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Detergent Solubilization, Purification, and Separation of Specificities of HLA Antigens from a Cultured Human Lymphoblastoid Line, RPMI 4265*

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HLA antigens have been purified to homogeneity after detergent solubilization from RPMI 4265, a human lymphoblastoid line. The inhibition of cytotoxicity assay for HLA antigen was modified, using preincubation with bovine serum albumin of antigen samples containing detergent to prevent lysis of target cells by detergent. Solubilization was tested with many types of detergents. A polyethyleneglycol oleyl ether nonionic detergent mixture, Brij 99:Brij 97 (2:1) was selected for solubilization, since it selectively solubilized HLA antigens, had a low absorbance at 280 nm and was uncharged. HLA antigens were then purified by *Lens culinaris* lectin affinity chromatography and Bio-Gel A-5m filtration. The antigen specificity HLA-A2 was separated from specificities HLA-B7,12 by isoelectric focusing. Purified HLA antigens contained a subunit of M_r = 44,000 with NH_2-terminal glycine, and a subunit of M_r = 12,000, β_2-microglobulin, with NH_2-terminal isoleucine.

HLA antigens, the major histocompatibility antigens of humans, carry the immunologic determinants involved in transplantation rejection. Two closely linked loci in the major histocompatibility complex, *HLA-A*, formerly called the LA or first locus, and *HLA-B*, formerly called the 4 or second locus, each code for biochemically similar antigens, which are co-dominantly expressed on the cell surface of many tissues. (For simplicity, the *A* and *B* locus will be called *HLA*.) The products and genetic arrangement of the major histocompatibility complex of higher vertebrates are highly homologous. Other closely linked genes in the major histocompatibility complex code for immune response, disease susceptibility, immune cell interactions, serum complement components, developmental abnormalities, and B cell-specific alloantigens (Ia) (1, 2). Many products of the major histocompatibility complex seem to function in the immune system, or are cell surface structures, or both.

HLA antigens are the target of killing in an *in vivo* analogue of graft rejection, the cell-mediated lympholysis assay. In the assay, responder lymphocytes which have been cultured with target lymphocytes from another individual, recognize target cells by virtue of their HLA antigen specificities, and kill them (1, 3, 4). Recently, it was shown that one biological function of H-2 antigens, the murine homologue of HLA, may be to be recognized as an altered self-antigen during viral infection or transformation, *e.g.* killer cells may recognize either a virally modified form of H-2 antigens, or a complex of H-2 antigens with a viral product (5-8). Alternatively, independent recognition of both H-2 types and a foreign antigen may be required for cell-mediated lympholysis, as a general rule, since it is required for both rejection of grafts (9), and killing of virally infected cells. It has been suggested that HLA antigens share certain structural features with immunoglobulins (10-12), and therefore it was predicted that they may have a function in the immune surveillance system.

Further structural information about HLA antigens may be of importance in the understanding of their function on the cell surface. Most of what is now known about the chemistry of HLA is based on studies of papain-solubilized HLA antigens, since they are much easier to purify than detergent-soluble HLA antigens. The preparation of large quantities of HLA antigens has been facilitated by the use of cultured lymphoblastoid lines such as RPMI 4265, used in this study, which have a greatly enhanced amount of HLA antigens on their cell surface (20- to 50-fold more than peripheral blood lymphocytes) (13) and are a reproducible source of antigen. HLA antigens, when solubilized with detergents, contain a glycoprotein subunit of 44,000, the gene for which is on chromosome 6 (14), containing the unique HLA antigenic specificities, and a subunit of M_r = 12,000, β_2-microglobulin, the gene for which is on chromosome 15 (15), and which is common to all specificities (10, 11, 16-20). When membranes are treated with papain, water-soluble HLA antigens are released containing a M_r = 34,000 glycoprotein and β_2-microglobulin (21, 22). 3 M KCl has also been reported to solubilize a native molecule (23). However, KCl solubilization requires whole cells and has been suggested to be due to autolysis by cell sap (24). Moreover, the size of the 3 M KCl product is considerably smaller than that of the detergent-solubilized molecule.

The structure of detergent-soluble HLA antigens is of considerable interest, since their structure is presumably very similar to that of native HLA antigens in the membrane, differing only in that bound lipid is replaced by bound detergent. The portion of the glycoprotein subunit of detergent-soluble HLA which is cleaved from papain-solubilized HLA antigens has been found to contain cysteines involved in dimer

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formation (10, 25) and a detergent binding region (16). Detergent-solubilized HLA and H-2 antigens have been partially purified by biochemical techniques (16, 26–28), and a pure mixture of HLA specificities was obtained by these techniques and an additional β_2-microglobulin affinity column step[1] (12, 29, 30). The assay of detergent-solubilized HLA antigens was facilitated by the inclusion of bovine serum albumin preincubation in the assay procedure to prevent lysis of target cells by detergent (16). Detergent soluble H-2 and HLA antigens have been prepared on a small scale by immunological techniques such as precipitation of radio-labeled antigens with alloantisera or β_2-microglobulin affinity chromatography (12, 16, 29–31). The present report describes in detail the assay and detergent solubilization of HLA antigens, their purification to homogeneity by large scale biochemical techniques, and the separation of HLA antigenic specificities by isoelectric focusing.

MATERIALS AND METHODS

Detergents – Detergents were kindly given by the companies indicated: Amine 0 (CIBA-GEIGY Co., Saw Mill River Rd., Ardsley, N. Y.); Triton X-100, Triton X-114, and Triton QS-15 (Rohm and Haas Co., Philadelphia, Pa.); Teric 17A25 (ICIANZ, Nicholson St., Melbourne, Australia); G-1292, G-1790, G-2127, Renex 20, Brij 30, Brij 35, Brij 56, Brij 96, Brij 97, Brij 98, Brij 99, Tween 21, G-263, G-271, Atlox 3409-F, and Atlox 3403-F (Atlas Chemicals Division, ICI America Inc., Wilmington, Del.). Large amounts (8 pounds) of four lots each of Brij 97 and Brij 99 were obtained from Emulsion Engineering, Elk Grove Village, Ill. Solutions (10%) of several lots were allowed to stand in the cold room for 2 weeks. One lot of Brij 97 precipitated, probably due to saturated fatty alcohol adduct contaminants, and was discarded. The lots with lowest A_{280} were selected for use in HLA antigen purification (Brij 97: Lot 7140B; Brij 99: Lot 4162B). Hydrophilic-lipophilic balance numbers were found in *McCutcheon's Detergents* (32) or in product descriptions from ICI and Rohm and Haas. The hydrophilic-lipophilic balance number assigned to a mixture of two detergents was calculated as a weighted average (33). Deoxycholic acid and cholic acid were obtained from Sigma, sodium glycocholate, and sodium taurochenodeoxycholate from Research Plus, and sodium octyl sulfate from Eastman.

Reagents – The following reagents were employed: Bio-Gel A-5m, 200 to 400 mesh; Bio-Gel A-50m, 100 to 200 mesh; and gel electrophoresis reagents (Bio-Rad); ninhydrin, hydrindantin, and SDS[2] (Pierce Chemical Co.); dithiothreitol, Tris, and Hepes (Sigma); and proteins: thyroglobulin, γ-globulin, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, β-galactosidase, fumarase, phosphorylase *a*, lysozyme, hemoglobin, bovine serum albumin, and ovalbumin (Sigma); β-lactoglobulin (Schwarz/Mann); myoglobin (Pentex); catalase (Worthington); and myosin (the kind gift of Dr. Lloyd Waxman, The Biological Laboratories, Harvard). D-[^{3}H]Glucose (New England Nuclear) was used as a total column volume marker. Other reagents were reagent grade or the best grade obtainable.

HLA Antigen Assay – The ^{51}Cr inhibition of cytotoxicity assay (34–36) in microtiter plates (37) was used with modifications. Antisera were used at the following concentrations to give 60% ^{51}Cr release, except for FS which when used undiluted gave 40% release: Davis (HLA-A2), 1:3; BC (HLA-A3), 1:2; and FS (HLA-B12), 1:1; were the kind gifts of Dr. Bernard Amos, Duke University. Jackson (HLA-B7), NIH 2-54-702-17-01, 1:8, was obtained from the Division of Transplantation Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health; Sanderson (HLA-B7) was the kind gift of Dr. Arnold Sanderson, McIndoe Memorial Research Laboratory, East Grinstead, England. Sanderson was used in model experiments to determine whether 30% albumin interfered in the HLA assay, but since it did not react with the "short" HLA-B7 of RPMI 4265 cells, was not used in inhibition assays. Lymphocytes were prepared by Ficoll-hypaque gradient centrifugation (38), and were washed once with dextrose/gelatin/Veronal/saline (DGV buffer) (Gibco), and resuspended in 100 μl of autologous serum:DGV buffer (1:1). Na^{51}Cr-chromate, 10 mCi/ml in saline (0.9% NaCl solution) (New England Nuclear) (50 μl) was added and cells were labeled for 2 to 4 h at 37°, with blending on a Vortex mixer every half-hour. Immediately before use, cells were washed three times with DGV buffer (centrifugation at 400 × *g*) and resuspended to 2 × 10^6 cells/ml. Antigen samples containing detergent were stood with the appropriate volume of 30% albumin, 0.5% NaN_3 in saline (Sigma), then diluted 2-fold serially in 50 μl of DGV buffer in a master dilution microtiter plate. Antigen samples (except for column fractions) were assayed in duplicate. Aliquots (5 μl) were transferred to another plate for each HLA antigen specificity being assayed, 5 μl of antiserum was added (all reagents in the cytotoxicity assay were added with multiple dispenser syringes (Hamilton Co., Reno, Nev.)), and the covered plates were incubated for 2 h at 37°. ^{51}Cr-labeled lymphocytes (5 μl) were added and incubated 1/2 h at 37°. Complement (rabbit serum, Gibco) diluted 1:4 with 0.14 M NaCl, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01 M Tris/HCl, pH 8.0 (100 μl) was added and incubated 1/2 h at 37°. This large (100 μl) volume prevented nonspecific inhibition of complement by albumin or by material in the antigen solution, and did not lower the titer of the HLA alloantisera. An aliquot (50 μl) of DGV buffer 20 mM EDTA in 0.14 M NaCl, 10 mM sodium phosphate, pH 7.4 (1:1), containing 1 drop/20 ml of erythrocytes from the Ficoll-hypaque gradient as a marker for the cell pellet, was added and the plates were centrifuged at 400 × *g* for 5 min. Aliquots (100 μl) were withdrawn and counted in a Nuclear Chicago 1185 counter. The inhibitory titer is the inverse of the dilution of antigen, in the assay volume after target cell addition, giving 50% inhibition of ^{51}Cr release. Controls with no antigen and no alloantiserum (AB serum used instead) were included and an HLA antiserum (BC) for an antigen (HLA-A3) not found on RPMI 4265 cells was included as a control for nonspecific inhibition.

Protein Assays – Column effluents were monitored for protein by measuring the A_{280}/A_{310} ratio. For more accurate determination, protein was measured using a micromodification of the ninhydrin assay (39), since nonionic detergents interfered with the Lowry assay (40). To samples containing 1 to 30 μg of protein in 100 μl in a tube (7 × 70 mm) which had been washed with glacial acetic acid, were added 30 μl of 6.75 N NaOH. Samples were heated at 95° for 2 h, cooled, and 25 μl of glacial acetic acid was added. Deaerated ninhydrin reagent (39) (200 μl), modified to contain 6 mg/ml of hydrindantin, was added, and an aspirator vacuum was applied and the tube immediately agitated for 10 s. Samples were heated 20 min at 95°, cooled, 400 μl of 50% ethanol was added, agitated to oxidize residual hydrindantin, and the A_{570} was measured. Albumin was used as a protein standard. Since Ampholines were present in Pools i, ii, and iii, even after extensive dialysis, protein was precipitated before assay by adding 0.5 volume of acetone and 0.1 volume of 100% w/v trichloroacetic acid, standing on ice for 1 h and centrifuging at 1500 × *g* for 5 min. An HLA antigen sample (Pool C-1) of similar concentration was also precipitated to control for the percentage of protein precipitation.

Phosphate Determination – Samples were ashed, hydrolyzed (41), and assayed by the ascorbic/molybdate procedure (42). The micromoles of phosphate were multiplied by 775 to calculate the micrograms of phospholipid.

Centrifugation to Determine HLA Solubility – Centrifugation of samples of about 50-μl volume was done in 12-ml tubes at 100,000 × *g* maintaining the centrifuge at the above speed for 5 min. Since the average sedimentation distance was short (0.5 mm), centrifugation for longer times would have sedimented soluble proteins, *e.g.* a protein of 1.45 S would sediment 0.5 mm in 1 h at 100,000 × *g*.

Lectin Affinity Column Preparation – The lectin from *Lens culinaris* beans, purchased at a local grocery, was purified (43) and coupled (44) to Bio-Gel A-50m using a modified procedure. The mixture of phytohemaglutinin I and II, 300 mg in 75 ml of 0.1 M $NaHCO_3$, 0.2 M methyl α-D-mannopyranoside, 0.1 mM $CaCl_2$, 0.1 M $MnCl_2$, was coupled to 75 ml of Bio-Gel A-50m after activation with 600 mg of CNBr. Coupling was 85% complete after 10 h and was stopped with 2 ml of 1.0 M Tris/HCl, pH 8.0.

Isoelectric Focusing in Sucrose Gradient – Electrofocusing was performed in a specially designed, waterjacketed column (500 × 20 mm) with integral stopcock fitted with male Luer outlet (Kontes Custom Drawing 028030-01) and a top adapter (Kontes Catalogue No. K-42820) joined with O-ring and clamp. The bottom outlet was connected with 5/8 inch inside diameter Tygon tubing to a 125-ml

[1] R. J. Robb, D. L. Mann, and J. L. Strominger (1976) *J. Biol. Chem.* **251**, 5427–5428.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; Brij 98:96, Brij 98:Brij 96 (2:1); Brij 99:97, Brij 99:Brij 97 (2:1); DGV buffer, dextrose/gelatin/Veronal buffer; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HLB, hydrophilic-lipophilic balance; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

reservoir, and the tubing and reservoir were filled with 50% w/v sucrose, 2% phosphoric acid, which was admitted into the column to a level 10 mm above the bottom of the waterjacket and the stopcock was closed. Six milliliters of 0.75% pH 4 to 6 Ampholine, 0.75% pH 5 to 7 ampholine, 1 mM dithiothreitol, 1.5% Brij 97-99 (Solution I) containing 45% sucrose were layered on top. Then a linear gradient of 69 ml of 40% sucrose in Solution I and 81 ml of 5% sucrose in Solution I was formed in the column. The sample, 49 ml of Pool C-2, was included in the 5% sucrose solution. Then 9 ml of 2.5% sucrose in Solution I were layered on, and overlaid with 4% ethylenediamine to the top of the column, on top of which was placed a membrane made from dialysis tubing, taking care to exclude bubbles. The membrane was inserted to prevent the solutions inside the column from moving due to hydrostatic pressure when the stopcock was opened. The O-ring and adapter were placed on top and clamped. The adapter was connected with Tygon tubing to a 125-ml reservoir, and this part of the assembly was filled with 4% ethylenediamine. The two reservoirs were placed at approximately equal heights, and platinum electrodes were inserted in the 50% sucrose, 2% phosphoric acid (anode), and 4% ethylenediamine (cathode). The stopcock was opened, and the voltage was set at 400 V for 4.5 days, during which time the current dropped from 1.3 to 0.4 mA. The column was cooled by a circulating waterbath at 4° connected to the waterjacket, and the column was placed in the coldroom to minimize temperature differences between the jacketed and unjacketed areas of the column. At the completion of focusing, the stopcock was closed, and the ethylenediamine and phosphoric acid reservoirs were disconnected. A piece of Tygon tubing containing 60% sucrose was connected to the stopcock outlet, excluding bubbles, and the column was eluted with a peristaltic pump, at a flow rate of 0.5 ml/min, and 1.4-ml fractions were collected.

Isoelectric Focusing in Acrylamide Gels—Samples were applied to the top of, and focused (45) in acrylamide gels (9 × 0.4 cm) containing 1.2% pH 5 to 7 ampholine and 0.1% Triton X-100. Triton X-100 was used rather than Brij 99:97 because the latter detergent contains antioxidants which required larger amounts of persulfate to be used in polymerization, and because persulfate might cause oxidation of the unsaturated bonds in the detergent.

RESULTS AND DISCUSSION

Solubilization of HLA Antigens with Detergent

Assay of HLA Antigens in Presence of Detergents—In the inhibition of cytotoxicity assay for HLA antigens, HLA antigens bind to HLA antibodies, preventing the antibodies from binding lymphocyte target cells of appropriate HLA antigen specificity. In the presence of complement, the ^{51}Cr-labeled lymphocyte target cells are lysed, releasing intracellular ^{51}Cr into a 400 × *g* supernatant. Since detergents also lyse ^{51}Cr-labeled lymphocytes, a method was sought of avoiding detergent-mediated lysis of lymphocytes. Bovine serum albumin was tested for its ability to prevent lysis after preincubating it with 3-fold serial dilutions of detergents (Fig. 1). Albumin proved very effective in preventing lysis, presumably because detergents were bound to its fatty acid binding sites. For example, in the absence of albumin, concentrations of the nonionic detergent Brij 30 greater than 3^{-6}% caused cytolysis. When an equal volume of 30% albumin was added, a concentration of detergents 80-fold greater (3^{-2}%) was required to cause lysis. Therefore, when compared to the alternative of diluting an antigen in detergent solution to avoid lysis, the addition of albumin renders the assay for antigens 80-fold more sensitive. From the data in Fig. 1, the stoichiometry of detergent binding was calculated to be 2.8 mol of detergent/mol of albumin for Brij 35, and 3.6 mol/mol for Brij 30. Multiple binding sites on albumin for fatty acids have also been found (46). The method for preventing detergent lysis by albumin was general for all types of detergents tested. Ten volumes of 30% albumin added to 1 volume of 1% detergent prevented lysis by all the nonionic, cationic, anionic, and bile salt detergents listed in Fig. 5 and Table I. The minimum amount of 30% albumin required to prevent lysis was determined for the detergents most used in this paper. The number of volumes required per volume of 1% detergent solution were sodium cholate, 2; Brij 96, 8; Brij 98, 2; Triton X-100, 10.

It was also important to establish that albumin did not interfere with the ability of serum and complement to lyse target cells. When 30% albumin was added to dilutions of antiserum, it did not change the amount of lysis obtained in the standard assay procedure, which included 100 μl of 4-fold diluted complement (Fig. 2). If instead, 5 μl of undiluted complement was added to give approximately the same final concentration, partial inhibition by albumin was detected. A large (100 μl) volume of complement was therefore used in the standard assay to dilute the effect of albumin as well as other substances in the antigen sample which might inhibit complement. Likewise, in the standard assay procedure, it was found that 30% albumin did not affect the ability of HLA antigens to inhibit serum and complement-mediated lysis (data not shown).

Parameters Affecting Solubilization of HLA Antigens with Detergent—To find conditions where maximal HLA antigen activity and minimum total protein would be solubilized, various parameters affecting solubilization were extensively investigated. The time dependence of solubilization was investigated, using whole cells rather than membranes, because they could be centrifuged more rapidly. Solubilization with the nonionic detergent G-2127 was carried out at 4°, was 85% complete after 5 min, and fully complete after 30 min. The conditions of 4° and 30 min were adopted for further experiments.

The pH dependence of detergent solubilization was investigated with two polyethyleneglycol oleyl ether nonionic detergents, Brij 96 and Brij 98 (Fig. 3). Solubilization of HLA-2 was maximal above pH 7.5, with half-maximal solubilization occurring at pH 5.7 for Brij 96 and pH 6.6 for Brij 98. The isoelectric point of HLA-A2 is between pH 5.2 and 6.0 (see below). It has been reported that membrane vesicles have a minimal volume near pH 4, suggesting that the apparent isoelectric point of the vesicles is near this pH (47). Therefore, both HLA-A2 antigens and the membrane as a whole are negatively charged at pH values of 7.5 and above where solubilization is maximal. Charge repulsion may have some importance in solubilization. Charge repulsion also seems to play a role in the distribution of freeze-fracture intramembrane particles. These particles aggregate when the pH is lowered to 5.5 or when the NaCl concentration is increased to 0.15 M (48).

When detergent solubilization was carried out in the presence of 0.14 M NaCl, 0.01 M Tris/HCl, pH 8.0, 30% less HLA was solubilized than in 0.01 M Tris/HCl alone. Also the addition of NaCl to a 100,000 × *g* detergent supernatant caused flocculent precipitation. However, after HLA had been highly purified, it was completely soluble in 0.5 M NaCl, 2% Brij 99; Brij 97 (2:1), 0.01 M Tris/HCl, pH 8.0, as judged by gel filtration.

Solubilization of HLA antigen was dependent on Brij 98 concentration, and reached a plateau at a 1:1 ratio of detergent to membrane protein (Fig. 4). The ratio of Brij 98 to membrane protein, rather than the absolute concentration of detergent, was the important criterion in determining the amount of HLA solubilized, as was determined in other experiments where the protein concentration was much higher. This was because during solubilization, detergent is bound to HLA antigens and other membrane proteins (16, 49). It should be noted that the critical micelle concentration is 2 orders of magnitude

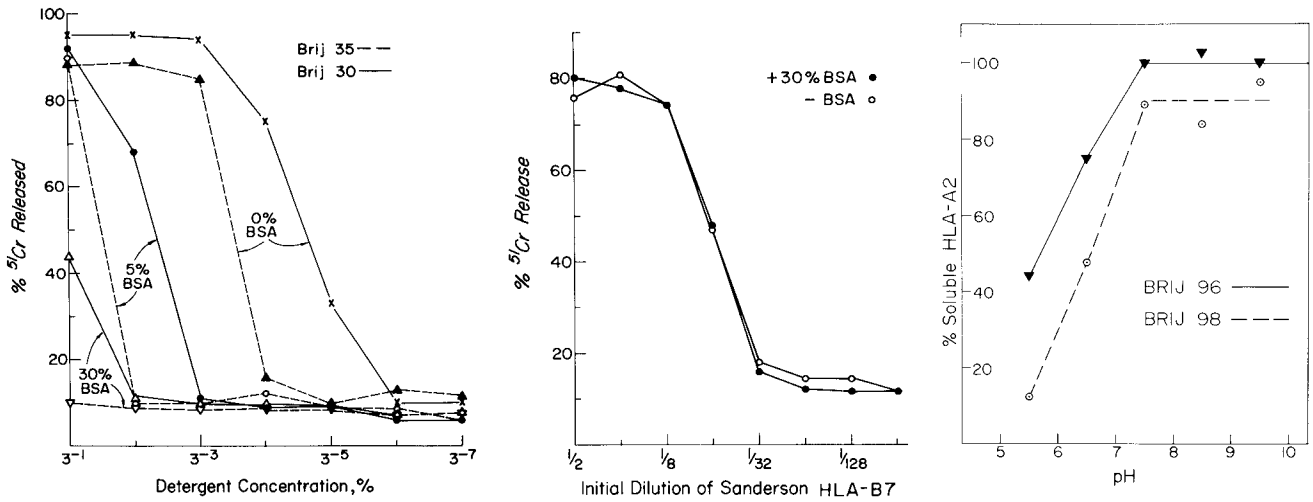

FIG. 1 (*left*). Protection against detergent cytolysis by preincubation with bovine serum albumin (*BSA*). Aliquots (10 μl) of 30%, 5%, or 0% albumin in saline were added to 10 μl of 3-fold serial dilutions of Brij 30 or 35. After 10 min at 37°, 5 μl of ^{51}Cr-labeled lymphocytes were added, and the remaining steps were carried out as described under "HLA Antigen Assay" ("Materials and Methods").

FIG. 2 (*center*). Lack of effect of 30% bovine serum albumin (*BSA*) upon a cytotoxic titration. The assay was carried out essentially as described under "HLA Antigen Assay" ("Materials and Methods") except that 30% or 0% albumin in saline was used instead of antigen, and 2-fold dilutions of Sanderson (HLA-B7) antiserum were used instead of a uniform antiserum dilution.

FIG. 3 (*right*). pH profile of detergent solubilization. Membranes were centrifuged at 100,000 × *g* for 1 h and resuspended in distilled water by homogenization to 26.5 mg/ml. To 60 μl of membranes were added 30 μl of the following buffers (0.2 M): pH 5.5, sodium acetate; pH 6.5, sodium cacodylate; pH 7.5, sodium Hepes; pH 8.5, Tris/HCl; pH 9.5, sodium glycine. Detergent, 20 μl of 10% Brij 96 or 10% Brij 98 was added, and tubes were stood 1/2 h at 4° and 20-μl aliquots were withdrawn to assay for pH stability. The remainder was centrifuged at 50,000 × *g* for 2 h, and the supernatant withdrawn. Supernatants and uncentrifuged controls were assayed for HLA-A2 antigenic activity. The controls showed that HLA was completely stable over the entire pH range tested.

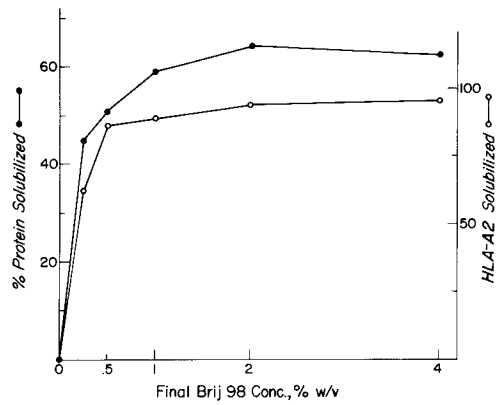

FIG. 4. Effect of detergent concentration on HLA antigen solubilization. To 25 μl of detergent was added 25 μl of membranes (final protein concentration of 6 mg/ml in 0.01 M Tris/HCl, pH 8.0). After 30 min at 4°, the mixture was centrifuged as described under "Materials and Methods," and supernatants were assayed for HLA-A2 antigen and protein.

lower than the concentration (0.5%) required for solubilization and therefore, the existence of detergent micelles is not sufficient for solubilization.

Since little was known about what detergent was best suited for use in the solubilization and purification of HLA antigens, a wide variety of detergents was tested. The presence of HLA antigenic activity in a 100,000 × *g* supernatant was used as an initial criterion of solubility and this was confirmed by gel filtration in later experiments. Nonionic detergents are commercially available with many different types of lipophilic groups, usually attached to polyethyleneglycol chains of a defined average length. In the emulsifier industry, an empirical value called the hydrophilic-lipophilic balance number is assigned to each detergent on the basis of comparison to standard detergents. The hydrophilic-lipophilic balance number is used to predict the solubilization and emulsification properties of the detergent. The structure of the hydrophobic group of a detergent can exert a lesser, but definite effect on its ability to solubilize a given substance. Hydrophilic-lipophilic balance numbers are on a scale of 0 to 20 with 20 most hydrophilic, 0 most hydrophobic, and 10 equally hydrophilic and hydrophobic. The hydrophilic-lipophilic balance number can also be accurately predicted from the relative weight of hydrophobic and hydrophilic moieties (hydrophilic-lipophilic balance = % hydrophile/5) (50, 51). Most detergents with low hydrophilic-lipophilic balance numbers (11 and below) are not truly water-soluble although they can be dispersed. Some detergents with higher hydrophilic-lipophilic balance numbers are also insoluble, generally those with long, saturated fatty alcohol hydrophobic groups. Only detergents which formed clear solutions were tested for their ability to solubilize HLA antigens.

A general pattern emerged when solubilization was plotted as a function of the hydrophilic-lipophilic balance number of the detergent (Fig. 5). Detergents with several different types of hydrophobic group structures were tested, and some scatter in the data exists which is probably attributable to these differences. The five detergents which gave between 95 and 100% solubilization all had hydrophilic-lipophilic balance between 12 and 14.3. Most but not all of the other detergents in this range were nearly as effective at solubilization. The effect of hydrophilic-lipophilic balance number alone was demonstrated in a series of detergents which contain oleyl alcohol lipophilic groups and only differ in the length of their polyethyleneglycol side chains. The amount of solubilization decreased as the hydrophilic-lipophilic balance number increased from 14.3 to 16.2. Detergents with oleyl alcohol lipo-

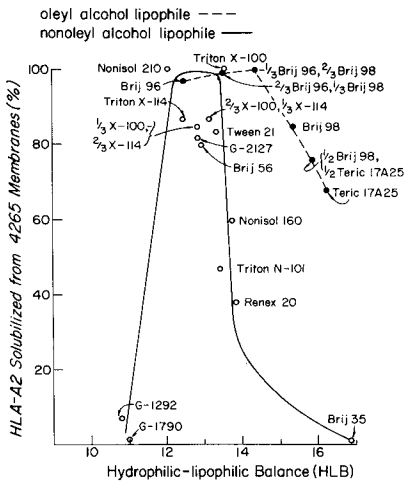

FIG. 5. Solubilization of HLA with nonionic detergents. Membranes (25 μl, 3.9 mg/ml in 0.01 M Tris/HCl, pH 7.5) were mixed with 25 μl of 1% nonionic detergent in 0.01 M Tris/HCl, pH 7.5, or with 25 μl of 1% ionic detergent (see Table I) in 0.01 M Tris/HCl, pH 7.5, 0.14 M NaCl, and stood at 4° for 1/2 h. They were centrifuged as described under "Materials and Methods" and the supernatant was withdrawn for HLA-A2 assay. HLA activity was found in pellets of membranes which had been treated with nonionic detergents which did not solubilize HLA activity (G-1292, 76%; Brij 35, 35%).

TABLE I

Solubilization of HLA with ionic detergents

Procedures are described in the legend to Fig. 5. Ionic detergents which did not solubilize HLA activity (sodium octyl sulfate, sodium dodecyl sulfate, amine O, Triton Q S-15, Atlox 3403-F, and Atlox 3409-F) were tested to determine whether this result was artifactual, due to failure of albumin to prevent detergent lysis of target cells. Control assays in which AB serum was substituted for the HLA-A2 antiserum showed detergent-mediated lysis did not occur. Pellets treated with ionic detergents which did not solubilize HLA antigenic activity were resuspended, assayed, and found not to contain HLA antigenic activity.

Class	Detergent	HLA solubilized
		%
I. Bile salts	Sodium cholate	94
	Sodium deoxycholate	100
	Sodium glycocholate	87
	Sodium taurochenodeoxycholate	107
II. Alkyl cationics	G-263	93
	G-271	39
	Sodium octyl sulfate	<2
	Sodium dodecyl sulfate	<2
III. Alkyl anionics	Amine O	<2
	Triton QS-15	<2
IV. Nonionic-ionic blends	Atlox 3409-F	<2
	Atlox 3403-F	<2
	1/2 Brij 56, 1/2 Sodium Cholate	98
	1/2 Brij 96, 1/2 Sodium Cholate	107

philic groups seemed to differ from other detergents tested, being relatively better solubilizers at high hydrophilic-lipophilic balance than other detergents. Nonionic detergents did not denature HLA antigens, since, when activity was not in the supernatant, it was found in the pellet. Hydrophilic-lipophilic balance numbers have also been found to be useful in predicting what detergents would solubilize the D-alanine carboxypeptidase of *Bacillus subtilis* (52).

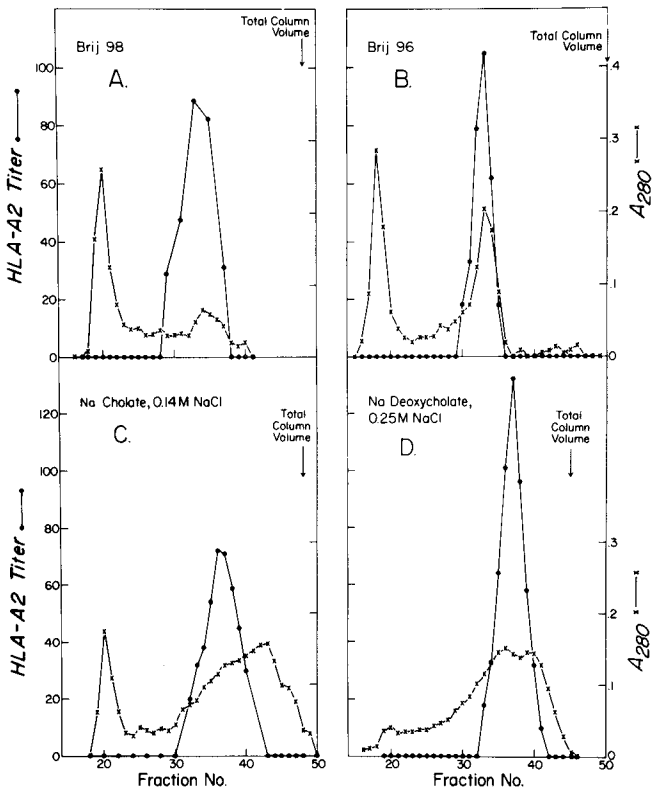

FIG. 6. Bio-Gel A-5m filtration of HLA solubilized with various detergents. To 250-μl membranes, 7 mg/ml, were added 50 μl of 10% detergent; 10 M Tris/HCl, pH 8.0, was added to a final concentration of 0.03 M, and 2.8 M NaCl was added to bring to the concentrations noted in the figure. The sample was centrifuged at 100,000 × *g* for 1/2 h and the sample was applied to a Bio-Gel A-5m column (42 × 1.1 cm) eluted with the above buffer plus 0.5% detergent, 1 mM EDTA, 0.02% NaN_3 at a flow rate of 3 ml/h.

Ionic detergents were also tested for their ability to solubilize HLA antigens (Table I). Four different bile salts, including sodium cholate and sodium deoxycholate, gave complete solubilization. Some alkyl cationics solubilized HLA antigens in active form, while SDS and sodium octyl sulfate did not, but solubilized almost all the membrane protein; no activity remained in the pellet, and therefore, HLA antigen was solubilized in a nonantigenic form. Alkyl anionics also failed to solubilize HLA antigenic activity. Two commercially available nonionic-ionic blends failed to solubilize HLA, but nonionics (Brij 56 and Brij 96) and a bile salt (sodium cholate) which successfully solubilized separately were also effective as a mixture.

It was concluded that two different modes of solubilization exist, resulting in solubilization in active or inactive antigenic form. SDS which solubilizes in inactive form is known to unfold and bind to the entire length of polypeptide chains (53). On the other hand, detergents which solubilize in active form, such as Brij 99:97 do not bind to the hydrophilic portion of HLA molecules which is solubilized by papain, and presumably only bind to the portion of the remaining polypeptide which normally binds to membrane lipid (16, 49).

As a further criterion of solubility, detergent-solubilized HLA antigen preparations were filtered on Bio-Gel A-5m (Fig. 6). The four detergents in Fig. 6 and Triton X-100 (data not shown) all solubilized HLA antigens in a form which was included in the gel. However, the detergents varied in the amount of protein solubilized which chromatographed in Bio-

TABLE II

Molecular weight of HLA antigens determined by gel filtration in presence of various detergents

HLA antigens were solubilized with the appropriate detergent and subjected to gel filtration as described in the legend to Fig. 6. The column was calibrated with bovine thyroglobulin, bovine catalase, bovine β-lactoglobulin, and equine myoglobin. In each gel filtration, the total column volume was determined with D-[^{3}H]glucose.

Detergent	M_r of detergent-soluble HLA
Brij 98	460,000
2/3 Brij 98, 1/3 Brij 96	410,000
Brij 96	380,000
Triton X-100	330,000
2/5 Sodium deoxycholate, 3/5 Brij 96, 0.14 M NaCl	260,000
Sodium cholate, 0.14 M NaCl	230,000
Sodium deoxycholate, 0.28 M NaCl	160,000

Gel A-5m in the same position as HLA antigens. For example, deoxycholate solubilized most membrane proteins, while Brij 98 appeared to solubilize HLA antigens selectively, since much less of the membrane protein was included in the gel, and much protein was left in the excluded volume, presumably aggregated. This is presumably because as shown in Fig. 5, Brij 98 has an hydrophilic-lipophilic balance which is at one end of the hydrophilic-lipophilic balance scale in which solubilization occurs, and the hydrophilic-lipophilic balance optimum for other proteins may not extend so far into the hydrophilic end of the scale, making them insoluble in Brij 98.

The molecular weights estimated by gel filtration depend on the type of detergent used. Molecular weights ranged from 460,000 in Brij 98 to 160,000 in deoxycholate (Table II). The molecular weights were much higher than 56,000, the sum of molecular weights of the M_r = 44,000 and 12,000 subunits (see below). The gel filtration data could be consistent either with binding of a micelle of variable size depending on detergent, or aggregation, the extent of which depends on the detergent. Later studies in the detergent Brij 99:97 showed that in certain preparations of HLA, material at high M_r (640,000 to 1,000,000) was present in addition to the majority of HLA at M_r = 410,000 (25). Studies with the cross-linking reagents 4-mercaptobutyrimidate and Lomant's reagent (dithiobis(succinimidyl propionate)) showed that the higher molecular weight material was aggregated, while the M_r = 410,000 material was monomeric, containing only one M_r = 44,000 and one M_r = 12,000 subunit (25). Therefore, the differences in molecular weight must be due to differences in the size of the detergent micelle bound to the antigen. The variability in size of HLA in different detergents also correlates with variability in size of detergent micelles, since micelles of nonionic detergents are much larger than those of deoxycholate and cholate (49). In a previous report (16), it was shown that during gel filtration in Brij 98:96, HLA antigens chromatographed at an M_r only slightly higher than that of the major phospholipid peak. This is consistent with HLA antigens being bound to a micelle of only slightly different size than that to which phospholipid is bound.

The binding of a detergent micelle with large Stokes radius and low density (49) (ρ = 1.06 for Brij 99:97) is consistent with the $s_{20,w}$ = 5.2 S[3] determined by sedimenting HLA in a sucrose gradient (Fig. 7), which is considerably lower than $s_{20,w}$ = 16.4 S for a globular protein of M_r = 410,000. Sucrose gradient sedimentation of HLA antigens showed that HLA in detergent can be separated from phospholipid, which remains near the top of the gradient. Presumably detergent covers the area of the molecule previously covered by phospholipid. This is important in purification, since if phospholipid remained bound it could cause charge heterogeneity in isoelectric focusing. In the final form of the purification procedure, phospholipid was removed from HLA in the lectin affinity column step.

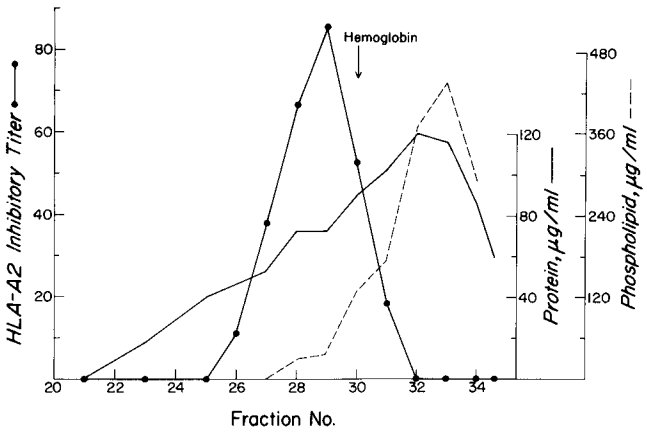

FIG. 7. Sedimentation of HLA in a sucrose gradient. HLA was sedimented in a 3 to 15% sucrose gradient (54) containing 0.3% Brij 98:96, 0.01 M Tris/HCl, pH 8.2, at 120,000 × *g* for 17 h at 4°. Equine hemoglobin was sedimented in a parallel gradient.

Selection of Detergent for Use in Purification – After examination of many detergents for possible use in the solubilization and purification of HLA antigens, the oleyl ether polyethyleneglycol nonionic detergent mixture, Brij 98:Brij 96 (2:1) was chosen. Although bile salts solubilized HLA antigens excellently, they were discarded since they would interfere with separations based on charge, such as ion exchange chromatography and isoelectric focusing. Detergents such as Triton X-100 and Nonidet P-40 were also eliminated, since their hydrophobic moiety, nonylphenol, has a high extinction coefficient at 280 nm which would make monitoring protein at this wavelength impossible.

The mixture of Brij 98:Brij 96 (2:1) was chosen because it selectively solubilized HLA antigens (as judged by gel filtration), was uncharged, and had a low absorbance at 280 nm. In later work Brij 99 and 97 were used, which have the same chemical formulas as 98 and 96, respectively, but which contain an oleyl alcohol from a purer commercial source.

Purification of Detergent-solubilized HLA Antigens

All procedures were carried out at 4°. HLA antigens were stored on ice, except that for periods of storage longer than 1 day, antigen preparations were frozen and stored at −80° with no loss in activity.

Step 1: Membrane Preparation – Frozen RPMI 4265 cells (HLA-A2; B7,12) (301 g) were quickly thawed in a 37° bath and placed on ice. Since the frozen cells were usually acidic (pH 5 to 6), the pH was rapidly adjusted to pH 7.5 with 1 M Tris. The volume was adjusted to 1200 ml with 0.14 M NaCl, 0.01 M Tris/HCl, pH 7.5. The cells were homogenized with three strokes of a Teflon pestle homogenizer and centrifuged at 3,000 rpm (1,500 × *g*) for 5 min in a Sorvall GS-3 rotor. The supernatant solution was carefully decanted, and the pellet was extracted twice more as described above. The three supernatants (2,810

[3] Bound detergent would lower ρ and introduce some uncertainty in the $s_{20,w}$ value. Allowing for a large amount of detergent (M_r = 350,000) bound to M_r = 56,000 of protein, the $s_{20,w}$ value would be raised to 5.8 S.

ml) were pooled and centrifuged in the 300-ml bottles of the Sorvall GSA rotor at 13,000 rpm (27,000 × *g*) for 1 h. The majority of membranes were pelleted, and were washed once by resuspending with homogenization in 700 ml of 0.01 M Tris/HCl, pH 7.5, and centrifugation for 1.5 h at 13,000 rpm. The membranes remaining in the first 13,000 rpm supernatant, comprising 20% of the total membrane HLA activity, were pelleted by centrifugation in an IEC A-170 rotor at 39,000 rpm (160,000 × *g*) for 1 h. They were washed by resuspending in 140 ml of 0.01 M Tris, pH 7.5, and centrifugation at 160,000 × *g* for 1.5 h. The washed membranes were resuspended with homogenization in 450 ml of 0.01 M Tris/HCl, pH 8.0.

Step 2: Detergent Solubilization—A 20% solution of Brij 99:Brij 97 (2:1) (63 ml) was added to the membranes while stirring to give a ratio of detergent:protein of 2:1. Membrane protein had been quickly estimated by boiling an aliquot in 1% SDS and measuring the A_{280}/A_{310} (1 $A_{280-310} \cong 0.6$ mg/ml). Dithiothreitol (1 mM) was added and was included in all subsequent buffers to prevent formation of disulfide-bonded dimeric and oligomeric forms of HLA antigens (25). After 30 min at 4°, the material was centrifuged at 39,000 rpm (160,000 × *g*) for 1 h in an IEC A-170 rotor. The supernatant solution was carefully decanted, avoiding a white viscous layer on top of the pellet.

Step 3: Lectin Affinity Chromatography—The supernatant solution after detergent solubilization was made 0.01 mM in $MnCl_2$ to avoid demetallization (55) of the lectin and applied at a flow rate of 30 ml/h to a *L. culinaris* lectin affinity column (16 × 2.2 cm) (300 mg of lectin coupled to 75 ml of CNBr-activated Bio-Gel A-50m). After washing with 225 ml of 0.01 M Tris/HCl, pH 8.0, 1 mM dithiothreitol, 0.01 mM $MnCl_2$, 0.05% Brij 99:97 (wash buffer), the HLA activity was eluted with 700 ml of wash buffer containing 2% methyl mannoside at a flow rate of 70 ml/h (Fig. 8). Slow elution was essential, since release of HLA from the lectin binding sites occurred slowly. NaCl was not used in the wash buffer because it caused partial elution of HLA from the column. Brij 99:97 was used at the low concentration of 0.05% in the elution buffer because it is retained during the subsequent ultrafiltration step. Fractions were assayed for HLA activity. Late wash fractions and elution fractions containing high HLA activity/A_{280} unit (Pool II, Fig. 8) were pooled and saved, while later flow-through fractions and early wash fractions containing low specific activity of HLA antigens (Pool I, Fig. 8) were pooled and rechromatographed on the same column, after it had been re-equilibrated with wash buffer. Chromatography was repeated until no HLA activity could be detected in the flow-through fractions and it was all recovered during elution with methyl mannoside. No evidence was found for a carbohydrate-free form of HLA antigens (28). Usually, one or two rechromatographies were required. It was important not to omit rechromatography, if required, because aggregated HLA (and, when dithiothreitol was omitted, disulfide-linked HLA) which was detected during the subsequent gel filtration step had a higher affinity for the lectin column than monomeric HLA and was a much higher proportion of the HLA activity in the first methyl mannoside elution than in subsequent elutions (25). The pools from each column were combined and concentrated on a PM-10 membrane in a 500-ml Amicon apparatus, and then dialyzed overnight against 0.02 M Tris/HCl, pH 8.0, 1 mM dithiothreitol.

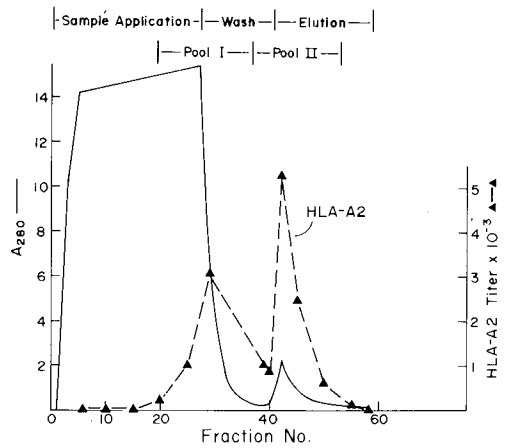

FIG. 8. Lens culinaris lectin affinity chromatography. The lectin affinity column was eluted with 300 ml of wash buffer and detergent-solubilized HLA antigens were applied to the affinity column and chromatographed as described in the text. Fractions of 24 ml were collected.

Step 4: Gel Filtration—Gel filtration was conducted on a 750 ml (117 × 2.9 cm) column which was packed with a 2:1 slurry of Bio-Gel A-5m, 200 to 400 mesh, in 0.02 M Tris/HCl, pH 8.0, 0.02% NaN_3, 0.1 mM EDTA, 1 mM dithiothreitol, 2% Brij 99:97, with flow at 15 ml/h controlled during packing with a peristaltic pump. Flow was adjusted to the same rate after packing by hydrostatic pressure. The column was checked by chromatographing myoglobin, and if band distortion was noticed, a new column was packed. The sample from the lectin step was divided into three equal portions which were separately chromatographed on the Bio-Gel A-5m column (Fig. 10). A sharp peak of HLA-A2 activity was detected at M_r = 400,000, and sharp HLA-B7 and HLA-B12 peaks were detected at slightly higher molecular weight (a reproducible, small difference). This same molecular weight (see Table II) was also found in the absence of dithiothreitol, showing dithiothreitol had not caused dissociation of hypothetical multimeric forms of HLA antigens, but merely stabilized the native monomeric form, which can be oxidized during purification to multimeric forms (25). Seventy to eighty-five per cent of the HLA-A2 and HLA-B7,12 antigens were in the monomeric peak at about M_r = 400,000. A smaller amount of HLA activity, apparently in an aggregated form, was found in a shoulder at higher molecular weight. The aggregated material did not dissociate upon rechromatography on Bio-Gel A-5m, in the presence of 1 mM dithiothreitol, nor upon rechromatography at pH 9.0, and therefore the aggregation cannot be due to disulfide bonds. Aggregated, non-disulfide-bonded forms of HLA were also found in the absence of dithiothreitol (25). The fractions were pooled as indicated in Fig. 9, and the HLA-containing fractions (Pool C) were concentrated 4-fold with a PM-10 membrane in an Amicon apparatus.

Step 5: Rechromatography on Bio-Gel A-5m—Portions of the material from Step 4 (15 ml) were subjected to a second Bio-Gel A-5m filtration (Fig. 10). Fractions containing HLA activity were placed in three pools (C-1, C-2, C-3). SDS-gel electrophoresis showed that Pool C-2 and C-3 contained only M_r = 44,000 and 12,000 polypeptides. Pool C-1 contained contaminants and was concentrated 4 times, rechromatographed, and pooled as above into C-1, C-2, and C-3. The remaining C-1 could not be further purified by rechromatography, but was sufficiently pure for applications such as sequencing after preparative SDS-gel electrophoresis.

Much better resolution during Bio-Gel A-5m filtration was obtained when the column was equilibrated with 2% Brij 99:97

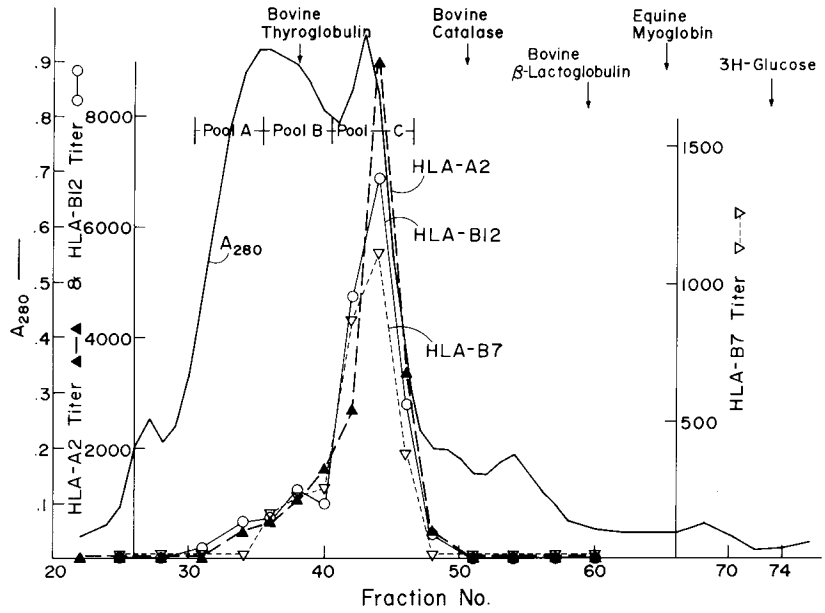

FIG. 9. Bio-Gel A-5m filtration. The material from the lectin affinity column was filtered on Bio-Gel A-5m as described in the text. The column was calibrated with the proteins indicated in the figure and [^{3}H]glucose was used as a total column volume marker.

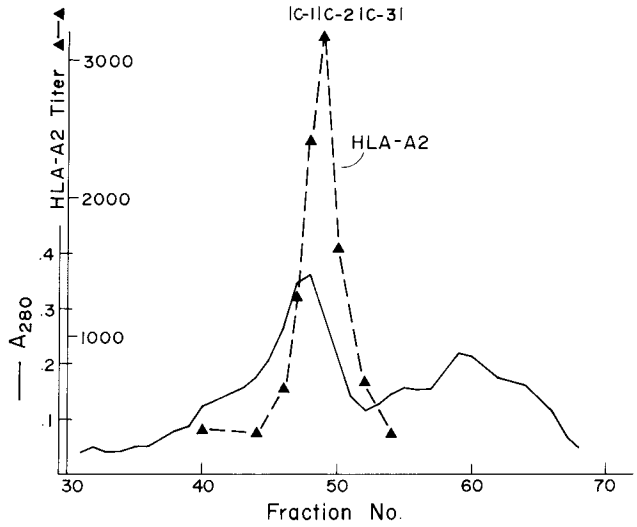

FIG. 10. Second Bio-Gel A-5m filtration. The material from *Pool C* of the first Bio-Gel A-5m column was filtered on the same Bio-Gel A-5m column a second time and placed in Pools C-1, C-2, and C-3 as indicated.

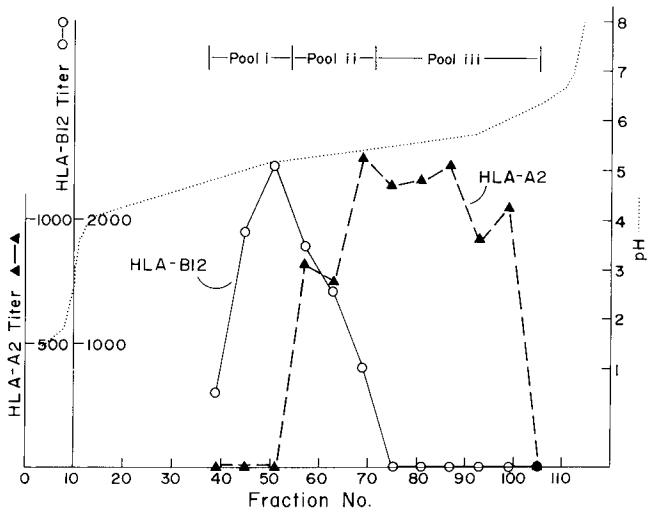

FIG. 11. Isoelectric focusing in a sucrose gradient. Half of Pool C-2 was focused in a sucrose gradient in a specially designed column as described under "Materials and Methods." After elution, the pH of each fraction was measured and 5 μl of each fraction was mixed with 200 μl of 30% albumin:DGV (1:1) and assayed for HLA activity.

than with 0.2% or 0.5% Brij 99:97. This requirement for high detergent concentrations was only noticed when applying samples of highly concentrated protein, *i.e.* 8 mg/ml, and at lower protein concentrations, such as 1 mg/ml, 0.5% Brij 99:97 could be used. Peak broadening at low detergent:protein ratios was probably due to inclusion of two or more protein molecules in the same detergent micelle, in other words, aggregation. Using a higher salt concentration in the eluant, *i.e.* the addition of 0.1 M NaCl, also caused decreased resolution. Eluting the column at the above flow rate or slower was also essential for obtaining good resolution, since the molecules being separated have small diffusion constants and equilibrate with the included volume slowly. The sample applied to the column was in 15 ml or less, and its detergent concentration was not in excess of 8%, since its viscosity at higher concentrations would cause band spreading.

Step 6: Separation of HLA Specificities by Isoelectric Focusing – Isoelectric focusing in a sucrose gradient was carried out as described under "Materials and Methods" in a specially designed column. HLA-A2 focused as a band containing several peaks from pI 5.2 to 6.0, while HLA-B12 focused from pI 4.8 to 5.4, with a peak at pI 5.1 (Fig. 11). HLA-B7 focused in the same region as HLA-B12, as is shown in the purification summary (Table III). Separation of the specificity HLA-A2 from mixture of specificities HLA-B7,12, was achieved in the isoelectric focusing column, although the region pI 5.2 to 5.4 contained an overlap of the HLA-A2 and the HLA-B7,12 antigen peaks. Separation of papain-solubilized antigens on DEAE-cellulose showed a similar pattern (56). Pools were made of the region containing "pure" HLA-B7,12 antigen mixture, the region containing the mixture of HLA-A2; B7 and B12 and the region containing pure HLA-A2 (Pools i, ii, and iii, respectively), and the pools were dialyzed against three changes of 4 liters of 0.1 M NaCl, 10 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM dithiothreitol for 5 days, then against 20

TABLE III

Purification and separation of specificities of detergent-soluble HLA antigens from RPMI 4265 cells

Step	Volume	Total protein	HLA-A2				HLA-B7				HLA-B12				HLA-A3 (specificity control)
			Total units, ($\times 10^{-3}$)	Specific activity	Purification	Yield	Total units, ($\times 10^{-3}$)	Specific activity	Purification	Yield	Total units ($\times 10^{-3}$)	Specific activity	Purification	Yield	Total units
	ml	*mg*			*-fold*	%			*-fold*	%			*-fold*	%	
Cell homogenate	900	25,000	437	17.5		100	133	5.3		(100)	412	16.5		100	n.d.
1. Membranes	466	5,130	480	96	(1)	110	146	28.4	(1)	110	460	89.7	(1)	111	n.d.
2. Detergent supernatant	486	3,400	456	134	1.4	105	132	39	1.4	99	437.5	129	1.4	106	276
3. Lectin affinity chromatography	34.3	274	500	1,820	19	114	128	467	16.4	96	284	1,036	11.6	69	0
4. Bio-Gel A-5m chromatography															
Pool A	141	71.5	23			5	0				18				0
Pool B	94	69.5	69			16	15.4				37				0
Pool C	158	71.8	229	3,200	33.4	52	89	1,240	43.7	67	152	2,117	23.6	37	0
5. Bio-Gel A-5m rechromatography (total)		26.7	137.5	5,150	54	32	68	2,550	90	51	139	5,210	58	32	n.d.
Pool C-1	42	7.74	33.3	4,300	44	8	25.7	3,300	116	19	63	8,140	90.7	15	n.d.
Pool C-2	99	16.3	91.5	5,600	58	21	42	2,600	91.6	32	70	4,290	47.8	17	n.d.
Pool C-3	95	2.66	12.7	4,800	50	4	0			0	6	2,260	25.2	0	n.d.
6. Isoelectric focusing[a]															
Pool i	12.3	3.5	1.7				18.4	5,300	186	14	66.4	19,100	213	16	0
Pool ii	12.3	5.21	25.9				18.7				34.1				n.d.
Pool iii	26.9	7.54	77.7	10,300	107	18	0				0				0

[a] Twenty-nine per cent of the material in Step 5 was used in Step 6. Volume, total protein, total units, and yield are calculated as if all material from Step 5 was used.

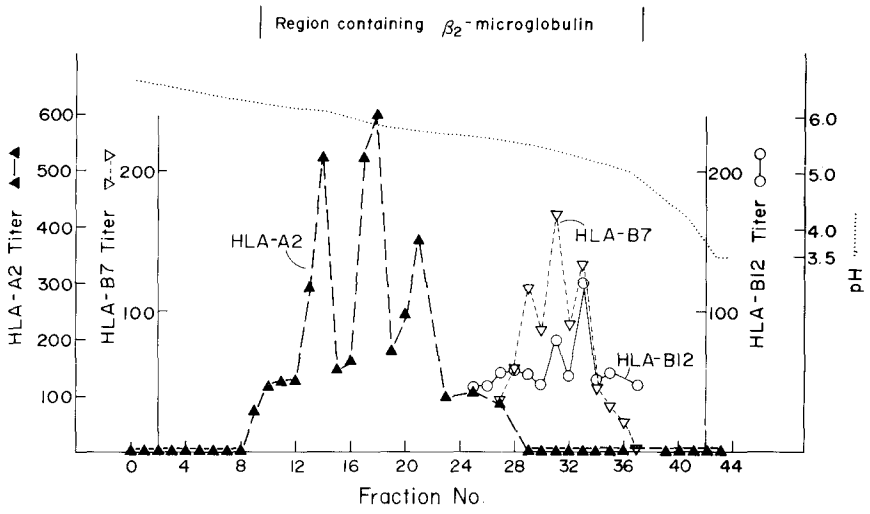

FIG. 12. Isoelectric focusing in an acrylamide gel. Samples were focused as described under "Materials and Methods." Gel slices (2 mm) were eluted into 0.2 ml of 0.05 Tris/HCl pH 8.2, 0.2 mM EDTA, 0.5% NaN_3 for 3 days at 4° before assaying for β_2-microglobulin (18) and HLA antigens. A parallel gel was sliced and eluted into distilled water to determine the pH gradient.

mM Tris/HCl, pH 8.0, 0.01 mM EDTA, 0.1 mM dithiothreitol for 2 days, and were then concentrated in the dialysis sack 4-fold using Sephadex G-200 to absorb buffer. As shown in the purification summary (Table III), Pool iii contained only HLA-A2 antigen activity, and was pure on SDS-slab gels (Fig. 13). Pool i was essentially "pure" HLA-B7 and HLA-B12, and was either slightly contaminated with HLA-A2, to the extent of 5% of the protein, as calculated from the specific activity of pure HLA-A2, or HLA-B7 and HLA-B12 antigen showed slight cross-reactivity with the HLA-A2 antiserum, Davis. Purified, papain-solubilized HLA-B7,12 shows slight reactivity with HLA-2 antiserum.[4]

In isoelectric focusing in the sucrose gradient column, which does not have as great a resolving power as an acrylamide gel, broad HLA activity peaks which were suggestive of multiple peaks were found. In isoelectric focusing gels containing Triton X-100 (Fig. 12) all antigens focused as three peaks. This is presumably since HLA antigens are heterogeneous in their

[4] P. Parham, personal communication.

sialic acid content, and normally contain either 1, 2, or 3 residues of sialic acid per molecule (57).

Attempts were made to improve focusing separations by digesting off sialic acids with *Vibrio cholera* neuraminidase, and thereby removing heterogeneity due to sialic acids. The thiobarbituric acid assay (58) showed that all sialic acid could be removed. However, isoelectric focusing separations in sucrose gradients were worse than before sialic acid removal. When focusing was attempted in 4% acrylamide gels containing detergent, the neuraminidase-treated sample did not enter the gel, and was therefore highly aggregated. Although it was possible that this was a result of the pH of 5.8 which was used during neuraminidase treatment, this is considered unlikely, since untreated HLA-B7 and -B12 antigens which focused at the lower pH of 4.8 to 5.4 showed no aggregation. Neuraminidase treatment of erythrocytes caused aggregation of freeze-fracture intramembrane particles in membrane ghosts (48) and therefore the behavior of detergent-soluble proteins after sialic acid removal closely corresponds to the behavior of proteins in membrane ghosts.

Purification Summary—The purification is summarized in Table III. Membrane preparation gave a 5.4-fold purification over the crude homogenate. Detergent solubilization gave a 1.4-fold purification. The amount of truly soluble protein is much lower than indicated by the amount of purification in this step, since the detergent-soluble supernatant is cloudy, containing highly aggregated material which is removed during the next step. Nonspecific inhibitory material was found in steps up to detergent solubilization; all of this material was removed during the lectin affinity chromatography step. Lectin affinity chromatography is the best purification step, giving a 14-fold increase in specific activity. Including dithiothreitol in this step improved the purification, presumably because non-glycoproteins could become disulfide-linked to glycoproteins which are bound to the column. Bio-Gel A-5m chromatography and rechromatography gave a purification of 3- to 5-fold, and resulted in homogenous (Pool C-2 and C-3) and near-homogenous (Pool C-1) mixtures of HLA antigenic specificities. During this step, HLA antigens were separated from a B cell-specific glycoprotein with subunits of M_r = 29,000 and 34,000 (59, 60) which chromatographs at a higher molecular weight. Separation of the antigen specificity HLA-A2 from the specificities HLA-B7,12 was achieved by isoelectric focusing. Papain-solubilized HLA-2 can be separated from an HLA-B7, 12 mixture by DEAE-cellulose chromatography. DEAE-cellulose chromatography of detergent-soluble HLA antigens was not as effective for separation of specificities, and the detergent-soluble antigens eluted at much lower salt concentrations than the papain-solubilized antigens, presumably because the detergent micelle sterically hindered binding of charged groups on the HLA molecule. The yields and purification factors after the final step for HLA-A2 were 18%, 107-fold; HLA-B7, 14%, 186-fold; HLA-B12, 16%, 213-fold. The total amount of HLA antigen obtained is best estimated from Step 5 as 27 mg, or 9 mg/100 g of cells. This was better than the yield of 5 mg after anti-β_2-microglobulin column purification of detergent-solubilized HLA antigens from J. Yoder cells (29), and considerably better than the yield of 2 mg reported for papain-solubilized HLA from the same cell line, RPMI 4265 (56). However, the papain-solubilized HLA purification has been improved, so that yields of 9 mg/100 cells are currently obtained.[4] The overall purification is similar to other values reported for the detergent and papain products (29).

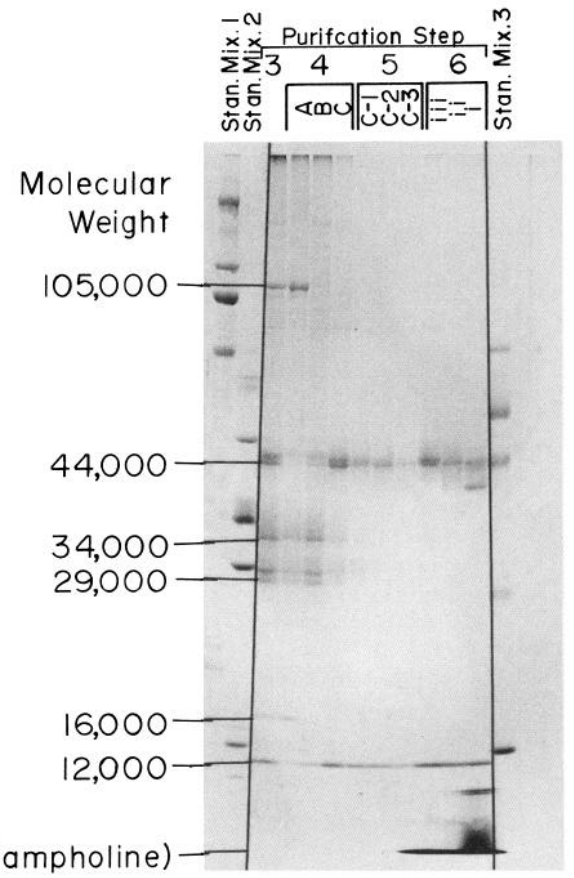

FIG. 13. SDS-gel electrophoresis of purification steps. Aliquots of Steps 3 to 6 were lyophilized and extracted with 100 μl of 95% acetone to remove detergent, boiled 5 min in sample buffer containing 5% 2-mercaptoethanol, and electrophoresed (61) in 9 to 12% linear acrylamide gradient slab gels. Standards, from top to bottom, and their molecular weights were for Mixture 1, myosin (220,000) β-galactosidase (130,000), and phosphorylase (94,000); in Mixture 2, catalase (60,000), fumarase (49,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), and myoglobin (17,200); and in Mixture 3, bovine serum alumin (68,000), γ-globulin, H chain (50,000), ovalbumin (43,000), γ-globulin, L chain (25,000), and lysozyme (14,300).

SDS Gels of Purified HLA Antigens

SDS-gels of purified HLA antigens, and of various steps during the purification, are shown in Fig. 13. The lectin column pool (Step 3) contained the HLA antigen M_r = 44,000 and 12,000 subunits, and in addition, four other putative lymphocyte cell surface glycoproteins: polypeptides of M_r = 105,000 and 16,000, which elute in Pool A of the Bio-Gel A-5m column, and polypeptides of M_r = 34,000 and 29,000, of the B lymphocyte-specific antigen (59, 60), which elute predominantly in Pool B of the Bio-Gel A-5m column. After Step 5 (Pool C-2) HLA contains a doublet band at M_r = 44,000 and a band at M_r = 12,000, which has been shown to be β_2-microglobulin. The purified HLA-A2 (Pool iii) and HLA-B7, 12 (Pool i) contain a M_r = 44,000 and 12,000 band, although in the case of Pool i, some proteolysis to M_r = 39,000 occurred during a 2-month storage in the cold room. Before storage, only M_r = 44,000 and 12,000 bands were found. It has been shown earlier that the M_r = 44,000 and 12,000 bands are precipitated by incubation with HLA antisera followed by incubation with sheep anti-human serum (16).

NH_2-terminal Analysis—NH_2-terminal amino acids were determined by dansylation of Pool C-2, hydrolysis, and chromatographic analysis on polyamide plates (62). Dansyl-isoleucine and dansyl-glycine were found in similar amounts. Isoleucine is the NH_2-terminal amino acid of β_2-microglobulin (63) and glycine is the NH_2-terminal amino acid of the M_r = 34,000 subunit of all papain-solubilized HLA antigenic specificities which have been examined (64, 65). To determine from which subunits the NH_2-terminal amino acids were derived, Pool C-1 was dansylated, electrophoresed on SDS-gels, the fluorescent bands corresponding to the M_f = 44,000 and 12,000 subunits were cut out, eluted, hydrolyzed, and analyzed. The M_r = 44,000 subunit yielded dansyl-glycine, the M_r = 12,000 subunit yielded dansyl-isoleucine, and a blank slice from the same gel yielded no dansyl-amino acids. The detergent-soluble HLA preparations are therefore chemically homogeneous. NH_2-terminal sequencing of the first five amino acids of detergent-soluble HLA antigens (66) confirmed that the NH_2-terminal sequences of detergent-soluble and papain-solubilized (65) HLA antigens are identical and that therefore, the COOH-terminal region of the M_r = 44,000 subunit is responsible for binding to the cell membrane. Thus, detergent-solubilized HLA antigens have been obtained in homogenous, chemically pure form by the criteria of SDS-gel electrophoresis and NH_2-terminal analysis, and have been shown to contain subunits of M_r = 44,000 and 12,000.

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