This antiserum did not produce any precipitation arcs on crossed immunoelectrophoresis of standard of isolated, reduced and alkylated J chain (28).

**RESULTS**

**Isolation of Two Molecular Forms of Protein HC Immunoreactivity from Plasma—** Crossed immunoelectrophoresis ofnormal human plasma with use of a rabbit antiserum raised against human protein HC isolated from urine reveals that at least two molecular forms of protein HC are present in human plasma (Fig. 1a). A similar precipitation pattern was obtained when the normal plasma was replaced by plasma obtained by plasmapheresis of patients with IgA M components (Fig. 1b).

Such an IgA M component-containing plasma was used as starting material for isolation of the various molecular forms of protein plasma HC. Table I summarizes the yields of the three purification steps, as measured by quantitative crossed immunoelectrophoresis with use of isolated low and high molecular weight plasma protein HC components as standard proteins (see below).

Virtually all protein HC immunoreactivity was precipitated from human plasma by the addition of ammonium sulfate to a final saturation of 55%. When the redissolved proteins of the precipitate were applied in suitable amounts to an immunosorbent column containing antibodies against human urinary protein HC all immunoreactive molecules were absorbed to the column at neutral pH. Molecules eluted from the column at pH 2.2 were subjected to gel chromatography on a column of Ultrogel AcA 22 (Fig. 2). The eluate was continuously monitored for protein by measuring its absorb-

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**FIG. 1.** Crossed immunoelectrophoresis of (a) normal human plasma, (b) IgA M component-containing human plasma, (c) isolated LMW plasma protein HC component, (d) isolated HMW plasma protein HC component, and (e) concentrated normal human urine with the use of a polyclonal rabbit antiserum raised against isolated urinary protein HC. An agarose gel electropherogram of normal human plasma is shown above for comparison.

| Table I | Yields in the isolation of low and high molecular weight protein HC from human plasma |
|---------|--------------------------------------------------------------------------------------|
|         | LMW plasma protein HC* | HMW plasma protein HC* |
|         | Total protein | Yield | Total protein | Yield |
| Human plasma | 4.4 | 100   | 103 | 100 |
| Ammonium sulfate precipitation | 4.7 | 107 | 45 | 44 |
| Immunosorption | 3.8 | 86 | 32 | 31 |
| Gel chromatography (Ultrogel AcA 22) | 2.4 | 55 | 16 | 16 |

* Determined by quantitative crossed immunoelectrophoresis with use of isolated LMW and HMW plasma protein HC as standard proteins.
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The sample (3 ml) was applied to a column (2.5 x 30 cm) in 0.05 M Tris-HCl buffer, pH 7.4, with 0.1 M NaCl and 2 mM EDTA. Fractions of 5 ml were collected at a flow rate of 10 ml/h. The distribution in the column of Ultrogel AcA 22 demonstrated that the isolated LMW plasma protein HC material showed that it had the same elution volume as the LMW protein HC. The isolated LMW plasma protein HC immunoreactivity was demonstrated by crossed immunoelectrophoresis of the isolated LMW plasma protein HC, IgA, and albumin by electroimmunoassay and the fractions were combined into two pools as indicated by the horizontal bars.

Characterization of the LMW Plasma Protein HC Immunoreactivity—Crossed immunoelectrophoresis of the isolated LMW plasma protein HC material showed that it had the same electrophoretic mobility as the anodal protein HC immunoreactivity of native plasma (Fig. 1, a and c). Gel chromatography of the isolated LMW material on a column of Ultrogel AcA 22 demonstrated that the isolated material had the same elution volume as the LMW protein HC immunoreactivity of native plasma. Part of the solution of the LMW plasma protein HC immunoreactivity was desorbed from an anti-protein HC immunoreagent. The elution volume of protein HC, IgA, and albumin was determined by electroimmunoassay. Two dominating protein peaks, one containing HMW and one LMW material, were obtained. The major part of the protein HC immunoreactivity was also eluted in the fractions constituting these two protein peaks. The fractions of the peak containing the HMW material reacted not only with the antiserum against protein HC but in addition with an antiserum against IgA. The fractions of the second protein peak only reacted with the antiserum against protein HC. A small amount of albumin immunoreactivity was found in the fractions eluted just before those of the second protein peak.

The fractions of the two dominating protein peaks were combined as indicated in Fig. 2. The two pools were concentrated by pressure ultrafiltration and constituted about 95% of all the UV-absorbing material eluted from the Ultrogel column.

SDS-polyacrylamide gel electrophoresis of the reduced LMW material gave a narrow stained zone corresponding to a polypeptide chain with an apparent $M_r \sim 31,000$ (Fig. 3). The position in the gel of the zone coincided with that of the polypeptide chain of reduced urinary protein HC. The unreduced material also formed a narrow stained zone in the gel close to the position of the reduced material and at the same position as unreduced urinary protein HC.

Analytical agarose gel electrophoresis of the LMW plasma protein HC material showed that it migrated as a relatively wide protein band in the $\alpha_1$-globulin zone, indicating charge heterogeneity (Fig. 4). However, the charge heterogeneity of protein HC isolated from the urine of a patient with tubular proteinuria was still greater. A difference in the charge heterogeneity of native LMW plasma protein HC and urinary protein HC could also be demonstrated by crossed immunoelectrophoresis of native human plasma and urine (Fig. 1, a and e).

The amino acid composition of the LMW plasma protein HC material was essentially identical with the one of urinary protein HC (Table II). The hexosamine and sialic acid content of the two protein preparations were also nearly the same (Table II).

The NH$_2$-terminal amino acid sequence of the LMW
plasma protein HC material was established by automatic degradation in a Sequencer of the completely reduced and carboxymethylated material. A unique amino acid sequence up to residue 31 could be identified and no evidence was found of more than one amino acid residue at any of the positions of the NH₄-term minus of the polypeptide chain. The sequence obtained (GPVPTPDINIQVQENFBISRIYGKWYNLAI) was identical with the NH₂-terminal one of urinary protein HC (6).

The carboxyl terminus of the LMW plasma protein HC material was studied by carboxypeptidase digestions. Arginine was the only amino acid released upon digestion of the material with carboxypeptidase B (0.4 mol of Arg/mol of protein). When the material was digested with carboxypeptidase Y, four amino acids were released in the molar amounts shown in Table III. This table also demonstrates that similar results were obtained when urinary protein HC was digested with the two carboxypeptidases.

Completely reduced and ¹⁴C-carboxymethylated LMW plasma protein HC (2.0 mg) was treated with CNBr and then fractionated on a column of Sephacryl S-200. The absorbance at 280 nm and the radioactivity of the eluted fractions are shown in Fig. 5A. The material of peak CN7 was rechromatographed on the same column (Fig. 5B) and then lyophilized. Amino acid analysis demonstrated that it lacked homoserine and had an amino acid composition similar to the one of the corresponding carboxyl-terminal CNBr fragment of urinary protein HC (Table II). Digestion with carboxypeptidase B released only one amino acid, arginine, from the material.

Performic acid oxidation of 200 μg of LMW plasma protein HC material followed by electrophoresis at pH 6.5 on a thin layer cellulose plate revealed the presence of four ninhydrin-positive substances with anodal mobility. The first was situated close to the application site, the second had the mobility of free cysteic acid, the third had the mobility of oxidized glutathione, and the fourth had even greater anodal mobility. When 400 μg of LMW plasma protein HC material was oxidized and subjected to electrophoresis at pH 6.5, two of the four released substances could be isolated in amounts sufficient for determination of their amino acid composition. The substance close to the application was found to be a cysteic acid-containing peptide with an amino acid composition nearly identical with the one of a peptide of the same electrophoretic mobility released upon oxidation of urinary protein HC (29). The substance with cysteic acid mobility was identified as cysteic acid by automatic amino acid analysis.

### Table II

| Constituent | Plasma LMW protein HC | CN7a | Protein HC | CN7a* |
|-------------|-----------------------|------|------------|-------|
| Lysine      | 10.0                  | 9.4  |            |       |
| Histidine   | 3.1                   | 3.6  |            |       |
| Arginine    | 8.8                   | 8.6  | 1.6        |       |
| Cysteineb   | 3.9                   | 3.9  | 1.6        |       |
| Aspartic acid| 14                    | 14   | 1.6        |       |
| Threoninec  | 15.4                  | 13.9 |            |       |
| Serine      | 9.5                   | 9.9  |            |       |
| Glutamic acid| 22.3                  | 20.7 | 4.7        |       |
| Proline     | 11.5                  | 11.0 | 3.9        |       |
| Glycine     | 13.2                  | 12.0 | 2.0        |       |
| Alanine     | 9.3                   | 8.7  | 1.0        |       |
| Valine      | 10.4                  | 9.5  | 1.5        |       |
| Methionine  | 3.3                   | 4.4  |            |       |
| Isoleucine  | 11.1                  | 11.9 | 1.5        |       |
| Leucine     | 12.2                  | 11.2 | 0.9        |       |
| Tyrosine    | 7.4                   | 7.8  |            |       |
| Phenylalanine| 6.4                   | 7.3  |            |       |
| Tryptophan  | 1.7                   | 1.4  |            |       |
| NH₂-terminal Gly | ND<sup>a</sup> | Gly | Ala       |       |
| COOH-terminal | Arg                 | Arg  |            |       |

<sup>a</sup> Taken from Ref. 29.

<sup>b</sup> Determined as cysteic acid after performic acid oxidation of the native proteins, or as carboxymethylcysteine in the CNBr fragments.

<sup>c</sup> Values obtained after extrapolation to zero hour hydrolysis.

<sup>d</sup> Determined after hydrolysis with 3 M p-toluene sulfonic acid (18).

<sup>e</sup> Not determined.

### Table III

Amino acids released from LMW plasma protein HC and urinary protein HC by carboxypeptidases Y and B

| Carboxypeptidase Y | LMW plasma protein HC | Urinary protein HC |
|--------------------|-----------------------|--------------------|
| 1 min              | Pro (1.1), Ile (1.0), Leu (1.1), Arg (1.0) | Pro (1.0), Ile (1.0), Leu (1.0), Arg (1.0) |
| 3 min              | Pro (1.7), Ile (1.9), Leu (1.1), Arg (1.0) | Pro (1.5), Ile (1.7), Leu (1.2), Arg (1.0) |
| Carboxypeptidase B |                       |                    |
| 5 h                | Arg                   | Arg                |
150 μg of completely reduced and 14C-carboxymethylated LMW plasma protein HC and urinary protein HC were digested with trypsin for fingerprint analysis as described under "Experimental Procedures." The digests were fractionated on cellulose thin layer plates by electrophoresis and chromatography. The distribution patterns of the two peptide mixtures (Fig. 6) were very similar but a few differences could be observed.

Lyophilized LMW plasma protein HC material as well as solutions thereof (1 mg/ml) had the same yellow-brown color as urinary protein HC. The absorption spectra of the two protein preparations were also similar (Fig. 7) with maxima at 278 nm, inflexion points at 289 nm, and considerable absorptions at wavelengths longer than 320 nm where aromatic amino acids do no absorb. The molar extinction coefficients at 280 nm were determined to be 3.8 × 10^4 M⁻¹ cm⁻¹ for the LMW plasma protein HC material and 6.9 × 10^4 M⁻¹ cm⁻¹ for the urinary protein HC.

Characterization of the HMW Plasma Protein HC Immunoreactivity—Gel chromatography of the isolated HMW material on an Ultrogel AcA 22 column showed that its elution volume was the same as that of the HMW protein HC immunoreactivity of native plasma which was eluted after dimeric plasma IgA, but before monomeric IgA.

Crossed immunoelectrophoresis of the isolated HMW plasma protein HC material demonstrated that its electrophoretic mobility was identical with that of the cathodal protein HC immunoreactivity of native plasma (Fig. 1, a and c). When the isolated HMW material was analyzed by crossed immunoelectrophoresis with the use of an intermediate gel containing antiserum against human IgA α-chain, all of the protein HC immunoreactivity remained in the intermediate gel (Fig. 8d). This contrasted with the result when the isolated LMW material was analyzed by the same technique (Fig. 8b). When the HMW material was applied to an immunosorbent column containing antibodies against human IgA α-chain, all material was retained on the column. In control experiments, when the material was applied to a similar immunosorbent in which the antibodies were replaced by antibodies against human IgG γ-chain, no material was retained on the column. Double radial immunodiffusion of the isolated HMW and LMW plasma protein HC, normal plasma, and isolated LMW plasma protein HC (d). The center well contained rabbit antiserum against urinary protein HC.

The native unreduced isolated HMW plasma protein HC material barely penetrated the gel on SDS-polyacrylamide gel electrophoresis. After reduction, however, there were only two heavily stained polypeptide bands could be observed in the gel in positions corresponding to Mr = 23,000, 54,000, and 90,000. One faint band was also observed in a position corresponding to Mr = 31,000. The polypeptide bands were transferred to nitrocellulose sheets and subjected to immunochemical analysis by use of monospecific rabbit antiserum against human light immunoglobulin α-chains, IgA α-chains, and protein HC. The anti-α-chain antiserum reacted only with the 23,000-Da
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FIG. 10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of isolated HMW plasma protein HC followed by transfer of the polypeptide chains to nitrocellulose sheets and immunochemical analysis. Isolated HMW plasma protein HC was boiled for 5 min in 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol prior to its application in four consecutive slots in the SDS-polyacrylamide gel. After the electrophoresis, one-fourth of the gel was stained directly with the nonspecific protein stain Coomassie brilliant blue (lane a) while the polypeptides of the rest of the gel were transferred to nitrocellulose sheets by a procedure which simultaneously removed all SDS. Polypeptides reacting with rabbit antiserum against urinary protein HC (lane b), IgA α-chains (lane c), or light immunoglobulin κ-chains (lane d) were then visualized by use of 125I-labeled protein A followed by autoradiography.

polypeptide chain, while the anti-α-chain antiserum reacted strongly both with the 54,000-Da and the 90,000-Da polypeptide chains. The anti-protein HC antiserum reacted strongly with the 90,000-Da polypeptide chain and weakly with the 31,000-Da polypeptide chain (Fig. 10). The same electrophoretic and immunochemical patterns were obtained whether the reduction of the HMW complex was carried out in the presence of 6 M guanidinium chloride or not.

SDS-polyacrylamide gel electrophoresis of the pure IgA M component isolated from the same plasma as the HMW and LMW protein HC immunoreactivities gave only two polypeptide bands in positions corresponding to the molecular masses of 23,000 and 54,000 Da (Fig. 3b). This pure IgA M component and pure urinary protein HC were used to construct standard curves on densitometric scanning of the 23,000-, 31,000-, and 54,000-Da polypeptide chains obtained on SDS-polyacrylamide gel electrophoresis of the isolated HMW plasma protein HC complex. The molar ratios obtained for the 23,000-, 31,000-, and 54,000-Da polypeptide chains were 1.80:0.37:1.

No J chain could be demonstrated in the isolated HMW protein HC component by a sensitive electroimmunoassay (28). No color of the HMW plasma protein HC complex at a protein concentration of 1 mg/ml could be observed and its absorption spectrum was virtually identical with that of the isolated IgA M component.

Immunochemical Quantitation of LMW and HMW Plasma Protein HC in Biological Fluids—Several dilutions of isolated urinary protein HC, LMW plasma protein HC, and HMW plasma protein HC were prepared with each of the following different diluents: 0.15 M NaCl; 0.075 M barbital buffer, pH 8.6, with 2 mM EDTA; barbital buffer with 10 mg/ml of BSA; and, finally, normal human plasma from which all protein HC immunoreactivity had been removed by its passage through a sorbent column with insolubilized antibodies against protein HC. Before this protein HC-free plasma was used as diluent, it was concentrated to normal plasma protein concentration by pressure ultrafiltration and then diluted with an equal volume of barbital buffer.

Electroimmunoassay standard curves were produced for each of these isolated protein preparations for each type of diluent with use of a polyclonal rabbit antiserum raised against isolated urinary protein HC. The assays were run with 5 V/cm at 15 °C for 15 h with 3% (w/v) polyethylene glycol 6000 in the agarose gel. The standard curves varied for each protein preparation with the diluent used (Figs. 11 and 12).
However, dilution with protein HC-free plasma and bovine serum albumin solution produced identical dose-response curves. The standard curve for urinary protein HC was identical with that for LMW plasma protein HC for each diluent while it differed markedly from that for HMW plasma protein HC for each diluent (Figs. 11 and 12).

On the basis of the above-mentioned observations, quantitative crossed immunoelectrophoresis was chosen to measure separately, but simultaneously, the LMW and HMW protein HC molecules in biological fluids. Separate standard curves were produced for the LMW and the HMW material and both the two-standard solutions and the samples were diluted with protein HC-free plasma. The concentrations of LMW and HMW protein HC molecules in 13 plasma samples from healthy Caucasian adults were measured by this method. The results were for LMW protein HC: mean, 20.3 mg/liter, S.D., 3.2 mg/liter, range, 13.6–26.0 mg/liter; and for HMW protein HC: mean, 293 mg/liter, S.D., 176 mg/liter, range, 36–620 mg/liter.

Quantitative crossed immunoelectrophoresis of plasma samples from 11 registered blood donors with selective IgA deficiency demonstrated in all cases the absence of the cathodal immunoprecipitate representing the HMW protein HC, but normal concentration of the LMW protein HC.

**DISCUSSION**

Both the LMW and HMW plasma protein HC components are known to display considerable charge heterogeneity by earlier immunochemical studies (1, 3, 30). The HMW plasma protein HC component has been shown to possess IgA immunoreactivity (1) and the charge heterogeneity of normal polyclonal IgA therefore probably contributes to the charge heterogeneity of the HMW protein HC component in normal plasma. In the present work on the isolation of the different protein HC components from human plasma, the starting material was chosen in order to minimize the charge heterogeneity of the protein HC components. Therefore, plasma from only one single person with monoclonal rather than polyclonal IgA was used.

The LMW plasma protein HC component has similar or identical amino acid composition as isolated urinary protein HC and contains almost identical amounts of hexosamine and sialic acid. It has the same NH₂- and carboxyl-terminal amino acid sequences and a very similar molecular weight as the urinary protein. Fingerprint analysis of the two molecules shows that the distribution patterns of the tryptic peptides are almost identical and both release small amounts of cysteic acid and cysteic acid-containing polypeptides on performic acid oxidation. A solution of the plasma protein has a yellow color just as a solution of the urinary protein. The LMW plasma protein HC component isolated in this work and the urinary protein HC isolated and described earlier are thus very closely related. However, two clear-cut differences between the proteins were observed. First, the charge heterogeneity of the LMW plasma protein HC was considerably less than that of the urinary protein. This was demonstrated both by analytical agarose gel electrophoresis of the isolated proteins and by immunoelectrophoretic studies of unfractionated material. It should be noticed that the LMW plasma protein HC was isolated without use of ion-exchange chromatography which might easily decrease the charge heterogeneity of an isolated protein compared to its charge heterogeneity in the starting material. Second, the molar extinction coefficient at 280 nm of the plasma protein was considerably lower than that of the urinary protein. Although the molar extinction coefficient at 280 nm for different preparations of urinary protein HC has been found to vary considerably, it has for all preparations exceeded that observed for the LMW plasma protein HC. Since the molar extinction coefficient calculated from the amino acid composition of protein HC according to the procedure of Wetlaufer (31) is considerably lower than those observed both for the plasma and urinary protein preparations, it is tempting to speculate that the differences are due to the chromophores which give the various protein HC preparations their yellow color. The reason that urinary protein HC is more heterogeneous in charge and has a higher molar extinction coefficient than its plasma equivalent might then be that it carries more and more heterogeneous chromophore material.

Gel chromatography of both the isolated HMW plasma protein HC component and unfractionated normal human plasma demonstrated that the hydrodynamic volume of this plasma protein HC component was slightly bigger than that of monomeric IgA and considerably smaller than that of dimeric IgA. Since antisera against the heavy chain of human IgA retained all the HMW plasma protein HC molecules on immunosorbent analysis and on crossed immunoelectrophoresis with intermediate gel, all these molecules must possess one part which is immunochemically related to protein HC and one which is immunochemically related to IgA. SDS-polyacrylamide gel electrophoresis of the unreduced and completely reduced isolated HMW protein HC component disclosed that it contained at least three different types of disulfide-bridged polypeptide chains. One of the polypeptide chains had the apparent molecular weight and immunoreactivity of a light immunoglobulin chain and one had the molecular weight and immunoreactivity of an immunoglobulin α-chain. The third type of polypeptide chain had an apparent molecular mass of 90 × 10² Da and reacted with antisera both to protein HC and to α-chain. The molar ratio between the light and heavy immunoglobulin chain of the HMW protein HC component was determined by densitometric scanning of the polyacrylamide gel with the use of a standard consisting of an IgA M component purified from the same plasma as the HMW protein HC component. The molar ratio was observed to be about 1.8 in contrast to the ratio of 1 normally found in immunoglobulins. Therefore, on the basis of the gel chromatographic, the immunochemical, and the SDS-polyacrylamide gel electrophoresis results, the HMW plasma component with protein HC immunoreactivity seems to be composed of two light immunoglobulin chains, one “normal” heavy immunoglobulin α-chain and one 90,000-Da polypeptide chain carrying both protein HC and α-chain immunoreactivity.

Whether the 90,000-Da chain is composed of one complete α-chain (M, 54,000) and one complete protein HC polypeptide chain (M, 31,000) firmly linked to each other is not known, but the observed apparent molecular weights are compatible with this possibility. Whether the linkage between the two immunoreactive parts of the 90,000-Da chain is formed by a peptide bond or not must be decided by further investigations.

The presence in the isolated HMW protein HC component of small amounts of a polypeptide chain with the same immunoreactivity and apparent molecular weight as plasma LMW protein HC and urinary protein HC may be explained in three ways. Because this polypeptide chain may be a contamination from the isolation procedure, it may be released as a consequence of a partial cleavage of the 90,000-Da chain upon reduction or it may be a regular constituent of a small percentage of the normal HMW plasma protein HC component.

It is of considerable interest that the protein HC immunoreactivity cannot be released from the IgA immunoreactivity.
by complete reduction since this means that the protein HC-IgA complex is of another type than the disulfide-linked complexes formed between, e.g., IgA and J chain (32) or IgA and α1-antitrypsin (33). The stability of the protein HC-IgA complex and its general presence in human plasma suggest that it may play a role in the humoral immune system and warrant further investigations of this possibility.

The complete absence of the protein HC-IgA complex concomitantly with a normal concentration of the LMW protein HC component in the plasma samples from all investigated individuals with selective IgA deficiency demonstrates that IgA-secreting cells are necessary for the production of the protein HC-IgA complex. It cannot be decided, however, whether the complex is produced in IgA immunocytes or formed in the extracellular fluid.

Previous attempts to quantitate the plasma concentration of protein HC or α1-microglobulin have given widely different results and the reported mean plasma concentration has varied between 2 and 98 mg/liter (1, 11). No reports concerning the separate quantitation of the LMW and the HMW protein HC species present in human plasma have been published.

In all previously used procedures for the determination of the plasma concentration of protein HC or α1-microglobulin, standard solutions containing the isolated urinary protein HC species have been used (1, 3, 4, 8, 11, 34-40). This is probably one of the major reasons for the great variation in the reported results, since the dose-response curve for the HMW protein HC species and the LMW urinary protein HC most probably differ markedly regardless of the immunochemical procedure used for their quantitation. For example, as demonstrated in the present work, LMW protein HC gives on electroimmunoassay about 20 times higher response by weight than HMW protein HC. It is also evident, that unless the HMW and LMW protein HC species are separately quantitated, the same immunochemical response can be obtained for samples having greatly varying concentration ratios between the HMW and LMW protein HC molecules.

These standardization problems are taken into account in the quantitative crossed immunoelectrophoresis method proposed in the present work by the use of separate standards of isolated LMW and HMW protein HC in the simultaneous quantitation of both LMW and HMW protein HC present in human plasma. In this procedure, it is assumed that the cathodal precipitation arc obtained on crossed immunoelectrophoresis using antiserum against protein HC represents solely the HMW protein HC-IgA complex and the anodal arc solely the LMW free protein HC. The assumption regarding the cathodal precipitation arc seems to be fully justified since the use of an intermediate gel with anti-IgA antibodies completely suppresses this precipitate. The assumption concerning the anodal precipitation arc is more difficult to completely verify since such a verification would demand evidence that no molecular species other than LMW free protein HC contribute to the anodal precipitate. However, the assumption seems at least to be a very good approximation since the yield calculations for the isolation of LMW free protein HC from plasma show that at least 55% of the area below the anodal precipitation arc is accounted for by LMW protein HC and since no significant amounts of any other protein HC species with anodal mobility were observed during the isolation of molecules with protein HC immunoactivity from plasma.

The present work on quantitation of protein HC also demonstrates that the composition of the solution used to dilute the test and standard samples may influence greatly the results. For example, the ratios of the precipitation heights obtained on electroimmunoassay of equally concentrated solutions of isolated protein HC in 0.15 M NaCl, 0.075 M barbital buffer, and 0.075 M barbital buffer with 1% (w/v) BSA are about 1:1.7:2.1. Although the reasons for the influence of the dilution medium on the quantitation of protein HC by electroimmunoassay are unknown, it is highly probable that the results of other immunochemical methods used to measure protein HC will also be influenced by the composition of the dilution medium. We therefore believe that dilution of test and standard samples on immunochemical quantitations of protein HC generally should be done in such a way that the overall compositions of the test and standard samples are as similar as possible. In the present study, protein HC-free human plasma was used for dilution of both the test and standard samples.

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