the sensitivity. Thus, the dual-wavelength technique, which measures deviations from a null-balance point, offers equivalent or greater sensitivity for the detection of small pH perturbations.

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Chemical, Physical–Chemical, and Immunological Properties of Papain-Solubilized Human Transplantation Antigens†

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ABSTRACT: Papain-solubilized HLA-A, -B, and -C antigens have been isolated from cadaveric spleens. The isolated material was homogeneous and comprised subunits with the apparent molecular weights 33 000 and 12 000. Amino acid analyses of a mixture of HLA antigen heavy chains obtained from a great number of spleens with different HLA antigen phenotypes revealed a composition that is very similar to that of individual HLA-A and -B antigens. Likewise, the NH_2-terminal 30 residues of the HLA-antigen heavy chain mixture were virtually identical with that recorded for individual specificities. The circular dichroism spectra for the isolated HLA antigens and for free β_2-microglobulin revealed similarities with spectra recorded for immunoglobulin chains and domains. The HLA-antigen heavy chain may contain an appreciable amount of β structure. Antibodies raised against free β_2-microglobulin react better with β_2-microglobulin in free form than when bound to the HLA-A, -B, and -C antigen heavy chains. This is due to the fact that free β_2-microglobulin can bind a maximum of four Fab fragments simultaneously, whereas the HLA-antigen-associated β_2-microglobulin can bind only two Fab fragments without dissociating from the heavy HLA-antigen subunit.

The HLA[1] antigens are recognized by T-killer cells in the graft vs. host reaction (see Thorsby, 1974). It is the hydrophilic portion of the membrane-integrated HLA antigens that directly participates in this recognition event. To obviate the need for detergents in dealing with biologically active transplantation antigens, several groups of workers have shown that limited proteolysis yields a water-soluble fragment which can be isolated (Sanderson & Batchelor, 1968; Mann et al., 1969; Miyakawa et al., 1971; Cresswell et al., 1973; Peterson et al., 1974). Miyakawa et al. (1971) and Turner et al. (1975) have previously detailed methods to isolate papain-digested HLA antigens. In both studies the starting materials consisted of large quantities of in vitro grown lymphoblastoid cells. Because of the high cost of cultured cells, we have developed methods to obtain papain-solubilized HLA antigens in

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[1] For brevity, HLA-A, -B, and -C antigens are called HLA antigens throughout this article.

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chemical amounts from cadaveric spleens. The present report outlines the purification procedure. The chemical, physical-chemical, and immunological properties of the highly purified HLA antigens demonstrate that they are suitable for structural studies.

Materials and Methods

Antisera. Antisera against β_2-microglobulin were raised in rabbits by injections of the immunogen first into the popliteal lymph nodes and then booster injections were given intradermally. The free β_2-microglobulin was isolated to homogeneity essentially as outlined elsewhere (Berggård & Bearn, 1968). Antisera raised against highly purified detergent-solubilized (Trägårdh et al., 1979) and papain-solubilized HLA antigens were prepared as described above. In some cases, the antibodies directed against β_2-microglobulin were removed by passage of the antiserum over a Sepharose 4B column containing covalently bound β_2-microglobulin (Cuatrecasas, 1970). After this treatment, the antisera were specific for the heavy HLA-antigen chain.

Special Materials. Papain (60 units/mg) was obtained from Kebo AB (Stockholm). Ampholines were purchased from LKB Produkter AB (Stockholm). All other chemicals used were of analytical grade or better.

Membrane Preparation. Crude membrane fractions were prepared from cadaveric spleens within 3 days post mortem. Usually about 750 g of spleen tissue (wet weight) was repeatedly freeze-thawed and minced in 1000 mL of 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and subjected to homogenization in a Virtis homogenizer. Nuclei, unbroken cells, and debris were removed by centrifugation at 10000*g* for 10 min. The supernatant fraction was then subjected to centrifugation at 105000*g* for 60 min. The pellet was resuspended in 500 mL of the Tris-HCl buffer and the centrifugation procedure was repeated. The resulting pellet comprised the crude membrane fraction.

Papain Solubilization of the HLA Antigens. The crude membrane fraction was adjusted to 10 mg of total protein per mL in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 0.05 M cysteine, and 0.05 M EDTA.[2] Papain was added to a final concentration of 3 mg/mL. Digestion was carried out at 37 °C for 60 min, which in preliminary tests had been shown to be the optimal time period to maximize the release of water-soluble HLA antigens. After the digestion had been terminated by the addition of 0.055 M iodoacetic acid (final concentration) and the pH had been adjusted to 8 with NaOH, insoluble material was removed by centrifugation at 105000*g* for 60 min. The supernatant, containing the solubilized HLA antigens, was used as the starting material for the isolation of the HLA antigens.

Concentration of proteins during the isolation procedure was accomplished by ultrafiltration (Berggård, 1961). The recoveries of the HLA antigens after ultrafiltration always exceeded 90%.

Immunological Techniques. Immunoelectrophoresis was carried out as described (Scheidegger, 1955). β_2-Microglobulin was determined with a solid phase radioimmunoassay (Evrin et al., 1971), and indirect immunoprecipitations were carried out as detailed elsewhere (Östberg et al., 1976). Fab fragments were prepared from rabbit β_2-microglobulin antibodies and from normal rabbit IgG (Vahlquist & Peterson, 1973). Antibodies against β_2-microglobulin were used in preparing Sepharose 4B immunosorbent columns (Cuatrecasas, 1970).

Electrophoresis and Isoelectric Focusing. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as described by Laemmli (1970). Two-dimensional isoelectric focusing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis were performed as outlined by O'Farrell (1975). In the isoelectric focusing step, 6% polyacrylamide gels in the pH interval 3.5 to 10 (Ampholine, LKB Produkter AB, Stockholm) were used.

Amino Acid Analysis. Quantitative amino acid analyses were performed on a Beckman 121 M amino acid analyzer on extensively reduced and alkylated HLA antigen heavy chains. The heavy chain was separated from β_2-microglobulin by gel chromatography on a Sepharose 6B column equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 6 M guanidine hydrochloride. Tryptophan was estimated spectrophotometrically (Edelhoch, 1967).

NH_2-Terminal Sequence Determination. Automatic sequencing was carried out in a Beckman 890 C sequencer using the Beckman 122974 fast protein Quadrol program with 1.0 M Quadrol. All reagents and solvents for the sequencer were obtained from Beckman Instruments. Dithioerythritol, 15 μg/mL, was added to the extracting solvent, chlorobutane. The dried, reduced, and alkylated HLA-antigen heavy chains were dissolved in anhydrous trifluoroacetic acid and placed in the sequenator cup. Prior to application of the sample, a single cycle of the automatic procedure was carried out to verify delivery of all reagents and solvents. During this procedure, the cleavage acid, heptafluorobutyric acid, was omitted in order to clear the delivery lines of traces of the acid from previous runs. After using the Beckman sample application subroutine 92772, a blank cycle without the cleavage acid was carried out to condition the protein film and to extract any remaining extraneous materials such as buffer salts and free amino acids that reduce the background accompanying the first cycle.

The anilinothiazolinone derivatives, maintained under nitrogen in the refrigerated fraction collector of the sequencer, were dried under a stream of nitrogen and converted to the corresponding phenylisothiohydantoins in 0.2 mL of 1 N HCl containing 0.1% ethanethiol at 80 °C extracted with two 0.7-mL portions of ethyl acetate and were subsequently dried at 40 °C under nitrogen. The remaining aqueous layer was similarly reduced to dryness. The dried phenylthiohydantoins were dissolved in 50 μL of methanol, and aliquots were analyzed on a Waters high-pressure liquid chromatograph. The C18 column was equilibrated with 0.01 M sodium acetate buffer, pH 5.1, containing 18% acetonitrile. Elution was carried out with a 14-mL linear gradient from 18 to 45% in the sodium acetate buffer followed by another 18 mL of the limiting buffer.

All phenylisothiohydantoins were well resolved with the exceptions of isoleucine-phenylalanine and valine-methionine. Other aliquots of the phenylthiohydantoins were subjected to hydrolysis for 4 h in 6 M HCl containing 0.1% stannous chloride at reduced pressure to convert the derivatives back to the parent amino acids, which were analyzed on the Beckman 121 M amino acid analyzer. The recovery was computed for each step by including norvaline or norleucine as internal standards.

Analytical ultracentrifugations were performed in a Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature control unit and an electronic speed control device. Except where otherwise stated, all experiments were performed in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The centrifuge was operated at 60 000 rpm for sedimentation

[2] Abbreviations used: EDTA, ethylenediaminetetraacetate; CD, circular dichroism.

Table I: Purification of Papain-Solubilized HLA Antigens[a]

fractionation step	total protein (mg)	HLA antigens[b] (mg)	yield (%)	purity
papain-solubilized protein	13100[c]	6.4	100	0.049
1st DEAE-Sephadex chromatogr	595[c]	5.2	81	0.87
2nd DEAE-Sephadex chromatogr	61[c]	3.9	61	6.4
Sephadex G-200 chromatogr	10.5[c]	3.1	48	29.5
immunosorbent chromatogr	3.0[d]	2.9	45	97
Sephadex G-100 chromatogr	2.6[d]	2.7	42	104

[a] Crude membrane protein (21 800 mg) was subjected to papain solubilization. [b] Determined with a β_2-microglobulin radioimmunoassay with HLA antigens as the standard. [c] Determined by the Folin–Lowry method. [d] Estimated from the optical density at 280 nm under the assumption that an HLA antigen solution of 1 mg/mL has an optical density of 2.0 at 280 nm. Quantitative amino acid analyses on HLA-antigen samples were in agreement with this value.

velocity analyses. The sedimentation equilibrium experiments were performed by the meniscus depletion technique of Yphantis (1964) or by the modification of the Yphantis technique described by Chervenka (1970). The same techniques were used to examine the molecular weight of the heavy HLA antigen chain in 0.02 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride. Calculations of apparent weight average molecular weights were performed with values for the partial specific volumes obtained from the amino acid compositions (Edsall, 1943).

Determinations of Diffusion Coefficients, Molecular Weights, and Frictional Ratios. These measurements and the calculations were carried out as described (Karlsson et al., 1972).

Measurements of CD Spectra. CD spectra were measured with a Jasco Model J-20 spectropolarimeter. The proteins studied were dissolved in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. The spectra were recorded at room temperature using protein concentrations of 0.1–1.0 mg/mL and cells with from 10- to 1-mm path lengths were used. The optical density never exceeded 1.5. The results are given as reduced mean residue ellipticity, $[\theta]$, vs. wavelength. Each curve represents the average of at least three measurements. The parameter $[\theta]$ was computed in the usual manner (Björk & Tanford, 1971). The mean residue weights were calculated from the amino acid compositions and were found to be 114 for the HLA antigen heavy chains and 118 for β_2-microglobulin.

Other Methods. Radioactive labeling with ^{125}I was performed with the chloramine-T method (Hunter & Greenwood, 1962). Protein was determined with the Folin–Lowry method (Lowry et al., 1951).

Results

Purification of Papain-Solubilized HLA Antigens. A typical isolation procedure is summarized in Table I. The 105000*g* supernatant containing the papain-solubilized membrane proteins was subjected to chromatography on a column of DEAE-Sephadex. Figure 1A shows that all of the β_2-microglobulin emerged as a single, relatively broad peak just in front of the main 280-nm absorbance peaks. Fractions containing β_2-microglobulin were pooled and concentrated, as indicated in the figure.

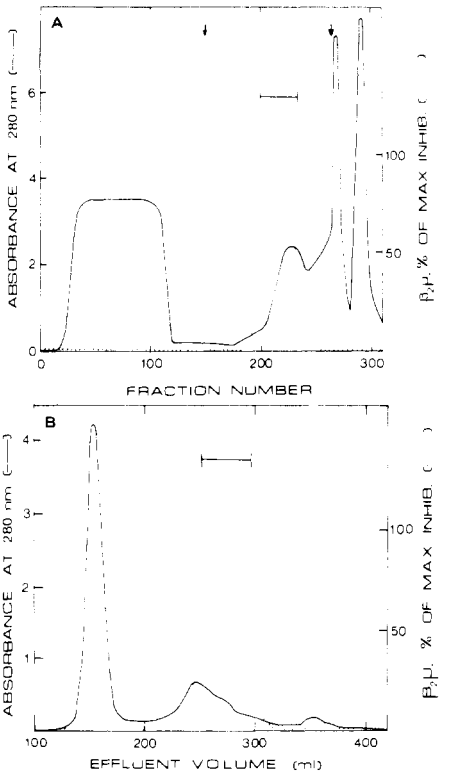

FIGURE 1: Purification of papain-solubilized HLA antigens. (A) First chromatography on DEAE-Sephadex of papain-solubilized spleen cell membrane molecules. The column (40 × 6 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. After application of the sample (containing 3100 mg of total protein and 6.4 mg of HLA antigen), which had been dialyzed exhaustively against the equilibrating buffer, elution was performed first with 1200 mL of the equilibrating buffer followed by a 2000-mL linear NaCl gradient from 0.05 to 0.6 M NaCl and finally with 500 mL of 1.0 M NaCl in the 0.02 M Tris-HCl buffer, pH 8.0. The arrows indicate the buffer changes. Fractions of 12 mL were collected at a flow rate of 60 mL/h. (B) Gel chromatography on Sephadex G-200 of the HLA-antigen-containing fraction obtained after the second DEAE-Sephadex chromatography step (see text). The column (146 × 2 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The sample, containing 61 mg of total protein and 3.9 mg of HLA antigens, was eluted at a flow rate of 8 mL/h, and fractions of 2.7 mL were collected. The distribution in the effluent of HLA antigens was monitored by a radioimmunoassay measuring β_2-microglobulin. The fractions containing the HLA antigens were pooled as indicated by the bars.

The β_2-microglobulin-containing fraction obtained from the first DEAE-Sephadex chromatography step was dialyzed against 0.02 M Tris-HCl buffer, pH 7.2, containing 0.05 M NaCl and rechromatographed on a DEAE-Sephadex column (26 × 6 cm) equilibrated with the dialysis buffer. Elution was performed with a 1500-mL linear NaCl gradient from 0.05 to 0.5 M. The β_2-microglobulin-containing material occurred in between the two main protein peaks and was eluted at a NaCl concentration of about 0.25 M.

After the two ion-exchange chromatography steps, the HLA-antigen (and thus, the β_2-microglobulin) containing fraction was subjected to gel chromatography on a column of Sephadex G-200. Figure 1B shows that the protein emerged as one dominating peak in the void fraction and several minor peaks in the included volume of the column. The HLA antigens were eluted at a K_{av} of about 0.4, as revealed by the fact that β_2-microglobulin occurred in this position. The β_2-microglobulin-containing fractions were pooled, as indicated by the bar in Figure 1B.

The HLA-antigen-containing gel chromatography fraction was applied on to a Sepharose 4B column containing covalently

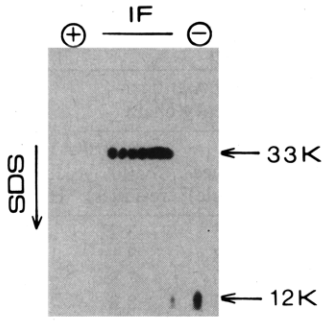

FIGURE 2: Two-dimensional isoelectric focusing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis of a preparation of highly purified, papain-solubilized HLA antigens. From the migration positions of molecular weight markers run separately, the values denoted by the arrows in figure were obtained.

bound antibodies against β_2-microglobulin. Protein which did not bind to the antibodies was eluted with the equilibrating buffer, 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl. β_2-Microglobulin-containing material was desorbed from the column by including 3 M $MgCl_2$ in the equilibrating buffer. The desorbed material was dialyzed against 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 NaCl, concentrated, and subjected to chromatography on a column (100 × 1 cm) of Sephadex G-100 equilibrated with the dialysis buffer. The protein eluted from the column occurred in two peaks. A small amount of material occurring in the void fraction comprised negligible amounts of β_2-microglobulin, whereas the symmetrical, main protein peak, emerging at a K_{av} of about 0.25, was eluted coincidentally with the β_2-microglobulin as revealed by radioimmunoassay determinations. The main protein peak was pooled and concentrated and represented highly purified HLA antigens.

Purity and Homogeneity of the Isolated HLA Antigens. It was obvious from the last purification step that the HLA-antigen-containing fraction was homogeneous in size. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis demonstrated that the HLA-antigen-containing fraction contained but two types of polypeptide chains (cf. Figure 2). The fastest migrating component, β_2-microglobulin, displayed a molecular weight of 12 000, whereas the larger chain had an apparent molecular weight of 33 000. No additional polypeptide chains were visualized.

The size homogeneity of the HLA antigens is not paralleled by a corresponding charge homogeneity as revealed by two-dimensional isoelectric focusing in urea and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The HLA antigen heavy chain gave rise to several distinct protein zones of similar if not identical molecular weight (Figure 2). That all the protein zones represented HLA antigen heavy chains was demonstrated by repeating this experiment with the HLA antigens labeled with ^{125}I. The radioactive material was mixed with an antiserum against β_2-microglobulin, and immune complexes were isolated and subjected to two-dimensional isoelectric focusing/sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The radioactive material gave rise to the same polypeptide pattern as shown in Figure 2.

Immunological Characteristics of the Isolated HLA Antigens. Immunodiffusion analyses with antibodies directed against β_2-microglobulin revealed that the papain-solubilized, highly purified spleen membrane HLA antigens gave a precipitin line displaying complete immunological identity with highly purified, detergent-solubilized platelet membrane HLA antigens (Trägårdh et al., 1979). Likewise, the absorbed antiserum specific for the heavy HLA antigen chain could not distinguish between the detergent-solubilized and the proteolytically derived HLA antigens. These data are consistent with the view that the papain-solubilized, HLA-antigen fragments do not exhibit an immunologically recognizable conformation differing from that of the intact, detergent-solubilized molecules.

Table II: Physical Characteristics of Papain-Solubilized HLA Antigens

sedimentation constant, $s^0_{20,w}$ (S)	3.8
Stokes' molecular radius (Å)[a]	32
diffusion constant (× 10^7 cm^2 s^{-1})[a]	6.3
frictional ratio (f/f_0)	1.31
mol wt	
sedimentation–equilibrium[b]	46 000
sedimentation–equilibrium (6 M Gdn·HCl)[c]	35 000
sedimentation–Stokes' radius	47 000
gel chromatography[d]	32 000; 12 000
electrophoresis[e]	33 000; 12 000
partial specific volume (mL/g)[f]	0.71
ellipticity at 217 nm (deg cm^2 $dmol^{-1}$)	−7500

[a] Estimated by analytical gel chromatography. [b] Average from three determinations at speeds of 26 000 and 30 000 rpm. [c] The value is for the isolated HLA-antigen heavy chain. [d] Determined in 6 M guanidine hydrochloride (Gdn·HCl) on reduced and alkylated polypeptide chains. [e] Data from sodium dodecyl sulfate–polyacrylamide gel electrophoresis. [f] The partial specific volume for the polypeptide portion of the HLA-antigen heavy chain is 0.71 and for β_2-microglobulin 0.72. These data were obtained from the amino acid compositions. It has been assumed that the HLA-antigen heavy chain carries 10% carbohydrate by weight (cf. Parham et al., 1977).

Immunoelectrophoretic analyses of the purified HLA antigens revealed that antisera directed against β_2-microglobulin and against the HLA antigen heavy chain recognized but single precipitin arcs. The HLA antigens exhibited a higher anodal electrophoretic mobility than free β_2-microglobulin.

Some Physical–Chemical Properties of the Papain-Solubilized HLA Antigens. Some physical and chemical properties of the highly purified, papain-solubilized HLA antigens are summarized in Table II. Sedimentation velocity analyses were carried out at protein concentrations ranging from 0.04 to 0.2%. The HLA-antigens behaved as a single homogeneous component in the ultracentrifuge within the concentration range tested. The sedimentation constant was only moderately dependent on the protein concentration. The Stokes' radius for the HLA antigens was determined by gel chromatography on calibrated columns of Sephadex G-200. The value obtained, 32 Å, was somewhat smaller than that for albumin, used as a marker in these experiments. The frictional ratio (f/f_0) 1.31 indicates that the papain-solubilized HLA antigens may not be spherical, but, as glycoproteins often have a high degree of hydration, it is likely that the calculated frictional ratio may be compatible with a rather globular structure.

Molecular weights were estimated by sedimentation equilibrium ultracentrifugation at two concentrations. The values obtained seemed to be independent of concentration. Linear relationships between ln C and r^2 suggested that the papain-solubilized HLA antigens were homogeneous. As can be seen in Table II, molecular weights measured by sedimentation equilibrium ultracentrifugation and calculated from the sedimentation constant and analytical gel chromatography data were in good agreement.

The two HLA antigen subunits, extensively reduced and alkylated, behaved as 32 000- and 12 000-dalton polypeptide chains on analytical gel chromatography on a column of Sepharose 6B equilibrated with 6 M guanidine hydrochloride and on sodium dodecyl sulfate–polyacrylamide gel electro-

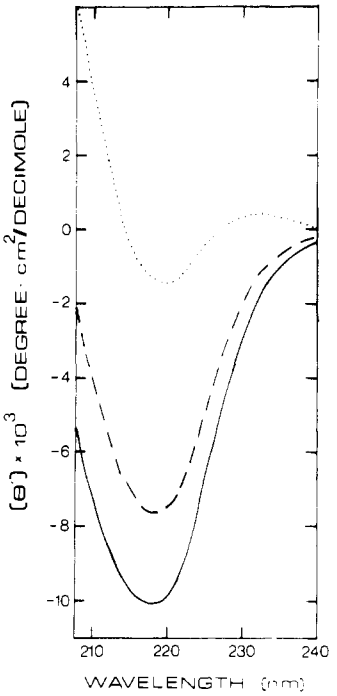

FIGURE 3: Measurements of the circular dichroism of β_2-microglobulin (···) and papain-solubilized HLA antigens (- - -). The theoretical curve for the HLA antigen heavy chain (—) was obtained by subtracting the curve found for β_2-microglobulin from the curve for the HLA antigens.

phoresis. The gel chromatography procedure was also used at the preparative scale, omitting the reduction and alkylation steps, to obtain the HLA antigen heavy chain free from β_2-microglobulin. The HLA-antigen heavy chain material was pooled, concentrated, and subjected to sedimentation–equilibrium ultracentrifugation in 6 M guanidine hydrochloride. The examined material appeared homogeneous and the ln C vs. r^2 plot gave a straight line. It can be seen in Table II that the molecular weight for the HLA-antigen heavy chain measured by sedimentation–equilibrium ultracentrifugation agrees reasonably well with the values obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel chromatography.

The conformations of the highly purified HLA antigens and of free β_2-microglobulin were investigated by circular dichroism measurements. Both for the HLA antigens and for β_2-microglobulin the CD curves show relatively little detail as can be seen in Figure 3. For the HLA antigens there is a major negative band at 217 nm with a reduced mean residue ellipticity of about −7500°. Due to scarcity of material, measurements of the fine structure in the aromatic region were precluded. β_2-Microglobulin displayed two well-resolved bands: a small positive one at 234 nm and a main negative band at 218 nm.

Despite numerous attempts, we have failed to isolate the HLA-antigen heavy chain free from β_2-microglobulin under nondenaturing conditions. To gain insight into the conformation of the HLA-antigen heavy chain, a theoretical curve was calculated by subtracting the experimentally found ellipticity for β_2-microglobulin from the curve found for the HLA antigens. This theoretical curve, shown in Figure 3, has as its main feature a pronounced band at 217 nm.

Amino Acid Composition and NH_2-Terminal Sequence Analysis of the Papain-Solubilized HLA Antigens. The amino acid composition of the HLA-antigen heavy chain is presented in Table III. Methionine and cysteine are the least frequent amino acids, whereas glutamic acid constitutes almost 17%

Table III: Amino Acid Composition of the Papain-Solubilized HLA-Antigen Heavy Chain[a]

amino acid	HLA-antigen heavy chain (residues/molecule)[c]	HLA-antigen heavy chain (residues/100 residues)	residues/100 residues HLA-A2[b]	residues/100 residues HLA-B7[b]
Lys	8.5	3.2	4.3	3.7
His	8.3	3.1	4.7	3.5
Arg	20.9	7.8	7.7	8.8
Asp	23.6	8.8	7.9	9.7
Thr[d]	19.6	7.3	7.6	6.2
Ser[d]	16.7	6.2	5.2	5.2
Glu	44.9	16.7	13.9	14.8
Pro	14.1	5.2	4.4	5.4
Gly	20.9	7.8	7.8	8.0
Ala	21.3	7.9	8.7	8.0
CM-Cys[e]	3.6	1.3	1.6	1.6
Val[f]	12.0	4.5	6.4	5.0
Met	3.3	1.2	1.4	1.1
Ile[f]	6.8	2.5	1.7	2.6
Leu	18.3	6.8	6.3	6.4
Tyr	12.3	4.6	4.8	4.5
Phe	6.0	2.2	2.9	2.3
Trp[g]	8.1	3.0	3.2	3.1

[a] Except where noted, all figures are average values of 24-, 48-, and 72-h hydrolyses. [b] Data taken from Terhorst et al. (1976). [c] Calculated on the assumption that the polypeptide portion of the HLA-antigen heavy chain has a molecular weight of 30500. [d] Values were obtained by extrapolation to zero-time hydrolysis. [e] Determined after extensive reduction and alkylation in 6 M guanidine hydrochloride. [f] Seventy-two hour hydrolysis value only. [g] Determined spectrophotometrically.

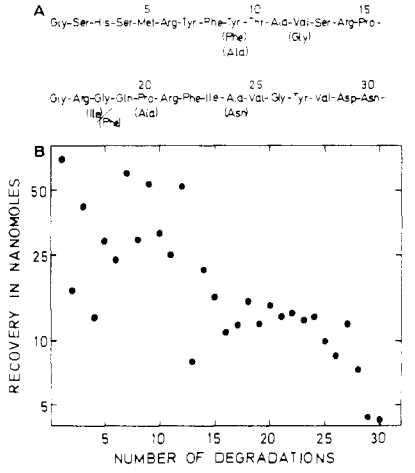

FIGURE 4: The NH_2-terminal sequence of the HLA antigen heavy chain as determined by automated sequence analysis. (A) The sequence represents amino acid residues found in highest amount for each degradation cycle. When multiple amino acid residues were obtained, those present in minor amounts are given within parentheses. (B) The yields of the phenylthiohydantoin derivatives for each degradation cycle are given. Approximately 80 nmol of material was subjected to analysis.

of the amino acid content. The amino acid compositions for papain-solubilized HLA-A2 and HLA-B7 antigens, determined by Terhorst et al. (1976), are included in the table for comparison. Although the HLA-antigen preparation examined here represents a mixture of several HLA-antigen specificities, most probably derived from all three HLA antigen loci, it is remarkable how similar the amino acid compositions are between the heterogeneous mixture and the two defined A- and B-loci products.

The NH_2-terminal sequence of the isolated HLA-antigen heavy chain, determined by automated sequencer analysis, is shown in Figure 4A. For each cycle, unambiguous identi-

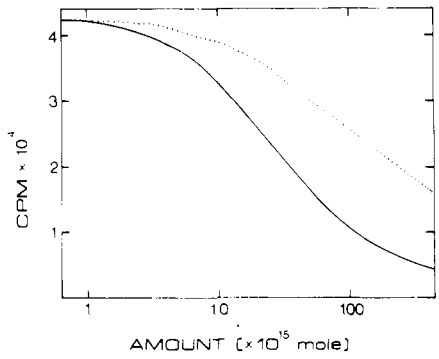

FIGURE 5: Inhibition curves of free β_2-microglobulin (—) and papain-solubilized, highly purified HLA antigens (···) in a β_2-microglobulin radioimmunoassay.

fication of a main amino acid residue was obtained. In some positions, multiple residues occurred (Figures 4A and 4B). This is not surprising as the analyzed material comprised molecules with various allotypes. However, the main sequence is identical with that published for an HLA-B7 antigen (Terhorst et al., 1977) with the exceptions that alanine and arginine were the residues found in positions 11 and 21, respectively, and that glutamine and asparagine rather than glutamic acid and glutamine occurred in positions 19 and 30, respectively.

Reactivity of Antibodies against β_2-Microglobulin with HLA Antigens. To obtain quantitative information about the difference in reactivity of anti-β_2-microglobulin antibodies with free and HLA-antigen-bound β_2-microglobulin, radioimmunoassay determinations were performed. Figure 5 shows that highly purified HLA antigens were about tenfold less efficient in molar amounts than the free β_2-microglobulin in competing with the ^{125}I-labeled, free β_2-microglobulin for binding to the antibodies. The inhibition curves for the two materials were not quite parallel. To further study the mechanism behind these observations, ^{125}I-labeled β_2-microglobulin and ^{125}I-labeled HLA antigens were separately mixed with excessive amounts of normal rabbit IgG Fab fragments and Fab fragments directed against β_2-microglobulin. The mixtures were subjected to sucrose density gradient centrifugation and to gel chromatography. Figure 6 (a and b) shows that free β_2-microglobulin and the HLA antigens in the presence of normal rabbit IgG displayed the expected sedimentation constants of 1.6 S and 3.8 S, respectively. In the presence of anti-β_2-microglobulin Fab fragments, β_2-microglobulin displayed a sedimentation constant of about 8.4 S (Figure 6c). Regardless of the amount of Fab fragments added, β_2-microglobulin never attained a higher sedimentation constant. This result suggests that, when all the antigenic sites of β_2-microglobulin are simultaneously occupied by Fab fragments, the resulting protein complex has a sedimentation constant of 8.4 S. When the same type of analysis was performed with the ^{125}I-labeled HLA antigens, three distinct radioactive peaks were evident (Figure 6d). On increasing the concentration of the anti-β_2-microglobulin Fab fragments, the relative amount of radioactivity in peaks IA and IIIA increased until peak IIA was virtually absent. To analyze the distribution of the HLA-antigen heavy chain in the gradient, aliquots from each fraction were mixed with rabbit antibodies specifically directed against the HLA-antigen heavy chain. Immune complexes containing intact antibodies, i.e., also the Fc region, were precipitated by the addition of *Staphylococcus aureus* Cowan I bacteria (Östberg et al., 1976). It can be seen in Figure 6d that the HLA antigen heavy chain was present only in peaks IIA and IIIA. Aliquots from each peak were also

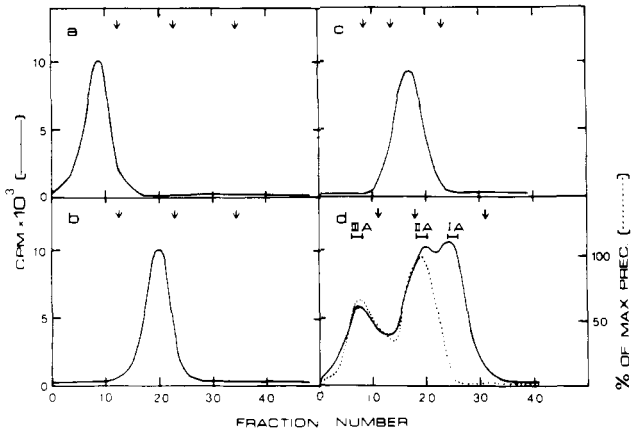

FIGURE 6: Sucrose density gradient ultracentrifugations of free ^{125}I-labeled β_2-microglobulin (a) and ^{125}I-labeled papain-solubilized, highly purified HLA antigens (b) in the presence of 0.3 mg of Fab fragments derived from normal rabbit IgG. ^{125}I-labeled β_2-microglobulin and ^{125}I-labeled HLA antigens were also analyzed by sucrose density gradient ultracentrifugation in the presence of 0.3 mg of Fab fragments prepared from rabbit anti-β_2-microglobulin IgG antibodies (c and d). The arrows denote from left to right the sedimentation positions of ^{131}I-labeled, retinol-binding protein (2.3 S), bovine serum albumin (4.4 S), and IgG (6.7 S) in a and b and bovine serum albumin, IgG, and secretory IgA (10.9 S) in c and d. For further explanations, see text.

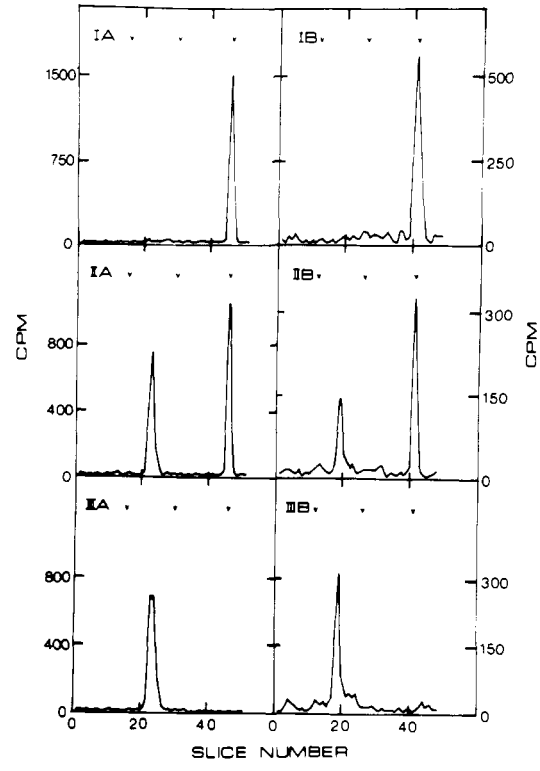

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ^{125}I-labeled material derived from the peaks depicted in Figures 6 and 8.

subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Figure 7 shows that peak IA from the sucrose density gradient only contained β_2-microglobulin, whereas peak IIIA only contained the HLA-antigen heavy chain. Material in peak IIA comprised both HLA-antigen subunits.

The mixtures of Fab fragments and ^{125}I-labeled β_2-microglobulin and HLA antigens, respectively, were separately subjected to gel chromatography on columns of Sepharose 6B. In the presence of Fab fragments derived from normal rabbit IgG, β_2-microglobulin and the HLA antigens exhibited the expected elution behavior, as can be seen in Figure 8a and 8b.

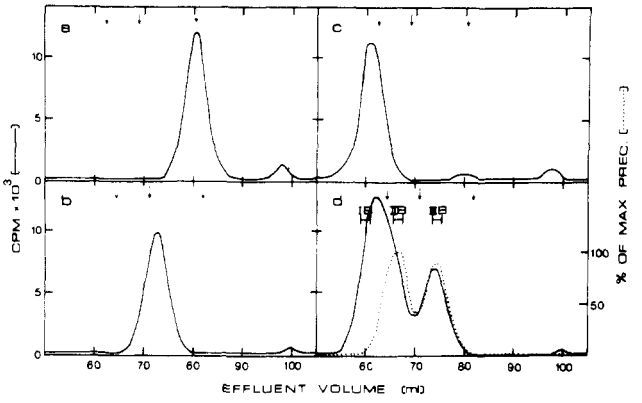

FIGURE 8: Gel chromatography on columns (108 × 1 cm) of Sepharose 6B of free ^{125}I-labeled β_2-microglobulin (a) and ^{125}I-labeled, highly purified papain-solubilized HLA antigens (b) in the presence of 0.3 mg of Fab fragments derived from normal rabbit IgG. ^{125}I-labeled β_2-microglobulin and ^{125}I-labeled HLA antigens were also subjected to gel chromatography after the addition of 0.3 mg of Fab fragments prepared from the IgG fraction of a rabbit antiserum directed against β_2-microglobulin (c and d). The columns were equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Fractions of 2.1 mL were collected at 20-min intervals. The arrows denote the elution positions of ^{131}I-labeled IgG, bovine serum albumin, and β_2-microglobulin. For further details, see the text.

In large excess, the Fab fragments derived from anti-β_2-microglobulin antibodies formed a relatively homogeneous complex with β_2-microglobulin (Figure 8c). However, the ^{125}I radioactivity emerged as one broad, early eluted peak and one later eluted peak when labeled HLA antigens were examined in the presence of the anti-β_2-microglobulin Fab fragments (Figure 8d). Indirect immunoprecipitations of HLA-antigen heavy chain containing material were performed on the gel chromatography fractions obtained. Figure 8d shows that HLA-antigen heavy chains occurred at two elution positions. The tail part of the first eluted radioactive peak and the second peak contained HLA-antigen heavy chains. Material in the fractions denoted IB, IIB, and IIIB in the figure were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Figure 7 shows that the earliest eluted fractions containing radioactivity (IB in Figure 8) only contained β_2-microglobulin and that the last eluted radioactive peak (fraction IIIB) comprised exclusively the HLA-antigen heavy chain. The intermediate fraction contained both HLA-antigen chains (Figure 7).

Table IV summarizes some physical characteristics determined for the various immune complexes. The sedimentation coefficients were determined by the sucrose density gradient centrifugation experiments. Stokes' molecular radii have been calculated from the gel chromatography analyses. From these values, the molecular weights for the complexes have been estimated. It is evident from the table that β_2-microglobulin can simultaneously bind a maximum of four Fab fragments. The situation for the HLA antigens is more complex. It appears that, when HLA-antigen-associated β_2-microglobulin binds more than two Fab fragments, it dissociates from the HLA-antigen heavy chain, which consequently occurs in free form (fraction III).

Table IV: Physical Characteristics of Immune Complexes Consisting of Fab Fragments Directed against β_2-Microglobulin and HLA Antigens and Free β_2-Microglobulin, Respectively

	free β_2-microglobulin	HLA antigens[a]		
		I	II	III
sedimentation constant (S)	8.4	8.8	7.0	3.3
Stokes' radius (Å)	61	61	48	28
mol wt[b]	214000	224000	141000	37000
ratio (Fab/$\beta_2\mu$)[c]	4.0:1	4.2:1	1.9:1	

[a] The HLA antigens gave rise to three distinct fractions on gel chromatography and sedimentation velocity analyses. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed that fraction I contained β_2-microglobulin free from the HLA-antigen heavy chain. Fraction II contained intact HLA antigens and fraction III free HLA antigen heavy chains. [b] Calculated from the sedimentation constants and the Stokes' radii. [c] To calculate the number of Fab fragments bound per molecule of β_2-microglobulin, it was assumed that the molecular weights for the Fab fragments, β_2-microglobulin, and the HLA antigens were 50000, 12000, and 46000, respectively.

Discussion

The rather simple isolation procedure adopted gives highly purified HLA antigens in a reasonable yield. The main advantage of the purification procedure is that large amounts of solubilized macromolecules can be used as the starting material. Turner et al. (1975) have previously described an efficient purification procedure for papain-solubilized HLA antigens derived from lymphoblastoid cells. Although these workers started out with a highly purified membrane preparation, their isolation scheme involved about the same number of fractionation steps as described here. Miyakawa et al. (1971) have also described a relatively efficient isolation procedure for papain-solubilized HLA antigens, although documentation of the purity of the isolated material is largely lacking.

The isolated HLA antigens were shown to be homogeneous in size as revealed by several techniques. This is in contrast to Turner et al. (1975) who obtained a 37000-dalton fragment of the HLA-B7 chain rather than a 34000-dalton fragment. This discrepancy is most easily explained on the assumption that the proteolysis with papain was not as extensive as that employed here. Alternatively, the B7 antigen may be slightly different than other B-series molecules. The size homogeneity obtained here contrasted remarkably with the charge heterogeneity. It is the heavy HLA-antigen subunit that exhibits this heterogeneity. Since it is the heavy subunit that is cleaved by papain, an enzyme with a broad substrate specificity, the possibility exists that the heavy chain is "frayed" in its COOH-terminal end (Terhorst et al., 1976). However, papain digestion of the intact, detergent-solubilized HLA antigens does not significantly increase the charge heterogeneity (Trägårdh et al., 1979). This is in keeping with the observation that papain-solubilized HLA antigens displaying the antigenic specificities A-2 and B-7 have identical COOH-terminal amino acid residues (Henriksen et al., 1976). The charge heterogeneity is therefore most probably due mainly to variations in the content of sialic acid. For human and murine transplantation antigens of defined antigenic specificities evidence has been presented demonstrating that neuraminidase treatment produces molecules of much greater charge homogeneity than the untreated controls (Parham et al., 1974; Kvist et al., 1977).

The amino acid composition of the isolated HLA-antigen heavy chain agrees remarkably well with the amino acid compositions published for HLA antigens of defined antigenic specificities (cf. Terhorst et al., 1976; Henriksen et al., 1976). These data give credence to the view that the HLA antigens, at least from the A and B loci, are very similar in structure regardless of antigenic specificity. This notion is also supported by the NH_2-terminal sequence analysis and by cleavages of the HLA antigen heavy chain from pooled antigenic specificities with acid, cyanogen bromide, and various proteolytic enzymes. These analyses demonstrate that all heavy chains have certain amino acid sequences that are invariant (un-

published observations). This makes HLA antigens from cadaveric spleens an attractive source for further structural work in as much as milligram quantities of the HLA antigens may easily be obtained.

Molecular weight determinations of the isolated HLA-antigen subunits and of the intact molecule were in excellent agreement. Previous molecular weight estimates have relied upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In view of the difficulty of obtaining good molecular weight estimates with this method when glycoproteins are analyzed, it is reassuring to note that sedimentation–equilibrium ultracentrifugations yielded molecular weight data in close agreement with those of the electrophoretic procedure. The physical–chemical characteristics of the highly purified papain-solubilized HLA antigens did not differ in any significant way from those of globular, water-soluble glycoproteins.

Preliminary studies have shown that the HLA antigens in their primary structure display typical immunoglobulin-like disulfide bridges (Peterson et al., 1975; Terhorst et al., 1977). This feature is in keeping with the suggestion that HLA antigens may be evolutionarily related to the immunoglobulins (Gally & Edelman, 1972; Bodmer, 1972; Peterson et al., 1974). The present data on the circular dichroism of the HLA-antigens may lend some support to this view. Thus, the most prominent CD band for the HLA antigens and their subunits is situated at 217–218 nm. A band at this position, generally of a magnitude in between those of β_2-microglobulin and the HLA antigens, is common to all immunoglobulins, the Fab and Fc fragments, heavy and light chains, and isolated immunoglobulin domains (cf. Dorrington & Tanford, 1970; Björk et al., 1971) with the exception of the pFc fragment (Litman et al., 1970; Dorrington et al., 1972). This band is generally ascribed to an n $\rightarrow$ p transition of the β-structured backbone conformation (Litman et al., 1970; Cathou et al., 1968), and the theoretically computed curve for the heavy HLA antigen chain suggests that this subunit has an appreciable content (about 40%) of β structure (cf. Chen et al., 1972), whereas at most a few percent (less than 10) of α-helical structure is consistent with the data. It is interesting to note that free β_2-microglobulin also seems to contain β structure and the spectrum recorded for this protein agrees well with a previous study (Karlsson, 1974).

The theoretical CD curve computed for the HLA-antigen heavy chain was used since methods to separate the two HLA-antigen subunits under nondenaturing conditions were not available. This theoretical curve is meaningful only if β_2-microglobulin when free and when bound exhibits the same conformation. To investigate this, the reactivity of antibodies raised against free β_2-microglobulin was examined. The antibodies recognized free β_2-microglobulin considerably better than they recognized β_2-microglobulin bound to the heavy HLA-antigen subunit. The reason for this is that, to simultaneously bind more than two Fab fragments, β_2-microglobulin has to dissociate from the HLA-antigen heavy chain. Thus, the immunological reactivity of β_2-microglobulin does not argue for the free form being different from the bound molecule.

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Mr. Kjell Andersson and Mr. Jörgen Eriksson skillfully assisted in this study. Dr. Ingvar Sjöholm kindly performed the CD measurements.

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Interaction of a Bivalent Ligand with IgM Anti-Lactose Antibody†

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ABSTRACT: A model system has been developed for the study of the interaction between bivalent ligands and multivalent antibody. This system utilizes modified β_2-microglobulin as the carrier of the reactive lactosyl groups and equine anti-lactose IgM antibody. Chemical modification of the carrier allows conjugation of two such groups to cysteinyl residues at positions 25 and 81 with a potential separation of 20 nm. Each lactosyl group contains a 2,4-dinitrophenyl moiety which serves as a sensor of the binding of the group to an antibody site and permits binding measurements by fluorescence quenching. Examination by analytical ultracentrifugation has demonstrated no significant cross-linking by the bivalent ligand of either the IgM antibody or the monomer (IgMs) derived from it. Binding constants for complex formation between the bivalent and monovalent ligands and IgM, IgMs, and Fabμ have been determined by fluorescence titrations. These measurements also established that the bivalent ligand was bound to IgM and IgMs primarily as a cyclic complex. The small enhancement factor (ca. threefold) for the binding of the bivalent ligand compared with the monovalent one was attributed to the loss of configurational entropy associated with the formation of the cyclic complex. The complex of bivalent ligand and IgM antibody was effective in the depletion of complement in contrast to the complex with the small monovalent ligand. It is suggested that this antigen-mediated effector activity is the consequence of a distortion of the normal planar structure of the IgM molecule. The structural perturbation would consist of a bending of some of the $F(ab')_2$ regions out of the plane containing the $(Fc\mu)_5$ core due to the cross-linking of adjacent $F(ab')_2$ regions by the bivalent ligand.

The interaction of soluble multivalent antibody with a multivalent particle containing identical antigenic determinants is a characteristic feature of the expression of biological function by humoral antibody. This feature is found in the interaction of IgG and IgM antibodies, for example, with viruses, bacteria, and malignant cells. It is in this context that the full potential of multivalent antibody to form complexes ("functional affinity") is evident (Karush, 1976) and in which conformational changes associated with effector functions would be revealed.

The thermodynamic and structural characterization of multivalent complex formation can, probably, be achieved most readily with relatively simple, soluble systems involving a bivalent ligand capable of forming a 1:1 cyclic complex. This capability depends on appropriate spacing between the two reactive groups of the ligand because of the apparent restriction on the distance of closest approach between the combining sites of a bivalent antibody (Werner et al., 1972). This separation is probably limited to a minimum value of ~9 nm.[1] The failure of several bivalent ligands unable to extend to this minimum distance to exhibit bivalent interaction with 7S anti-lactose antibody has pointed to the significance of the restriction (Gopalakrishnan & Karush, 1974).

The major published study of the formation of monomeric and higher cyclic bivalent complexes was carried out by Archer & Krakauer (1977) with equine anti-2,4-dinitrophenyl (Dnp[2]) antibodies of the IgG and IgG(T) classes. The bivalent ligands were linear poly(ethylene glycol) polymers, with Dnp groups attached to their ends, covering a molecular weight (number average) range of 25 000 to 75 000. This elegant and thorough study revealed that, with random coil ligands in this range, there was a large loss of configurational entropy associated with ring closure. This entropic term gave rise to a closure factor of the order of magnitude of 10^{-5} for the conversion of the linear 1:1 complex, for example, to the cyclic form. Nevertheless, it was found by measurement of light scattering that the predominant species in an equimolar mixture (5×10^{-6} M) of IgG antibody and ligand (58 000) was the cyclic monomeric form. Its stability was derived from the relatively

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[1] A recent study by R. Luedtke, C. Owen, and F. Karush (manuscript in preparation) has confirmed and extended the findings of Werner et al. (1972). In both studies, the nanosecond monophoton technique was used to detect energy transfer between a fluorescent donor bound at one antibody site and an acceptor group bound to the other site of the same molecule. In the earlier study, however, the single inter-heavy-chain disulfide of the rabbit IgG antibody had been reduced and alkylated. This cleavage introduced the possibility that the separation observed with the modified antibody was not characteristic of the native antibody. In the recent experiments, this ambiguity was eliminated by the re-formation of the interchain disulfide in the course of preparation of the hybrid IgG antibody.

[2] Abbreviations used: Dnp, 2,4-dinitrophenyl; EK, *N*-(N^{α}-acetyl-N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside; iodo-EK, *N*-(N^{α}-iodoacetyl-N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside; PBS, 0.15 M NaCl, 0.02 M phosphate, pH 7.4; TLC, thin-layer chromatography; BSA, bovine serum albumin; bis(EK), bivalent ligand in which EK groups are attached through the S atoms of the cysteine residues at positions 25 and 81 of β_2-microglobulin; $NaDodSO_4$, sodium dodecyl sulfate.

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