THE STRUCTURE AND BEHAVIOR OF CORONAVIRUS A59 GLYCOPROTEINS

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INTRODUCTION

Coronavirus A59 contains three structural proteins. The location of these proteins in relation to the viral envelope and RNA are illustrated in the model shown in Figure 1. A nucleocapsid protein, N, (mw $\simeq$ 50k) forms the nucleocapsid together with the RNA genome. Surrounding the nucleocapsid is a lipoprotein membrane which contains the envelope glycoproteins E1 (mw $\simeq$ 23k) and E2 (mw $\simeq$180k and 90k). E1 is an integral membrane protein, the bulk of which lies within the viral membrane and probably spans the lipid bilayer. E2 is a peripheral glycoprotein which forms the characteristic peplomers that are associated with this virion.

In this report I will describe some features of the behavior of these viral glycoproteins and propose models for the structure of E1 and E2.

CHARACTERIZATION OF E1 AND E2 BASED ON INCORPORATION OF DIFFERENT RADIOLABELED PRECURSORS

Inasmuch as these studies depended on the use of radiolabeled compounds, I would like to first review the relative incorporation of several radiolabeled precursors into E1 and E2. This information is summarized in Table 1.

Methionine is incorporated into E1 to a greater relative extent than into the other structural proteins, compared with other labeled amino acids such as valine, leucine, arginine, glutamic acid and mixtures of amino acids. Seventy percent of the radiolabeled methionine incorporated into A59 virus was found in E1 and only approximately 10% in E2. In contrast, when A59 virus was labeled, with valine, only 40% of the label in the virus was incorporated into E1 and 20% into E2. E2 was highly labeled by both
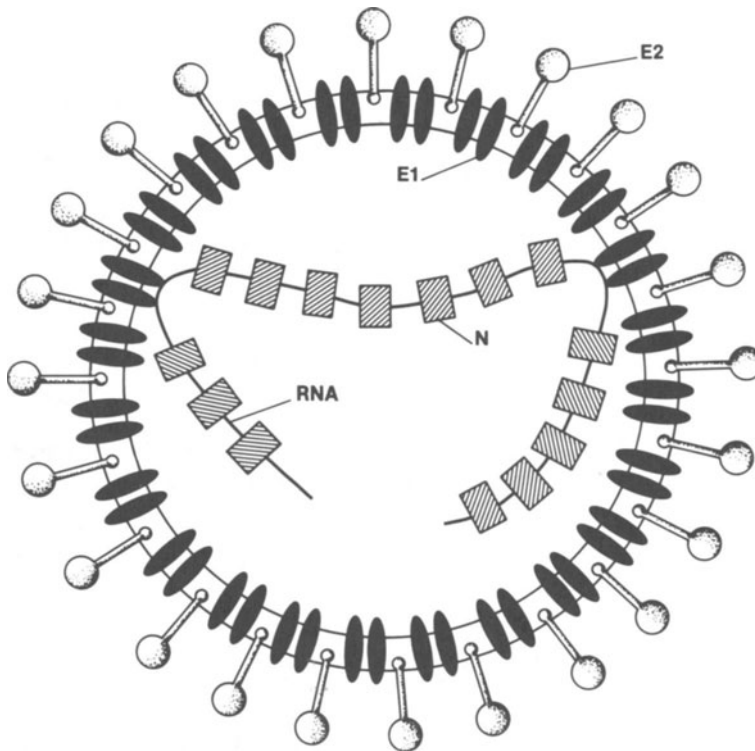
Fig. 1. A schematic model of coronavirus A59 (Sturman et al., 1980)

radiolabeled fucose and glucosamine, whereas E1 was not labeled by fucose and only to a small extent by glucosamine. The ratio of incorporation of glucosamine relative to valine in E1 was about one tenth of that in E2. Thus E1 is the principal viral structural protein which becomes labeled with methionine, whereas E2 is the principal glycoprotein which can be labeled with glucosamine and the only one which can be labeled with fucose. These

Table 1. Radiolabeling Characteristics of Coronavirus A59 Glycoproteins

Designation	Relative extent of labeling with		
	Methionine	Fucose	Glucosamine
E1	++++	–	+
E2	+	++++	++++

differences have been used in following the behavior of E1 and E2 in the virus and after solubilization with SDS or NP40.

CONFORMATIONAL TRANSITION AND AGGREGATION OF THE PEPLOMERIC GLYCOPROTEIN

Several investigators have shown that the thermal lability of coronavirus infectivity is pH dependent. Pocock and Garwes (1975) reported that transmissible gastroenteritis virus infectivity was most stable at pH 6.5 at 37°C and least stable at pH 8. Alexander and Collins (1975) obtained similar results in their study of the effect of pH on the infectivity of avian infectious bronchitis virus.

I will describe the results of experiments with A59 virus which provide evidence that pH dependent thermal lability of coronavirus infectivity is associated with a conformational change in E2 and aggregation of the peplomeric glycoprotein.

In Figure 2, the pH dependence of A59 virus infectivity at 37°C is illustrated. Virus infectivity was measured after 24 hours incubation over a pH range from 4.0 to 8.0 at 4°C and 37°C. Incubation was carried out in a buffer prepared from boric acid, citric acid, diethylbarbituric acid and phosphoric acid (Johnson and Lindsey, 1939). The ionic strength was adjusted to approximate that of physiological saline and fetal bovine serum was added to a final concentration of 10%. At 4°C virus infectivity was stable over much of this pH range, but at 37°C the virus was markedly inactivated above pH 6.5 and below pH 5.0. At 37°C virus infectivity was most stable between pH 6.0 and 6.5.

Analysis of the kinetics of thermal inactivation at 37°C revealed that at pH 8.0 there was a 50% loss in A59 virus infectivity in less than 1 hour. In contrast at pH 6.0 the half life of virus infectivity was 24 hours.

The effect of thermal inactivation at pH 8.0 on the structural proteins of the virus was analyzed by SDS-polyacrylamide gel electrophoresis. A marked change in the electrophoretic mobility of E2 was observed. A typical gel profile is shown in Figure 3. In this experiment $[^3H]$ glucosamine-labeled A59 virus was held at pH 6.0, while $[^{14}C]$ glucosamine-labeled virus was inactivated at pH 8.0 at 37°C for 2 hours. Then the samples were mixed, solubilized in SDS at 25°, and coelectrophoresed.

In the virion at pH 6.0, E2 is found in two forms, as 180k (GP180) and 90k (GP90) dalton species which produce identical tryptic peptide patterns (Sturman and Holmes, 1977). GP90 can be produced from GP180 by treatment of virions with trypsin without significant loss of infectivity.

The control pattern in Figure 3 shows both 90k and 180k molecular weight forms of E2 as well as a small amount of aggregate which did not enter the resolving gel. The electrophoretic mobility of E2 from virions
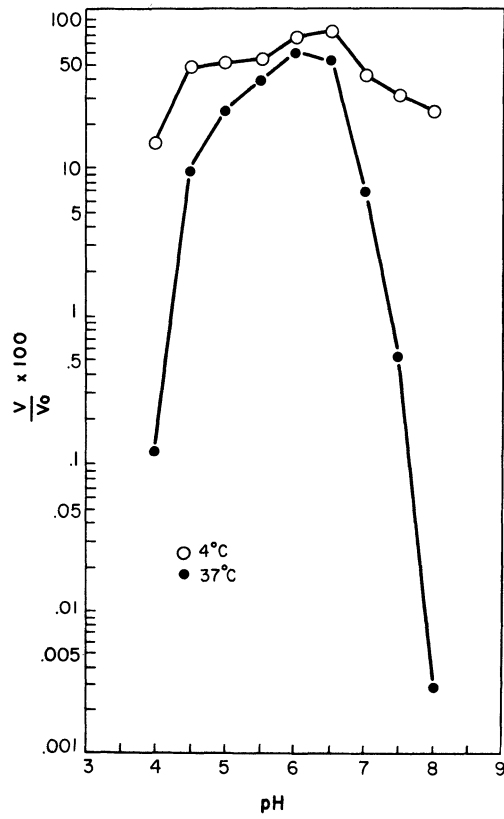
Fig. 2. Survival of A59 virus infectivity after 24 hrs at 4°C or 37°C
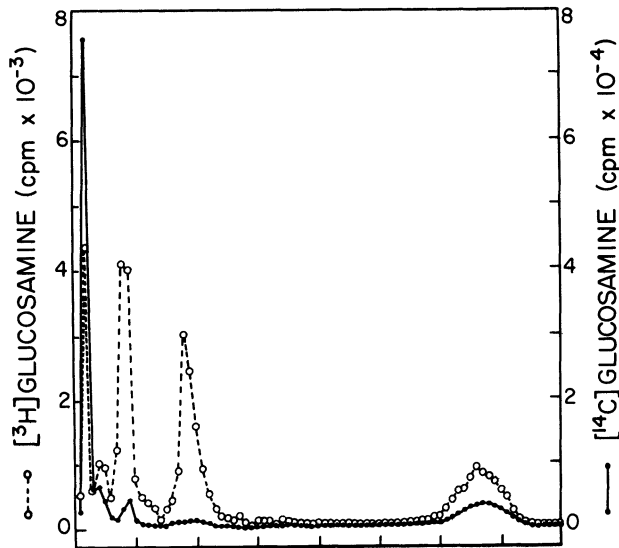as a function of pH.

Fig. 3. Co-electrophoresis of the glycoproteins of [^{3}H] glucosamine-labeled, control (pH 6.0, 25°C, O——O) and [^{14}C] glucosamine-labeled, inactivated (pH 8.0, 37°C, 2 hrs, ●——●) A59 virions.

inactivated at pH 8.0 at 37°C was quite different. Very little of this glycoprotein comigrated with the 90k and 180k molecular weight forms from native virions. Most of the E2 from pH 8.0 inactivated virus formed aggregates which remained at the top of the gel.

The other structural proteins, N and E1 were not affected. E1 from pH 6.0 and pH 8.0 treated virus comigrated as a single peak, and in other experiments, the electrophoretic mobility of amino acid labeled nucleocapsid protein was found to be unaltered after pH 8.0 inactivation of virus.

The distribution of E2 between monomeric forms and aggregates after incubation of virus at pH 6.0 and 8.0 at 4°C and 37°C is shown in Table 2.

About 80% of E2 remained as monomers when virus was held at 4°C at either pH 6.0 or pH 8.0, or at 37°C at pH 6.0. In contrast, after incubation of virus at 37°C at pH 8.0 for 2 hours, more than 80% of this glycoprotein was recovered in the form of aggregates at the top of the gel.

Table 2. Aggregation of Virion-Associated E2

Condition[a]		GP 180/90	Aggregate
pH	°C	(%)[b]	(%)[b]
6	4°	82	18
	37°	88	12
8	4°	80	20
	37°	17	83

[a] Virions incubated for 120 min in 100mM NaCl, 1 mM EDTA, 50 mM Tris
Maleate at the indicated pH and temperature.
[b] SDS gel electrophoretic distribution of [^{14}C] glucosamine-labeled E2.

E2 appears to contain both intramolecular disulfide bonds and free sulfhydryl groups (Sturman, L. S. and Holmes, K. V., in preparation). Both must be retained to maintain the native configuration of the peplomeric glycoprotein. As shown in Table 3 the sulfhydryl blocking reagent, p-chloromercuribenzoate (PMB), enhanced E2 aggregation. Incubation of virus in the presence of PMB (10 mM) for 30 minutes at 37°C at pH 8.0 caused 91% of the peplomeric glycoprotein to become aggregated, compared with 34% in controls.

Coronavirus structural proteins can be isolated by disruption of purified virus with the nonionic detergent NP40 at 4°, and the solubilized envelope glycoproteins and viral nucleocapsid can be separated by sucrose density gradient sedimentation (Sturman et. al., 1980).

As with virion associated E2, aggregate formation could be induced in NP40-solubilized E2 by incubation at pH 8.0 at 37°C (Table 4). Only a small proportion of the isolated solubilized glycoprotein became aggregated at pH 6.0 at 4°C or 37°C or at pH 8.0 at 4°C. However, by 2 hours at pH 8.0 at 37°C a substantial amount of aggregation had taken place. PMB enhanced aggregation of isolated E2 as well.

During inactivation of virus at pH 8.0 a portion of the 90k dalton form of E2 was released from the virus. However, none of the 180k dalton species was detached. Cleavage of GP180 with trypsin prior to inactivation at pH 8.0 resulted in release of increased amounts of GP90. A greater amount of E2 was released also as a consequence of disulfide bond reduction and sulfhydryl group blockage (Sturman and Holmes, in preparation). Recent evidence indicates that the two approximately 90k molecular weight products of proteolytic cleavage of GP180 can be distinguished. Hopefully future studies on these two parts of the peplomeric glycoprotein will provide

Table 3. Enhancement of Aggregation of Virion-Associated E2
by 30 Min Incubation with p-Chloromercuribenzoate (PMB)[a]

PMB (mM)	GP 180/90 (%)[b]	Aggregate (%)[b]
None	66	34
10	9	91

[a] Virus treated at pH 8 for 30 min at 37°C in 100 mM NaCl, 1 mM EDTA, 50 mM Tris Maleate, pH 8 with or without PMB.
[b] SDS gel electrophoretic distribution of [^{14}C] glucosamine-labeled E2.

information about the different functional domains within this large molecule.

A MODEL OF E2

A model of E2 based on these findings and results of our earlier investigations (Sturman, 1977; Sturman and Holmes, 1977; Sturman et al., 1980) is shown in Figure 4. E2 is probably attached to the viral envelope through a short hydrophobic region which penetrates the outer lipid bilayer. Carbohydrate side chains (represented by "lollipops") are present on both parts of the large hydrophilic portion of the molecule. Synthesis of E2 is sensitive to tunicamycin (Holmes, personal communication) and therefore the carbohydrate moieties are most likely N-glycosidically linked to the protein. One trypsin sensitive site is exposed near the middle of the amino acid chain. Proteolytic cleavage produces two species which have similar electrophoretic mobilities on SDS polyacrylamide gels. The portion

Table 4. Aggregation of NP40-Solubilized E2

Condition[a] pH	°C	GP 180/90 (%)[b]	Aggregate (%)[b]
6	4°	89	11
	37°	88	12
8	4°	82	18
		57	43

[a] NP-40 solubilized E2 incubated for 120 min in 100 mM NaCl, 1 mM EDTA, 50 mM Tris Maleate at the indicated pH and temperature.
[b] SDS gel electrophoretic distribution of [^{3}H] fucose-labeled E2.
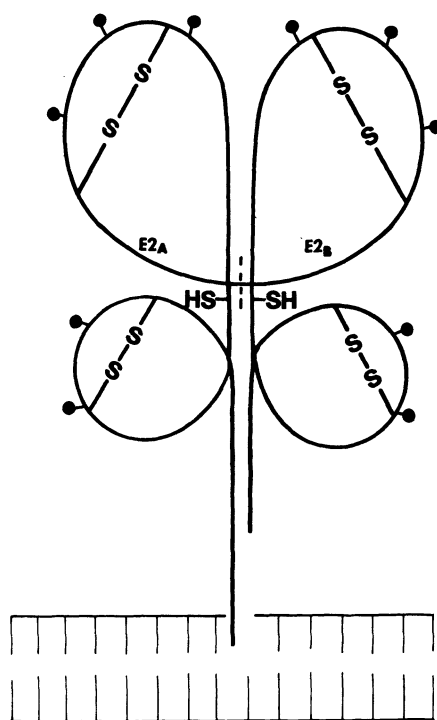
Fig. 4. A model of E2, the peplomeric glycoprotein.

attached to the viral envelope has been designated E2$_A$. The free segment E2$_B$. The condition of disulfide and sulfhydryl groups in E2 appears to be of paramount importance in determining the conformational state of the molecule.

ANOMALOUS BEHAVIOR OF E1 IN SDS-POLYACRYLAMIDE GELS

Let us turn now to the smaller envelope glycoprotein, E1. E1 is substantially different from E2. In the intact virion only a small portion of E1 is susceptible to proteases. Pronase or bromelain treatment of virus results in a decrease in the apparent molecular weight of E1 from 23k to 18k and loss of the carbohydrate containing region (Sturman, 1977; Sturman and Holmes, 1977). Thus most of the E1 molecule is concealed within the viral membrane, except for a small glycosylated portion which extends outside the lipid envelope.

E1 exhibits anomalous behavior on SDS–polyacrylamide gel electrophoresis. After heating to 100°C in SDS, the electrophoretic mobility of E1 is altered and several new forms of lower mobility are produced (Sturman, 1977). β-mercaptoethanol (β-MSH) and dithiothreitol (DTT) exaggerate the

effects of heating. These changes in E1 are shown in Figure 5. Figure 5A shows the profile obtained on a 10% cylindrical gel when [^{35}S] methionine-labeled A59 viral proteins were solubilized in SDS at 25°C. E1 migrated as a broad band with an average apparent molecular weight of 23k. The same pattern was obtained when the sample in SDS was treated at 37°C in the presence of 5% β-MSH. However, multiple (three to four) bands of E1 could be resolved in this region when run on slab gels with reducing agent under the same conditions. Heating the sample at 100°C in the absence of reducing agent resulted in the appearance of species with apparent molecular weights of 38k and 60k and a concomitant reduction in the amount of the 23k dalton species. This is shown in Figure 5B. A diffuse increase in the amount of labeled material in the upper region of the gel is also seen. If the sample was heated at 100°C in the presence of reducing agent, the amount of 23k dalton E1 was reduced further and a greater proportion was found near the top of the gel. The pattern obtained thus depended on the conditions employed in preparation of the sample: the temperature and the concentration of reducing agent. However, heating per se was not required to produce changes in the electrophoretic mobility of E1. Freezing also resulted in the appearance of the 38k dalton species. Prolonged treatment of virions with 7M guanidine followed by 9M urea and then 1% SDS all at 25°C in the presence of 1mM β-MSH, resulted in the same changes in electrophoretic mobility of E1 as heating at 100°C in the presence of a high concentration of reducing agent. This suggests that E1 is not completely denatured in SDS at 25°C or 37°C. Heating and reduction of disulfide bonds may promote unfolding of a region of E1 which was incompletely denatured and thus facilitate interactions between hydrophobic domains of the molecule.

INTERACTION OF E1 WITH THE VIRAL NUCLEOCAPSID

Studies with NP40-solubilized E1 illustrates that the conformation of this molecule determines its interaction with viral RNA.

E1 can be solubilized by NP40 at 4°C and separated from E2 and the viral nucleocapsid by sucrose density gradient sedimentation (Sturman et al., 1980). A profile of separated viral components labeled with [^{3}H] fucose and [^{35}S] methionine is shown in the upper panel of Figure 6. The large upper peak consists of [^{35}S] methionine-labeled E1. Below this in the gradient is a peak which contains most of the [^{3}H] fucose label and a small amount of [^{35}S] methionine. This is E2. On the high density cushion is a third peak which contains a large amount of [^{35}S] methionine and a small amount of [^{3}H] fucose. This consists of the nucleocapsid protein N, plus the viral RNA, and a small amount of incompletely solubilized E2. If the mixture of NP40-disrupted virions was incubated at 37°C for 30 minutes before sedimentation, a strikingly different pattern of labeled comonents was obtained. This is shown in the lower panel of Figure 6. The peak of [^{35}S] methionine-labeled E1 was not found at the top of the gradient. A new peak of [^{35}S] label was detected above the high density cushion, and the amount of radiolabeled material on the cushion was markedly decreased. Recovery of the new complex was incomplete due to its adherence to the
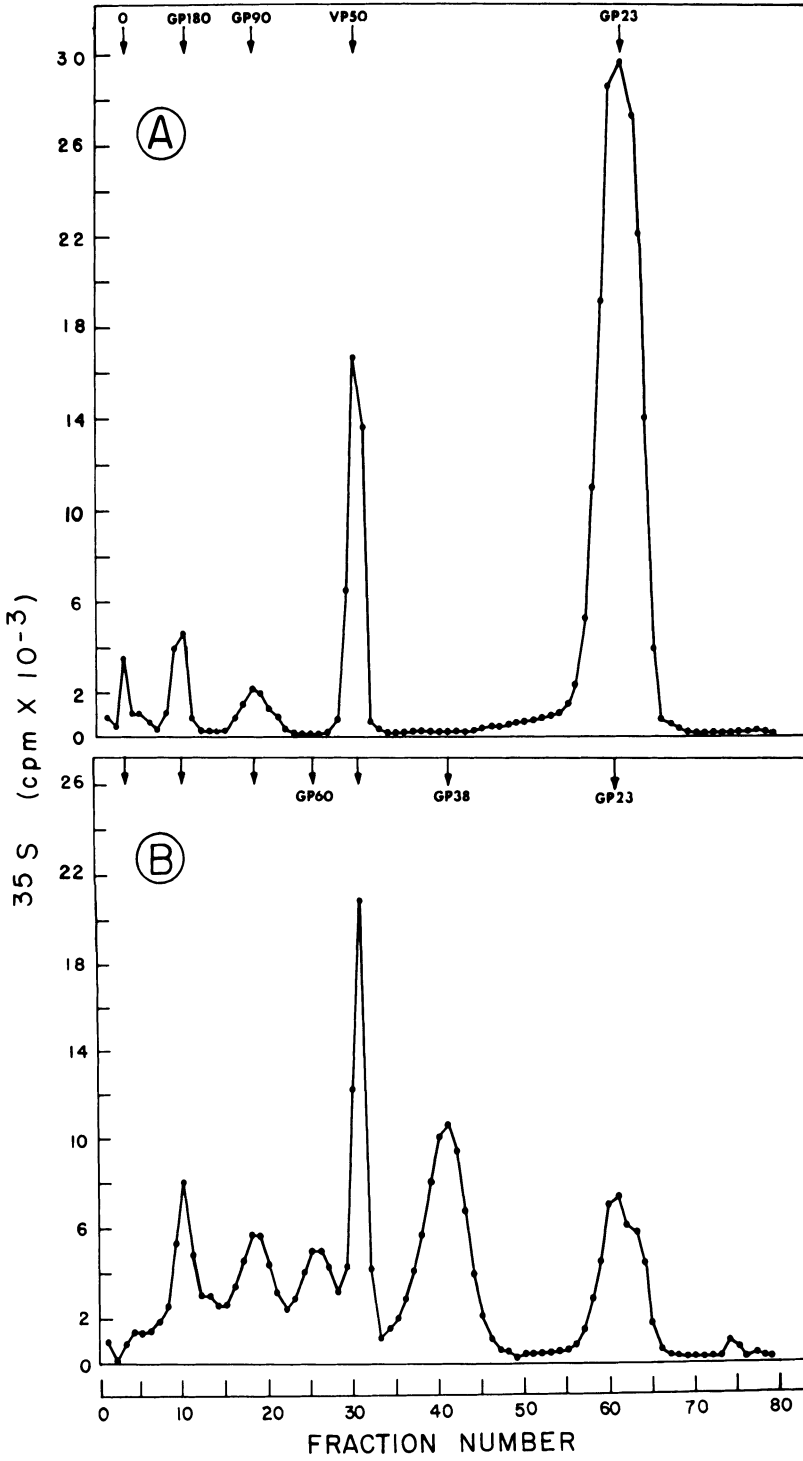
Fig. 5. Effect of boiling on the SDS–PAGE profile of E2. (A)25°C; (B)100°C.
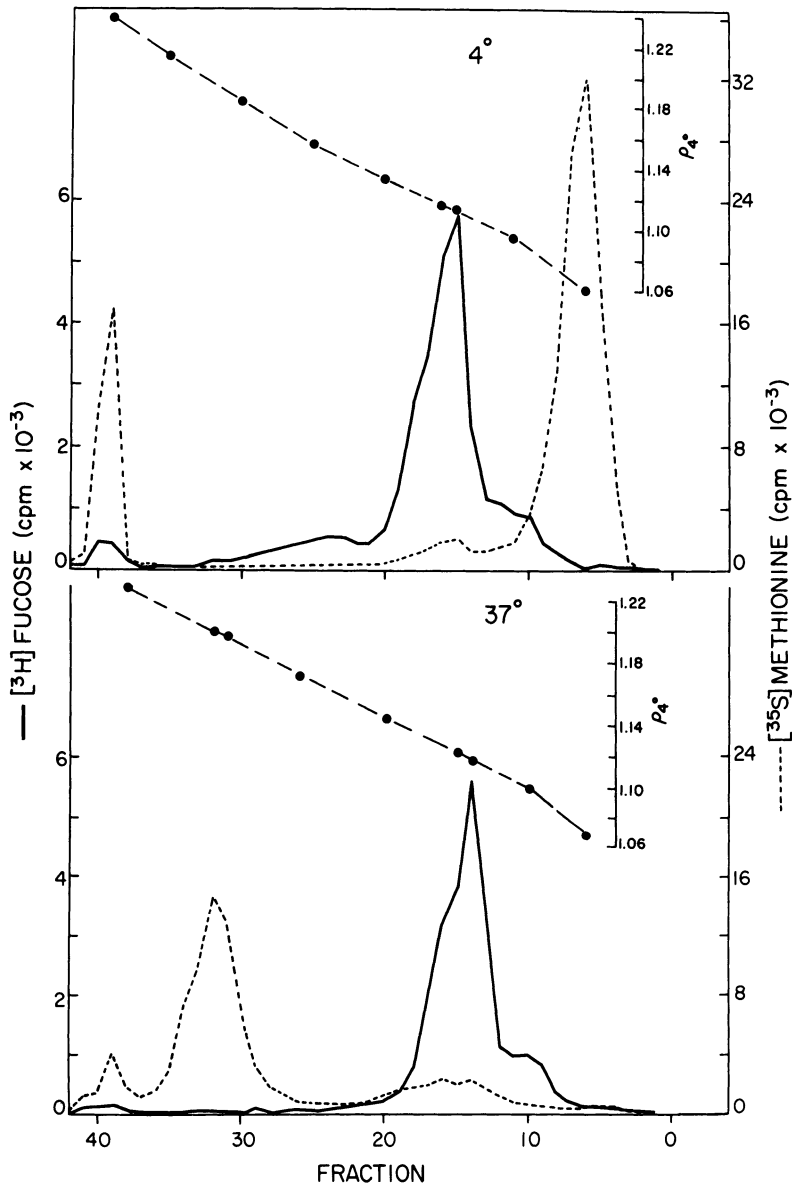
Fig. 6. Sucrose gradient (15-50%) distribution of A59 structural proteins after disruption of the viral envelope with 0.25% NP40 at 4°C and incubation at (upper panel) 4°C; (lower panel) 37°C for 30 min. (Sturman et al., 1980)

centrifuge tube. SDS gel electrophoretic analysis of the new peak revealed that it contained both E1 and N proteins. Experiments with [^{3}H] uridine-labeled virus showed that viral RNA was also present in this complex.

The E1-N-RNA complex could be reconstructed from E1 and viral nucleocapsids which had been isolated separately at 4°C. This is shown in Figure 7. E1 labeled with [^{35}S] methionine and nucleocapsids labeled with [^{3}H] uridine were isolated from virions by treatment with NP40 at 4°C, mixed and incubated at 4°C or 37°C for 30 min. The sedimentation profile obtained after components were mixed at 4°C is shown in Figure 7A and after incubation together at 37°C in Figure 7B.

Further experiments (Sturman et al., 1980) showed that the conformation of NP40-solubilized E1 was altered as the temperature was raised from 4°C to 37°C. At 37°C E1 bound to the RNA in the viral nucleocapsid.

A MODEL OF E1

These studies have led to a provisional model for E1 which is shown in Figure 8. This membrane glycoprotein appears to possess three domains: a small glycosylated hydrophilic region which projects outside the viral envelope, a hydrophobic portion within the membrane, and a third domain which may be associated with the viral RNA in the nucleocapsid inside the viral envelope. Because of the effects of β-MSH and DTT on E1 aggregation, disulfide bonds are placed within the hydrophobic region of the molecule.

DIFFERENCES IN CARBOHYDRATE SIDE CHAINS ON E1 and E2

There are significant differences in the carbohydrate side chains on E1 and E2. E1 and E2 glycopeptides have been analyzed by polyacrylamide gel electrophoresis after borate-ester formation using the method described by Weitzman et al. (1979). Borate ions react with neutral sugars converting them to charged species. The number of borate ions bound to a glycopeptide is a function of the composition, sequence and linkages of the carbohydrates. The electrophoretic patterns of pronase derived glycopeptides from E1 and E2 obtained on tris-borate gels are shown in Figure 9. Borate-glycopeptide complexes of E2 shown in Figure 9A produce a complex pattern of multiple peaks in the upper and middle regions of the gel. At least six components can be resolved by double labeling. The pattern obtained from E1 glycopeptides shown in Figure 9B is quite different. E1 borate-glycopeptide complexes exhibit less variety and are found in the lower third of the gel.

It was mentioned previously that synthesis of E2 is sensitive to tunicamycin. Nevertheless, in the presence of tunicamycin, A59 virus particles are still produced, although reduced in amount. These particles do not possess spikes, but they appear to contain the usual proportions of nucleocapsid and E1. SDS-polyacrylamide gel analysis of such virus is shown in Figure 10. Figure 10A is the profile of control virus labeled with [^{3}H]
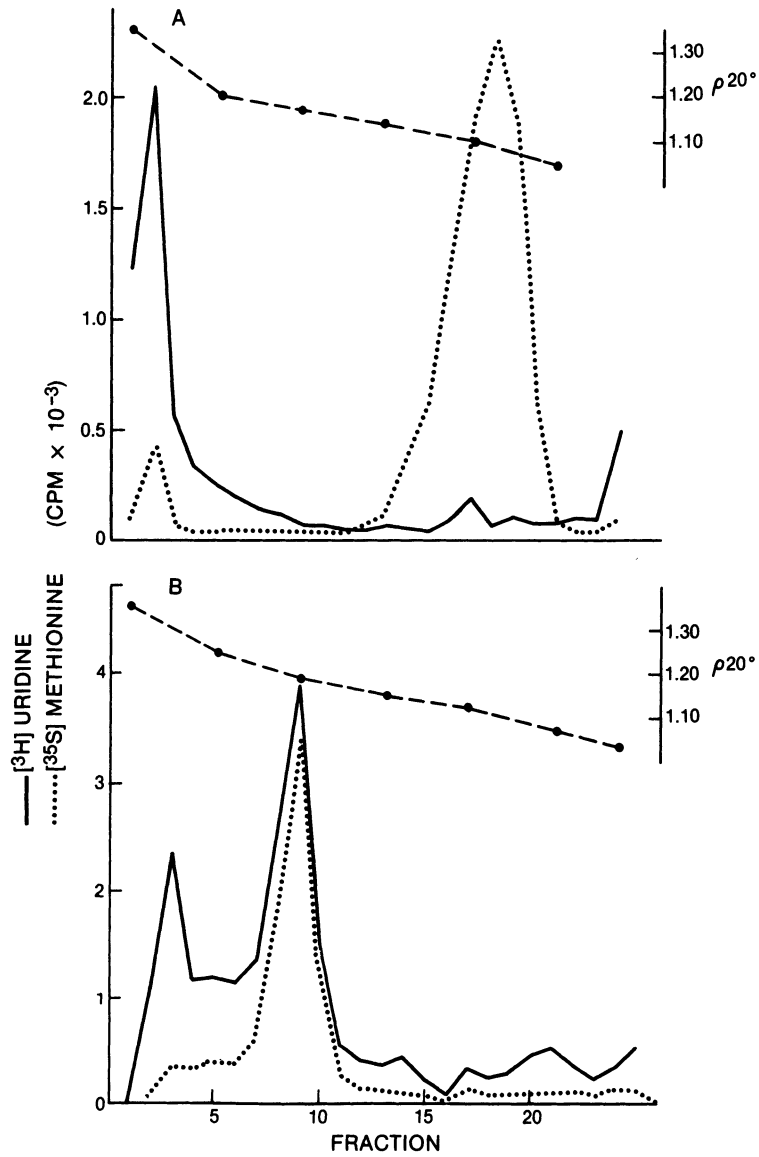
Fig. 7. Temperature-dependent association of isolated E1 and nucleocapsid. [^{35}S] methionine-labeled E1 and [^{3}H] uridine-labeled nucleocapsid were isolated at 4°C, mixed, incubated at (A) 4°C or (B) 37°C for 30 min, and then sedimented into 15 to 50% sucrose gradients (Sturman et al., 1980)
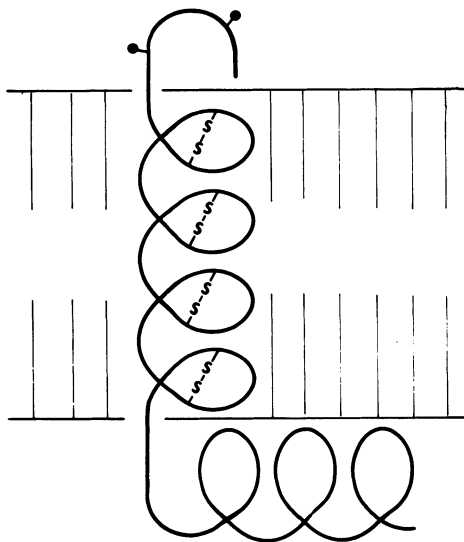
Fig. 8. A model of the membrane glycoprotein, E1.

glucosamine and [^{35}S] methionine. All three structural proteins can be
seen: E1, N and E2. Figure 10B is the profile of virions similarly labeled,
grown in the presence of 1 μg/ml tunicamycin. There is no E2. E1 and N
are found in the same relative proportions as in the control virus. Glycosy-
lation of E1 is not sensitive to tunicamycin. E1 is labeled to the same
degree with glucosamine as in the control and has the same apparent
molecular weight. The peplomeric glycoprotein, E2, thus appears not to be
required for virus maturation. Envelopment of the nucleocapsid may be
determined solely by interaction of viral RNA with the reactive domain of
the membrane glycoprotein, E1.

Studies by Neimann and Klenk (personal communication) which will be
described later at this meeting show that the carbohydrate composition of
E1 is different from that of viral glycoproteins with N-glycosidically linked
side chains. Their investigations suggest that the carbohydrate linkages in
E1 are of O-glycosidic nature. This is consistent with the resistance of E1
glycosylation to tunicamycin.

It should be quite apparent from this report that we are just beginning
to learn something about the coronavirus glycoproteins. We need to find out
a great deal more about their structure, functional domains, and their
dynamic interactions. Our future understanding of the molecular archi-
tecture of these viral glycoproteins will undoubtedly add to our compre-
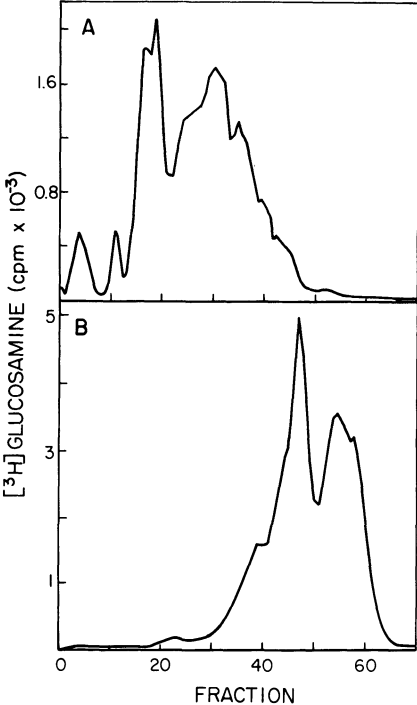hension of their roles in infection and disease.

Fig. 9. Tris-borate gel electrophoresis of (A) E2, (B) E1 glycopeptides.
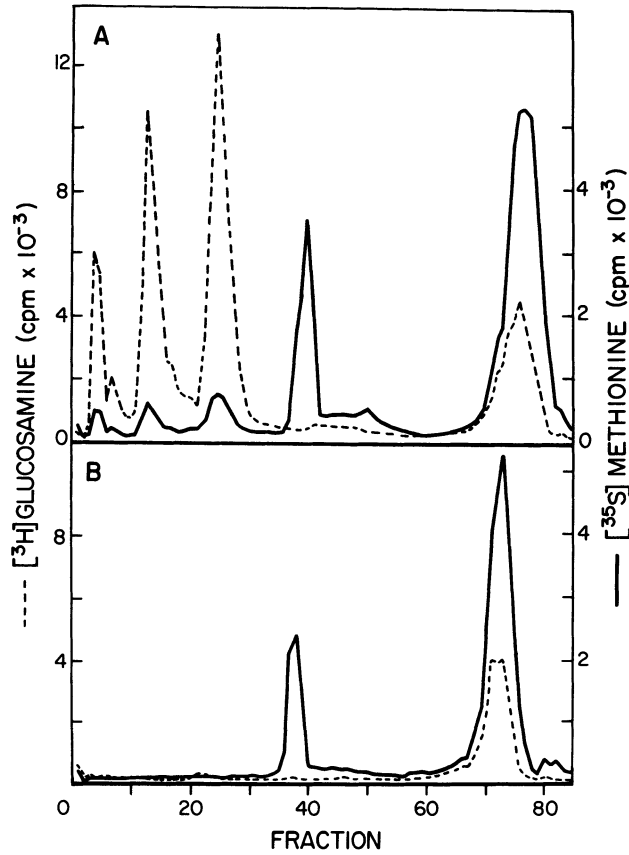
Fig. 10. SDS-polyacrylamide gel electrophoresis profiles of [^{3}H] glucosamine labeled, and [^{35}S] methionine-labeled structural proteins of corona-virus A59. (A) control virus; (B) virus grown in the presence of 1.0 µg/ml tunicamycin.

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