Murine *Histocompatibility-2 (H-2)* Alloantigens. Purification and Some Chemical Properties of a Second Class of Fragments (Class II) Solubilized by Papain from Cell Membranes of $H\text{-}2^b$ and $H\text{-}2^d$ Mice*

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ABSTRACT: A smaller glycoprotein *H-2* alloantigen fragment (class II) released from cell membranes of mouse spleen cells of $H\text{-}2^b$ and $H\text{-}2^d$ genotypes by limited papain digestion, was separated from a larger *H-2* alloantigen fragment (class I) by Sephadex G-150 chromatography and purified by the following procedures: CM-Sephadex, DEAE-Sephadex, and Sephadex G-75 chromatography, and polyacrylamide disc gel electrophoresis. The eluates from the latter procedure showed an increase of about 100–300-fold in specific activity in the inhibition assay. Chemical analyses showed that the class II alloantigen fragments were glycoproteins containing 72 ($H\text{-}2^d$) to 82% ($H\text{-}2^b$) protein and 6 to 7% neutral carbohydrate. Only material from the $H\text{-}2^b$ genotype showed significant glucosamine (approximately 6%). Amino acid analysis of the disc gel purified fractions from the two *H-2* genotypes showed marked similarity; however, there were differences to the extent of 4 moles % in serine, 2 moles % in aspartic acid, and about 1 mole % in valine, isoleucine, leucine, and tyrosine. An estimate of the molecular weight by Sephadex chromatography and sucrose gradient centrifugation gave a value of approximately 33,000, while the class I *H-2* glycoproteins were found to have a molecular weight of 66,000. Of the ten or so possible specificities for each particular genotype the class II *H-2* fragments apparently carried only one *H-2* specificity, *i.e.*, H-2.2 for the $H\text{-}2^b$ fragment, and H-2.31 for the $H\text{-}2^d$ fragment.

Mouse *H-2* alloantigens are membrane located macromolecules bearing the immunological specificities which are determined by the *H-2* locus, and which are involved in transplantation rejection. The *H-2* locus has a number of different alleles, each of which determines a different combination of antigenic specificities (*cf.* Amos, 1964; Snell and Stimpfling, 1966; Shreffler, 1966).

We have previously reported on the properties of *H-2* alloantigens solubilized from their membrane site by the action of papain. This solubilization process produced at least two classes of antigenically active fragments for each of the two mouse strains examined, $H\text{-}2^b$ and $H\text{-}2^d$. For one type of fragment (which we called class I) we described a purification method and some chemical properties (Shimada and Nathenson, 1969).

In the present study we describe a method for the purification, and some chemical and serological properties of the second class of *H-2* alloantigen fragments (class II) solubilized by papain.

Materials and Methods

Mice homozygous for the *H-2* allele were purchased from Jackson Laboratories, Bar Harbor, Maine, or were obtained from the breeding colonies of Dr. Frank Lilly, Albert Einstein College of Medicine, Bronx, N. Y. The strains used were the same as reported earlier (Shimada and Nathenson, 1969).

Assay of *H-2* alloantigenic activity was carried out as described previously (Nathenson and Davies, 1966; Shimada and Nathenson, 1969) by the method of inhibition of immune cytolysis (Sanderson, 1964; Wigzell, 1965). Systems permitting detection of the following *H-2* specificities are described in detail elsewhere (Shimada and Nathenson, 1969): H-2.4,10,13; H-2.3; H-2.3.8; H-2.4; H-2.31; H-2.2; H-2.5; H-2.33 and H-2.28. For identification of the group of possible specificities H-2.6, 14, 27, 28, 29 sera raised in B10.BR ($H\text{-}2^k$) mice immunized with cells of C57BL/6 ($H\text{-}2^b$) mice were tested on B10.D2 ($H\text{-}2^d$) cells. For detection of H-2.8, sera raised in C57BL/10 ($H\text{-}2^b$) mice immunized with H-2G ($H\text{-}2^g$) cells were tested on B10.BR cells, and for detection of H-2.10, sera raised in C57BL/6 ($H\text{-}2^b$) mice immunized with tumor cells of Meth A ($H\text{-}2^d$) were tested on cells of I strain ($H\text{-}2^i$) mice.

Crude particulate fractions of mouse spleen cells of C57BL/6 and DBA/2 animals and papain-solubilized digests of these particulate fractions were prepared as described previously (Shimada and Nathenson, 1969).

Preparative polyacrylamide gel electrophoresis in 7.5% gels (either 0.9 cm × 15 cm or 1.5 cm × 20 cm) was carried out according to the method of Davis (1964). After the electrophoresis the gels were frozen and cut crosswise into 0.2- or 0.25-cm wide slices and extracted as described previously (Shimada and Nathenson, 1969).

Total protein was determined by the method of Lowry *et al.* (1951), amino sugar by a modification of the method of Elson and Morgan (Reissig *et al.*, 1955), total carbohydrate by a modification of the orcinol method (Francois *et al.*, 1962),

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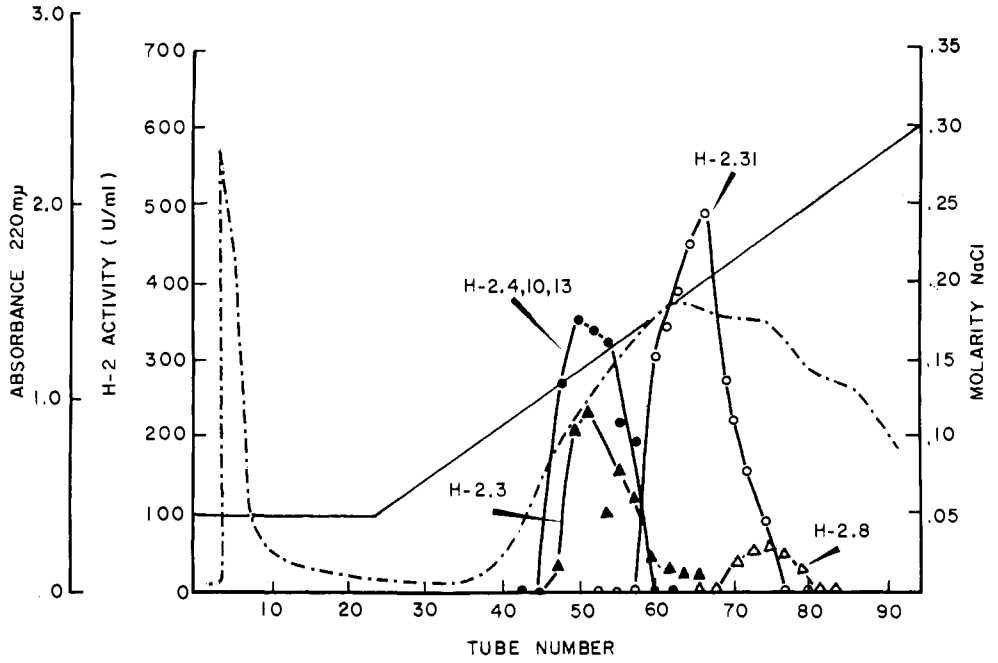

FIGURE 1: DEAE-Sephadex A-25 column chromatography of *H-2*^{d} alloantigens. The pooled fraction from the CM-Sephadex column of material from 12,000 spleens in 6.3 ml was applied to a 2.5 × 22 cm DEAE-Sephadex A-25 column equilibrated with 0.01 M Tris-HCl–0.05 M NaCl buffer, pH 8.4, and eluted with the same buffer for fraction 1–24, at which time a linear gradient was begun which reached 0.30 M NaCl at tube 95; 10.5 ml/fraction was collected. Elution rate was 22 ml/hr. Tubes were monitored for absorbance 220 mμ (– – – –); H-2.4,10,13 (—●—●—); H-2.31 (—○—○—); H-2.3 (—▲—▲—); H-2.8 (—△—△—) antigenic inhibitory activity.

TABLE I: Purification of *H-2* Alloantigen Fragments (Class II) from *H-2*^{b} and *H-2*^{d} Sources.[a]

	H-2^{b}				*H-2*^{d}			
		H-2.2 Activity				H-2.31 Activity		
Purification Step	Total Protein (mg)	Total Units (× 10^{4})	Sp Act. (units/mg)	Purifcn	Total Protein (mg)	Total (× 10^{4})	Sp Act. (units/mg)	Purifcn
Extract	90,000	1,440	160	1	114,000	3,540	311	1
Crude particulate fraction	31,024	1,044	330	2.08	31,320	2,571	820	2.64
Papain solubilized fraction	10,624	57.6	54		13,662	122	88	
Ammonium sulfate fraction (50–75%)	917	42.0	460	2.88	1,085	101	940	3.02
Sephadex G-150 eluate	93	16.4	1,800	11.3	324	18.3	565	1.82
CM-Sephadex eluate	55.2	12.9	2,340	14.2	110	17.0	1,540	4.96
DEAE-Sephadex eluate	9.2	5.1	5,550	34.6	25.6	11.7	4,650	15.0
Sephadex G-75 eluate	3.1	3.5	11,500	72.0	8.6	5.1	5,960	19.2
Disc gel electrophoresis eluate								
Fraction I	0.21	0.7	35,500	222	0.50	1.56	31,800	102.4
Fraction II	0.22	1.7	75,000	468	0.65	0.84	13,000	41.9

[a] For the *H-2*^{b} preparation, 8000 spleens of C57BL/6 mice were used; for the *H-2*^{d} preparation, 12,000 spleens of DBA/2 mice were used.

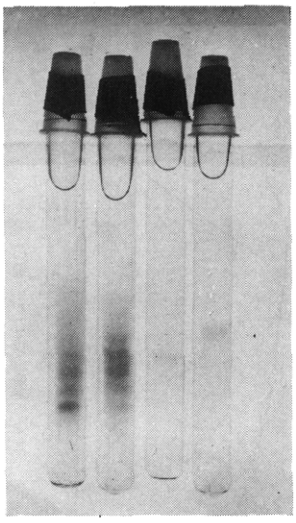

FIGURE 2: Disc electrophoresis of *H-2*^{b} samples on polyacrylamide. Photograph shows four gels stained with coomasie blue 0.25% and run according to the method of Davis (1964). The DEAE-Sephadex fraction was applied to the first gel, the G-75 Sephadex fraction to the second gel, and the disc gel, fractions I and II to the third and fourth gels.

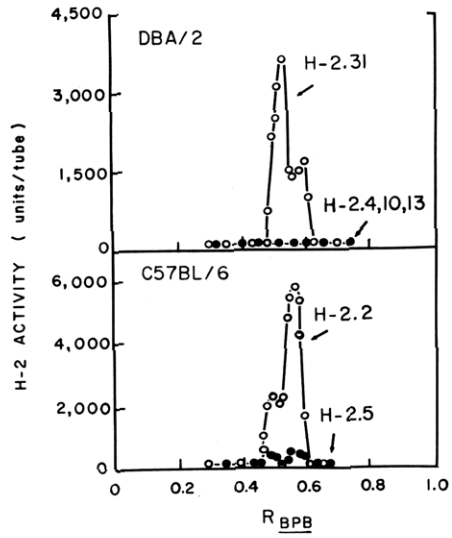

FIGURE 3: Preparative disc gel electrophorogram of *H-2*^{b} and *H-2*^{d} alloantigen Sephadex G-75 fractions. The *H-2*^{b} fraction was applied to a 9.5 × 150 mm (7.5%) gel and the *H-2*^{d} fraction to a 15 × 200 mm (7.5%) gel. The gels were run according to the method of Davis (1964) until the bromophenol blue reached the end, then were sliced and processed as described under Methods section. The eluted fractions were assayed for *H-2* activity as follows: H-2.31 (—○—○—); H-2.4, 10,13 (—●—●—) for *H-2*^{d}, and H-2.2 (—○—○—); H-2.5 (—●—●—) for *H-2*^{b}.

and sialic acid by the method of Warren (1959). Details of standards used for these methods have been given previously (Shimada and Nathenson, 1969). Amino acid analyses (Hubbard, 1965; Spackman *et al.*, 1958) were performed as described previously (Shimada and Nathenson, 1969), Chemicals were commercial products of analytic reagent grade or better.

Results

Purification of Alloantigens. We prepared samples of antigenically active material from *H-2*^{b} and *H-2*^{d} spleens using the first five stages of the method of Shimada and Nathenson (1969) which included preparation of the cellular extract, preparation of the membrane fraction, solubilization with papain, ammonium sulfate fractionation, and Sephadex G-150 column chromatography (Table I). During the chromatography on a 5 × 135 cm Sephadex G-150 column, the *H-2* alloantigens separated into two classes. Class I fragments eluted first from the column, while class II fragments eluted later (*cf.* Figures 5 and 6, Shimada and Nathenson, 1969).

The pooled fractions comprising class II were concentrated by ultrafiltration (Diaflo, Amicon Corp., Lexington, Mass.), dialyzed against 0.01 M $Na\text{-}PO_4$ buffer, pH 6.25, and applied to a CM-Sephadex C-50 column (2.5 × 30 cm). More than 90% of the *H-2* activity passed through the column without adhering, and was purified 1.5–2.5-fold by this step (Table I, step 6).

The antigen was then concentrated and dialyzed against 0.01 M Tris-HCl buffer, pH 8.4, containing 0.05 M NaCl, and applied to a DEAE-Sephadex A-25 column (2.5 × 22 cm) equilibrated with the same buffer. The alloantigen fragments were eluted at the concentration of 0.15–0.20 M NaCl in a linear salt gradient (Figure 1). By this chromatographic method, a two- to threefold purification could be achieved. As seen in Figure 1, the H-2.31 class II fragment was clearly separated from the class I fragments of *H-2*^{d} alloantigens H-2.4,10,13 or H-2.3. The *H-2*^{b} class II fragments containing H-2.2 could also be separated from H-2.5 and H-2.33, but not quite so completely as was the case for H-2.31 (data not shown).

The next purification step (Table I) was column chromatography on Sephadex G-75 (1.5 × 119 cm) in 0.15 M NaCl–0.01 M Tris-Cl, pH 8.4. The pooled fraction containing the antigenic activity was 1.4–2-fold higher in specific activity than the starting material.

The final purification was accomplished by electrophoresis on polyacrylamide gels carried out as described in the Methods section. The stained protein pattern of a G-75 eluate electrophoresed on an 0.6 × 10 cm analytic gel (Figure 2, gel 2) revealed five or six bands, but eluted samples from the larger unstained gels showed that only two of these bands had antigen activity (Figure 3). The major peak for the *H-2*^{d} material (H-2.31) had an R_{BPB} = 0.53 (R_{BPB} = migration distance of *H-2* activity/migration distance of bromophenol blue), and the major peak of the *H-2*^{b} material (H-2.2) had an R_{BPB} = 0.56. The two active fractions from both sources were eluted, dialyzed, and freeze dried. When reelectrophoresed in the same system used for their final fractionation, they ran as single bands (*cf.* Figure 2, gels 3 and 4). The homogeneity of the fractions of highest activity from both sources was suggested by the finding that upon electrophoresis in urea, only one protein staining band was seen.

Chemical Analyses. The general chemical characteristics of the Sephadex G-75 and disc gel electrophoresis purified alloantigen fractions were determined (Table II). A dry weight estimate was carried out only on the G-75 fractions because of the small amounts available at the more purified stages. Even for the analysis of the Sephadex G-75 purified sample analysis, complete removal of water was not possible, since heating would have altered the alloantigenic activity thereby preventing further purification studies.

TABLE II: Chemical Analysis of Class II *H-2* Alloantigens.

	Protein[b] (%)	Neutral Carbohydrate[c] (%)	Glucosamine[d] (%)	Sialic Acid[e] (%)
A. Percentage of Dry Weight[a]				
Sephadex G-75 *H-2*^{b}	83.5	7.0	4.9	2.1
Sephadex G-75 *H-2*^{d}	72.5	5.8	0.8	0.8
B. Amount with Respect to Protein				
H-2^b Fractions				
Sephadex G-75	1.000	0.089	0.052	0.022
Disc gel peak I	1.000	0.058	0.066	0.013
Disc gel peak II	1.000	0.081	0.085	0.023
H-2^d Fractions				
Sephadex G-75	1.000	0.080	0.011	0.011
Disc gel peak I	1.000	0.042	0.014	0.004
Disc gel peak II	1.000	0.048	0.021	0.008

[a] Dry weight determined on lyophilized powder. [b] Protein value was determined as the mg equivalent to bovine plasma albumin by the method of Lowry *et al.* (1951). Since tyrosine content is a major factor in this color reaction, our values would be approximately 20–30% lower than the true value since the tyrosine content of bovine plasma albumin is *ca.* 5 moles %, whereas our *H-2*^{b} Sephadex fraction had tyrosine about 4 moles %, and *H-2*^{d} Sephadex fraction had about 3 moles %. However since tryptophan content is also a factor in the Lowry color reaction, and we have no tryptophan analyses, we cannot state for certain the true protein value. [c] Determined by the orcinol method of Francois *et al.* (1962) using mannose as standard. [d] Determined by method of Reissig *et al.* (1955) using glucosamine as a standard after hydrolysis in 4 N HCl at 100° for 4 hr or from amino acid analysis using a factor of 2.46 to compensate for different hydrolysis conditions. Ninhydrin-positive peak found only at the glucosamine region. [e] Determined by the method of Warren (1959).

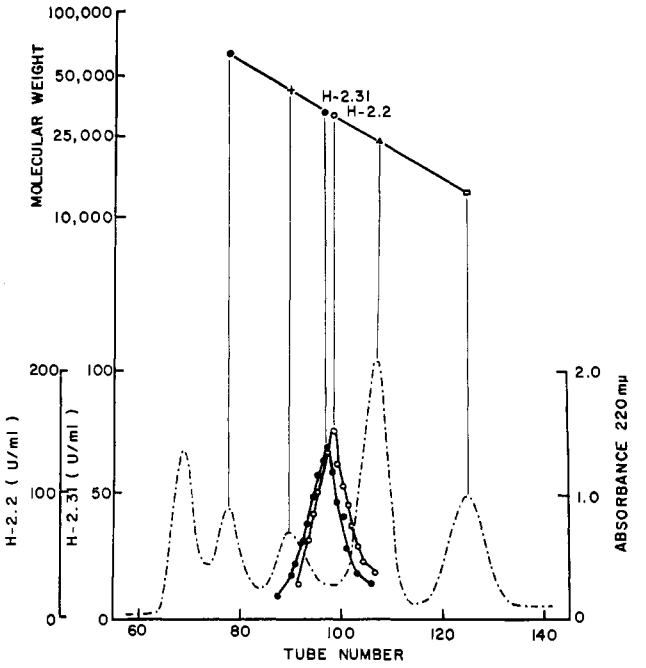

FIGURE 4: Determination of molecular weight of *H-2* alloantigens by Sephadex G-75 column chromatography. Purified isoantigens: *H-2*^{b} (H-2.2) (○) and *H-2*^{d} (H-2.31) (●); albumin 1.0 mg, mol wt 67,000 (⊙): Ovalbumin 1.0 mg mol wt 45,000 (+), chymotrypsinogen E, 1.0 mg, mol wt 25,000 (▲) and cytochrome *c*, 1.0 mg mol wt 12,400 (□), were dissolved in the buffer, centrifuged (1000*g*, 10 min) and the supernatant was applied to the Sephadex G-75 column (1.5 × 118 cm) which was previously equilibrated with the 0.01 M Tris-HCl buffer, pH 8.4, containing 0.15 M NaCl. The column was eluted with the same buffer at the rate of 1.25 ml/fraction per 20 min. Tubes were monitored for absorbance at 220 mμ (–·–·–); H-2.31 (—●—●—); and H-2.2 (—○—○—) antigenic inhibitory activity.

In samples from the Sephadex G-75 stage of purification the *H-2*^{d} material had 72% protein, 5.8% neutral carbohydrate, and the *H-2*^{b} material had 82% protein and 7.0% carbohydrate. Significant glucosamine was found only in the *H-2*^{b} fractions. In part B of Table II, in order to permit a direct comparison of the different fractions, we arbitrarily set the protein content at 1.000 and related the carbohydrate, glucosamine, and sialic acid to the protein value. Overall, the analyses of the disc gel fractions were quite similar to the analyses of the Sephadex G-75 fractions.

Amino Acid Analysis. The amino acid composition of the Sephadex G-75 fractions, and the more purified disc gel fractions were determined (Table III). Overall, the composition for the *H-2*^{b} glycoprotein and the *H-2*^{d} glycoprotein was similar. However, *H-2*^{d} glycoprotein was higher in aspartic acid content by about 2 moles %, in valine by 1.5 moles %, isoleucine by 0.8 mole %, and leucine content by about 1 mole %. The *H-2*^{b} glycoprotein, on the other hand, was higher in serine by an average of about 4 moles % and tyrosine by about 0.8 mole %. Differences in glycine are difficult to assess since glycine forms a part of the disc gel electrophoresis system and may not be completely removed by dialysis.

Molecular Weight. We estimated the molecular weight of the *H-2*^{b} and *H-2*^{d} fragments by gel filtration on Sephadex G-75 and sucrose gradient centrifugation. By Sephadex chromatography, the molecular weight of the *H-2*^{d} glycoprotein appeared to be slightly larger than that of the *H-2*^{b}, since these two fragments peaked two fractions apart when chromatographed as a mixture on Sephadex G-75 (Figure 4). The molecular weight markers, also contained in the samples applied to the column, showed a direct logarithmic relationship of the molecular weight to elution volume, and allowed us to estimate a molecular weight (Andrews, 1964) of approximately 34,000 for the *H-2*^{d} (H-2.31) glycoprotein and 33,000 for the H-2^b (H-2.2) glycoprotein. Results of sucrose gradient centrifugation (Figure 5) and estimation of the molecular weight by the method of Martin and Ames (1961) showed rather close agreement to these estimates, with values of around 30,000.

Immunological Specificity of H-2 Alloantigenic Glycoproteins. Each of the disc gel fractions was tested for several *H-2* alloantigenic specificities. The *H-2*^{b} material was strongly reactive

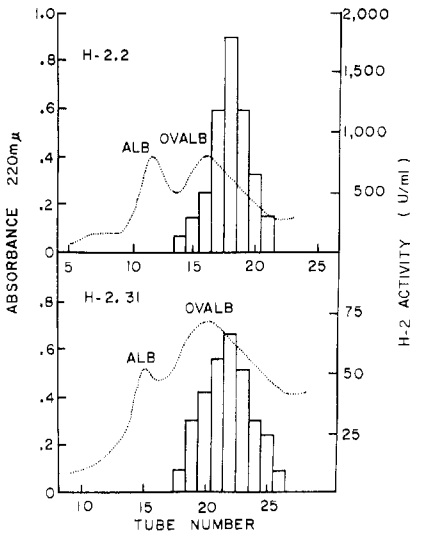

FIGURE 5: Determination of molecular weight of *H-2* alloantigens by sucrose gradient centrifugation. A mixture of *H-2*^{b} (H-2.2) or *H-2*^{d} (H-2.31) material and markers (albumin, mol wt 67,000; ovalbumin, mol wt 45,000 was applied to a 2.5–15% sucrose gradient in 0.01 M Tris-Cl–0.15 M NaCl, pH 8.4 buffer (5-ml tube, SW 50 rotor, Spinco). After centrifugation at 35,000 rpm for 18 hr, 3-drop fractions were collected and analyzed. The molecular weight was calculated according to the method of Martin and Ames (1961). Absorbance 220, dotted line; *H-2* activity, solid bar graph. The upper half of the figure shows the H-2.2 activity profile, and the lower half of the figure shows the H-2.31 activity profile.

TABLE III: Amino Acid Composition of Class II *H-2* Alloantigen Fractions.[a]

Amino Acid	*H-2*^{b} Sephadex G-75	*H-2*^{b} Disc Gel I	*H-2*^{b} Disc Gel II	*H-2*^{d} Sephadex G-75	*H-2*^{d} Disc Gel I	*H-2*^{d} Disc Gel II
Lys	6.2	6.5	5.9	6.8	6.8	6.8
His	1.4	1.7	1.7	2.0	1.5	1.8
Arg	5.2	3.8	4.3	3.0	3.3	3.2
Asp	11.4	9.9	10.1	11.6	11.9	12.0
Thr	6.7	6.6	6.3	7.0	6.4	5.4
Ser	7.6	12.0	8.7	6.1	6.3	6.2
Glu	12.7	12.1	12.8	13.5	12.8	13.5
Pro	5.1	4.7	5.6	3.0	4.8	4.8
Gly[b]	7.3	10.6	9.1	7.9	8.7	8.0
Ala	7.0	7.9	7.4	7.1	7.4	6.8
½-Cys	2.5	1.6	2.6	1.7	2.2	1.6
Val	5.6	4.5	4.7	6.9	6.3	6.3
Met	1.9	1.4	1.9	1.8	1.2	1.5
Ile	4.8	3.8	4.4	5.6	4.9	5.1
Leu	7.1	6.1	7.6	8.9	8.3	8.7
Tyr	3.7	3.5	4.1	3.0	2.9	3.1
Phe	4.2	3.0	3.6	4.0	4.1	4.3

[a] Data expressed as moles of amino acid residue/100 moles of amino acid residues recovered in analysis. Tryptophan was not determined. All results were from consecutive runs on the analyzer. [b] Glycine content is subject to some uncertainty since glycine is used as a part of the buffer system for disc gel electrophoresis (Davis, 1964) and may bind to proteins.

for the H-2.2 alloantigenic specificity, yet gave very weak reaction with an antiserum detecting the set of specificities H-2.6, 14, 27, 28, 29, and almost no reaction in systems detecting H-2.5 and H-2.33 (about 0.1% of the activity of highly purified H-2.5 material, class I (*cf.* Shimada and Nathenson, 1969). It was reactive in a system testing for H-2.2, 22, yet unreactive in a system testing for H-2.22 alone. Therefore, we have concluded that it is mainly reactive for the H-2.2 specificity. In a series of control tests the *H-2*^{b} material was found unreactive in test systems detecting H-2.3; H-2.3, 8; H-2.4, 10, 13; H-2.8 and H-2.31, as would be expected since none of these specificities are determined by the *H-2*^{d} genotype. The *H-2*^{d} material, on the other hand, was reactive in tests for H-2.31 and negative in tests for H-2.4, 10, 13, H-2.3, H-2.8, and the set of specificities H-2.6, 14, 27, 28, 29. The control tests were negative for the specificities H-2.2; H-2.2, 22; H-2.5; H-2.33 and H-2.22, specificities which are genetically determined by the *H-2*^{b} and absent in the *H-2*^{d} genotype.

Discussion

We have previously reported (Shimada and Nathenson, 1969) that the soluble supernatant from the limited papain digest of crude membrane preparations of spleen cells from two mouse strains, *H-2*^{d} and *H-2*^{b}, contained at least two classes of fragments carrying alloantigenic activity as judged by our assessment with a limited number of monospecific *H-2* test systems. One class of fragments (class I) had an approximate molecular weight of 66,000 and probably carried several of the alloantigenic specificities found in that strain. It was glycoprotein in composition with approximately 85–90% protein, and 10% carbohydrate, including neutral sugar, glucosamine, and sialic acid.

The isolation procedure for the class II fragments was the same as for the class I fragments during the preparation of membranes, solubilization, and ammonium sulfate fractionation. During Sephadex G-150 chromatography, the two classes of fragments were separated. The class II fragments were further purified by CM-Sephadex C-50, DEAE-Sephadex A-25 and Sephadex G-75 chromatography and disc gel electrophoresis. The purification on a protein basis with respect to the crude membrane fraction for the H-2.2 specificity from the *H-2*^{b} genotype was about 470-fold and about 100-fold for the H-2.31 specificity from the *H-2*^{d} genotype.

The chemical analyses show that the class II *H-2* alloantigen fragments are glycoproteins, containing mostly protein (72–83%), neutral carbohydrate, glucosamine, and sialic acid. The fragments carrying H-2.2 contain around four times as much glucosamine as the fragments carrying H-2.31, and this is certainly the most striking difference between these moieties.

An examination of the amino acid composition shows a general similarity between the two purified class II preparations. However, differences between these glycoproteins were found to the extent of 4 moles % for serine, about 2 moles % for aspartic acid, and from 0.5 to 1.5 moles % in the content of valine, leucine, isoleucine, and tyrosine.

The overwhelming impression of the data from this work and our previous report (Shimada and Nathenson, 1969) is that, in actual fact, the amino acid analyses of all fragments, both class I and class II, are quite similar to each other when examined with respect to unrelated mouse proteins whose amino acid composition is known (*e.g.*, mouse IgG light chains). Such a general overall similarity in amino acid compositional analyses also fits with other findings on the alloantigenic fragments from human cell lines, where reasonably close amino acid compositional analyses were demonstrated between mouse class I *H-2* and human class I HLA alloantigens (Mann and Nathenson, 1969).

The conclusions from this study and our previous work (Shimada and Nathenson, 1969) is that chemical determinants of the *H-2* alloantigenic specificities are part of glycoprotein fragments which can be released from the cellular membranes by papain digestion. For each of the mouse strains studied, one of the *H-2* specificities is so constituted that papain cleaves the molecule carrying it in such a way that the class II fragment is formed. The papain treatment generates also a larger fragment (class I) which our preliminary data suggest (Cullen and Nathenson, 1969) may carry two or greater of the other *H-2* specificities determined by that genotype. Thus, the glycoprotein fragments released by the solubilization process of papain digestion may carry either one or more sites of a single *H-2* specificity, or one or more sites of several *H-2* specificities. The relationship of these fragments to each other *in situ* in the cell membrane at present is not yet clear.

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