H-2 Histocompatibility Alloantigens. Some Biochemical Properties of the Molecules Solubilized by NP-40 Detergent†

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ABSTRACT: The nonionic detergent, NP-40, solubilizes *H-2* alloantigens in a form which can be isolated by indirect immune precipitation, and which most probably reflects the native form of the antigen. *H-2* alloantigen glycoproteins were isolated by indirect precipitation from cells grown in the presence of labeled amino acid or monosaccharide precursors. A single peak of specific *H-2* activity was found during fractionation by molecular sieve chromatography on Bio-Gel 15m in 0.5% NP-40. This peak represented an aggregate with an elution behavior of a protein of molecular size of 380,000. Analysis of the molecular weight of the *H-2* alloantigens after disruption of this aggregate with sodium dodecyl sulfate and 2-mercaptoethanol showed a molecular size of 43,000 for the $H\text{-}2D^d$ product and 47,000 for the $H\text{-}2K^d$ product as measured by electrophoresis on polyacrylamide–sodium dodecyl sulfate gels or Sephadex chromatography in sodium dodecyl sulfate. If disruption was carried out without 2-mercaptoethanol, two forms of the antigen were recovered, the larger (tentatively called a "dimer") having a molecular size twice that of the smaller (tentatively called a "monomer"), which was identical with the size found with reduction. The *H-2* antigen fragments solubilized by papain digestion rather than by detergent have a molecular weight of about 39,000 for the $H\text{-}2D^d$ product, 44,000 for the larger $H\text{-}2K^d$ product, and 29,000 for the smaller $H\text{-}2K^d$ product. The molecular weights determined by electrophoresis or molecular seive chromatography in sodium dodecyl sulfate differ from and are more reliable estimates than the originally reported molecular sizes (Shimada, A., and Nathenson, S. G. (1969), *Biochemistry 8*, 4048).

Histocompatibility alloantigens are the cellular products of histocompatibility genes, and as such carry the immunological determinants involved in tissue transplant rejection (Snell and Stimpfling, 1966; Lengerova, 1969). In the mouse, approximately 30 genetic loci controlling histocompatibility have been described, and genetic analysis postulates that as many as 400 may exist (*cf.* Lengerova, 1969). One system, that involving the *H-2* genes, is more important than the others because differences in the *H-2* gene products elicit the strongest graft rejections. For this reason, *H-2* is considered the major histocompatibility system in the mouse.

Although the physiological role of the *H-2* antigens has not yet been uncovered, the genes controlling these antigens exhibit an extraordinary degree of polymorphism. More than 25 different haplotypes[1] exist among the various laboratory mouse strains, and numerous others are being found among wild mice (Klein and Shreffler, 1971). Additional interest in these genes and their cellular products arises from the recent findings that one of several genes controlling susceptibility to Gross leukemia virus (Lilly, 1970) and one or more genes controlling immune responses to certain antigens (Benacerraf and McDevitt, 1972) are either closely linked to the *H-2* genes or identical with them.

A knowledge of the structure of the *H-2* histocompatibility antigens is of key importance for understanding their genetic organization, the basis of their exceptional immunogenic properties, and their preference for a membrane environment. Two closely linked genes, *H-2K* and *H-2D* (Snell *et al.*, 1971; Shreffler *et al.*, 1971), control the molecular expression of these products which are glycoproteins (Shimada and Nathenson, 1969) tightly integrated into the lipid membrane matrix, and which carry their antigenic determinants in characteristic array for each different *H-2* haplotype.

Because of their importance in immunogenetics, the *H-2* alloantigens have been the subject of many biochemical studies (*cf.* Davies, 1968; Reisfeld and Kahan, 1971; Haughton and Nash, 1969; Nathenson, 1970). In previous work (Shimada and Nathenson, 1969), *H-2* alloantigen glycoprotein fragments solubilized from cell membranes by proteolytic digestion were found to be of two size classes, of molecular weight *ca.* 65,000 and *ca.* 40,000, and were approximately 90% protein, 4% neutral carbohydrate, 3–4% glucosamine, and 1% sialic acid. Because of their water-soluble state these fragments could be purified and partially characterized. By contrast, the intact *H-2* alloantigen glycoprotein molecules solubilized by detergents such as the Triton series (Kandutsch and Stimpfling, 1963; Hilgert *et al.*, 1969) rather than by enzymes have been difficult to handle; and available technology was not sufficient to allow extensive purification.

Recently, we described (Schwartz and Nathenson, 1971a,b) an isolation procedure using the nonionic detergent Non-Idet P-40 (NP-40) to solubilize presumably intact *H-2* alloantigen molecules from radiolabeled mouse tumor cells which, when combined with indirect immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis, permitted

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[1] The haplotype designation, indicated by a superscript, *e.g.*, $H\text{-}2^d$, identifies the two *H-2* genes (*H-2K* and *H-2D*) present on a single chromosome. More explicit designation of these two genes is also achieved through the use of superscripts, *e.g.*, for $H\text{-}2^d$, $H\text{-}2K^d$ and $H\text{-}2D^d$. This type of designation is especially useful for showing the composition of cross-over haplotypes, *e.g.*, for $H\text{-}2^h$, $H\text{-}2K^k$ and $H\text{-}2D^b$, indicating that for $H\text{-}2^h$ the *H-2K* gene was from the $H\text{-}2^k$ haplotype and the *H-2D* gene was from the $H\text{-}2^b$ haplotype. Each *K* gene and each *D* gene determines one unique, "private" specificity, *e.g.*, for $H\text{-}2D^d$, specificity H-2.4. (For more information, see Klein and Shreffler, 1972.)

TABLE I: *H-2* Alloantisera.

Serum Designation	Source	Recipient Strain (*H-2* Type)	Donor Cells (*H-2* Type)	Private Specificities Detectable[a] With $H\text{-}2^b$	With $H\text{-}2^d$
SN 21	SGN[b]	C57/B10 ($H\text{-}2^b$)	Meth-A ($H\text{-}2^d$)	Control	4 (*H-2D*), 31(*H-2K*)
C-33	NIAID[c]	B10.D2 × A ($H\text{-}2^{d/a}$)	B10.A(5R) ($H\text{-}2^i$)	33 (*H-2K*)	None
AS 477	Jax[d]	B10.D2 × A ($H\text{-}2^{d/a}$)	B10.A(2R) ($H\text{-}2^h$)	2 (*H-2D*)	None
SN 26	SGN	BALB/c ($H\text{-}2^d$)	EL-4 ($H\text{-}2^b$)	2 (*H-2D*), 33 (*H-2K*)	Control
AS 377	Jax	AKR.M × B10 ($H\text{-}2^{m/b}$)	B10.A ($H\text{-}2^a$)	None	4 (*H-2D*)
AS 382	Jax	A/wySn × B10.129 ($H\text{-}2^{w/b}$)	B10.D2 ($H\text{-}2^d$)	None	31 (*H-2K*)

[a] Designation in parentheses in two last columns is the particular gene (*i.e.*, *H-2D* or *H-2K*) of the haplotype determining this specificity. For example, H-2.4 is the *H-2D* gene product of the $H\text{-}2^d$ allele, or $H\text{-}2D^d$. [b] Alloantiserum produced in our own laboratory, used as control serum with syngeneic tumor cells. [c] Alloantiserum obtained from the *H-2* alloantiserum bank, Transplantation Immunology Branch, National Institute of Allergy and Infectious Diseases. [d] Alloantiserum was the gift of Drs. G. D. Snell and M. Cherry, Jackson Laboratories, Bar Harbor, Me.

purification and isolation of these radiolabeled antigen molecules.

The present paper describes some of the results of preliminary characterization studies on these NP-40-solubilized *H-2* alloantigens, and compares their properties with those of antigen fragments isolated from cell membranes by papain digestion.

Materials and Methods

Tumor Cell Lines. Meth-A is the chemically induced ascites fibrosarcoma maintained in BALB/cJ ($H\text{-}2^d$) mice. EL-4 is a chemically induced ascites lymphosarcoma maintained in C57BL/6J ($H\text{-}2^b$) mice. MTC is the ascites mastocytoma P815 maintained in DBA/2J ($H\text{-}2^d$) mice.

Radiolabeling of Tumor Cells. Tumor cells in ascitic fluid were removed from the mice, immediately placed in Hank's balanced salt solution lacking calcium and magnesium (BSS), containing 0.5 unit/ml of heparin, and washed free of contaminating blood elements and heparin. Meth-A and MTC cells were labeled at a density of 10^7 cells/ml in Joklik modified Eagle's medium containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 50–100 μCi/ml of [^{3}H]fucose or 6 μCi/ml of [^{14}C]fucose, or 5–10 μCi/ml of choline–methyl [^{14}C]chloride (New England Nuclear, Boston, Mass.). EL-4 cells were radiolabeled at 5×10^7 cells/ml in Fischer's medium containing 10% fetal calf serum, 10% rabbit serum (Grand Island Biological Co., Grand Island, N. Y.), and 50–100 μCi/ml of [^{3}H]fucose. When [^{14}C]leucine was used, cell cultures were incubated with 5–10 μCi/ml. All cell types were incubated in suspension culture at 37° for 4 hr.

Preparation of Papain-Solubilized Alloantigen. Radiolabeled, papain-solubilized antigen was prepared from intact Meth-A cells as described previously (Schwartz and Nathenson, 1971b).

Preparation of NP-40-Solubilized H-2 Alloantigen. NP-40 solubilized cell extracts were prepared as described in detail previously (Schwartz and Nathenson, 1971a). The detergent Non-Idet P-40, from Shell Chemical Co., is an alkylaryl–ethylene oxide adduct.

Source of Alloantisera. Presumptively monospecific or nearly monospecific antisera were generously supplied by Drs. G. D. Snell and M. Cherry at the Jackson Laboratory, Bar Harbor, Maine; or by the *H-2* alloantiserum bank, Transplantation Immunology Branch, National Institute of Allergy and Infectious Diseases; or were made by methods previously published (Snell, 1968). Table I lists the alloantisera used in these experiments.

Indirect Precipitation Assay. Indirect precipitation was performed as described previously (Schwartz and Nathenson, 1971a).

Cytotoxic Inhibition Assay. This assay, performed as described (Nathenson and Davies, 1966), could be carried out only when the number of cytotoxic inhibition units was sufficient to be detectable after a 1:1000 dilution. Dilution of column effluents to reduce detergent concentrations to 0.0005% or less was required to prevent nonspecific lysis of target cells. The necessary quantity of antigen was sometimes obtained by cochromatography of nonlabeled, detergent-solubilized material along with specifically isolated radiolabeled antigen.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoresis, a modification of the method of Shapiro *et al.* (1967), was performed as described previously (Schwartz and Nathenson, 1971a). When samples were treated with 2-mercaptoethanol, a 1% final concentration was used.

Other Materials and Methods. Protein was determined by the method of Lowry *et al* (1951). Chemicals were reagent grade or better.

Results

Studies on the Molecular Size of the NP-40-Solubilized Antigen. To provide a rough estimate of the molecular size of the *H-2* antigen as solubilized by the detergent NP-40, we fractionated the labeled, detergent-solubilized extracts of Meth-A ($H\text{-}2^d$) cell membranes on an agarose 15m column in 0.5% NP-40 buffer. Assays of cytotoxic inhibition and indirect precipitation gave identical patterns, as shown in Figure 1. The position of standard molecular weight markers included with several column runs showed a linear relationship between the logarithm of the molecular weight and elution volume. The markers used were porcine thyroglobulin (mol wt (669,000), *Escherichia coli* β-galactosidase (mol wt 520,000), and rabbit muscle aldolase (mol wt 160,000). Using the positions of these markers, the approximate "molecular

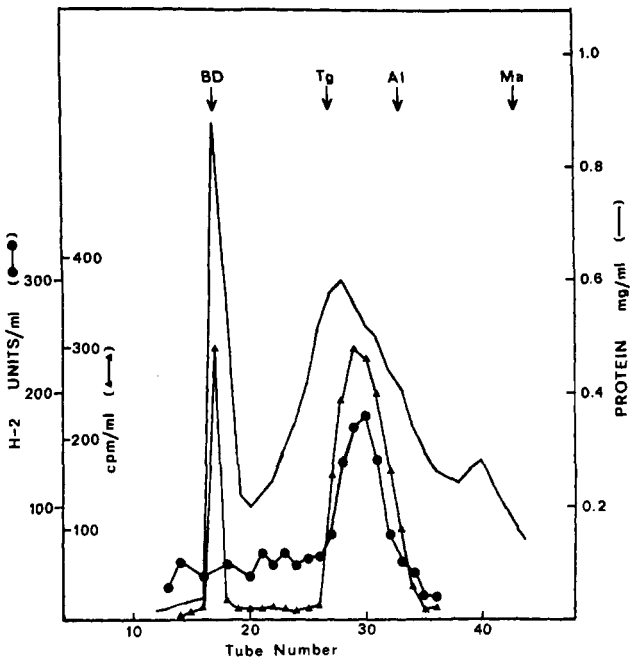

FIGURE 1: Chromatography of NP-40-solubilized *H-2* alloantigen on Bio-Gel A-15m. NP-40-solubilized extract (14 mg of protein) obtained from L-[^{3}H]fucose-labeled Meth-A cells was mixed with NP-40-solubilized extract (8 mg) from BALB/c spleen, and applied to a column (0.96 × 110 cm) of Bio-Gel A-15m (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) which had been equilibrated with 0.5% NP-40, 0.9% NaCl, 0.01 M Tris-HCl buffer (pH 7.5), and 1.5 mM $MgCl_2$. Elution was carried out with the same buffer at 4° and 2.2-ml fractions were collected. The flow rate was 4.5 ml/hr. Aliquots of each tube were assayed for protein (—), *H-2* units (●) in the inhibition of immune cytolysis assay (Nathenson and Davies, 1966), and *H-2* activity (▲) as tested by the indirect precipitin assay (Schwartz and Nathenson, 1971a). The position of molecular weight markers in other runs on the column are indicated: BD (Blue Dextran, which gives the excluded volume); Tg (thyroglobulin, which has a mol wt of 667,000); Al (rabbit muscle aldolase, which has a mol wt of 160,000); and Ma (mannose, which gives the position of the included volume).

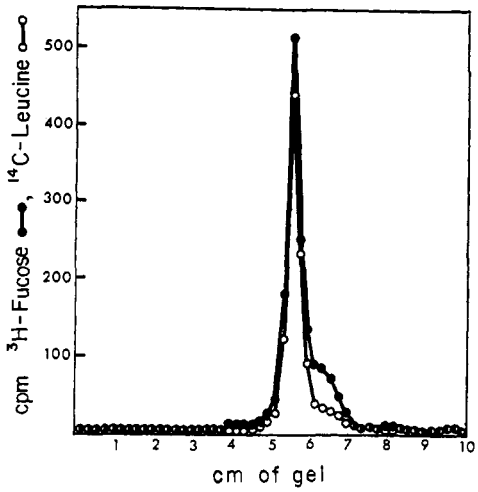

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of a precipitate of doubly labeled [^{3}H]fucose and [^{14}C]leucine alloantigen reacting with serum anti-H-2.33. A specific precipitate of antibody with antigen from NP-40-solubilized EL-4 (*H-2^b*) cells was electrophoresed on a 10% polyacrylamide gel as described in the Methods section. The fucose radioactivity (●) (which labels the carbohydrate portion) and the leucine radioactivity (○) (which labels the protein portion) showed complete coincidence, thus suggesting the glycoprotein nature of the *H-2* alloantigens isolated from NP-40-solubilized cell extracts.

weight" of the peak of *H-2* activity could be calculated to be 380,000.

Studies with Carbohydrate and Amino Acid Labeled NP-40-Solubilized Antigen. Previous studies on *H-2* alloantigens solubilized from spleen cell membranes by papain digestion had shown that the antigen was glycoprotein in nature (Shimada and Nathenson, 1969). The proof of specificity of the antigen isolated from NP-40-solubilized extracts and the indirect precipitation procedure was established previously (Schwartz and Nathenson, 1971a). To confirm that the alloantigen solubilized by detergent was also a glycoprotein and to establish the validity of all experiments using labeled fucose, we analyzed doubly labeled alloantigen.

An [^{3}H]fucose and [^{14}C]leucine labeled preparation was precipitated with a specific alloantiserum. Figure 2 shows that when this precipitate was solubilized in sodium dodecyl sulfate and reduced with 2-mercaptoethanol, the electropherogram showed one major peak for both the amino acid and the monosaccharide label. Such results support the conclusion that NP-40-solubilized material is in fact glycoprotein.

In a further analysis of the carbohydrate chains of the antigens, fucose labeled glycopeptides from both the NP-40-solubilized and papain-solubilized antigens were found to be identical in size as judged by Sephadex G-50 column chromatography (Figure 3).

Papain-solubilized *H-2* alloantigens are lipid free (Shimada and Nathenson, 1969), but there could be lipid associated with the NP-40-solubilized alloantigen. In a preliminary series of experiments, we utilized [^{14}C]choline as a tracer to ascertain whether phospholipid was present. Cells labeled with [^{14}C]choline and [^{3}H]fucose were solubilized with detergent, and the extract was chromatographed on Bio-Gel 15m. The material from the high molecular weight region of the column was freeze-dried and extracted with chloroform–methanol (2:1). Only the [^{14}C]choline label was extracted, thus showing that all the ^{14}C-labeled material was in lipid extractable form. The indirect precipitation assay was used on the column fractions to determine whether any [^{14}C]choline was associated with *H-2* antigen.

As always, the indirect precipitation assay was controlled by comparison of radioactivity found in precipitates made using a specific alloantiserum with that found in precipitates made using normal mouse serum. In the column fraction with peak antigen activity there were [^{3}H]fucose (26,500 cpm) and [^{14}C]choline (54,400 cpm). The specific precipitate made from this fraction contained 517 cpm of [^{3}H]fucose, while the nonspecific control for the same fraction was 82 cpm, giving the highly significant specificity ratio of 6.25. The [^{14}C]choline counts in the same precipitates were 5 cpm for the specific and 7 cpm for the nonspecific control. Thus, the presence of choline-containing lipid bound to the antigen was ruled out. Of course, the absence of choline does not preclude the presence of phospholipid other than lecithin, or of neutral lipid, or of the detergent itself.

The Molecular Size of Monomeric H-2 Alloantigens. The relatively large apparent "molecular weight" of the *H-2* antigen solubilized by NP-40 and analyzed by gel filtration in the same relatively mild detergent could be lowered by the use of more vigorous dissociation procedures. NP-40-solubilized material was isolated by indirect precipitation and treated

TABLE II: Molecular Weight Estimations of NP-40-Solubilized Glycoprotein Molecules.[a]

	5% Polyacrylamide Gels		10% Polyacrylamide Gels		
	SDS[a] Only	SDS + 2-ME[a]	SDS Only	SDS + 2-ME	Av
H-2.4 (*H-2*^{d}, *D* gene)	43,000 (5)[b]	43,000 (3)	42,000	45,000	43,000[c]
H-2.31 (*H-2*^{d}, *K* gene)	46,000 (2)	48,000	48,000	46,000	47,000

[a] [^{3}H]Fucose labeled *H-2* antigen from Meth-A was solubilized by NP-40 and then coprecipitated with marker [^{14}C]immunoglobulin by indirect immunoprecipitation. The precipitates were dissolved in 2% sodium dodecyl sulfate (SDS) or in 2% sodium dodecyl sulfate + 1% 2-mercaptoethanol (2-ME), and electrophoresed on sodium dodecyl sulfate gels of 5 or 10% polyacrylamide. The molecular weight values were determined by comparison with the ^{14}C-labeled marker (Shapiro *et al.*, 1967). [b] Numbers in parentheses indicate the number of independent estimations. [c] Average molecular weight to nearest thousand.

with sodium dodecyl sulfate, sodium dodecyl sulfate + 2-mercaptoethanol, or sodium dodecyl sulfate + 2-mercaptoethanol with carboxymethylation, and the size of the resulting preparations was then determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or sodium dodecyl sulfate molecular seive chromatography. The molecular weight markers used were labeled immunoglobulin components produced by the mouse myeloma MPC-11 (a gift of Dr. M. D. Scharff).

As shown in Figure 4, precipitated H-2.4 activity showed two major peaks when treated with sodium dodecyl sulfate and electrophoresed. The molecular weights found were 88,000 for the larger peak and 43,000 for the smaller peak. However, the same sample treated with sodium dodecyl sulfate but also reduced with 2-mercaptoethanol showed only one peak, of molecular weight of about 43,000 (Figure 5).

The molecular weights found for the alloantigenic specificities H-2.4 (*D* gene) and H-2.31 (*K* gene) after dissociation in sodium dodecyl sulfate or dissociation–reduction in sodium dodecyl sulfate and 2-mercaptoethanol are summarized in Table II. Both 5 and 10% polyacrylamide gels were used for the analysis.

The smaller sized molecule can almost certainly be considered a monomeric form of the antigen, since reduction and carboxymethylation produced no smaller subunits detectable by sodium dodecyl sulfate gel electrophoresis. In addition, reduced and carboxymethylated H-2.4 glycoprotein when examined by sodium dodecyl sulfate column chromatography on Bio-Gel P300 or Sephadex G-200 also showed a single

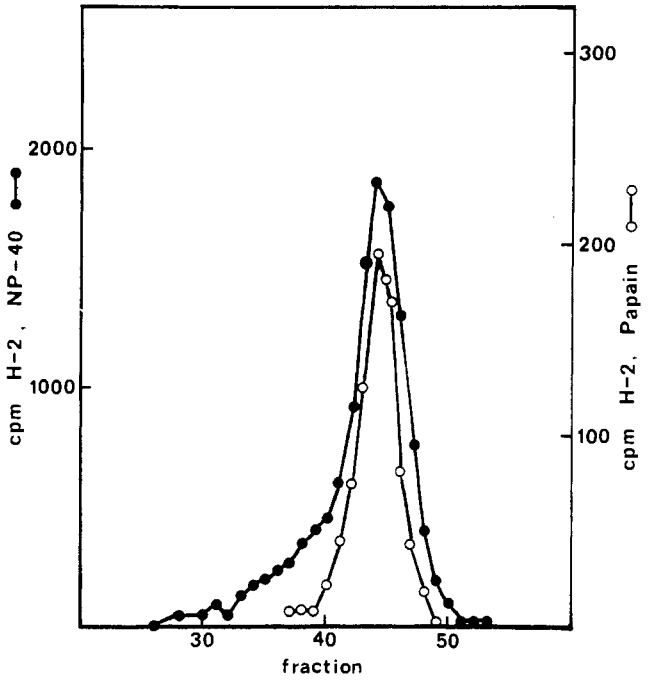

FIGURE 3: Comparison of glycopeptides from NP-40- and papain-solubilized *H-2* alloantigen from EL-4 (*H-2*^{b}) by Sephadex column chromatography. Pronase digestion of specific precipitates of *H-2* alloantigens (either NP-40 solubilized or papain solubilized) was carried out as described previously (Muramatsu and Nathenson, 1970). Aliquots were chromatographed on a Sephadex G-50 column (115 × 0.9 cm) in 0.15 M NaCl, 0.01 M Tris-Cl, pH 7.4, at a flow rate of 2 ml/hr, and 20 drops per fraction were collected. The Blue Dextran (excluded volume) eluted from the column at tube 26, and free fucose eluted at tube 61. Two column runs are superimposed. In this graph it can be seen that the glycopeptide peak from the NP-40-solubilized (●) and the papain-solubilized (○) alloantigens were identical.

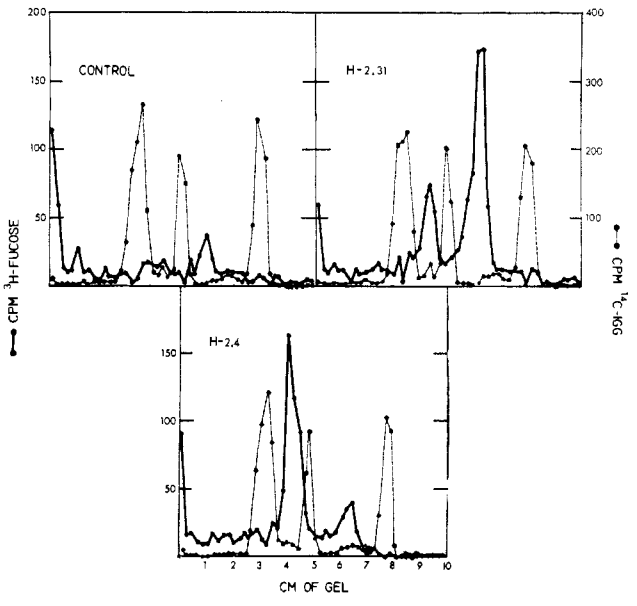

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of specific *H-2* precipitates without reduction with mercaptoethanol. NP-40-solubilized [^{3}H]fucose-labeled MTC (*H-2*^{d}) *H-2* alloantigens (●—●) were coprecipitated with ^{14}C-labeled IgG (●- - -●) as marker, dissolved in 2% sodium dodecyl sulfate, boiled for 1 min, and electrophoresed on 5% polyacrylamide gels. The marker peaks are complete IgG molecules, heavy–light half-molecules and light chains. Two major peaks for both *H-2* specificities tested were seen. The molecular size estimates are given in Table II.

TABLE III: Molecular Weight Estimations of *H-2* Glycoprotein Fragments and Comparison with Molecular Weights of Intact Molecules.[a]

	5% Polyacrylamide Gels		10% Polyacrylamide Gels			Mol Wt of Intact	
	SDS Only[a]	SDS + 2-ME[a]	SDS Only	SDS + 2-ME	Av[b]	Molecule[c]	Difference
H-2.4 (*H-2*^{d}, *D* gene)							
class I only	35,000 (3)[d]	41,000 (2)	38,000 (2)	41,000	39,000	43,000	4,000
H-2.31 (*H-2*^{d}, *K* gene)							
class I	43,000 (2)	45,000	41,000	46,000	44,000	47,000	3,000
class II	31,000	30,000	27,000	27,000	29,000	47,000	18,000

[a] [^{3}H]Fucose-labeled *H-2* antigen from Meth-A or MTC tumor cells was solubilized by papain digestion as previously described (Schwartz and Nathenson, 1971b), and coprecipitated with marker [^{14}C]immunoglobulin by indirect immunoprecipitation. The precipitates were dissolved in 2% sodium dodecyl sulfate (SDS) or 2% sodium dodecyl sulfate (SDS) + 2-mercaptoethanol (2-ME), and electrophoresed on 5 or 10% polyacrylamide gels. The molecular weight values were determined by comparison with the ^{14}C-labeled markers (Shapiro *et al.*, 1967). [b] Average value to nearest thousand. [c] Values taken from Table II. [d] Numbers in parentheses indicate number of independent estimations.

monomeric peak corresponding to a molecular weight of 43,000 (data not shown).

The larger molecular weight peak observed when samples were dissolved in sodium dodecyl sulfate but not reduced is tentatively referred to as a dimeric form. Dissociated but unreduced alloantigen always showed both monomers and dimers, although the proportion of dimer to monomer varied according to the alloantigen specificity and the mouse strain examined (data not shown).

The Relationship between H-2 Alloantigens Solubilized by NP-40 and by Papain. In a previous study (Shimada and Nathenson, 1969), it was found that papain solubilization produced two classes of glycoprotein fragments bearing *H-2* alloantigenic activity. It was important therefore to compare the molecular sizes of the papain-solubilized fragments to those of the intact molecules solubilized by NP-40. Indirect precipitates from a papain-solubilized extract of Meth-A cells were prepared and electrophoresed on sodium dodecyl sulfate–polyacrylamide gels along with IgG marker proteins. Preparations containing alloantigen bearing specificity H-2.31 were seen to have the two-peak distribution pattern defined previously for that specificity (Figure 6, upper half). Preparations containing alloantigen bearing specificity H-2.4 were seen to have their characteristic single peak (Figure 6, lower half). The same preparations reduced by mercaptoethanol treatment had electrophoretic patterns identical with those for the unreduced material. The molecular size, estimated by co-electrophoresis with myeloma protein markers, is shown in Table III. The large H-2.31 peak migrated as a protein of about 44,000 mol wt and the smaller peak as a protein of about 29,000 mol wt. The single H-2.4 peak migrated with a mol wt of about 39,000.

In order to directly compare the molecular weight of the papain- and NP-40-solubilized materials, several different mixtures were subjected to polyacrylamide gel electrophoresis.

Indirect precipitates containing [^{3}H]fucose-labeled H-2.4 alloantigens solubilized by papain and [^{14}C]fucose-labeled H-2.4 alloantigens solubilized by NP-40 were dissolved and reduced in sodium dodecyl sulfate and 2-mercaptoethanol and combined. The result of the electrophoresis of this mixture is shown in the upper part of Figure 7. The NP-40-solubilized antigen, when reduced to monomer form, is slightly but definitely larger than the glycoprotein fragment solubilized by papain. The difference in mol wt is about 5000.

In another experiment, [^{14}C]fucose-labeled NP-40-solubilized antigen was isolated by indirect precipitation, digested by papain, and then mixed with [^{3}H]fucose-labeled papain-solubilized alloantigen. This combination was dissolved and reduced with sodium dodecyl sulfate and 2-mercaptoethanol, and the result of the electrophoresis of this mixture is shown in the lower part of Figure 7. The papain treatment of the NP-40-solubilized molecule results in a fragment that is the

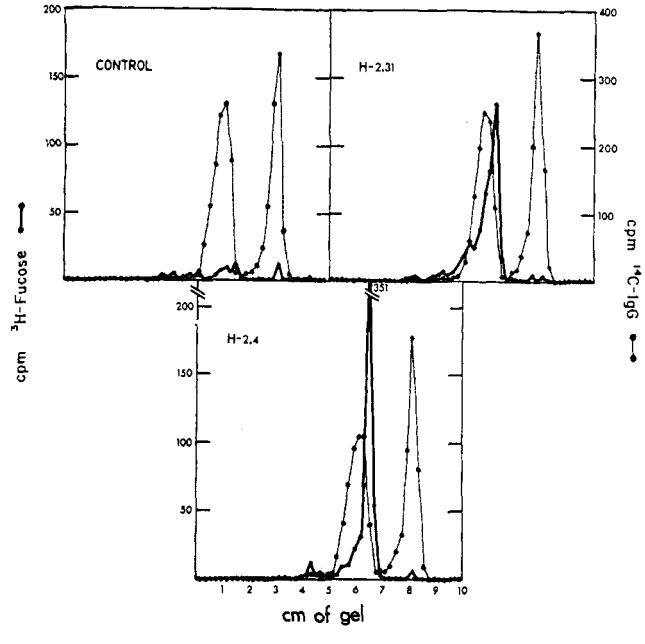

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of alloantigens after treatment with 2-mercaptoethanol. NP-40-solubilized [^{3}H]fucose-labeled Meth-A (*H-2*^{d}) alloantigens (●—●) were coprecipitated with [^{14}C]IgG (●- - -●) as marker, dissolved in 2% sodium dodecyl sulfate and 1% 2-mercaptoethanol, and electrophoresed on 5% polyacrylamide–sodium dodecyl sulfate gels. The marker peaks are heavy and light chains. Only the "monomer" forms of the *H-2* alloantigens are revealed. The molecular size estimates are given in Table II.

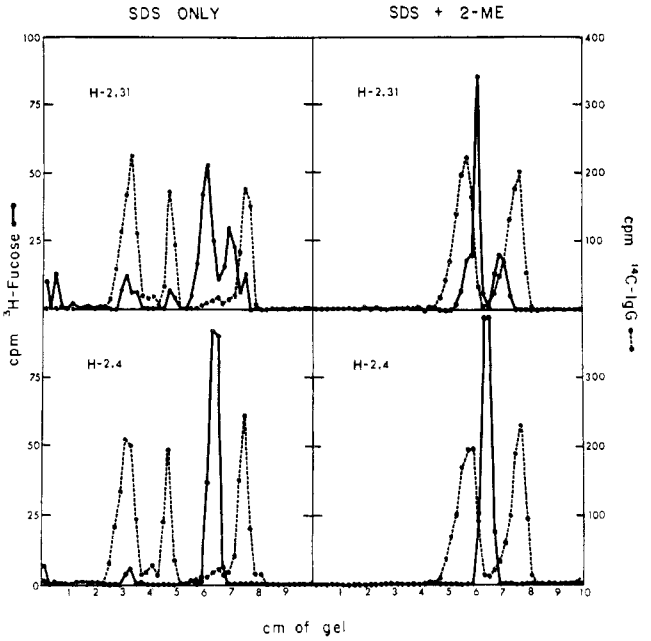

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide electrophoresis of papain-solubilized *H-2* alloantigens. [³H]Fucose-labeled alloantigens (●—●) and ¹⁴C-labeled MPC-11 IgG (●- - -●) as marker were coprecipitated, dissolved in 2% sodium dodecyl sulfate or 2% sodium dodecyl sulfate plus 1% 2-mercaptoethanol, and coelectrophoresed on 5% polyacrylamide–sodium dodecyl sulfate gels. The left-hand panel shows the patterns for H-2.31 (top) and H-2.4 (bottom), dissolved in sodium dodecyl sulfate alone. The markers represent molecular sizes of 150,000 (intact IgG), 77,000 (heavy and light chain), and 22,000 (light chain). The right-hand panel shows H-2.31 (top) and H-2.4 (bottom) dissolved in sodium dodecyl sulfate plus 2-mercaptoethanol. The markers from immunoglobulin are 55,000 (heavy chains) and 22,000 (light chains). The molecular weights of approximately 35,000 for H-2.4 and 43,000 for the larger H-2.31 and 30,000 for the smaller H-2.31 peaks did not decrease in the presence of 2-mercaptoethanol. These findings indicate that these molecules contained no interpolypeptide chain disulfide bonds (*cf.* Table III).

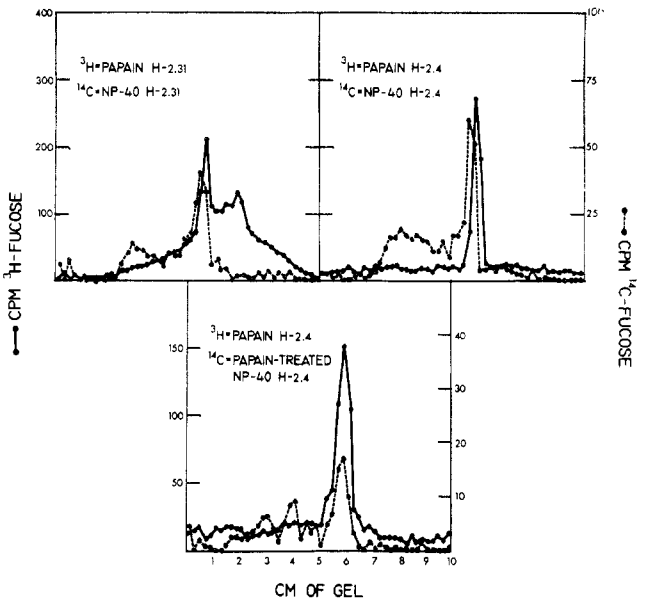

FIGURE 7: Comparison by sodium dodecyl sulfate–polyacrylamide electrophoresis of papain-solubilized and NP-40-solubilized *H-2* allontigens. Papain-solubilized [³H]fucose-labeled H-2.31 (upper left panel) and H-2.4 (upper right panel) were indirectly coprecipitated with NP-40-solubilized [¹⁴C]fucose H-2.31 and H-2.4, respectively. In addition (lower panel), an indirect precipitate of NP-40-solubilized [¹⁴C]fucose-labeled H-2.4 was digested with crystalline papain and mixed with an indirect precipitate of crude papain-solubilized [³H]fucose-labeled H-2.4. All precipitates were dissolved in sodium dodecyl sulfate plus 2-mercaptoethanol and electrophoresed on 5% polyacrylamide–sodium dodecyl sulfate gels. The two top gel patterns show that the NP-40-solubilized molecules are larger than the fragments solubilized by papain, while the bottom pattern shows that papain treatment of the NP-40-solubilized antigen results in a new fragment that is the same size as the original papain-solubilized fragment. See Table II for further discussion.

same size as the papain fragment prepared directly from cell membranes without intervening detergent solubilization. Thus, the molecules and fragments solubilized by the two methods are closely related, and it appears that papain releases the major portion of the native monomer.

Discussion

Water-insoluble macromolecules present unique problems in purification, in analysis of components, and in measurements of relatively simple parameters such as molecular weight. Such problems have been encountered in the analysis of the insoluble *H-2* antigens released from their normal membrane environment by the nonionic detergent NP-40.

While studies on *H-2* glycoprotein fragments solubilized by papain cleavage methods have yielded a certain amount of chemical information (Nathenson *et al.*, 1970), it is important to extend this information to intact antigen molecules. The data from studies of the water-soluble *H-2* fragments, combined with new knowledge concerning insoluble *H-2* macromolecules, should help to form a coherent idea of the structure and organization of these moieties in the membranes.

The experiments in this paper were made possible because of the development of an indirect precipitation method which permitted isolation of the *H-2* molecules from the NP-40-soluble extract and avoided some of the difficulties of purification by traditional chemical techniques. The specificity of this reaction was previously documented (Schwartz and Nathenson, 1971a), and the method showed that fucose was an integral part of the native antigen, just as it had been demonstrated to be a part of the papain-digested fragment, and that therefore fucose could be used as a radiolabel marker for the native antigen.

The results presented in this article point out several basic properties of the native *H-2* glycoproteins. First, these antigens are composed of glycoprotein subunits with molecular weights in the range of 43,000–47,000 depending on the genetic determinant. Second, the antigens may exist at least partially of dimers linked by disulfide bonds. Third, the dimers, and possibly a variable portion of monomers, may form an aggregate by noncovalent binding (of mol wt ~400,000), which represents the actual native form of the antigen. We have also been able to conclude that the papain-solubilized glycoprotein bearing the *H-2* antigenic sites includes the major portion (75–85%) of the native *H-2* glycoprotein subunit.

The conclusion that *H-2* alloantigens may exist as aggregates in the cell membrane can only be tentatively presented since there is a possibility that the NP-40 treatment creates these aggregates as artifacts; also, the measurements of the aggregate size should be viewed with caution, since accuracy of measurement of molecular weight by gel filtration depends on the compositional and conformational homogeneity of the

unknown substance as well as the markers (Fish *et al.*, 1970). This system is certainly not ideal in that respect, and the molecular weight may vary somewhat from the estimate of 380,000. However, the conclusion that the size of *H-2* alloantigen solubilized by nonionic detergents is relatively large compared to the size after extreme disruption is consistent with previous findings of Hilgert *et al.* (1969) who showed agarose 0.5m chromatographic patterns of antigen solubilized with Triton X-114 similar to those we observed. Of course, the definitive description of the aggregate must also await complete characterization of its components, since if it contains nonspecific materials not labeled with fucose, glucosamine, amino acid, or choline precursors, these certainly contribute to its molecular weight, and would make estimates of the number of monomers in each aggregate difficult to determine.

Nonetheless, some nonspecific components have been ruled out by this work. The antigen–antibody precipitate was free of lipid-soluble [^{14}C]choline, suggesting that the aggregated form of the antigen was free of adsorbed lecithin. The absence of lecithin further argues against nonspecific adsorption of other membrane lipids. We have not explored the possibility that the detergent itself is found in the aggregate.

The evidence for the molecular size of the monomeric form of the *H-2* glycoprotein is quite dependable, because the use of sodium dodecyl sulfate and reduction equalizes charge and conformational differences (Shapiro *et al.*, 1967; Fish *et al.*, 1970). Thus, the molecular weights of 43,000 for the H-2.4 monomer and 47,000 for the H-2.31 monomer obtained in sodium dodecyl sulfate are reliable. The finding of reproducible differences of molecular weight of the *H-2D* and *H-2K* products is of considerable interest with regard to the genetic evolution of the *H-2* genes and their interrelationships. This finding is substantiated by experiments (Schwartz, Cullen, and Nathenson, 1973,[2] showing electrophoretic separation of the $H\text{-}2D^d$ and $H\text{-}2K^d$ gene products radiolabeled with different isotopes.

The evidence for the existence of dimers arises from the observation that when immune precipitates are dissociated by boiling in sodium dodecyl sulfate, two radioactive peaks with molecular weights in a ratio of 2:1 are found by polyacrylamide gel electrophoresis. When precipitates are dissociated in sodium dodecyl sulfate and 2-mercaptoethanol, only a single peak is found. The finding of a peak approximately twice the size of monomer on electrophoresis without reduction suggests the presence of a "dimer" linked by disulfide bonds. Reduction of these bonds produces monomers. Whether the dimer has functional significance is not presently known.

The comparative studies on the papain-solubilized *H-2* fragments and the detergent-solubilized *H-2* molecules have suggested that the larger papain fragments are relatively faithful images of the native glycoproteins. The small (2500–6000) molecular weight difference between the papain fragments and the intact molecules shows that the papain fragments represent the major portion of the native molecule. However, since the loss of a small portion of the antigen confers aqueous solubility to the papain fragment, it seems reasonable to assume that this region carries or confers some properties necessary for membrane integration of the molecule. This contribution might be a particularly hydrophobic region, or a region that is involved or influential in bringing about aggregation into large molecular weight complexes, which then perhaps have the hydrophilic areas sequestered.

Further evidence that papain-solubilized and detergent-solubilized antigens are not profoundly different in composition could be inferred from studies of their glycopeptides. Pronase digestion of both papain-digested and NP-40-solubilized antigen produced glycopeptides which were identical in size (Figure 3), and no additional fucose-containing molecules of different size were found. This suggested that there were no resolvable fucose-containing sugar chains on the native NP-40 molecule other than those found on papain fragments. Also, it is unlikely that there is contamination of the specific *H-2* precipitates by non-*H-2* glycoproteins similar in size to the *H-2* molecule since such non-*H-2* molecules might be expected to have separable glycopeptides. For example, the glycopeptides produced by Pronase digestion of the *H-2* and *Tla* gene products can be readily separated by molecular seive chromatography (Muramatsu and Nathenson, 1973).

Thus, the present studies show that intact *H-2* antigens are similar to the papain-cleaved fragments which were studied previously. The glycoprotein nature of the fragments was confirmed by this work and the native molecules were likewise shown to be glycoproteins whose molecular weights (43,000 for the $H\text{-}2D^d$ gene product and 47,000 for the $H\text{-}2K^d$ gene product) were not more than 20% greater than the papain-derived fragments. As noted, the *H-2D* and *H-2K* gene products were slightly different in molecular weight. Our present methods have overcome some of the difficulties inherent in handling and analyzing membrane macromolecules, and appear adequate for more precise determination of primary structure with the aim of describing the structures involved in the formation of antigenic sites and the properties important for integration in the membrane.

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Equilibrium Kinetic Study of the Catalytic Mechanism of Bovine Liver Glutamate Dehydrogenase†

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ABSTRACT: The catalytic mechanism of glutamate dehydrogenation with bovine liver glutamate dehydrogenase was studied by equilibrium kinetics utilizing α-[^{14}C]ketoglutarate to measure glutamate ⟷ α-ketoglutarate and [^{14}C]NAD(P)H to measure NAD(P)$^+$ ⟷ NAD(P)H rates in 0.1 M Tris-acetate at pH 8.0 and 25°. Increasing glutamate:α-ketoglutarate concentration resulted in increase in both glutamate ⟷ α-ketoglutarate and NAD$^+$ ⟷ NADH rates to plateau values while NADP$^+$ ⟷ NADPH rates were moderately depressed at elevated substrate levels (glutamate above about 0.04 M). Moderate depression of glutamate ⟷ α-ketoglutarate rates was obtained at elevated NAD$^+$:NADH concentration (NAD$^+$ greater than about 1 mM). The results are compatible with an alternative order of reactant addition and with enhancement of reactant binding by enzyme-bound reactant resulting in a decrease in the respective dissociation rates in ternary or quaternary complexes as compared to binary. Increasing concentrations of glutamate:NADH and α-ketoglutarate:NAD$^+$ resulted in marked and moderate inhibition, respectively, in both substrate and coenzyme interchanges, supporting the formation of the unreactive complex enzyme–NADH–glutamate and less facile formation of enzyme–NAD$^+$–α-ketoglutarate. Substrate interchange was usually considerably less than twice coenzyme interchange suggesting that chemical transformation may be of the same order of magnitude as the slower dissociation rates. NADP ⟷ NADPH rates were two–three times greater than glutamate ⟷ α-ketoglutarate rates below about 0.02 M glutamate, suggesting that under these conditions substrate rather than coenzyme dissociation may be rate limiting. Minimum estimates of dissociation constants from reciprocal plots of equilibrium rates and substrate concentrations were 66–360 μM (α-ketoglutarate), 1.8–4.5 μM (NADH), and 700–910 μM (NH_4^+). Simultaneous threefold depression of glutamate ⟷ α-ketoglutarate and NAD ⟷ NADH rates from maximum values at elevated glutamate:NH_4^+ concentrations suggested the possibility of simultaneous binding of both ligands in an unreactive or less reactive enzyme complex, resulting in decrease in the rate of chemical transformation.

Equilibrium kinetics studied by isotopic exchange is a powerful tool in the elucidation of enzyme mechanism (Boyer, 1959; Boyer and Silverstein, 1963; Silverstein 1963; Silverstein and Boyer, 1964a,b; Fromm *et al.*, 1964; Silverstein and Sulebele, 1969b,c). We have extended the application of equilibrium kinetics to the elucidation of the mechanism of enzyme modifier action and of the allosteric mechanism of regulatory enzymes which are of fundamental importance in metabolic control (Silverstein and Sulebele, 1966, 1967, 1969a,d, 1970a,b; Silverstein, 1970a–c). We have initially explored the application of equilibrium kinetics to a study of the mechanism of modifier action for the enzymes pig heart mitochondrial malate dehydrogenase (Silverstein and Sulebele, 1966, 1967, 1970b) and horse liver alcohol dehydrogenase (Silverstein and Sulebele, 1967; Silverstein, 1968, 1970a).

Subsequent equilibrium studies have been done with bovine liver glutamate dehydrogenase (L-glutamate:NADP$^+$ oxidoreductase (deaminating), EC 1.4.1.3) which is an important, well-studied allosteric enzyme at the interphase between amino acid and carbohydrate metabolism which catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonium ion (Frieden, 1963, 1964). The active oligomer has a mol wt of about 312,000 or 320,000, contains six apparently identical subunits arranged compactly in two identical triangular layers, and reversibly forms linear aggregates with molecular weights of up to several million (Eisenberg, 1970; Cassman and Schachman, 1971). The molecular weight per active center is 57,000 by spectrophotometric titration (Egan and Dalziel, 1971). Many studies have been made of the initial rate of catalytic activity and of the effect of various allosteric effectors on it and the state of ag-

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