Murine Histocompatibility-2 (*H-2*) Alloantigens. Purification and Some Chemical Properties of Soluble Products from $H\text{-}2^b$ and $H\text{-}2^d$ Genotypes Released by Papain Digestion of Membrane Fractions*

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ABSTRACT: Membrane-located murine *H-2* alloantigens were solubilized by limited papain digestion of crude membrane fractions of mouse spleen cells of $H\text{-}2^b$ and $H\text{-}2^d$ genotypes. The major active fragments of the papain digest were purified by the following procedures: ammonium sulfate fractional precipitation, column chromatography on Sephadex G-150, CM-Sephadex, and DEAE-Sephadex, and polyacrylamide disc gel electrophoresis. The disc gel eluates were essentially homogeneous when reelectrophoresed on polyacrylamide at pH 4.3 or in sodium dodecyl sulfate. The final products from each mouse genotype were 300 to 700 times more active than the starting material in the quantitative inhibition assay. Chemical analyses showed that the active alloantigen fragments were glycoproteins containing about 90% protein. The electrophoretically pure $H\text{-}2^b$ material contained *ca.* 5% neutral carbohydrate, 4.5% glucosamine, and 1.2% sialic acid. The identical fractions of $H\text{-}2^d$ genotype contained *ca.* 4% neutral carbohydrate, 3% glucosamine, and 1% sialic acid. There was no phosphate nor detectable lipid. Amino acid analyses of these same fractions showed almost identical patterns except for a higher arginine and glutamic acid content for the $H\text{-}2^d$ alloantigen.

An estimate of the molecular weight of these major alloantigen-bearing fragments was of the order of 65,000–75,000 as judged by Sephadex column chromatography and by sucrose gradient centrifugation.

Mouse *H-2* alloantigens are membrane-located products having the immunological determinants involved in transplantation rejection. They are genetically controlled by the complex *H-2* locus, and form the major transplantation system in the mouse (*cf.* Snell and Stimpfling, 1966; Amos, 1964; Shreffler, 1966).

There are greater than 20 defined alleles of the *H-2* locus, and each allele determines a different combination of antigenic specificities. Recombination between the genetic determinants of the *H-2* specificities has permitted the construction of a linear genetic map (*cf.* Snell and Stimpfling, 1966). Therefore, extraction and purification of the membrane-located cellular products of the *H-2* gene and elucidation of their chemical architecture is of considerable interest from the standpoint of understanding the molecular representation and control of the genetic information. Knowledge of the chemical nature of the *H-2* alloantigens is, in addition, of immense practical importance for rational design of studies to solve the problem of tissue graft rejection.

Methods for preparing cell-free fractions containing histocompatibility antigens have been described some years ago (Billingham *et al.*, 1956; Kandutsch and Reinert-Wenck, 1957; Kandutsch, 1960); however, the insoluble nature of the antigens so prepared, their extreme lability, and the lack of a rapid and quantitative assay method has held back rapid progress on the study of their chemistry.

Various workers, using different test systems and different methods for extraction, and a variety of cell sources, have clearly established that nearly all of the *H-2* alloantigen activity is associated with membraneous cell fractions (Kandutsch and Reinert-Wenck, 1957; Billingham *et al.*, 1958; Herzenberg and Herzenberg, 1961; Davies, 1962; Manson *et al.*, 1962; Basch and Stetson, 1963; Dumonde *et al.*, 1963; Hilgert *et al.*, 1964; Herberman and Stetson, 1965; Ozer and Wallach, 1967). However, due to different methods of preparation, assay system, and cell source, there is still some question concerning whether certain *H-2* specificities are located entirely on external membranes or on internal membranes as well (*cf.* review by Davies, 1968).

Since purification would be greatly aided if the antigen could be obtained in soluble form, many methods for solubilizing the membrane-located *H-2* antigens have been reported, including butanol extraction (Kandutsch, 1960; Manson *et al.*, 1964), nonionic detergents (Kandutsch, 1960), chelating agents (Edidin, 1966), ultrasound (Kahan, 1965), and lipases (Kandutsch, 1960). A certain very small proportion (about 1%) of histocompatibility activity has also been found in cell extracts in water-soluble form by Haughton (1965), a finding further borne out in our own work (Nathenson and Davies, 1966b).

In preliminary studies we described a procedure for solubilizing *H-2* alloantigens from spleen or tumor cell membranes by proteolytic digestion, using crude ficin (Nathenson and

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TABLE I: Alloantisera and Alloantigenic Specificities Detected.[a]

Antiserum	Possible *H-2* Alloantibodies	Target Cell	Possible *H-2* Alloantigen Specificities Detected
1. B10.A anti-Meth A; *H-2*^{b} anti-*H-2*^{d}	Anti-31	B10.D2 (*H-2*^{d})	31
2. C57BL/10 anti-Meth A; *H-2*^{b} anti-*H-2*^{d}	Anti-3,4,8,10,13,31	B10.BR (*H-2*^{k})	3, 8
3. (AKR × C57BL/6) F_1 anti-Meth A; (*H-2*^{k} × *H-2*^{b}) F_1 anti-*H-2*^{d}	Anti-4,10,13,31	B10.BR (*H-2*^{k})	4, 10, 13
4. B10.D2 anti-E.L. 4; *H-2*^{d} anti-*H-2*^{b}	Anti-2,5,22,23	B10.BR (*H-2*^{k})	5
5. (A.CA × B10.D2) F_1 anti-B10.A;[b] (*H-2*^{f} × *H-2*^{d}) F_1 anti-*H-2*^{a}	Anti-1,5,11,25	C57BL/10 (*H-2*^{b})	5
6. (BALB/C × C3H) F_1 anti-E.L. 4; (*H-2*^{d} × *H-2*^{k}) F_1 anti-*H-2*^{b}	Anti-2,22,33	H2H (*H-2*^{h})	2
7. (B10.A(5R) × DBA/2) F_1 anti-C57BL/10;[b] (*H-2*^{i} × *H-2*^{d}) F_1 anti-*H-2*^{b}	Anti-2	C57BL/10 (*H-2*^{b})	2
8. H-2G anti-E.L. 4;[c] *H-2*^{g} anti-*H-2*^{b}	Anti-33	C57BL/10 (*H-2*^{b})	33
9. (A.BY × WB/Rc) F_1 anti-A.SW;[d] (*H-2*^{b} × *H-2*^{w}) F_1 anti-*H-2*^{d}	Anti-1,3,19	B10.D2 (*H-2*^{d})	3
10. (C3H.SW × AKR.M) F_1 anti-B10.A;[d] (*H-2*^{b} × *H-2*^{m}) F_1 anti-*H-2*^{a}	Anti-4	B10.D2 (*H-2*^{d})	4
11. (A.CA × B10.BR) F_1 anti-A.SW;[d] (*H-2*^{f} × *H-2*^{k}) F_1 anti-*H-2*^{s}	Anti-3,19,28	C57BL/10 (*H-2*^{b})	28
12. (B10.A(2R) × DBA/1) F_1 anti-C57BL/10;[d] (*H-2*^{h} × *H-2*^{q}) F_1 anti-*H-2*^{b}	Anti-22,23	H.TG (*H-2*^{u})	22

[a] Column 1 contains the mouse strains used for immuniza tion and the source of tissue (spleen or tumor cells) used as the antigen. Column 2 contains the possible alloantibodies such an immunization could produce according to the published Table I (Snell *et al.*, 1964). Using the target cell listed in column 3, the p ossible detected specificities are listed in column 4, which for H-2.2, H-2.4, H-2.5, and H-2.33 are monospecific detection systems. Antiserum 2 could detect either H-2.3 or H-2.8 or both. Antiserum 3 could detect any one or a combination of the specificities H-2.4, H-2.10, H-2.13. Except for antiserums 6 and 9, the detected specificities must be of the *H-2* family since the mouse strain producing the antiserum and the target cell are congenic with respect to the *H-2* locus. [b] Gift of Dr. George Snell, Jackson Labs, Bar Harbor, Maine. [c] Absorption by liver of strain B10.BR. [d] Obtained from the *H-2* antisera bank maintained by the Transplantation Immunology Branch, National Institutes of Health, Bethesda, Md.

Davies, 1966a) or "autolysis" (Nathenson and Davies, 1966b). Though of a low yield, soluble fractions so obtained could be partially purified 30-fold by biochemical fractionation techniques. The material at this stage had the properties of glycoprotein without any detectable lipid.

In extending these studies, we found that both the autolytic and ficin digestion methods suffered from a considerable variation in reproducibility with respect to the strain of origin of the starting material (Nathenson, 1968). The desire to use as well-defined and reproducible a procedure as possible led us to investigate the use of crystalline papain as the solubilization agent (Nathenson and Shimada, 1968).

The present paper describes the technique whereby crystalline papain has been used to solubilize *H-2* alloantigens from spleen cell particulate preparations of two genetically defined mouse genotypes, *H-2*^{b} and *H-2*^{d}, and reports on the serological properties of such soluble digests. We further present a fractionation scheme that provides a 300- to 600-fold purification of some of the alloantigen-bearing fragments. These still retain biological activity with respect to antibody binding activity, the induction of cytotoxic antibody, and the acceleration of skin graft rejection. Some aspects of chemical composition of the purified preparations are given.

Materials and Methods

Mice. The following inbred mice, purchased from Jackson Laboratories, Bar Harbor, Maine, or obtained from the breeding colonies of Dr. Frank Lilly, Albert Einstein College of Medicine, were used: B.10A (*H-2*^{a}); C57BL/6, (*H-2*^{b}); DBA/2, BALB/C, B10.D2 new (H-2^d); H-2H (*H-2*^{h}); and AKR, B10.BR (*H-2*^{k}). Meth A (*H-2*^{d}) ascites tumor cells were maintained in BALB/C mice; E. L. 4 (*H-2*^{b}) tumor cells in C57BL/6 mice.

Alloantisera and Test System. Alloantigenic activity was detected and quantitated as described previously (Nathenson and Davies, 1966b) by the method of inhibition of immune cytolysis (Sanderson, 1964; Wigzell, 1965). This method provided reproducibility of ±10% for samples assayed in a single assay, but reproducibility was ±25% for samples tested in separate assays. When comparison between many fractions required assays on several different days, a standard antigen sample was included along with the unknowns and the measure of units was adjusted accordingly. For data in Tables III and IV assays were repeated at least once, and in most cases twice, and the mean of the determination was used. The alloantisera were either prepared according to Davies (1962), or

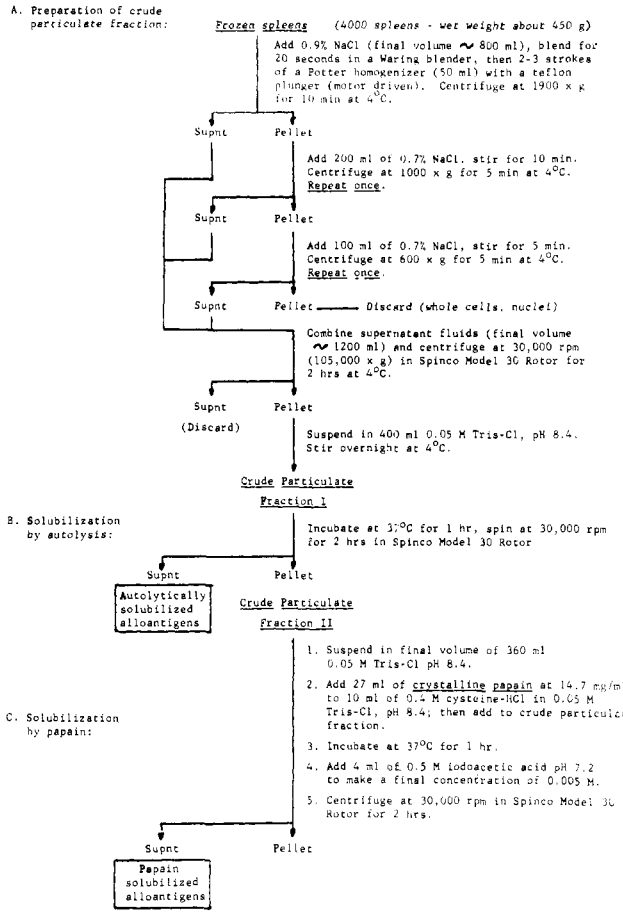

FIGURE 1: Flow scheme for preparation of soluble *H-2* alloantigens.

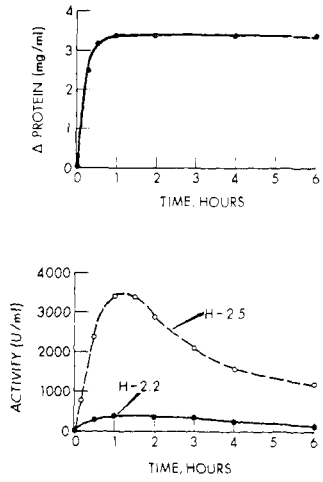

FIGURE 2: Time course of solubilization of *H-2*^{b} alloantigens by papain. Each time point represents supernatant activity of a tube which contained crude particulate fraction (after autolytic treatment) (9.0 mg), crystalline papain (0.3 mg), and cysteine (0.01 M–Tris-Cl, pH 8.4, 0.05 M) in 0.5-ml volume. Each tube was incubated for different times at 37° until the reaction was stopped by the addition of neutralized iodoacetic acid sufficient to make a final concentration of 0.02 M. Tubes were centrifuged in a Spinco 40.3 rotor at 40,000 rpm for 1 hr, and supernatant and pellet were separated. Supernatants were assayed for H-2.5 (○--○) and H-2.2 (●—●) (lower half of figure), and protein (upper half of figure). Amount of antigen present in crude particulate fraction: H-2.5 (15,000 U/ml) and H-2.2 (12,000 U/ml).

were gifts of Dr. George Snell, Jackson Laboratories, or were obtained from the *H-2* Antiserum Bank maintained by the Transplantation Immunology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health. They were used to detect alloantigenic specificities as described in Table I.

Chemical Methods and Materials. Total protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine plasma albumin as standard. Total phosphate was determined by the method of Lowry and Lopez (1946) with potassium monophosphate and disodium phenyl phosphate as standards. Absorbance in the ultraviolet range was measured in a Gilford Model 220 spectrophotometer. Total amino sugar was measured by a modification (Reissig *et al.*, 1955) of the Elson and Morgan method using glucosamine-HCl as standard after hydrolysis of samples at 100° for 4 hr in 4 N HCl in sealed glass tubes. Total carbohydrate was measured by a modification of the orcinol method (Francois *et al.*, 1962) with mannose as standard. Total sialic acid was measured by the micro method of Warren (1959) after hydrolysis of samples at 80° for 1 hr with a final concentration of 0.05 M H_2SO_4.

Before chemical analyses, every sample was dialyzed against two changes of a 2000-fold volume of distilled water and lyophilized. When sufficient material was available, the lyophilized samples were weighed and dissolved in distilled water.

Amino acid analyses (Spackman *et al.*, 1958; Hubbard, 1965) were carried out on a Beckman Model 120C amino acid analyzer equipped with the appropriate scale expansion card for the recorder so that 2×10^{-9} mole could be determined with ±3% precision. Samples of 50–100 μg of protein (Lowry *et al.*, 1951) were routinely analyzed after hydrolysis by 6 N HCl, *in vacuo* for 22 hr.

Papain (twice crystallized, and crude, type 1) was purchased from Sigma Chemical Co., St. Louis, Mo., and was used as described in the text. Sephadex, DEAE-, and CM-Sephadex were purchased from Pharmacia Fine Chemicals, Inc. All other reagents were analytical grade or better.

Results

The Crude Particulate Fraction of Alloantigens. A crude particulate fraction from spleen cells containing most of the alloantigenic activity was prepared by a modification of the method of Davies (1966). This procedure, outlined in the upper part of Figure 1, was utilized with 4000 frozen spleens from either C57BL/6 or DBA/2 mice. With smaller quantities of starting material a simple mincing of the spleens is carried out as the first step.

The crude particulate fraction of mouse spleen cells represents about 35% of the protein content of the original homogenate, yet contains about 90% of the alloantigenic activity of the original homogenate, thus providing approximately a 2.5-fold purification (*cf.* Tables III and IV).

Other tissues, such as lymph node, thymus, and liver, must be processed in a slightly different manner, especially with respect to the concentration of NaCl used to lyse the cells. Tumor cells also can be processed, but usually require treatment in distilled water to bring off the crude particulate fraction (Davies, 1966).

TABLE II: Distribution of *H-2* Alloantigenic Activity in Autolytic and Papain-Solubilized Fractions during Ammonium Sulfate Fractionation.[a]

	H-2[b] Alloantigens				H-2[d] Alloantigens			
	H-2.5		H-2.2		H-2.4,10,13		H-2.31	
Fractions	Units	%	Units	%	Units	%	Units	%
Autolytic								
0–50% $(NH_4)_2SO_4$	35,800	37.3	16,900	45.3	183,000	65	96,500	86.5
50–75% $(NH_4)_2SO_4$	61,100	62.7	20,400	54.7	93,300	35	13,800	13.5
Papain								
0–50% $(NH_4)_2SO_4$	50,000	6.7	21,600	9.4	44,000	1.5	11,900	4.1
50–75% $(NH_4)_2SO_4$	698,000	93.3	210,000	90.6	2,820,000	98.5	282,000	95.9

[a] Data taken from purification preparations used for Tables III and IV.

Solubilization of the Alloantigens. Two solubilization procedures were compared in our studies, *i.e.*, "autolysis" (Nathenson and Davies, 1966b) and papain digestion (Nathenson, 1968). We found that the latter method was superior to "autolysis" with respect to absolute yield of antigen and reproducibility, and we chose it as the best method for preparing large amounts of alloantigen from mouse strains for comparative serological and chemical studies. We also found differences in some of the properties of the products of these two methods with respect to solubility in ammonium sulfate and chromatography on Sephadex.

In order to examine the papain solubilization process in greater detail than reported previously (Nathenson, 1968), the release of alloantigen was studied with respect to time of digestion and enzyme concentration. In a time-course experiment (Figure 2) crude particulate membranes were incubated at 37° with papain (at an enzyme:crude particulate fraction ratio of 1:20). The reaction was stopped by the addition of iodoacetic acid and, after separation of soluble supernatant and insoluble pellet fractions by centrifugation at 105,000*g* for 1 hr, activities for several different *H-2*[b] specificities were examined. The H-2.5 (inhibitory) activity appearing in the supernatant rose to a maximum at about 1 hr (Figure 2) as did protein content. However, upon further incubation, H-2.5 activity progressively fell. This apparent destruction of alloantigenic activity has been noticed in all preparations so far tested in time course experiments, and most likely represents proteolytic breakdown of the alloantigen fragments after their release from the membrane site, or alteration of the fragments so that they no longer exhibit high antibody combining activity.

The H-2.2-reactive material solubilized by papain (Figure 2) was considerably lower in amount (2%) than H-2.5, for which about 25% of the activity on the membrane was solubilized. Testing with an anti-H-2.33 and an anti-H-2.6,14,27,28,29 antiserum showed that release of these activities was similar to H-2.5.

The features of solubilization by papain were examined further by testing the extent of solubilization with regard to enzyme concentration (Figure 3). Here a crude particulate fraction of *H-2*[b] origin was incubated with different amounts of activated crystalline papain for 2 hr. Up to about a 1:10 ratio of enzyme:crude particulate fraction, increasing amounts of enzyme gave increasing amounts of solubilized active material. However, greater enzyme concentration, in a manner similar to the longer incubation periods of Figure 2 gave lower yields of active material. Since at both higher enzyme concentrations and the longer time periods H-2.5 activity remaining in the crude particulate fractions also was lower than at the points of maximum solubilization (data not plotted), we can conclude that excess enzyme activity (either in amount or time allowed to act) destroyed the *H-2* alloantigenic activity not only in its soluble, but also in its membranous form.

Time-course experiments using "autolysis" for solubilization (*i.e.*, incubation of the crude particulate fraction at 37° in 0.05 M Tris-Cl buffer at pH 8.4) showed that maximum release of alloantigen was reached by 1 hr. No decrease in soluble activity occurred with further incubation for 8 hr. However, this method produced a relatively small amount of active material (*ca.* 5–10% of that released by the papain digestion method (*cf.* Tables III and IV) and gave variable results with material from different mouse strains.

Nonetheless, the products of autolysis were of considerable interest since some of their properties were different from those of the materials released by papain. As seen in Table II, less than 10% of the *H-2* alloantigenic activity released by papain precipitated in 50% saturated ammonium sulfate; on the

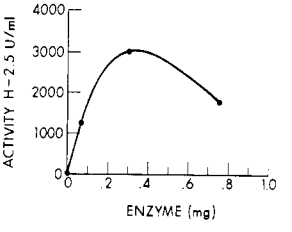

FIGURE 3: Solubilization of *H-2*[b] alloantigens by different concentrations of papain. Each point represents the supernatant activity of a tube with crude particulate fraction (after autolytic treatment) (9.0 mg), cysteine (0.01 M Tris-Cl, pH 8.4, 0.05 M), and papain in amounts indicated, in a final volume of 0.5 ml. After 2-hr incubation at 37°, iodoacetic acid (pH 8.4) was added to make a final concentration of 0.02 M. Activity in crude particulate fraction was 15,000 U/ml.

TABLE III: Purification of H-2^b Alloantigens.

Fractionation Stage	Protein (mg)	H-2.5 Activity				H-2.2 Activity			
		Units	Yield (%)	Sp Act. (units/mg)	Purifcn	Units	Yield (%)	Sp Act. (units/mg)	Purifcn
1. Extract	45,000	6,360,000	100	141	1	7,200,000	100	160	1
2. Crude particulate fraction									
Before autolysis	16,800	5,850,000	92	348	2.47	6,280,000	87.3	375	2.35
After autolysis	15,000	5,580,000	87.8	372		5,220,000	72.5	348	
After papain	10,300	3,360,000	52.8	326		3,520,000	48.8	342	
3. Autolytic supernatant	5,200	113,000	1.77	21.6		65,000	0.93	12.5	
4. Papain supernatant	5,310	1,010,000	15.9			288,000	4.00	54.2	
5. $(NH_4)_2SO_4$ (50–75%) fraction of papain supernatant	459	698,000	11.0	1,520	10.8	210,000	2.92	459	2.87
6. Sephadex G-150									
Pool I	146	595,000	9.36	4,080	29.0	61,500	0.85	421	2.63
Pool II	97	67,000	1.05	690	4.9	130,000	1.81	1,340	8.4
7. CM-Sephadex of G-150									
Pool I	57	560,000	8.8	9,750	69.2	55,000	0.76	957	6.0
8. DEAE-Sephadex									
Pool I	3.16	166,000	2.61	52,500	372	12,000	0.17	3,080	23.8
Pool II	2.92	133,900	2.09	45,000	319	14,800	0.21	5,060	31.7
9. Disc gel of DEAE-Sephadex pool I									
Fx 36	0.399	39,500		99,000	702	662		1,660	10.4
Fx 38	0.578	53,600	2.14	96,100	682	680	0.027	1,180	7.4
Fx 40	0.457	42,800		96,500	685	575		1,260	7.9

TABLE IV: Purification of $H\text{-}2^d$ Alloantigens.

Fractionation Stage	Protein (mg)	H-2.4,10,13 Activity				H-2.31 Activity			
		Units	Yield (%)	Sp Act. (units/mg)	Purifcn	Units	Yield (%)	Sp Act. (units/mg)	Purifcn
1. Extract	39,800	21,400,000	100	552	1	11,800,000	100	304	1
2. Crude particulate fraction									
Before autolysis	12,800	19,200,000	89.6	1,500	2.72	9,120,000	77.4	712	2.35
After autolysis	10,400	18,700,000	87.4	1,790	3.24	8,570,000	72.7	820	
After papain	7,150	4,120,000	19.2	576		6,310,000	53.5	882	
3. Autolytic supernatant	3,840	312,000	1.46	81		113,000	0.96	29.5	
4. Papain supernatant	4,560	2,970,000	13.9	652		307,000	2.60	67.4	
5. $(NH_4)_2SO_4$ (50–75%) fraction of papain supernatant	403	2,820,000	13.3	7,010	12.7	282,000	2.39	701	2.31
6. Sephadex G-150									
Pool I	154	2,740,000	12.8	17,700	32.1	254,000	2.15	1,650	5.44
Pool II	80	76,000	0.329	952		25,100	0.21	314	
7. CM-Sephadex of G-150									
Pool I	100	2,320,000	10.1	23,200	42.0	190,000	1.61	1,900	6.25
8. DEAE-Sephadex									
Pool I	6.5	980,000	4.25	148,000	268	32,000	0.27	4,920	16.2
Pool II	5.2	500,000	2.17	96,100	174	54,000	0.46	10,400	34.2
9. Disc gel of DEAE-Sephadex pool I									
Fx 36	0.415	74,300		179,000	324	3,380		8,140	26.8
Fx 38	0.548	104,000	1.21	190,000	344	4,680	0.09	8,360	27.5
Fx 40	0.441	81,000		184,000	334	2,730		6,200	20.4

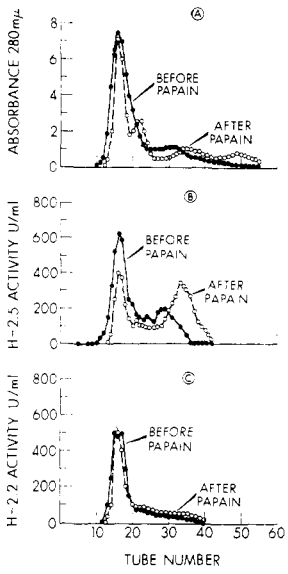

FIGURE 4: Demonstration of the effect of papain digestion on autolytically prepared *H-2*^{b} alloantigens. A preparation of *H-2*^{b} alloantigen prepared by autolytic digestion of crude particulate fraction was fractionated by addition of ammonium sulfate to 50% saturation. This precipitate was redissolved in 0.01 M Tris-Cl–0.15 M NaCl (pH 8.4) and an aliquot of approximately 50 mg (2.5 ml) was applied to a 92 × 1.5 cm column of Sephadex G-200 equilibrated with the above buffer; 50 mg of this material was also treated with crystalline papain (4.7 mg) for 2 hr at 37° in the Tris-Cl buffer containing 0.01 M cysteine. After addition of iodoacetic acid (final concentration 0.02 M) the preparation was dialyzed and applied to the column. The before papain and after papain patterns are plotted: (A) absorbance 280 mμ, (B) H-2.5 activity, and (C) H-2.2 activity.

other hand, in the autolytic supernatant, 65–87% of the activity of the specificities of the *H-2*^{d} genotype and 36–46% of the activity of the specificities of the *H-2*^{b} genotype were precipitated at that ammonium sulfate concentration.

Sephadex chromatography of autolytic and papain solubilized alloantigenic activity demonstrated clearly the differences between effective molecular size of these preparations. Papain-digested alloantigenic material clearly eluted in the included volume on Sephadex G-200 columns, whereas nearly 50–75% of autolytically prepared alloantigen was excluded from the gel.

We further found that this Sephadex-excluded material could also serve as a substrate for papain, and would be reduced in size by the treatment. Figure 4 shows the results of Sephadex G-200 chromatography of autolytic G-200-excluded material before and after digestion with papain. The 280-mμ absorbance diagram (Figure 4A) showed the appearance of two small peaks at tubes 35 and 50 (papain eluted at tube 50 on this column). The diagram of H-2.5 activity (Figure 4B) was most remarkable in revealing a striking difference brought about by papain: about half of the excluded activity was changed so that it eluted at tube 35 (roughly the position of bovine serum albumin). The effect of papain on H-2.2 activity (Figure 4C) was not at all as striking; however a small amount of activity was changed to a smaller fragment, but not nearly the amount of H-2.5.

Purification of Alloantigens. To follow the amount of *H-2* alloantigenic activity during purification, we chose two representative detection systems for each mouse strain. For *H-2*^{b} we chose to moniter H-2.2 and H-2.5, which, as will be shown, solubilize and purify as separate fragments. H-2.33 and H-2.28 which were also checked in some purification stages, fractionated with H-2.5. No other specificities which we checked purified with H-2.2.

For *H-2*^{d} alloantigens, we chose a system testing a set of specificities H-2.4, 10,13; and also an antiserum testing H-2.31. Spot checks of H-2.4 and H-2.3 showed a parallelism with H-2.4,10,13 during fractionation. We were unable to test all fractions with anti-H-2.4 and H-2.3 since these sera became available only after most of our studies were completed.

Tables III (*H-2*^{b}) and IV (*H-2*^{d}) list the steps of the entire process of solubilization and purification of *H-2* alloantigens from a typical run of 4000 spleens of the two mouse genotypes.

AUTOLYSIS. The crude particulate fraction was first subjected to autolysis which removed a considerable amount of trapped hemoglobin as well as a small amount of alloantigenic activity (the soluble autolytic fraction). This latter material, as already mentioned, had different molecular properties from the alloantigen solubilized by papain, and was removed for further studies.

PAPAIN DIGESTION. Papain digestion of the crude particulate fraction was carried out as outlined in the lowest part of Figure 1. A 1-hr incubation with 400 mg of papain was chosen for highest solubilization yield of *H-2*^{b} and *H-2*^{d} alloantigens although with other mouse strains this time may be different.

A consideration of *H-2*^{b} preparations (Table III) shows that H-2.5 activity was solubilized to an extent of about 18% of the activity present in the crude particulate fraction, while about 60% remained with the latter insoluble fraction; H-2.2, on the other hand, was solubilized to the extent of 5.5% with about 67% remaining in the crude membranes (Table III). H-2.33 and H-2.28 were solubilized in amounts similar to H-2.5, while H-2.22 was destroyed on the membranes and not solubilized at all.

The preparation of *H-2*^{d} alloantigens showed a similar solubilization picture, with some differences in yield. H-2.4,10,-13 activity was solubilized to the extent of 16%, with only 22% remaining in the crude membranes; on the other hand, only 3.6% of the H-2.31 activity was solubilized with nearly 74% remaining in the membranes (Table IV). Sera testing for H-2.3,8, H-2.3, and H-2.4 showed that these antigenic activities were similar in their solubilization yield to that of H-2.4-10,13.

AMMONIUM SULFATE FRACTIONATION. The papain digest supernatant fluids were next subjected to fractional ammonium sulfate precipitation. For the *H-2*^{b} isoantigens, the fraction precipitating at 75% saturation after removal of the fraction precipitated at 50% saturation contained about 70% of the activity for H-2.5 and H-2.2 with about a 7× purification (Table III). The 50–75% fraction for *H-2*^{d} alloantigens (Table IV) showed about 95% recovery of activity with about a 10× purification.

SEPHADEX COLUMN CHROMATOGRAPHY. Gel filtration of the ammonium sulfate purified alloantigens was the next purification step. *H-2*^{b} material chromatographed on a 130 × 5 cm column of G-150 Sephadex (Figure 5) separated into four reproducible protein peaks (absorbance 280 mμ), with the *H-2* activity eluting in the region of tubes 50–70. The H-2.5-reactive material formed a peak at tubes 59 and 60 with a slight shoulder. The H-2.2-reactive material showed a shoulder in

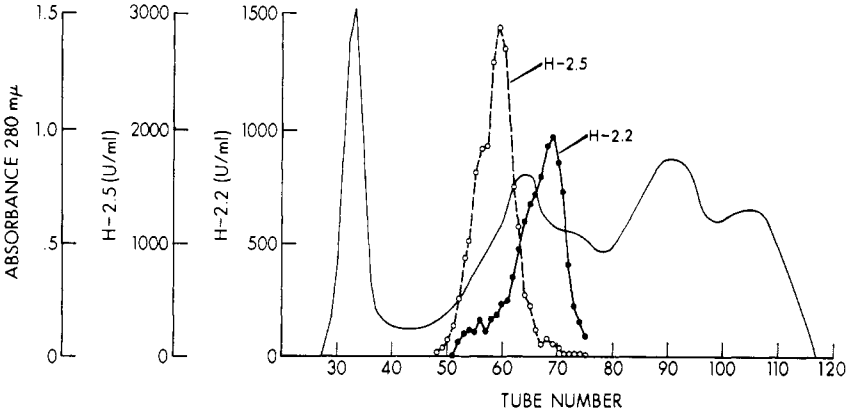

FIGURE 5: Sephadex G-150 chromatography of *H-2*^{b} alloantigens; 34.5 ml of ammonium sulfate fraction (50–75%) from a 4000 C57BL/6 spleen membrane papain digest was applied to a Sephadex G-150 column (130 × 5 cm) which had been equilibrated with, and was eluted with 0.01 M Tris-Cl–0.15 M NaCl (pH 8.4); 24.5 ml/tube was collected. Absorbance 280 mμ (—), *H-2* inhibitory activity for H-2.2 (●—●), and H-2.5 (○— —○) were determined on each tube.

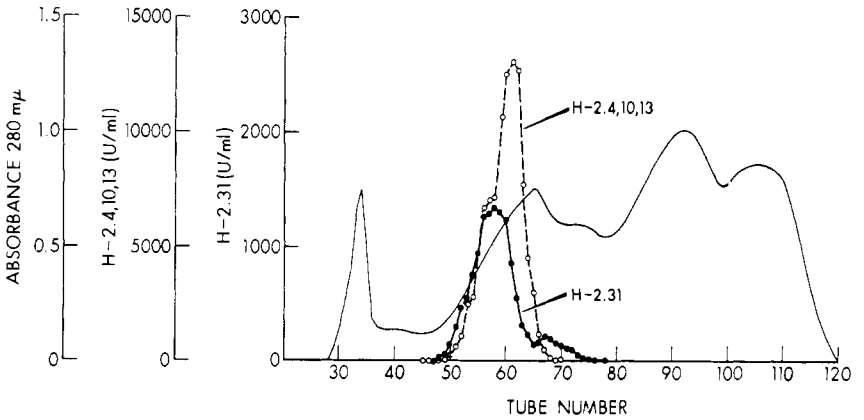

FIGURE 6: Sephadex G-150 column chromatography of *H-2*^{d} alloantigens. 34.5 ml of ammonium sulfate fraction (50–75%) from a 4000 DBA/2 spleen membrane papain digest were applied to a Sephadex G-150 column (130 × 5 cm) which had been equilibrated with and was eluted with 0.01 M Tris-Cl–0.15 M NaCl (pH 8.4); 24.5 ml/fraction was collected. Absorbance 280 mμ (—), *H-2* inhibitory activity for H-2,4,10,13 (○— —○) and H-2.31 (●—●) was measured on each tube.

common with the bulk of the H-2.5 material, but most of the H-2.2 material peaked at tubes 68–70, well separated from the H-2.5. This procedure, thus, clearly separated fragments carrying these two *H-2*^{b} specificities. H-2.33 and H-2.28 material eluted with inhibitory activity profiles identical with that of H-2.5. No specificities for which we tested chromatographed in a manner similar to H-2.2.

Sephadex G-150 chromatography of the *H-2*^{d} (Figure 6) alloantigen preparation showed a very similar protein profile (absorbance 280 mμ) to the *H-2*^{b} chromatogram. Analysis of the alloantigenic pattern showed a complex situation. H-2.4,-10,13-reactive material peaked at tubes 60–62, very similar to H-2.5 of the *H-2*^{b}, with a shoulder at tubes 56–58. H-2.31 material, peaked at tubes 58–60, clearly different than the major peak of H-2.4,10,13. Moreover, a very small H-2.31 peak in this particular column was seen at tubes 66–68. The amount of activity in this latter peak has been found to vary from preparation to preparation. Testing the fractions with antisera detecting H-2.3, H-2.4, and H-2.28 showed their pattern to be similar to that for H-2.4,10,13.

Pooled fractions (tubes 48–65) from the G-150 columns, when tested for specific activity (H-2.5 and H-2.4,10,13), showed a threefold purification over the previous stage.

CM-Sephadex Chromatography. The next purification stage was CM-Sephadex C-50 chromatography. The Sephadex G-150 column fractions were pooled into two fractions: 48–65 (I) and 66–76 (II). Each pooled fraction was separately subjected to CM-Sephadex chromatography. This paper describes the further purification of fraction I from both *H-2*^{b} and *H-2*^{d} alloantigens. The complete purification of the number II fractions forms the second paper in this series (K. Yamane and S. G. Nathenson, in preparation).

The CM-Sephadex chromatography was performed on columns (2.5 × 30 cm) equilibrated with 0.01 M sodium phosphate (pH 6.25). Fractions of 10 ml were collected after development of the column with the same buffer. At fraction 20, a linear gradient with respect to NaCl was begun, reaching a concentration of 0.3 M NaCl by tube 80. *H-2* alloantigenic activity was not retained by the column, but appeared in fractions 5–10, with a purification generally of about twofold, and recovery of about 95%. Essentially all of the reddish brown color of the fraction applied to the column was held at the top 1 cm of the column.

DEAE-Sephadex Column Chromatography. DEAE-Sephadex chromatography was used as the next purification stage (Table III and IV; Figures 7 and 8). The chromatography, performed as described under the figures, showed a protein (absorbance 220 mμ) pattern of three peaks. For the *H-2*^{b} alloantigens (Figure 7), H-2.5-reactive material eluted in the second of these peaks, *i.e.*, in tubes 32–52 with a peak at 38, while the small amount of H-2.2 material remaining at this stage eluted with a peak at tube 42. H-2.33 gave a very similar inhibitory activity profile (not plotted) to the H-2.5 profile.

For the *H-2*^{d} alloantigenic material, the protein (absorbance 220 mμ) profile was similar to that for *H-2*^{b} alloantigens. The second peak, that containing the H-2.4,10,13 antigenic activity, eluted in tubes 38–52, with a peak at tube 43. H-2.31 activity was spread from tubes 40 to 70.

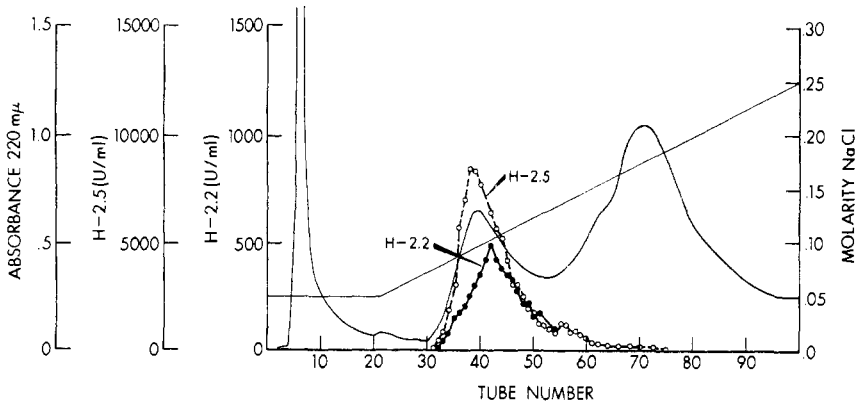

FIGURE 7: Column chromatographic separation of *H-2*^{b} alloantigens on DEAE-Sephadex. The pooled fraction from the CM-Sephadex column of material from 4000 spleens in 3.45 ml was applied to a 2.5 × 29 cm DEAE-Sephadex A-25 column equilibrated with Tris-Cl buffer (0.01 M)-NaCl (0.05 M, pH 8.4) and eluted with Tris buffer (0.01 M, pH 8.4)-0.05 M NaCl for fractions 1–20 at which time a linear gradient was begun which reached 0.27 M NaCl at fraction 108; 10.5 ml/fraction was collected. Elution rate was 24 ml/hr. Tubes were monitered for $A_{220\ m\mu}$ (—), and H-2.5 (O— —O), and H-2.2 (●—●) antigenic inhibitory activity.

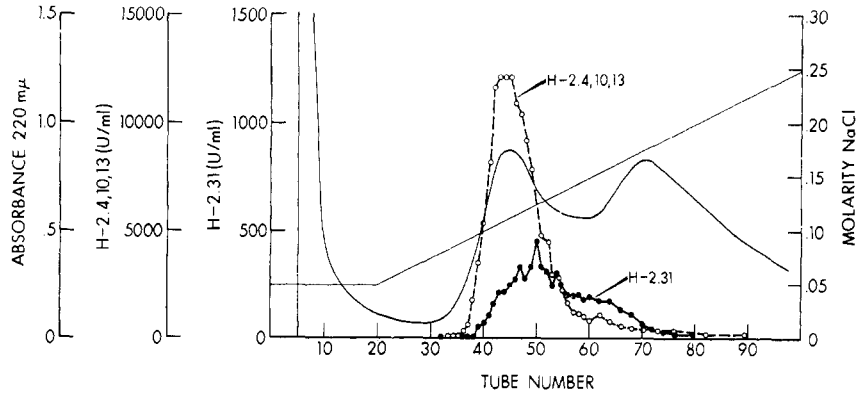

FIGURE 8: Column chromatographic separation of *H-2*^{d} isoantigens on DEAE-Sephadex. Conditions of chromatography were identical with those for Figure 7. CM-Sephadex-purified material from 4000 DBA/2 spleens was applied to the column. $A_{220\ m\mu}$ (—), H-2.4,10,13 (O— —O), and H-2.31 (●—●) were determined for tubes shown.

TABLE V: Chemical Analyses of Purified Alloantigen Fractions.

Fraction	Percentage of Dry Weight[a]				
	Protein[c]	Neutral Carbohydrate	Hexosamine	Sialic Acid	Phosphate
DEAE pool 1[a] (*H-2*^{b})	89.1	8.85	4.44	2.82	0.081
Disc gel[b] fractions (*H-2*^{b})					
Fx 38	90	5.36	4.43	1.25	*d*
Fx 40	90	5.41	4.36	1.32	
Disc gel[b] fractions (*H-2*^{d})					
Fx 36	90	3.08	2.88	0.930	
Fx 38	90	3.51	2.90	1.050	
Fx 40	90	5.14	3.10	0.910	

[a] Dry weight was determined on two separate samples after extensive dialysis against distilled water and removal of free water by lyophilization. Range for samples was ±3.5%. All other determinations were made at least in duplicate and related to dry weight. [b] Dry weight could not be determined on these samples due to small amounts available. To allow comparison we assumed a 90% value for the protein content of the dry weight and then related other determinations accordingly. [c] Protein content was estimated by the procedure of Lowry *et al.* (1951), and is expressed essentially, as the milligram equivalent of bovine plasma albumin. Since bovine plasma albumin and the *H-2* alloantigen glycoproteins have similar tyrosine content, and tyrosine is a major factor in the Lowry procedure, we can presume that our data are reasonably accurate. However, since we do not know the tryptophan content of the *H-2* alloantigen glycoproteins, and tryptophan also is a factor in the Lowry procedure, we cannot claim that our protein values reflect the true values. [d] Not determined.

Fractions from the columns were pooled into two fractions: fraction 1 contained tubes 35–47, and fraction 2, tubes 48–58. For H-2.5 the pooled fractions represented a 56% recovery of activity applied to the DEAE-Sephadex column with a fivefold purification for fraction 1. For H-2.2 a similar purification and recovery figure was seen; however most activity was recovered in the DEAE-2 rather than in DEAE-1 fraction. As mentioned above, H-2.2 was purified by a separate procedure which is reported elsewhere (K. Yamane and S. G. Nathenson, in preparation).

The situation for *H-2*^{d} alloantigens was similar, with a 64% recovery and sixfold purification for H-2.4,10,13 in the DEAE-1 fraction. H-2.31 likewise showed a sixfold purification but, like H-2.2, was found mostly in the DEAE-2 fraction.

Disc Gel Electrophoresis. Discontinuous electrophoresis on 7.5% polyacrylamide gels was the next step in the fractionation scheme. These gels were prepared and run according to the method of Davis (1964). For analytic runs, approximately 0.1–0.5 mg of protein was used per (0.6 × 10 cm) gel and, after the bromophenol blue marker reached the end of the gel, the latter was either stained with 0.25% coomassie blue or sliced into 2-mm fractions which were eluted with 0.15 M NaCl–0.01 M Tris-Cl (pH 8.4) buffer by extracting each slice three times overnight with shaking with 0.3–0.5 ml of buffer. The eluted material for each slice was then tested for protein and *H-2* alloantigenic activity. Preparative runs were made on 0.95 × 10 cm gels on which 2–3 mg of material was run.

A photograph of a five analytically stained electropherograms is shown in Figure 9 where all four of the pooled DEAE fractions and the DEAE starting material from *H-2*^{b} alloantigens were electrophoresed. Two heavy major bands and one minor band were seen on the gel on which DEAE fraction 1 was placed. Testing a comparable polyacrylamide gel which was sliced and extracted, the H-2.5 and H-2.33 activities were found to electrophorese in the region of the two major bands (R_F 0.38 and 0.40; R_F = migration of measured material/migration distance of the bromophenol blue marker) and one minor band (R_F 0.36). The recovery of activity in the bands with R_F 0.36, 0.38, and 0.40 represented about 80% of the activity applied to the preparative gel with nearly a twofold purification (Table III). The H-2.2 activity recovered in these fractions represented less than 20% of that recovered from the gel, since the majority of H-2.2 ran with an R_F of about 0.46.

The DEAE-1 fraction from *H-2*^{d} alloantigens when examined by disc gel electrophoresis showed a similar pattern, but the bands were not quite so clean. Here, the *H-2* activity was more spread out, but about 25% of the H-2.4,10,13-reactive material was recovered in three gel cuts (R_F 0.36, 0.38, and 0.40) with a purification of about a 1.3-fold.

Evidence for Homogeneity. The disc gel fractions (R_F 0.36, 0.38, and 0.40) of *H-2*^{b} and *H-2*^{d} strains were examined by re-electrophoresis at pH 9.3 (the original conditions) and in sodium dodecyl sulfate, phosphate at pH 7.1 (Shapiro *et al.*, 1967). Electrophoresis under the original conditions showed a single protein staining band with an R_F of the original band. The *H-2* activity was confined to the 2-mm band which stained for protein.

In the sodium dodecyl sulfate gels, the *H-2*^{b} fractions ran as a single protein-staining band (Figure 10), however *H-2*^{d} fractions had a minor (10%) component in addition to the major band of the same R_F as the *H-2*^{b} band. Low pH (pH 4.3) disc gel electrophoresis of *H-2*^{b} material showed a single component; and for *H-2*^{d} material showed a major band, running with the same R_F as the *H-2*^{b} fractions, and a faster running minor band (about 10% as intense). *H-2* activity could not be measured in the sodium dodecyl sulfate gels or low pH gels since the antigen activity is irreversibly lost at concentrations greater than 0.01% sodium dodecyl sulfate, or at a pH as low as pH 4.3.

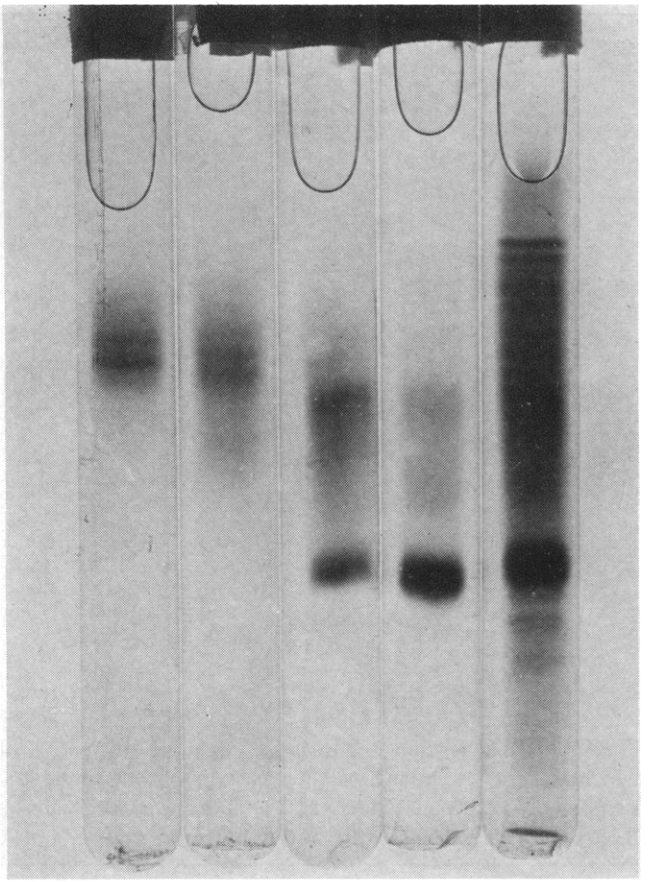

FIGURE 9: Analytic disc gel electropherograms of *H-2*^{b} alloantigen DEAE fractions. Of the five gels shown the first four from left to right represent material pooled from the following tubes of the DEAE-Sephadex column of Figure 7: DEAE-1 (35–47), DEAE-2 (48–58), DEAE-3 (59–78), and DEAE-4 (79–100). The starting material applied to the DEAE-Sephadex column is run in the gel on the far right. The bottom of the photo is the positive pole. The 0.6 × 10 cm gels were run according to the method of Davis (1964) until the bromophenol blue reached the end of the gel. Staining is with coomasie blue 0.25%.

General Chemical Composition. We examined the general composition of the DEAE-1 pool fractions and also of the polyacrylamide electrophoretic fractions (Table V). Dry weight determinations were carried out on two preparations of *H-2*^{b} DEAE pool I. When expressed as percentage of dry weight, the protein content was 89.1, neutral carbohydrate 8.85%, hexosamine 4.44%, sialic acid 2.82%, and organic phosphate 0.081%. The insignificant phosphorus content suggests that phospholipid is absent. One sample was extracted with chloroform–methanol and the concentrated extract was subjected to thin-layer chromatography. No lipid was detected with iodine vapor. This procedure would detect at least 5% by weight.

The polyacrylamide disc gel electrophoretic fractions were

TABLE VI: Amino Acid Composition of *H-2* Alloantigen Disc Gel Fractions.[a]

	H-2^b			H-2^d		
	Disc Gel Fractions			Disc Gel Fractions		
Amino Acid	36	38	40	36	38	40
Lys	4.42	4.38	4.78	4.48	5.24	4.69
His	2.89	3.10	3.00	2.72	2.80	2.80
Arg	4.75	4.85	4.85	6.08	5.63	5.95
Asp	9.66	9.53	9.28	9.80	9.60	9.68
Thr	7.46	7.28	7.40	7.11	7.28	7.23
Ser	6.74	6.43	6.24	6.18	5.65	5.76
Glu	12.71	12.68	12.56	13.91	13.67	13.47
Pro	6.88	6.84	7.08	6.63	6.67	6.42
Gly	7.28	7.09	7.13	7.21	7.25	7.32
Ala	7.02	7.02	7.00	7.01	7.20	7.27
Half-Cys	1.75	2.54	2.46	2.74	1.96	2.54
Val	5.78	5.59	6.06	4.96	5.10	5.22
Met	2.10	2.12	2.08	2.14	2.14	2.06
Ile	3.77	3.76	3.73	3.19	3.15	3.26
Leu	8.43	8.31	8.17	7.53	7.62	7.58
Tyr	4.94	5.09	4.96	4.71	4.72	4.40
Phe	3.42	3.66	3.54	3.59	4.14	4.32

[a] Amino acid residues are expressed as moles/100 moles of amino acids recovered after acid hydrolysis, except tryptophan, which was not determined. All disc gel analyses were single consecutive runs on the analyzer and represent only one 22-hr hydrolysis point. A Beckman Model 120C analyzer was used with increased sensitivity capability as described under Methods section. Recovery of all residues except tryptophan represented about 70–72% of average protein value before hydrolysis.

also examined for their general chemical composition. Since dry weight determinations could not be carried out, for comparative purposes we assumed that the protein consisted of 90% of the dry weight. *H-2^b* disc gel material contained about 5% neutral carbohydrate and 4.4% hexosamine, and *H-2^d* material contained carbohydrate in amounts ranging from 3 to 5% and hexosamine in amounts of about 3%. Sialic acid was about 1% in both strains. The hexosamine was presumably only glucosamine, since in the amino acid analysis runs, only the glucosamine peak was seen. The major neutral carbohydrate components were galactose and mannose. A complete study of the composition and structure of the carbohydrate is presently in progress.

Amino acid analyses were performed on the purified disc gel fractions from the *H-2^b* and *H-2^d* materials (Table VI). There is very little difference in the whole composition of the two strains. However, there are reproducible differences of about 1 mole % in the content of arginine and in the content of glutamic acid between the two strains.

Preliminary Estimate of the Molecular Weight of H-2 Alloantigens. Precise physical parameters of the purified alloantigens are presently being studied; however, an estimate of the molecular weight can be made from an analysis of the Sephadex elution behavior for the *H-2* alloantigen activities. In Table VII the gel filtration data are presented with the K_D (distribution coefficient) calculated from the peak tube for the particular alloantigen activity measured. The molecular weight for these alloantigens can be estimated by the method of Andrews (1964) giving values for H-2.5, H-2.4,10,13, and H-2.31 (large) of around 65,000–75,000; for H-2.2 and H-2.31 (small) of around 40,000. While such values are subject to revision when data from studies of sedimentation rate, diffusion coefficient and partial specific volume are available, they do provide some idea of the range of size to expect. The values do agree, furthermore, with molecular weight calculations of 66,000 ± 5,000 determined from sucrose gradient sedimentation velocity runs performed by the method of Martin and Ames (1961) (Figure 11).

Specificity and Biological Activity of Papain-Solubilized Alloantigens. The proper specificity of the papain-solubilized alloantigen preparations was established by testing the samples at several steps in the fractionation procedure. For example, *H-2^d* material after Sephadex G-150 chromatography containing 46,000 U/ml of H-2.4,10,13 alloantigenic reactivity was tested for and found not reactive for the following *H-2^b* specificities: H-2.5, H-2.2, and H-2.33. It did react for H-2.28, a specificity which it shares with *H-2^b* material. Pooled material after DEAE-Sephadex chromatography from *H-2^b* origin was also checked for and found lacking of the following *H-2^d* specificities: H-2.3,8, H-2.4,10,13, and H-2.31. It did contain H-2.28 which it shares with *H-2^b* alloantigens.

The capacity of the *H-2* alloantigen fragments from C57BL/6 to cause immunity to subsequent skin grafts has been tested elsewhere (D. L. Mann and S. G. Nathenson, in preparation). These results show that papain-solubilized alloantigen preparations from C57BL/6 mice injected either in Freunds' com-

TABLE VII: Gel Filtration Data and Estimate of Molecular Weight of *H-2* Alloantigens.[a]

	Distribution Coefficient, K_D	Mol Wt[b]
Alloantigen		
H-2.5	0.393	65,000 ± 3,000
H-2.2	0.515	39,000 ± 2,000
H-2.4,10,13	0.393	65,000 ± 3,000
H-2.31 (large)	0.357	76,000 ± 4,000
H-2.31 (small)	0.507	40,000 ± 2,000
Marker Proteins		
γ-Globulin (human)	0.179	160,000
Albumin (bovine)	0.364	67,000
Ovalbumin	0.487	45,000
Myoglobin (sperm whale)	0.707	17,800

[a] The G-150 Sephadex column (130 × 5 cm) was calibrated for V_0 and V_i using Blue Dextran (Pharmacia Inc.) and $Na_2Cr^{51}O_4$. K_D values were determined for marker proteins (Mann Research Labs, N. Y.) and for peak tubes of *H-2* alloantigen reactive material as indicated. Molecular weight estimates for *H-2* alloantigen glycoproteins were calculated by the method of Andrews (1964). [b] The range values reflect differences of ±1 fraction for the chromatography.

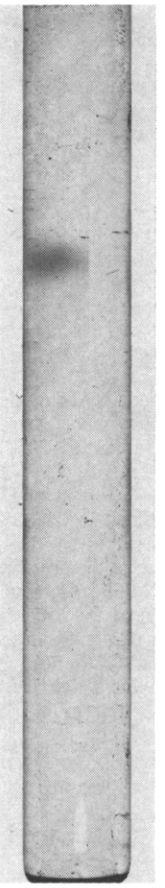

FIGURE 10: Electrophoresis of disc gel purified alloantigen on sodium dodecyl sulfate polyacrylamide. Fraction 38 eluted from preparative polyacrylamide electrophoresis of DEAE-1 was run on a 7.5% polyacrylamide gel in sodium dodecyl sulfate–phosphate buffer (Shapiro *et al.*, 1967). Positive pole is at the bottom of the photo.

plete adjuvant, or subcutaneously (9 or 18 doses of 1–10 μg) caused the acceleration of rejection of C57BL/6 skin grafts on B10.D2 new recipients (significant at the <0.05 levels).

Discussion

The purpose of this paper is the presentation of a method for solubilizing *H-2* alloantigens from spleen cell membranes, a method for the purification of such products, and preliminary results on their chemical and physical properties.

The first solubilization procedure which we applied to the crude membrane fraction was "autolysis." The product from this procedure contained about 2% of all of the tested specificities found in the $H\text{-}2^d$ and $H\text{-}2^b$ alloantigen membrane fraction, and perhaps may bear some similarity to preparations obtained by sonication (Kahan, 1965) and by buffer extraction (Haughton, 1964).

The autolytically solubilized material, however, was complex; and was separable into two major fractions by Sephadex G-200 chromatography: one excluded from the gel, and one included in the gel. The Sephadex G-200 excluded fraction had a molecular weight of the order of 1,000,000 as judged by Sepharose 4-B chromatography (unpublished observations) and could be digested with papain (Figure 4) to release H-2.5 alloantigenic fragments with an elution volume identical with that of alloantigen fragments released by papain digestion of the membranes directly (molecular weight of approximately 66,000). The second fraction found in the autolytic digest had a chromatographic behavior similar to the papain digest H-2.5 fragment even though papain had never been used.

The major solubilization procedure was performed by digestion of the membrane fraction with crystalline papain. We found that different specificities determined by the same genotype, were, in fact, solubilized with different efficiencies, a point to which we have previously called attention (Shimada and Nathenson, 1967). For the $H\text{-}2^b$ alloantigens, papain solubilized about 18% of H-2.5, while only 5% of H-2.2 activity was recovered in soluble form. This is remarkably different from the autolytic process where both specificities were solubilized in equivalent amounts. For the $H\text{-}2^d$ alloantigens, there was a similar situation in which specificities detected by anti-H-2.4,10,13 (and H-2.3,8, and H-2.4) were solubilized in relatively high efficiency as compared with the H-2.31 activity.

Redigestion of the crude particulate fraction with crystalline papain removed an additional 5% of the alloantigen H-2.4,-10,13, 2–3% of H-2.31; 10% of H-2.5, and 3% of H-2.2. We did not use this second digestion product in these studies since we wished to have as simple a product as possible. With the redigestion procedures, the total yield of active soluble antigen H-2.4,10,13 and H-2.5 was of the order of 25–30%; however, no activity remained in the crude membranes after the final digestion. Therefore, in no case were we ever able to prevent destruction of 70–75% of the antigenic activity–findings which fit with the demonstrated lability of the alloantigens to

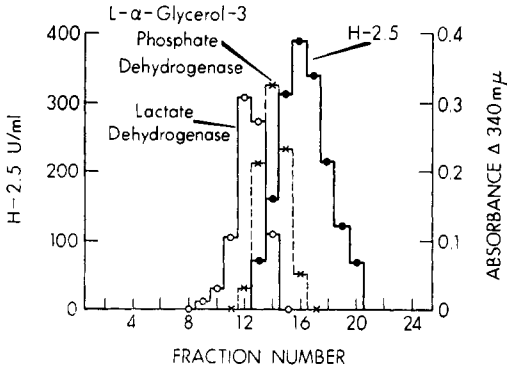

FIGURE 11: Sucrose gradient centifugation determination of molecular weight of *H-2*^{b} alloantigens. These experiments were carried out in a sucrose density gradient (5–20%) using a SW-39 rotor and a Spinco Model L ultracentrifuge. *H-2*^{b} alloantigens purified on DEAE-Sephadex (100 μl, 460 units of H-2.5), L-α-glycerol 3-phosphate dehydrogenase (mol wt = 78,000 ± 11,000, 50 μl, 20 μg), and lactate dehydrogenase (mol wt = 140,000 ± 15,000, 50 μl, 20 μg) were layered on the surface of the gradient, and the rotor was centrifuged at 4° for 16 hr at 38,000 rpm. Fractions (about 0.2 ml) were collected from the bottom of the tube, and the activities of H-2.5, L-α-glycerol 3-phosphate dehydrogenase (Beisenherz *et al.*, 1955), and lactate dehydrogenase (Neilands, 1955) were determined for each fraction. The molecular weight of the material having H-2.5 alloantigenic activity was determined by the method of Martin and Ames (1961).

proteolytic enzymes in general, and lability even to papain in excessive amount or digestion time.

There was a similarity of H-2.31 activity of the *H-2*^{d} genotype of H-2.2 activity of the *H-2*^{b} genotype with respect to solubilization efficiency. However, H-2.31 was solubilized as a fragment eluting on the G-150 Sephadex column earlier than the main region of H-2.4,10,13 and H-2.3,8 (Figure 6), whereas the H-2.2 major peak, eluted much later (Figure 5). On the other hand, as we reported earlier (Shimada and Nathenson, 1967), H-2.31 activity can also be found as the small form chromatographically similar to H-2.2 if in the original solubilization procedure, crude rather than crystalline papain were used. Further, if the large form were isolated and then digested again with crystalline papain at a higher concentration than originally, we found that the smaller fragment was formed. Thus, the H-2.31-carrying fragment is similar, but not identical with H-2.2-carrying fragment. It is clearly different from the fragment or fragments carrying H-2.4,10,13 and H-2.3,8.

The final yield of active alloantigen units for H-2.5 and H-2.4,10,13 from the first papain digest was of the same order of magnitude; that is, around 1–2% of the activity in the crude homogenate was recovered in the disc gel fractions. The greatest loss of activity was in the solubilization step, because thereafter each of the fractionation steps was 50–90% efficient.

The purification factor of about 350-fold for H-2.4,10,13 and 700-fold for H-2.5 allows one to estimate that these antigens comprise less than 1% of the protein of the cell membrane of an average cell. Adjusting for losses during solubilization and purification, the total amount of antigen in the average mouse spleen (average 10–20 mg of membrane protein dry weight) is of the order of 10 μg.

The disc gel electrophoretic pattern revealed that the purified alloantigen-bearing fragments from DEAE-Sephadex fractions consisted of several separate molecules. Each of these bands, upon reelectrophoresis at low pH, sodium dodecyl sulfate, and in urea, appeared homogeneous; however, rather than each band reacting for a different specificity, the separate bands carried most of the specificities tested for. For example, from *H-2*^{b} the fractions 36, 38, and 40 carried H-2.5, H-2.33, and H-2.28. For the *H-2*^{d} preparations, fractions 36, 38, and 40 carried H-2.4,10,13 and H-2.3,8. While a more detailed serological analysis is in preparation, the present data suggest that the molecular fragment forming each of the gel electrophoretic bands carries several specificities.

Additional evidence for this conclusion comes from studies with [^{14}C]leucine-labeled alloantigens (Cullen and Nathenson, 1969) which demonstrate that antibody against H-2.4,10,13 complexes alloantigen containing not only H-2.4,10,13 but also H-2.3,8; whereas H-2.31 remains uncomplexed. The H-2.3,8 are therefore on the same glycoprotein as H-2.4,10,13.

We have found that the papain digestion technique released two classes of water-soluble alloantigen fragments from each strain which we examined. One class of fragments (class I) had an approximate molecular weight of 66,000 and carried many of the alloantigenic specificities found in that strain (*e.g.*, H-2.5, H-2.33, and H-2.28 for *H-2*^{b} and H-2.3,8, H-2.4,10,13 for *H-2*^{d}). This was the major alloantigen fragment, and the one for which we have presented the data on purification and preliminary characterization. From each strain, however, a "minor" fragment (class II) was also found of smaller molecular size (about 30,000 molecular weight) and carrying only one of the specificities of that genotype (*e.g.*, H-2.2 for *H-2*^{b} and H-2.31 for *H-2*^{d}). Purification and characterization of this latter fragment is the subject of another paper (K. Yamane and S. G. Nathenson, in preparation).

By our methods we cannot directly determine the nature of the *in situ H-2* macromolecule or macromolecules from which these fragments are derived. It is possible for example, that all specificities are located on a single molecule, which by the action of papain is split into fragments of two sizes, one fragment carrying several specificities (class I), the other carrying only one specificity (class II). Or it is possible that each of these fragments comes from a separate but larger *in situ* molecule. We do know, for example, as mentioned before (*cf.* Figure 4), that there exists a form of the *H-2* alloantigen of very high molecular weight (of the order of 10^6) which can be released from membranes by "autolysis" and which can be degraded by papain to yield the 66,000 molecular weight glycoprotein fragments. Such molecules may represent the *in situ* macromolecules.

While our procedure most probably breaks up the *in situ* form of the *H-2* alloantigen, the availability of the resulting glycoprotein fragments carrying the antigenic sites for the *H-2* specificities allows us to approach the chemical definition of these antigenic properties. Although the measurement of dry weight on small amounts of material (which have not been subjected to extensive procedures to remove last traces of water) can be in error by 10–20%, we can still conclude that the major alloantigen fragments (class I) are glycoprotein, with the polypeptide portion comprising the major fraction (80–90%). The carbohydrate analyses (subject to some uncertainty due to lack of knowledge of the particular components present) show that there are small but significant differences between the alloantigen fragments of the *H-2*^{b} and *H-2*^{d} genotypes. The comparative amino acid analyses, further, point to

additional small but reproducible differences in content between the *H-2*^{b} and *H-2*^{d} material, for example, in the content of arginine and glutamic acid.

Over-all, the analyses of the glycoprotein fragments from the two *H-2* genotypes are quite similar. Such a finding might be expected, since these products are determined by different alleles of the same genetic locus, the *H-2* locus. Thus the chemical properties responsible for their antigenic identity might be expected to be subtle variations on a basic pattern, such as minor differences in polypeptide (*e.g.*, amino acid substitutions) or carbohydrate fine structure. For example, the allelic antigenic products of the Inv locus of the κ chain are identical except for a single amino acid interchange (Baglioni *et al.*, 1966; Terry *et al.*, 1969).

The chemical analyses of the murine alloantigens present a thought provoking contrast to the chemical character of alloantigens isolated from guinea pig tissues by the method of sonication (Kahan and Reisfeld, 1968). Of course, the methods of extraction, purification, and assay are different. However, it is quite interesting that the guinea pig preparations apparently lacked hexosamine which was tested for as part of the regular amino acid analysis. No direct hexosamine determinations were done, nor were quantitative assays for neutral carbohydrate. More striking was the finding that preparations from two histoincompatible guinea pig strains were very different in amino acid composition. It is pertinent, however, that the histocompatibility systems of the guinea pig are probably as complicated as in the mouse where so far 13 different histocompatibility genes have been documented. Therefore, since the histocompatibility systems of the guinea pig have not been genetically clarified, we do not know whether the products of the two strains examined are the result of single or multiple gene differences. Hence it is difficult to assess the meaning of the amino acid analytic data.

We feel that the products we have compared in our studies represent to a reasonable degree true allelic alloantigenic products of the *H-2* locus. The assay system, for example, utilizes antibody–target cell combinations which only detect *H-2* specificities, assuring that during purification, only the *H-2* alloantigens are being enriched. Furthermore, the papain solubilization procedure, which we know destroys even some of the *H-2* alloantigenic activity, also has been found to completely destroy products of the *H-7* locus (Summerell and Davies, 1968), and may destroy others. And finally, our purification procedure probably removes all non-*H-2* products which have been solubilized. For example, we (Lilly and Nathenson, 1967) previously found that the papain-solubilized product of the *H-6* locus was easily separated from the *H-2* products by Sephadex chromatography, and Davies *et al.* (1967) found that even the product of a gene closely linked to *H-2*, the TL antigen, was separable from *H-2* alloantigens by DEAE-cellulose chromatography.

As we have stated, our major interest behind the development of the techniques of solubilization and purification of *H-2* alloantigens reported herein has been our desire to establish the molecular correlate of the *H-2* alloantigenic specificities. However, a very important practical outcome of such information on transplantation antigens would be the potential usefulness for studies on the mechanism of tissue rejection, particularly with respect to altering graft rejection in man in order to permit practical, long-term allotransplant survival.

It has been particularly exciting, therefore, to find that our preparative methods can be transferred from the mouse system to other species. In addition to demonstrating that our solubilization methods are effective for guinea pigs (J. Halpern, B. R. Bloom, and S. G. Nathenson, unpublished observation), we have found that the process of papain digestion is also effective for solubilizing human HLA alloantigens (Mann *et al.*, 1968, 1969). The chemical and physical properties of the human and mouse alloantigens are, furthermore, quite similar.

We hope that the wide applicability of our method of solubilization and purification will permit the comparative examination between many species of the molecular and biological properties of these membrane located cell products.

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