Isolation and Partial Characterization of Papain-Solubilized Murine H-2 Antigens†

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ABSTRACT: Papain-solubilized murine transplantation antigens have been isolated from spleen cells. The isolated H-2 antigens, containing two types of subunits, were homogeneous in size but displayed charge heterogeneity. On isoelectric focusing under denaturing conditions the H-2 antigens resolved into several components. The most basic material (pI = 7.1) comprised exclusively the smaller subunit (β_2-microglobulin) and appeared homogeneous in charge. The more acidic material representing the larger, alloantigenic subunit gave rise to several peaks. Separate analyses of H-2K and H-2D antigens gave indistinguishable profiles suggesting that the charge heterogeneity was not preferentially of genetic origin. The heterogeneity was diminished after neuraminidase treatment of the H-2 antigens and it is concluded that variations in the content of sialic acid are responsible for at least part of the charge heterogeneity. The two types of subunits were separated under conditions which preserved most if not all of the antigenic determinants. Thus, the larger chain, free of β_2-microglobulin, reacted with alloantisera demonstrating that at least some alloantigenic determinants are expressed even in the absence of the small subunit. It is, however, likely that the alloantigenic chain undergoes a conformational change on dissociation from β_2-microglobulin since the reaction is not easily reversible. Moreover, the frictional ratio of the larger chain seems to be increased compared to the intact H-2 antigen.

The major histocompatibility complex controls several immunobiological phenomena (for a review, see Shreffler and David, 1975). The first discovered trait associated with this genetic region was its role in graft rejection. It is now well documented that two loci, H-2K and D, within the major histocompatibility complex code for the cell surface antigens which are the prime target molecules for the agressive T-lymphocytes that arise on allotransplantation (see Klein, 1974). Moreover, recent information suggests that the H-2K and D antigens may be modified or associated with other molecules, like virus antigens, so that syngeneic T-lymphocytes recognize the altered K and D antigens as foreign and T-killer cells develop against them (Doherty et al., 1976). In neither of these processes are the actual molecular events delineated.

It seems mandatory to isolate and characterize the H-2 antigens to get a better understanding of their physiological role. Much information is already available as to the molecular properties of the H-2K and D antigens (see Nathenson and Cullen, 1974; Peterson et al., 1977). The H-2 antigens are composed of two types of subunits. The smaller chain, β_2-microglobulin (Rask et al., 1974a; Silver and Hood, 1974), is invariant and appears not to be coded for by the major histocompatibility complex (Goodfellow et al., 1975). The larger chain carries the alloantigenic determinants and is thus a gene product of the H-2 locus (Nathenson and Cullen, 1974). Only this chain seems to be integrated into the membrane (Peterson et al., 1975). Therefore, since the H-2 antigens are membrane-bound proteins they have to be solubilized in various types of detergents (Shimada and Nathenson, 1971). Their isolation has mainly been achieved by indirect immunoprecipitation. This procedure gives only limited amounts of material since it is rather tedious to obtain large amounts of specific antisera. Moreover, the precipitated antigens have to be dissolved under drastic conditions. Although an immunosorbent purification method has been developed for the isolation of the detergent-solubilized H-2 antigens (Anundi et al., 1975) the mere presence of the detergent renders the H-2 antigens unsuitable for biological experiments.

Shimada and Nathenson (1969) described a successful isolation procedure for water-soluble H-2 antigens. They released the membrane-bound molecules by a combination of autolysis and limited papain digestion, and demonstrated that such H-2 antigens retained most if not all of the alloantigenic determinants. The isolation procedure described was tedious and the yield of highly purified material was low. We have therefore adopted a simplified isolation procedure which is reproducible and gives highly purified H-2 antigens in good yield. A method has also been developed to separate the H-2 antigen subunits and immunological, chemical, and physical-chemical characteristics of the isolated H-2 antigens and their subunits have been determined.

Materials and Methods

Mice. All mice were bred in our own mouse colony.

Antisera. Two alloantisera were used throughout this study. One of the antisera, against H-2K^d antigens, was obtained by injecting (B10.A × B10)F$_1$ mice (H-2K^{k}D^d × H-2K^{b}D^b) with B10.D2 (H-2K^{d}D^d) spleen and lymph node cells. The other antiserum, against H-2D^d antigens, was raised by immunizing (B10.BR × B10)F$_1$ mice (H-2K^{k}D^k × H-2K^{b}D^b) with spleen and lymph node cells from B10.A mice. The anti-H-2K^d serum reacted with Ia antigens as well as with H-2 antigens. The former reactivity was, however, much weaker and did not interfere at the dilutions of the antiserum which were usually employed. A series of alloantisera, listed in Table I, was used to examine the antigenicity of the isolated H-2 antigens. The antiserum against human B_2-microglobulin, which cross-reacted with mouse β_2-microglobulin, has been described (Rask et al., 1974a). A rabbit antiserum against a sample of highly purified H-2 antigens was produced by repeated lymph node and foot pad injections. The full characterization of this antiserum will be described elsewhere.

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TABLE I: Antigenic Specificities Theoretically Recognized by Various Alloantisera.[a]

Serum no.	Recipient	Donor	Antigenic specificities[b] Private K	Private D	Public
1	C3H	A/Sn		4	6, 13, 27, 28, 29, 41, 43, 44
2	(B10.AKM × A.SW)F_1	B10.A		4	35, 41, 43, 44
3	(B10.BR × B10)F_1	B10.A		4	13, 41, 43, 44
4	(A/Sn × C57B/6)F_1	Balb/c	31		34
5	(B10.A × C3H)F_1	B10.D2	31		27, 28, 29, 34, 36
6	(B10.A × B10)F_1	B10.D2	31		34
7	(C57B/6 × Balb/c)F_1	A/Sn	23		1, 11, 25, 45
8	(A/sn × Balb/c)F_1	C57B/6	33	2	35, 36, 39
9	(C57B/6 × CBA)F_1	B10.M	?	9	7, 37
10	(A/Sn × B10.D2)F_1	B10.BR		32	1, 5

[a] The specificities of the listed sera are the theoretical ones (see Klein, 1974). No attempts were made to document that the sera raised reacted with all the numbered specificities listed above. [b] H-2K^d antigens display specificities no. 31 (private) and 3, 8, 27, 28, 29, 34, 46, 47 (public). The numbered specificities for H-2D^d antigens are 4 (private) and 3, 6, 13, 27, 28, 29, 35, 36, 41, 42, 43, 44, 49 (public).

Special Materials. Sephadex G-200, G-100, DEAE-Sephadex A-50, and Sepharose 6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were prepared according to the instructions supplied. Guanidine hydrochloride was obtained from Sigma and treated with activated charcoal prior to use. Papain (twice crystallized, sp act. 17 U/mg) was purchased from Sigma Chemical Co. (St. Louis, Mo). Neuraminidase (*Clostridium perfringens,* sp act. 0.5 U/mg of protein using *N*-acetylneuraminic acid-lactose as the substrate) was obtained from the same source. Digestions with the latter enzyme were performed in a 0.1 M sodium acetate buffer (pH 5.0) at an enzyme to protein ratio of 1:25 (w/w). Lens culinaris hemagglutinin was isolated as described (Hayman and Crumpton, 1972). Ricin was purified from Castor beans by affinity chromatography on Sepharose 6B (Nicholson and Blaustein, 1972). Concanavalin A was a product of Pharmacia Fine Chemicals AB (Uppsala, Sweden). All other chemicals were of analytical grade or better.

Concentration of Proteins. Concentration of proteins during the isolation procedure was accomplished by ultrafiltration (Berggård, 1961). The recoveries of the H-2 antigens after ultrafiltration always exceeded 90%.

Immunological Techniques. The inhibition of the alloantiserum-induced lymphocytotoxicity as measured by the ^{51}Cr release (Sanderson, 1964; Wigzell, 1965) was used to trace the H-2 antigens during the isolation procedure. In some cases the occurrence of H-2 antigens in solubilized cell membrane molecule preparations or in highly purified preparations was monitored by the indirect immunoprecipitation technique. The details have been described (Östberg et al., 1976). β_2-Microglobulin was estimated by means of a solid-phase radioimmunoassay (Evrin et al., 1971).

Electrophoresis and Isoelectric Focusing. Preparative and analytical polyacrylamide gel electrophoresis was performed with gels consisting of 7% (w/v) acrylamide and 0.18% (w/v) *N,N*-methylenebisacrylamide (Eastman Kodak) in 0.4 M Tris-glycine buffer (pH 8.9). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as described (Laemmli, 1970). Isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis were carried out essentially as described by O'Farrell (1975). Separation in the first dimension was, however, not achieved by isoelectric focusing but by polyacrylamide gel electrophoresis at pH 8.9.

Radioactive Labeling. Solubilized macromolecules and highly purified H-2 antigens were labeled with ^{125}I or ^{131}I by a slight modification of the chloramine-T procedure of Hunter and Greenwood (1962). Splenocyte macromolecules were also labeled with [^{3}H]leucine, as described elsewhere (Östberg et al., 1976).

Analytical Gel Chromatography. The molecular weights of the H-2 antigens and their isolated polypeptide chains were determined by means of gel chromatography on Sepharose 6B (Fish et al., 1969). Stokes molecular radii were determined by analytical gel chromatography. The details have been described (Karlsson et al., 1972).

Determinations of Diffusion Coefficients, Sedimentation Constants, Molecular Weights, and Frictional Ratios. Apparent diffusion coefficients ($D_{20,w}$) were computed from Stokes radii by use of the Stokes–Einstein equation (Gosting, 1956). The sedimentation constants were determined by sucrose density gradient ultracentrifugation. Molecular weights were calculated from sedimentation coefficients, diffusion coefficients, and partial specific volumes ($\bar{v}$ was arbitrarily chosen as 0.72 since lack of material precluded actual measurements) by the equation of Svedberg. Frictional ratios (f/f_0) were calculated from Stokes radii, sedimentation constants, molecular weights, and partial specific volumes. The equations used are found in Svedberg and Pedersen (1940).

Other Methods. All spectrophotometric analyses were carried out on a Beckman DU-2 spectrophotometer. Protein concentrations in unpurified fractions were estimated by the modified Folin procedure of Lowry et al. (1951) with bovine serum albumin as the reference substance. More highly purified fractions were quantitatively assayed for protein by measuring the absorbance at 280 nm. Affinity chromatography columns, containing covalently bound lens culinaris hemagglutinin, were prepared according to Cuatrecasas (1970). The columns were equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Bound glycoproteins were desorbed by the addition of 10% α-methyl mannoside to the equilibrating buffer.

Results

Isolation of H-2 Antigens. A typical purification procedure is summarized in Table II. The starting material for the isolation of the H-2 antigens usually consisted of 500 spleens from adult male and female mice of the B10.D2 strain. A crude membrane pellet, containing approximately 2500 mg of protein, was isolated as described (Peterson et al., 1974). Papain digestion was accomplished according to a previously outlined procedure (Rask et al., 1974a). The solubilized, H-2 antigen-containing fraction, 548 mg of total protein, was subjected to gel chromatography on a Sephadex G-200 column (110 × 2.4 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0),

TABLE II: Purification of Papain-Solubilized H-2 Antigens.

Fraction	Total protein (mg)	H-2D^d antigen[a] (%)	Purity (%)
Solubilized material	548[b]	100	0.43
Sephadex G-200	231[b]	82	0.84
Affinity chromatography	11[b]	64	13.7
DEAE-Sephadex chromatography	2.8[b]	49	41.3
Preparative polyacrylamide gel electrophoresis	0.66[c]	28	100

[a] Measured by the inhibition of anti-H-2D^d alloantiserum-induced cytotoxicity. Similar amounts to those noted in the table were recorded for H-2K^d antigens measured with the same technique. [b] Determined by the Folin technique. [c] Estimated from the optical density at 280 nm.

containing 0.15 M NaCl. Material with H-2K^d and D^d antigenic properties was eluted as a broad peak with an apparent K_{av} of about 0.4. The elution position for β_2-microglobulin was identical with that for the alloantigens.

Since H-2 antigens are glycoproteins (see Peterson et al., 1977) we examined the possibilities of using affinity chromatography with concanavalin A, ricin, and lens culinaris hemagglutinin, respectively, for the further purification of the H-2 antigens. Lens culinaris hemagglutinin proved to be most advantageous, so the H-2 antigen-containing fraction obtained from the Sephadex G-200 chromatography step was subjected to affinity chromatography on this lectin column. Bound material was eluted with a 0.02 M Tris-HCl buffer (PH 8.0) containing 0.15 M NaCl and 10% α-methyl mannoside. Only 11 mg of the applied protein was bound to the column but this material contained more than 80% of the H-2 antigen-reacting material regardless of whether anti-H-2K^d or D^d alloantisera were used in the cytotoxicity assay. Similar results were also obtained with the β_2-microglobulin radioimmunoassay.

The H-2 antigen-containing fraction from the affinity chromatography step was dialyzed against 5 mM sodium phosphate buffer (pH 7.0) containing 0.05 M NaCl and subjected to ion exchange chromatography on a column (12 × 2 cm) of DEAE-Sephadex, equilibrated with the same buffer. Elution was performed at pH 7.0 with a 600-mL linear gradient of NaCl from 0.05 to 0.3 M. Apart from the breakthrough material the applied protein gave rise to a single, broad peak, which comprised all of the H-2 antigen-reacting material.

Preparative polyacrylamide gel electrophoresis at pH 8.9 was used as the final purification step. The DEAE-Sephadex chromatography fraction resolved into two well-separated protein peaks. The component with the slowest anodal modility comprised all of the H-2 antigen-reacting material. Evidence will be given below to certify that this material represents highly purified H-2 antigens.

Purity and Homogeneity of the Isolated H-2 Antigens. The homogeneity of the isolated preparation of the H-2 antigens was tested by gel chromatography on a column of Sephadex G-200. The applied sample emerged as a symmetrical protein peak from the column. The occurrence in the chromatogram of β_2-microglobulin and H-2 antigen-reacting material was determined. Both types of analyses demonstrated that β_2-microglobulin and H-2 antigenic material were eluted in positions identical with the protein peak. Thus, the specific "antigenicity" was constant over the entire protein distribution, indicating a high degree of purity and size homogeneity for the preparation tested.

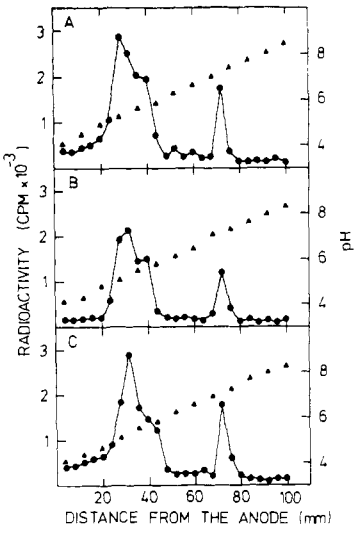

FIGURE 1: Isoelectric focusing in 6 M urea of ^{125}I-labeled H-2K^d and D^d antigens (A), H-2K^d antigens (B), and H-2D^d antigens (C). The experimental details are given in the text: (●) radioactivity; (▲) pH.

Although the isolated H-2 antigens appeared homogeneous on gel chromatography, polyacrylamide gel electrophoresis revealed charge heterogeneity. Some of this heterogeneity may have resulted from the partial dissociation of the two types of subunits. To examine if the H-2 antigens were heterogeneous even in the absence of protein–protein interactions ^{125}I-labeled material was subjected to isoelectric focusing in 8 M urea. The result is shown in Figure 1. Several radioactive components were apparent. Material from each peak was separately analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The peak with an apparent p*I* of 7.1 comprised exclusively material with an apparent molecular weight of 12 000. However, all other peaks also contained components which were homogeneous in molecular weight, i.e. 37 000. Thus, the small subunit, β_2-microglobulin, is homogeneous in size and charge whereas the larger chain, although homogeneous with regard to molecular weight, exhibits pronounced charge heterogeneity.

This heterogeneity may be explained in several ways. The proteolytic split may have occurred at different places leaving the antigen population heterogeneous in the COOH terminus. Partial deamidations may have occurred either in vivo or during the isolation procedure. One or more sialic acid residues may have been lost. The latter posibility was amenable to test. ^{125}I-Labeled H-2 antigens were digested with sialidase and then subjected to isoelectric focusing. The material was not homogeneous after this treatment but the heterogeneity was greatly reduced (not shown). Despite the fact that lack of material made it impossible to ascertain that all sialic acid had been removed, the data suggest that at least part of the charge heterogeneity depends upon a variable content of sialic acid.

The H-2 antigen preparation contained H-2K as well as H-2D molecules. The charge heterogeneity of the two types of antigens was examined. H-2K and D antigens were separately isolated from the ^{125}I-labeled H-2 antigen preparation by indirect immunoprecipitation with relevant alloantisera. The immune complexes were solubilized and subjected to isoelectric focusing. Figure 1 shows that H-2K and D antigens display a similar charge heterogeneity. The p*I* values for the various components are similar if not identical.

Subunit Separation of the Isolated H-2 Antigens. Previous studies have shown that H-2 antigens are composed of two

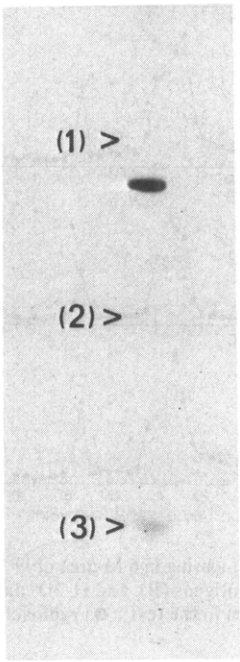

FIGURE 2: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of highly purified H-2 antigens. The *arrows* denote the migration positions of the marker proteins heavy (1) and light (2) immunoglobulin chains and β_2-microglobulin (3), run on a separate gel.

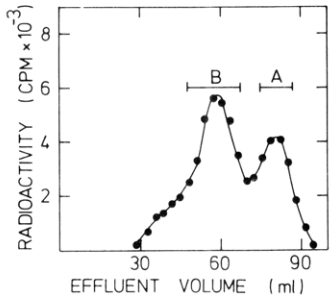

FIGURE 3: Gel chromatography on a Sephadex G-200 column (100 × 1 cm) of ^{125}I-labeled H2 antigens. The labeled protein was treated with 3 M NaSCN buffer for 3 h prior to the gel chromatography separation. The column was equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Before application of the sample 8 mL of the equilibrating buffer containing 3 M NaSCN was applied. Elution was performed with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl at a flow rate of 3 mL/h. One-milliliter fractions were collected. The *bars* denote fractions pooled for subsequent analyses.

types of subunits (Rask et al., 1974a; Silver and Hood, 1974). Figure 2 shows that the isolated H-2 antigen preparation contains both subunits. Thus, sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed two protein zones, which displayed the expected molecular weight behavior. Since no other molecules were detected, this result shows that the material was of high purity.

It appeared of interest to separate the two types of polypeptide chains under less denaturing conditions than provided by sodium dodecyl sulfate in order to be able to examine their chemical and immunological properties. Experiments to dissociate the chains in various concentrations of urea or at low pH were either unsuccessful or yielded separated polypeptides that retained little of their original antigenicity. However, ^{125}I-labeled H-2 antigens could be dissociated into the constituent polypeptide chains in the presence of 3 M NaSCN. The labeled material was subjected to the chaotropic ion during

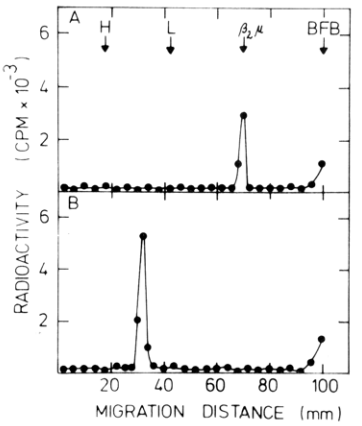

FIGURE 4: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the material in the two fractions (A and B) obtained after gel chromatography of ^{125}I-labeled, NaSCN-treated H2 antigens (see Figure 3). The *arrows* denote the marker substances, (H) heavy and (L) light immunoglobulin chains, ($\beta_2\mu$) β_2-microglobulin, and (BFB) bromophenol blue.

3 h, and then applied to a Sephadex G-200 column (100 × 1 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Prior to application of the sample 8 mL of 3 M NaSCN was allowed to enter the column. Elution was carried out with the Tris-HCl buffer. Figure 3 depicts the resulting chromatogram. It is evident that the two polypeptide chains were well separated. Fractions comprising the two types of subunits were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. It is evident from Figure 4 that both fractions were free from cross-contamination. Some properties of the isolated subunits will be described below.

Immunological Properties of H-2 Antigens and Their Subunits. The alloantigenic properties of the isolated H-2 antigens were examined. ^{125}I-Labeled H-2 antigens were separately reacted with anti-H-2K^d and D^d alloantisera. Incubations with normal mouse serum served as controls. Labeled antigen, bound to the antibodies, was isolated by indirect immunoprecipitation and subjected to gel chromatography on columns of Sepharose 6B equilibrated with 6 M guanidine-HCl. The results showed that both antisera reacted with molecules with the apparent molecular weights 37 000 and 12 000. Normal mouse serum did not react at all with the labeled material. However, only about two-thirds of the total material reacted with a mixture of the two alloantisera. The nonantigenic material comprised the same polypeptide chains as did the antigenic fraction as revealed by gel chromatography in 6 M guanidine-HCl. This observation and the fact that the proportion of nonalloantigenic reactive material in the H-2 antigen preparation varied considerably (from 20 to 65%) with the conditions employed for the ^{125}I labeling suggest that the nonantigenic material represents molecules in various stages of denaturation. Results identical with those described above were obtained when the ^{125}I-labeled H-2 antigens were reacted with a rabbit antiserum against β_2-microglobulin. Consecutive reactions of the labeled H-2 antigens, first with antiserum against β_2-microglobulin and then with H-2K^d and D^d alloantisera, revealed that antibodies against β_2-microglobulin precipitated all H-2K^d and D^d antigens. This could either mean that all molecules in the H-2 antigen preparation contained β_2-microglobulin or that only molecules containing β_2-microglobulin expressed the particular alloantigenic determinants. To distinguish between these two possibilities the free ^{125}I-labeled H-2 antigenic chain, isolated as described above, was separately mixed with a series of alloantisera (see Table I) to examine if any alloantigenic specificities were retained

TABLE III: Reactivity of the Isolated, ^{125}I-Labeled Alloantigenic H-2 Antigen Subunit with Alloantisera Recognizing Various Antigenic Specificities.

Serum no.[a]	% radioact. precipitated[b]
1	24.9
2	22.6
3	23.1
4	15.5
5	15.2
6	15.6
7	1.4
8	1.4
9	1.7
10	1.3
NMS	1.6

[a] The numbers refer to Table I. Sera no. 1 to 3 preferentially recognize H-2D^d antigen and sera no. 4 to 6 are directed against H-2K^d antigenic determinants. [b] The measurements were performed by indirect immunoprecipitations. A rabbit anti-H-2 antigen serum precipitated 58% of the radioactivity for this batch of labeled antigen.

by the free subunit. Indirect immunoprecipitations demonstrated that the various alloantisera with nominal specificities against H-2^d antigens precipitated a significant amount of the labeled H-2 antigen chain whereas alloantisera directed against irrelevant specificities did not recognize the H-2 antigen chain (Table III). Since data to be described below rule out that serum β_2-microglobulin bound to the ^{125}I-labeled alloantigenic chain, and thus complemented the molecule, the results strongly suggest that the interaction between the two subunits is not required for the expression of at least the private alloantigenic determinants.

Since proteolysis was used for the solubilization of the H-2 antigens some alloantigenic determinants might have been retained on that portion of the H-2 antigens which remained anchored to the membrane. To examine this possibility the H-2 antigen preparation was used to separately inhibit the anti-H-2K^d and D^d sera induced cytotoxicity. For both antisera the material was able to completely inhibit the cytotoxic action. It seems reasonable to conclude that all alloantigenic determinants, recognized by cytotoxic antibodies, were present on the H-2 antigen fragments derived by papain digestion.

Alloantisera recognized but restricted antigenic sites. Therefore, it was of interest to raise a heteroantiserum against the H-2 antigen preparation. A rabbit was immunized with the highly purified H-2 antigens. The resulting antibodies displayed excellent reactivity with the separated, ^{125}I-labeled H-2 antigen subunits. All ^{125}I-labeled preparations of whole H-2 antigens or separated subunits tested reacted better with the heteroantiserum than with the alloantisera suggesting that H-2 antigens express nonallotypic antigenic determinants which are less prone to derangement than the allotypic ones. A further check on the purity of the H-2 antigen preparation was obtained by reacting the heteroantiserum with ^{125}I-labeled, papain-solubilized macromolecules obtained from mouse spleens. Molecules precipitated by indirect immunoprecipitation were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only components with the apparent molecular weights 37 000 and 12 000 were evident. Moreover, [^{3}H]leucine labeled murine splenocyte macromolecules, solubilized with 1% Triton X-100, were allowed to react with the heteroantiserum. Also, in this case the immune precipitate contained but two components. The molecular weights were 47 000 and 12 000, which are the expected values for the subunits of intact H-2 antigens (see Peterson et al., 1977). Thus, also with regard to immunological criteria, the H-2 antigen preparation was of high purity.

TABLE IV: Physical Characteristics for Papain Solubilized H-2 Antigens and the Constituent Polypeptide Chains.

	H-2 antigens	A chain	B chain
Sedimentation constant (S)	3.9	3.1	1.65
Stokes molecular radius (Å)[b]	33	31	16
Diffusion constant ($\times 10^7$ cm^2 s^{-1})	6.3	6.7	13.0
Frictional ratio, f/f_0	1.24	1.32	1.13
Molecular weight			
Sedimentation-Stokes radius	51 500	38 600	12 500
Gel chromatography[c]		38 000	12 000
Electrophoresis[d]		37 000	12 000

[a] Separate analyses were performed for H-2K^d and D^d antigens. The values were indistinguishable. [b] Estimated by analytical gel chromatography. [c] Determined in 6 M guanidine hydrochloride on reduced and alkylated polypeptide chains. [d] Data from sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Physical Properties of the Two H-2 Antigen Subunits and the Intact Molecule. Some physical properties of papain-solubilized H-2 antigens and their separated subunits are summarized in Table IV.

Sedimentation velocity analyses were carried out in linear sucrose gradients with ^{125}I-labeled proteins. When intact H-2 antigens were examined the distribution of H-2K^d and D^d antigens was monitored by indirect immunoprecipitation with use of relevant alloantisera. The material behaved as a single homogeneous component and no separation between the different types of alloantigens could be detected. Also, the separate chains appeared homogeneous by sedimentation velocity analyses. The estimated sedimentation constants were highly reproducible and the standard error in six separate determinations was less than 3%.

The Stokes molecular radius for intact H-2 antigens (33 Å) was only slightly larger than that found for the alloantigenic chain (31 Å). The frictional ratio (f/f_0) for the alloantigenic chain (1.32) is somewhat high to be compatible with a globular structure for the molecule. Thus, on losing β_2-microglobulin the alloantigenic chain may change conformation and become somewhat asymmetric. Alternatively, the hydration may increase.

To distinguish between these two alternatives free β_2-microglobulin and β_2-microglobulin present in normal mouse serum were separately mixed with the isolated alloantigenic chain. After several hours of incubation at 37 °C the mixtures were analyzed by sucrose density gradient centrifugation. The sedimentation constant for the alloantigenic chain was unchanged (3.1 S) suggesting that either one or both of the H-2 antigen subunits had changed their conformations on dissociation so that recombination did not occur.

The molecular weights of the H-2 antigens and the constituent polypeptide chains were estimated by several methods. It can be seen in Table IV that the values obtained were in good agreement regardless of whether the analyses were performed under physiological or denaturing conditions.

Discussion

The purification procedure adopted for the H-2 antigens is very reproducible and the yield of highly purified material is reasonable. It has been estimated that a lymphocyte expresses about 5×10^5 molecules of transplantation antigens on the surface (Östberg et al., 1975). That would imply that the

starting material contained about 2.5 mg of H-2 antigens of which about one-fourth was recovered after the final purification step. This recovery is almost tenfold that obtained by Shimada and Nathenson (1969). The improved yield in the present study is largely dependent on the introduction of the lectin affinity chromatography step.

The isolated H-2 antigens contain, of course, a mixture of K^d and D^d antigens. Both types of antigens resolved into several components differing in charge but the overall patterns for the K^d and D^d antigens were similar if not identical. It seems likely that at least part of the heterogeneity depends on a variable content of sialic acid.

Previous studies have shown that the two H-2 antigen subunits are held together by noncovalent bonds only (Rask et al., 1974 a,b; Silver and Hood, 1974; Vitetta et al., 1975). The interaction is firm and no tendency toward dissociation of the two subunits is apparent even at concentrations of 10^{-9} M or less (unpublished observations). Therefore, separation of the two chains has so far only been achieved under denaturing conditions. However, exposure of the H-2 antigens to chaotropic ions, like NaSCN, abolished the interaction between the subunits. The two chains could be obtained free from each other by gel chromatography. The isolated components appeared "native" by several criteria. Neither chain in free form displayed any tendency toward aggregation and both chains retained, at least partly, their antigenic properties.

The highly purified H-2 antigens retained full antigenic activity. The cytotoxic action of alloantisera raised against H-2K^d and D^d antigens could, thus, be completely abolished by the papain-solubilized H-2 antigen preparation. It is well known that the alloantigenic determinants are very sensitive toward modification (Pancake and Nathenson, 1972). Consequently, it is not unexpected that iodine labeling will diminish the antigenicity of the H-2 antigens. Despite this modification the separated H-2 alloantigenic chain still reacted to a great extent with the alloantisera. This result signifies that the adopted subunit separation method indeed is rather nonderanging. Since the free alloantigenic chain retains antigenic determinants the association with β_2-microglobulin is obviously not required for the expression of all such determinants.

Scarcity of starting material precluded isolation of the H-2 antigens in quantities sufficient for high precision molecular weight determinations in the analytical ultracentrifuge. Molecular weights were therefore estimated by a combination of methods, such as analytical gel chromatography and sucrose density gradient centrifugation. The latter methods as well as sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel chromatography in 6 M guanidine hydrochloride, which have been used in the present study, are highly reproducible but not comparable in precision to sedimentation equilibrium ultracentrifugation. The methods employed measure different properties of the isolated proteins. Consequently, a combination of the various techniques should yield molecular weight estimates that are reasonably accurate. The results obtained agree satisfactorily for all methods.

The isolated alloantigenic chain displayed a rather high frictional ratio. This may indicate that the chain is more asymmetric in free form than when bound to β_2-microglobulin. That the alloantigenic subunit may undergo a conformational change on dissociation from β_2-microglobulin is also suggested by the observation that a mixture of the separated chains does not easily recombine to form the intact H-2 antigen.

References

Anundi, H., Rask, L., Östberg, L., and Peterson, P. A. (1975), *Biochemistry 14,* 5046.

Berggård, I. (1961), *Ark. Kemi 18,* 291.

Cuatrecasas, P. (1970), *J. Biol. Chem. 245,* 3059.

Doherty, P. C., Blanden, R. V., and Zinkerhagel, R. M. (1976), *Transplant. Rev. 29,* 89.

Evrin, P. E., Peterson, P. A., Wide, L., and Berggård, I. (1971), *Scand. J. Clin. Lab. Invest. 28,* 439.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244,* 4984.

Goodfellow, P. N., Jones, E. A., Van Heyningen, V., Solomon, E., Bobrow, M., Migginano, V., and Bodmer, W. F. (1975), *Nature (London) 254,* 267.

Gosting, L. J. (1956), *Adv. Protein Chem. 11,* 449.

Hayman, M. J., and Crumpton, M. J. (1972), *Biochem. Biophys. Res. Commun. 47,* 923.

Hunter, W. M., and Greenwood, F. C. (1962), *Nature (London) 194,* 495.

Karlsson, F. A., Peterson, P. A., and Berggård, I. (1972), *J. Biol. Chem. 247,* 1065.

Klein, J. (1974), Biology of the Mouse Histocompatibility-2 Complex, Berlin and New York, Springer Verlag.

Laemmli, V. K. (1970), *Nature (London) 227,* 680.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Nathenson, S. G., and Cullen, S. E. (1974), *Biochim. Biophys. Acta 344,* 1.

Nicholson, G. L., and Blaustein, J. (1972), *Biochim. Biphys. Acta 266,* 543.

O'Farrell, P. H. (1975), *J. Biol. Chem. 250,* 4007.

Östberg, L., Rask, L., Nilsson, K., and Peterson, P. A. (1975), *Eur. J. Immunol. 5,* 462.

Östberg, L., Sege, K., Rask, L., and Peterson, P. A. (1976), *Folia Biol. (Prague) 22,* 372.

Pancake, S., andNathenson, S. G. (1972), *J. Immunol. 110,* 1086.

Peterson, P. A., Rask, L., and Lindblom, B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 35.

Peterson, P. A., Rask, L., and Östberg, L. (1977), *Adv. Cancer Res. 24,* 115.

Peterson, P. A., Rask, L., Sege, K., Klareskog, L., Anundi, H., and Östberg, L. (1975), *Proc. Natl. Acad. Sci. U.S.A. 74,* 1612.

Rask, L., Lindblom, J. B., and Peterson, P. A. (1974a), *Nature (London) 249,* 833.

Rask, L., Östberg, L., Lindblom, J. B., Fernstedt, Y., and Peterson, P. A. (1974b), *Transplant. Rev. 21,* 85.

Sanderson, A. R. (1964), *Nature (London) 204,* 250.

Shimada, A., and Nathenson, S. G. (1969), *Biochemistry 8,* 4048.

Shimada, A., and Nathenson, S. G. (1971), *J. Immunol. 107,* 1197.

Shreffler, D. C., and David, C. S. (1975), *Adv. Immunol. 20,* 125.

Silver, J., and Hood, L. (1974), *Nature (London) 249,* 764.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon Press.

Vitetta, E. S., Uhr, J. W., and Boyse, E. A. (1975), *J. Immunol. 114,* 252.

Wigzell, H. (1965), *Transplantation 3,* 423.