A large scale purification of histocompatibility-2 (H-2) antigens from mouse liver is described. The antigens were solubilized by a limited papain digestion of a crude preparation of liver membranes (strain A/J) and purified by ion exchange chromatography, gel filtration, affinity chromatography, and isoelectric focusing. The overall degree of purification of H-2Kk was 1,300-fold and that of H-2Dd was 1,500-fold; approximately 8 mg of purified H-2* antigens were obtained from 1 kg of liver. The purification was followed by a sensitive radioimmunoassay in which H-2*-containing fractions were used to inhibit the binding of 125I-labeled H-2* to appropriate antisera. H-2Dd and H-2Kk co-purified through all the steps but the concentration of H-2Kk was 2- to 3-fold higher than that of H-2Dd in the liver homogenate as well as in the purified H-2 preparation. β2-microglobulin was initially present in a 3- to 10-fold excess over H-2 in the liver homogenate, but the purified H-2 preparation contained approximately 2 mol of alloantigenic heavy chain/mol of β2-microglobulin. Isoelectric focusing and disc-gel electrophoresis showed a charge heterogeneity of H-2, with a mean isoelectric point of pH 4.9. Electrophoresis on sodium dodecyl sulfate gels showed one band. Denaturing conditions were required to remove β2-microglobulin and small amounts of impurities from H-2. The amino acid sequence of the first 27 residues of the isolated heavy chains was determined.

The major histocompatibility complex of the mouse, the histocompatibility-2 (H-2) complex (1, 2), is composed of five regions defined by recombination. Two of these are the so-called K and D regions that code for H-2Kk and H-2Dd antigens. These two species of glycoproteins are integral components of the plasma membrane (3-5) and apparently play a major role as targets of graft rejection. It is unknown, however, whether this is part of their normal function. H-2 antigens are very polymorphic with numerous serologically defined antigenic specificities associated with alleles of different haplotypes. Structural studies have shown (6-9) that the antigens are composed of a glycoprotein with a molecular weight of approximately 40,000 associated with a smaller protein, β2-microglobulin, with a molecular weight of approximately 12,000. All of the antigenic determinants are associated with the larger of the two components (10).

Several investigators have shown that immunohistochemically and immunologically reactive H-2 antigens can be solubilized from cell membranes of lymphoid cells either by detergents (11) or by a limited proteolytic digestion (12-14), but the amount of material that has been obtained from this kind of tissue has been very limited. In contrast, the HLA antigens of human origin have been successfully purified in large quantities from tissue culture cells (15, 16). Hence, only limited studies on the primary structure have been carried out on H-2 antigens labeled in situ with radioactive amino acids and carbohydrates (17-20), but quite extensive investigations of HLA have been reported (21-24).

We have previously described the structural characterization of purified, papain-solubilized, H-2* antigens from mouse liver (25). In this paper we provide the detailed description of the purification and radioimmunoassay of H-2*. In addition, further immunochromical and structural studies of the purified H-2* are reported.

**EXPERIMENTAL PROCEDURES**

### Materials

**Mice**

A/J mice (H-2*), 6 to 8 weeks old, of mixed sex were purchased from the Jackson Laboratory, Bar Harbor, ME. H-2* is a recombinant of H-2d and H-2k haplotypes. Its transplantation antigens are H-2Dd and H-2Kk.

**Antigens—Alloantisera directed against the private specificities of H-2Kk (H-2.23) and H-2Dd (H-2.4) were produced for National Institute of Allergy and Infectious Diseases under contract with the Jackson Laboratory. The broad specificity alloantisera were prepared in this laboratory. Anti-H-2Kk antisera was produced in B10.D2 mice (H-2d*, H-2Kk*) after grafting of skin, followed by several injections of spleen cells from B10.A mice (H-2d*, H-2Kk*). Anti-H-2Dd antisera was similarly prepared in B10.BR mice (H-2d*, H-2Kk*). Rabbit anti-mouse IgG antisera and goat anti-rabbit IgG antisera were supplied by Microbiological Associates; rabbit anti-mouse β2-microglobulin antisera was a generous gift of Dr. T. Natori, Hokkaido University School of Medicine. The characteristics of this antisera have been described elsewhere (10).

**Rabbit Anti-H-2* Antiserum**

Each of two rabbits (female, white New Zealand) was injected in the footpads with 75 μg of purified H-2* antigen in complete Freund’s adjuvant and reinjected subcutaneously twice at 3-week intervals; the first time with 35 μg and the second time with 10 μg of antigen, both in incomplete Freund's adjuvant. 1en days after the last injection the animals were bled; the antisera had identical titers.

**Chemicals**

Dithiothreitol was purchased from Calbiochem and papain (twice crystallized) was from Worthington. Iodoacetamide was a product of Aldrich and was recrystallized from toluene/absolute ethanol (8:1 v/
v). The DEAE-cellulose, Cellex D, high capacity, and Tris base were obtained from Bio-Rad. Tris buffers with a pH value of 7.2 were prepared from Trizma preset crystals, manufactured by Sigma. Also, α-methyln-mannoside was a product of Sigma. Sephadex G-150 and concanavalin A-Sepharose were products of Pharmacia and ampholine, pH 4 to 6, was purchased from LKB. Na\(^{125}\)I (17 Ci/mg) was supplied by New England Nuclear Corp.

**Methods**

**Partial Purification of H-2 from Spleen**

H-2, partially purified from spleen of A/J mice, was used as the initial radiolabeled antigen for the radioimmunoassay of H-2. The purification of this material has been described elsewhere (9, 10).

Some of the properties of the partially purified H-2 from spleen are shown in Fig. 1. Panel A shows the characteristic two-component structure of H-2 (6–9) seen after SDS-polyacrylamide electrophoresis of a radiolabeled sample, and Panel B shows the linearity of the radioimmunoassay with increasing amounts of H-2. Panel C shows, by radioimmunoprecipitation, that the material contained both H-2D and H-2K. The alloantisera were used separately as well as in combination; in the latter case the amount of radioactivity precipitated was approximately equal to the sum of that precipitated by the individual antisera. This observation thus confirms earlier reports by others (10) that H-2D and H-2K are on different molecules.

The quantitation of H-2 throughout this study was based on a radioimmunoassay in which the degree of inhibition of the immunoprecipitation of \(^{125}\)I-H-2 by H-2-containing samples was used as a measure of the H-2 concentration. The technical aspects of the assay are discussed below. Fig. 1D shows a typical inhibition experiment in which increasing amounts of an unlabeled, partially purified, H-2 preparation were used to inhibit the immunoprecipitation of a constant amount of \(^{125}\)I-H-2 by a constant amount of an alloantiserum. The curve indicates that the immunoprecipitation of \(^{125}\)I-H-2 would be inhibited over 90% at infinite concentrations of unlabeled H-2.

**\(^{125}\)I Labeling and Radioimmunoassay of H-2**

**\(^{125}\)I Labeling—**H-2 was radio labeled by the chloramine-T method (27). Twenty to 30 μg of protein was reacted with 900 μCi of Na[\(^{125}\)I]. After addition of 1 mg of bovine serum albumin to the reduced incubation mixture, small molecular weight radioactive material was separated on a column (0.9 x 30 cm) of Sephadex G-50 (superfine).

The column was eluted with 100 ml of Tris-HCl (pH 7.2), 0.15 M NaCl, 0.05% bovine albumin, and fractions of 0.5 ml were collected. Two to 3 of the fractions containing the peak of the eluted radioactivity were pooled and the total radioactivity and the radioactive protein were precipitated in hot (90°C) trichloroacetic acid. The latter value was 85 to 90% for different preparations of \(^{125}\)I-H-2. The protein was normally labeled to a specific radioactivity of 2 to 9 x 10⁶ cpm/μg (approximately 70% counting efficiency).

**Radioimmunoassay of H-2—**\(^{125}\)I-H-2, approximately 20,000 cpm and 1 ng, was incubated overnight at 4°C with a mixture of H-2-containing material and sufficient antisera to bind approximately 20% of the radioactivity in the absence of unlabeled H-2. The incubation took place in 100 μl of RPMI-1640 medium (NIH media unit) containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 7.4, and 10% fetal bovine serum. After the incubation period, rabbit anti-mouse IgG antiserum was added in an amount equivalent to that of the alloantisera and the incubation was continued for 1 h at room temperature. During this time the total radioactivity of the samples was measured. To each sample, 2 ml of 10 mM Tris-HCl (pH 7.2), 0.15 M NaCl was added. The antigen double antibody complex was isolated by centrifugation, washed once by resuspension in the buffer, and reisolated. After removal of the supernatant the radioactivity of the pellet was measured. Uninhibited samples and samples in which normal mouse serum was used instead of antisera were used as standards and blanks, respectively. In the blank samples 1 to 2% of the total radioactivity was normally found in the precipitate.

The H-2 content of the material of interest was expressed by its degree of inhibition of the binding of \(^{125}\)I-H-2 to the antisem:

\[
\text{Inhibition} \% = \frac{(S-B) - (T-B)}{S-B} \times 100
\]

where the fraction: bound radioactivity/total radioactivity is calculated as follows: S, in the absence of inhibitor; T, in the presence of inhibitor; B, in the absence of inhibitor but with normal mouse serum instead of antisem.

A very reproducible quantitation of H-2 was obtained by measuring the inhibition, as described above, by serial dilutions of the samples of interest. The appropriate dilution factor of a particular sample was determined in preliminary inhibition experiments, and a stock dilution of the sample was made. Then, 5, 10, 20, 30, and 40 μl of the stock solution were used as inhibiting material in the radioimmunoassay. One inhibitory unit is defined as that amount of protein that inhibits the binding of \(^{125}\)I-H-2 to its antibody by 50% under the conditions described above. The assay of β2-microglobulin was done in an analogous fashion, but with rabbit anti-mouse β2-microglobulin antisem as the first and goat anti-rabbit IgG antisem as the second antisem.
Membrane Preparation—Livers were removed from approximately 1,000 A/3 mice (males or females, or both) and placed in cold Littlefield’s medium (0.05 M Tris-HCl (pH 7.2), 0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂). The average weight of the livers was 1 g. The livers were homogenized, 10 to 15 at a time, in 10 to 15 ml of Littlefield’s medium in a motor-driven tissue grinder with Teflon pestle (Arthur H. Thomas). This step, and all the following ones, were carried out at 0–5°C. The homogenate was centrifuged at 1,000 × g for 20 min. The pellet was discarded, and the supernatant was centrifuged at 30,000 × g for 60 to 90 min to give a pellet of the membrane fraction.

Sedimentation—The membrane material was resuspended in 0.05 M Tris-HCl, pH 8.4 (25°C), by homogenization; and the total volume (in milliliters) was brought to that of the wet weight (in grams) of the livers. A sample (0.5 to 1 ml) of the membrane suspension was dialyzed against 2,000 ml of distilled water, and the dry weight was determined after lyophilization. The concentration of solids (determined by dry weight) was adjusted to approximately 60 mg/ml, and dithiothreitol was added to a concentration of 10 mM. A limited digestion with papain then was carried out at 37°C for 1 h at a papain concentration of 0.16 unit/mg of solid material. After the incubation, iodoacetamide was added to a concentration of 22 mM, and the membrane suspension was cooled in an ice bath. Insoluble material was removed by centrifugation at approximately 150,000 × g for 1 h; the supernatant was dialyzed against 0.05 M Tris-HCl (pH 7.2, 25°C), 0.065 M NaCl with two to three changes of buffer.

DEAE-Cellulose Column—The solubilized, dialyzed material was loaded onto a column of DEAE-cellulose (4.5 × 33 cm) equilibrated with the dialysis buffer, and 90-ml fractions were collected. The column was washed with 1,000 ml of buffer after passage of the sample. Fractions with an absorbance (280 nm) higher than 1 were pooled, centrifuged at 5,000 × g for 15 min, and concentrated. The concentrated solution was dialyzed overnight against 2,000 ml of 10 mM Tris-HCl (pH 7.2, 25°C), 0.15 M NaCl and then centrifuged at 20,000 × g for 16 min.

Sepahed G-150 Column—The sample from the preceding step was loaded onto a column (6 × 106 cm) of Sephadex G-150 equilibrated with 0.05 M Tris-HCl (pH 7.2, 25°C), 0.5 M NaCl. Fractions of 20 ml were collected. After the sample was applied, the column was washed with the equilibrating buffer until the absorption (280 nm) of the eluate was below 0.1. The absorbed protein was eluted with 0.15 M NaCl with two to three changes of buffer. After concentration, the sample was stored at -20°C according to Lowry et al. (28).

Protein—Determinations of protein concentration were performed according to Lowry et al. (28).

Concentration of Solutions—H-2 containing solutions were concentrated under reduced pressure (80 p.s.i.) in stirred cells manufactured by Amicon with UM20 filters.

Electrophoresis and Gel Elution—Electrophoresis in polyacrylamide gels in the presence of SDS was carried out at pH 9.1 in 0.2 M glycine/Tris, 0.2% SDS (29). Disc-electrophoresis in the presence of 6 M urea was done according to the method of Ornstein (30) and Davis (31). The procedure for slicing the gels and the elution of slices was described earlier (28).

RESULTS

Purification of H-2—The preparation of crude liver membranes and their subsequent solubilization by limited papain digestion gave a recovery of more than 50% of the initial H-2 antigenic material (Table I).

A major consideration during the succeeding purification of H-2 was to avoid methods that separated proteins according to their charge since earlier studies by others (3, 10, 16, 37) have shown that H-2, as well as HLA antigens under such conditions, behave very heterogeneously. Hence, the solubilized H-2-containing material was passed through a DEAE-cellulose ion exchange column at a pH and ionic strength...
The proteins were purified as described under "Experimental Procedures." The numbers in the table are average values based on the results from several different H-2 preparations.

### Table II

| Purification step | Volume | Amount of protein | $10^6 \times$ total H-2 activity | Specific activity | Purification | Yield |
|-------------------|--------|-------------------|---------------------------------|-------------------|--------------|-------|
| Homogenate        | 1,400  | 131,800           | 10.28                           | 3.49              | 36.76        | 1.0   | 1.0 | 1.0 | 100 | 100 | 100 | 100 |
| Membrane          | 38,967 | 7.17              | 2.70                            | 19.71             | 154          | 69    | 279 | 1.0 | 1.0 | 1.0 | 100 | 100 | 100 | 100 |
| Solubilized       | 6,385  | 6.34              | 1.82                            | 7.65              | 993          | 285   | 1,198 | 12.7 | 10.6 | 4.3 | 61.7 | 52.2 | 20.8 | 4.8 |
| DEAE              | 3,935  | 5.91              | 1.31                            | 6.20              | 1,502        | 333   | 1,576 | 19.3 | 12.3 | 5.7 | 57.5 | 37.5 | 16.9 | 3.0 |
| G150              | 847    | 4.30              | 1.16                            | 1.61              | 5,070        | 1,370 | 1,901 | 60.1 | 30.7 | 6.8 | 41.8 | 33.2 | 4.4  | 0.6 |
| Concanavalin A    | 4      | 40.3              | 2.56                            | 0.68              | 63,524       | 16,873 | 25,558 | 814.4 | 624.9 | 91.5 | 24.9 | 19.5 | 2.8  | 0.03 |
| Electrofocusing   | 3      | 8.1               | 0.82                            | 0.34              | 101,235      | 41,975 | 50,617 | 1,297.9 | 1,554.6 | 181.4 | 8.0  | 9.7  | 1.1  | 0.006 |

which allowed the H-2 and the bulk of the protein to remain unabsorbed to the column material. The purpose of this step was to remove highly negatively charged material such as nucleic acids; that this was accomplished is evident from the ratio of the absorptions at 280 and 260 nm, 0.7 before and 1.4 after the DEAE-cellulose column.

The next step separated H-2 from the bulk of the proteins by gel filtration (Fig. 2). The H-2 activity was eluted from the column in a sharp peak. Other investigators (3) have reported H-2 activity associated with proteins of quite different sizes; such a pattern was not observed in the present study.

The following step, affinity chromatography on concanavalin A-Sepharose, was the most effective one (Fig. 3 and Table II). A 13-fold purification and a 60% yield was obtained. The lectin from *lens culinaris* (lentils) has been used by other investigators (38, 39) for the purification of HLA antigens and by our laboratory (9) for the purification of H-2 from spleen. However, during a preliminary study of the properties of this lectin and those of concanavalin A it was found that the latter gave a more complete separation of H-2 from impurities, with less H-2 appearing in the nonadsorbed fraction.

Various column chromatographic methods were tried after the concanavalin A step but all gave relatively large losses of activity. Isoelectric focusing proved to be the method of choice for the last step of the purification. In order to obtain a distinct separation of H-2 from impurities, it was necessary to use a pH gradient containing 4% ampholine. At lower concentrations, most of the proteins remained close together. Fig. 4 shows that the bulk of the protein as well as the activity was present in the electrofocusing column at pH values between 4.6 and 5.2, which gives a mean value of 4.9 for the isoelectric point of H-2. The absence of a sharp, well defined peak of H-2 is in agreement with an earlier report by Hess and Davies (37) who observed a heterogeneous distribution of activity after ion exchange chromatography, disc-gel electrophoresis and isoelectrofocusing. Turner et al. (16) also noticed a similar heterogeneity during the purification of HLA antigens. Only the distribution of H-2K$^\kappa$ is shown in Fig. 5. However, the distribution of H-2D$^\delta$ was identical, and even after a repeated electrofocusing in a very narrow pH gradient of the fractions containing most of the H-2 activity no separation of H-2D$^\delta$ and H-2K$^\kappa$ could be obtained.

H-2K$^\kappa$ and H-2D$^\delta$ co-purified through the various steps (Table II). The final purification of H-2K$^\kappa$ was approximately 1,300-fold with a yield of 8% of the initial activity, and H-2D$^\delta$ was purified approximately 1,500-fold with a 10% yield. The apparent ratio of H-2K$^\kappa$/H-2D$^\delta$ was 3:1 (Table II).
Purification of Liver H-2

I. Introduction

II. Methods

A. Isoelectric Focusing

B. Polyacrylamide Gel Electrophoresis

C. Immunoprecipitation

III. Results

A. Isoelectric Focusing

B. Polyacrylamide Gel Electrophoresis

C. Immunoprecipitation

IV. Discussion

V. Conclusion
H-2 (Fig. 7) These experiments suggest that over 70% of the material in the liver preparation and 66% of the spleen material could form a complex with the antisera.

β2-Microglobulin—β2-Microglobulin was present in a 3- to 10-fold excess over the alloantigenic chains of H-2 in the liver homogenate of several different preparations (Table II). Seventy per cent of the excess was removed when the membranes were isolated. A further loss of β2-microglobulin activity occurred when the preparation was concentrated and subjected to gel filtration. However, for the last three steps of purification, the ratio of heavy chain activity/β2-microglobulin activity was 3:1.

Precipitations of electrofocussed H-2 with a rabbit antisera directed against β2-microglobulin brought down less of the radiolabeled liver H-2 and less of the spleen H-2 than did precipitation with an alloantiserum against H-2Dd and H-2Kk (Fig. 6). This observation again suggests that the amount of β2-microglobulin associated with purified H-2 is less than 1:1.

For one preparation of carboxymethylated H-2 (see below), both the heavy chains and β2-microglobulin were subjected to amino acid analysis. A ratio of 1.5 mol of heavy chain/mol of β2-microglobulin was found.

Carboxymethylated H-2 and Separation of Heavy Chains—For chemical analyses of the alloantigenic heavy chains, electrofocussed H-2 was fully reduced and alkylated and subjected to gel filtration (25). This procedure separated the heavy chains from β2-microglobulin and small amounts of impurities. The carboxymethylated heavy chains were obtained in yields of 60 to 70%. Fig. 5D shows their homogeneous appearance by SDS-gel electrophoresis. Electrophoresis in the presence of urea resolved two proteins of slightly different charge (Fig. 5C). These were identified as H-2Kk (upper band) and H-2Dd (lower band) by immune complex formation and autoradiography (25).

Amino Acid Composition—The amino acid compositions of the fully reduced and carboxymethylated heavy chains of three different preparations of H-2k are shown in Table III. The compositions agree well, which indicates that the method of purification gives reproducible results. A total half-cystine content of 5 residues/mol (of 290 residues) was obtained. Analysis of partially carboxymethylated heavy chain showed that 1 of the 5 residues was present as free cysteine (Table IV). Analysis of the partially [14C]carboxymethylated sample after full reduction and alkylation with iodo[3H]acetic acid verified the content of 1 free cysteine and 4 half-cystine residues/mol. The latter are presumed to participate in two disulfide bridges.

For another preparation of [14C]carboxymethylated H-2, the specific activities of the heavy chains and β2-microglobulin were 11,260 and 4950 cpm/nmol, respectively. From the known cysteine content of β2-microglobulin (2 residues/mol) and the specific activities of the heavy chains and β2-microglobulin, a cysteine content of 4.5 residues/mol of heavy chain was calculated. This value is in agreement with those shown in Tables III and IV and indicates that the total number of residues/mol of heavy chain is close to 290.

NH2-terminal Sequence—Glycine was identified as the NH2-terminal residue of the heavy chains by dansylation (25). Three automated Edman degradations were performed on the carboxymethylated heavy chains. For the first (38 nmol of chain), glycine was identified at cycle 1 (15% yield) but no identification could be made at cycles 2 and 3. A second

| Table III | Amino acid composition of H-2Dd plus H-2Kk |
|-----------|-----------------------------------------|
| Amino acid | Preparation 1 | Preparation 2 | Preparation 3 |
| Cys (Cm)  | 4.7          | 4.7          | 4.4          |
| Asp       | 26.2         | 25.5         | 26.5         |
| Thr       | 17.3         | 16.9         | 16.3         |
| Ser       | 19.4         | 19.0         | 17.7         |
| Glu       | 43.1         | 40.9         | 43.7         |
| Pro       | 15.3         | 14.6         | 14.6         |
| Gly       | 35.5         | 31.4         | 32.7         |
| Ala       | 23.4         | 20.3         | 20.8         |
| Val       | 16.0         | 13.6         | 14.6         |
| Met       | 4.0          | 4.0          | 3.3          |
| Ile       | 8.0          | 7.8          | 7.7          |
| Leu       | 19.5         | 23.6         | 23.5         |
| Tyr       | 11.9         | 14.4         | 13.7         |
| Phe       | 8.8          | 8.3          | 8.2          |
| His       | 7.5          | 7.5          | 7.4          |
| Lys       | 10.7         | 11.4         | 10.8         |
| Arg       | 18.4         | 19.9         | 18.6         |

* Calculated on the basis of a total of 290 residues.

| Table IV | Determination of free and disulfide-bridged cysteine residues in H-2Dd plus H-2Kk |
|-----------|-----------------------------------------------|
| Cycle number | PTH-derivative | Yield | Method of identification |
| 1          | Gly             | 3.1   | MS, HPLC, AA             |
| 2          | Ser             | 0.3   | HPLC, AA                 |
| 3          | His             | 1.8   | HPLC, AA                 |
| 4          | Leu             | 2.8   | HPLC, AA                 |
| 5          | Arg             | 0.8   | HPLC, AA                 |
| 6          | Tyr             | 0.9   | HPLC, AA                 |
| 7          | Phe             | 2.0   | HPLC, AA                 |
| 8          | Val             | 2.2   | HPLC, AA                 |
| 9          | Thr             | 1.6   | HPLC, AA                 |
| 10         | Ala             | 1.8   | HPLC, AA                 |
| 11         | Val             | 1.1   | HPLC, AA                 |
| 12         | ( )             |       |                           |
| 13         | ( )             |       |                           |
| 14         | Pro             | 0.7   | HPLC, AA                 |
| 15         | Gly             | 1.0   | HPLC, AA                 |
| 16         | Leu             | 0.9   | HPLC, AA                 |
| 17         | Glu             | 0.7   | HPLC, AA                 |
| 18         | Lys             | 0.8 (0.2) | AA  |
| 19         | Pro             | 0.4   | HPLC, AA                 |
| 20         | Val             | 0.2   | HPLC, AA                 |
| 21         | Met             | 0.4   | HPLC, AA                 |
| 22         | ( )             |       |                           |
| 23         | ( )             |       |                           |
| 24         | ( )             |       |                           |
| 25         | ( )             |       |                           |
| 26         | ( )             |       |                           |
| 27         | ( )             |       |                           |

* MS, mass spectrometry; HPLC, high performance liquid chromatography; AA, amino acid analysis after hydrolysis with HI (35).

* Not identified in this analysis.

* Zorbax C-8 column.
degradation (19 nmol) was performed in the presence of 1% SDS (32). The initial yield increased to 35% but the repetitive yield was only 71%. Glycine, serine, and leucine were identified at cycles 1, 2, and 5, respectively. In the third degradation the preparation (31 nmol) was reacted with 4-SPITC (32, 33) in the presence of 0.5% SDS. The sequence of the first 27 residues was established (repetitive yield, 91%) with unidentified residues at positions 13, 14, 22, and 24 (Table V and Fig. 8). An automated degradation of electrophoresed H-2\(^b\) (2 mg) confirmed this sequence. One was that of the heavy chain (Fig. 8) and the other was that of \(\beta_2\)-microglobulin (43). Although \(\beta_2\)-microglobulin also was present in a 3-fold excess over H-2D\(^d\), has valine at this position. Similarly, the yield of valine-21 indicates that one of the polypeptide chains has a different residue at this position.

The failure to identify any PTH-derivatives at positions 13 and 20, respectively, the yields at these steps established their presence in the heavy chains as well (data not shown). The native H-2\(^b\) was much more soluble than the carboxymethylated chains and less than one-half the amount of material was required to obtain the same data. The NH\(_2\)-terminal sequences of H-2\(^b\) agree with the published sequences of intrinsically radiolabeled H-2K\(^k\) and H-2D\(^d\) (17-20, 44) with the following exceptions. Glycine, and no methionine, was found at position 1 and serine, instead of proline, was found at position 2. Histidine, in addition to valine, was detected at position 9 in only one degradation. The yield of valine at cycle 9 (Table V) indicated that H-2K\(^k\), present in a 3-fold excess over H-2D\(^d\), has valine at this position. Similarly, the yield of valine-21 indicates that one of the polypeptide chains has a different residue at this position. The failure to identify any PTH-derivatives at positions 13 and 14 as well as positions 22 and 24 could be due either to heterogeneity or to the fact that these are residues which are difficult to identify quantitatively, e.g. serine and arginine. Some lysine was detected at position 19 (Table V), but glutamine/glutamic acid was detected in much higher yield.

**DISCUSSION**

In this report, a procedure for the purification of pepsin-solubilized H-2 antigens that yields up to 10 mg of highly purified material has been described. The two most important factors in the purification scheme were the choice of liver as starting material and the introduction of a quantitative radioimmunoassay of H-2. The yield of purified H-2 was 81 \(\mu\)g/liver versus 1.3 \(\mu\)g/spleen (45). This 6-fold difference in yield appears to be due mainly to the difference in size of the two organs, since the specific activities of H-2 in the two membranes are comparable (data not shown). The choice of liver over spleen as a source for the antigen then has important practical consequences. In order to obtain a yield of H-2 from spleen equivalent to that from one preparation of 1000 livers, the expenditure of 5000 more mice and many more man-hours would be required.

Earlier studies in our laboratory (9) and by others (3, 37) have used the antibody-mediated cytotoxicity assay to monitor the purification of H-2. This assay is time-consuming; in addition, quantitative values are difficult to obtain. The use of a radioimmunoassay in the present study has overcome these difficulties and has made it possible to measure the relative amounts of H-2K\(^k\), H-2D\(^d\), and \(\beta_2\)-microglobulin at all stages of the purification (Table III). This technique indicated that the two transplantation antigens, H-2K\(^k\) and H-2D\(^d\), were not present in equal amounts in H-2 preparations purified from liver or from spleen. The 2- to 3-fold lower amounts of H-2D\(^d\) detected could not be attributed to a preferential loss during the purification since the recovery and increase in specific activity of H-2D\(^d\) closely followed that of H-2K\(^k\). The relative amounts of the two proteins eluted from ura gel (Fig. 5C and Ref. 25) support the immunochemical observation.

SDS-gel electrophoresis and disc-gel electrophoresis of iso-electric-focused H-2 in the presence of urea revealed only minor impurities in the preparation. Chemical analyses of the electrofocused H-2 and of the carboxymethylated chain confirmed the presence of only minor impurities. We conclude that the purity of the electrofocused material was approximately 90%.

The molecular weight of the pepsin fragments of the heavy chains was 37,800 as estimated by SDS electrophoresis and 37,000 if calculated from the sum of the molecular weights of the polypeptide chain and the carbohydrate moiety (25). These values are in agreement with those determined by Kvist et al. (45) wherein three different methods gave an average molecular weight of 37,900. Thus, although the heavy chains of detergent-solubilized H-2 and HLA both have molecular weights of approximately 45,000 (11, 24), pepsin cleavage yields heavy chain fragments of different molecular weights, namely 38,000 (H-2) versus 34,000 (HLA).

From the amino acid composition analyses and the limited sequence data, it appears that the primary structures of H-2 and HLA antigens are very similar (70% homology) in the first 27 residues for H-2 and HLA-B7 and 66% homology for H\(^a\) and H-2K\(^k\). Fig. 8) as are those of the H-2D and H-2K\(^k\) molecules (25). Comparative tryptic peptide analysis of allelic H-2K molecules and K and D region products of the same haplotype yielded only 30 to 45% identical peptides (46). However, this technique can overestimate the variability in the primary structure; for example, an interchange of 45 out of 300 amino acid residues in two related proteins could affect the properties of a large percentage of the tryptic peptides even though there is 85% sequence homology.

Comparison of our sequence data with published sequences obtained by using radiolabeling techniques demonstrates good agreement between the two. Two differences are, however, apparent. We identified glycine at position 1 of H-2\(^b\), whereas methionine was reported for radiolabeled H-2K and H-2D molecules (17-20). Recently, H 2K\(^k\) and H 2K\(^b\) were reanalyzed (44). No methionine could be detected at position 1 of either antigen; glycine was tentatively assigned to this position for both molecules. Glycine has been reported at position 1 for all HLA antigens examined to date. It is possible that this position is invariant in both species. Serine was identified at position 2 of H-2\(^b\) but proline has been reported for other H-
2 antigens. For another H-2 haplotype, we identified valine at position 2.² It appears then that this position is variable.

Two amino acids, valine and histidine, were identified at position 9 in one preparation of H-2*. Valine was reported at this position for H-2K and H-2D (18), whereas histidine was reported for H-2K and H-2D (20, 44). Position 9 is also variable in the HLA alloantigens (tyrosine, phenylalanine, or aspartic acid (47)) and in the chicken and guinea pig transplantation antigens (arginine or tyrosine (48)).

Serine and arginine have been reported at positions 13 and 14 for other antigens (41). No identifications were made for H-2* at these positions. However, serine and arginine can be difficult residues to detect and may be present in H-2* as well. Further work is required to assign these positions.

Position 21 has been reported to be arginine for both H-2D and H-2K molecules (49). For H-2*, valine was detected. Position 22 has been reported to be tyrosine or phenylalanine (44). Both residues are readily detectable; neither was identified in H-2*. Glutamic acid was identified at position 24 of H-2* (41). No identification was made for H-2*.

The amino acid interchanges at positions 2, 9, 21, 22, and 24 may be attributed to classical allelic variation. However, if the degree of variation observed at the NH₂ terminus is representative of variability within the entire polypeptide chain, then the H-2 antigens are more divergent than most allelic products. This degree of variation between the products of presumably allelic genes may indeed reflect the extraordinary polymorphism that has been described for the histocompatibility system. Recent results suggest that some of these features are shared by the immunoglobulin system. Extensive sequence differences have been found between the various group a and b allotypes in the rabbit (50). Observations by Strosberg (50) showed that certain rabbits may express three group a and three group b alleles after hyperimmunization. These findings in the rabbit suggest that the so-called “alleles” may represent pseudoallelic clusters. It is possible, as suggested by Bodmer (51), that the H-2 haplotypes are the products of different closely linked genes and the polymorphism resides in the control of the expressed gene. Experimental evidence in support of such a hypothesis has been recently reported (52). A methylcholanthrene-induced tumor of BALB/c (H-2d) origin expresses H-2 antigens nor-

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