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Characterization of a Coronavirus[1]

I. Structural Proteins: Effects of Preparative Conditions on the Migration of Protein in Polyacrylamide Gels

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Coronavirus A59 possesses four size classes of structural proteins which have apparent molecular weights measured by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of 23,000 (GP23), 50,000 (VP50), 90,000 (GP90), and 180,000 (GP180). VP50 is the only structural protein which is completely unaffected by protease treatment of intact virions. This species is the most highly labeled by polar amino acids such as glutamic acid and arginine and it is probably associated with the viral nucleocapsid. GP90, GP180, and GP23 are membrane-associated proteins. However, after protease treatment of virions, only 20% of the GP23 molecule is digested, whereas all of the GP90 and GP180 are removed. GP90 and GP180 appear to comprise most of the prominent layer of characteristic projections on the external surface of the viral envelope. The major portion of GP23 is presumed to lie within the lipid envelope, protected from protease digestion. GP23 and the protease resistant portion, p*18, exhibit anomalous behavior on SDS-PAGE. After heating to 100° in SDS the electrophoretic mobility of these polypeptides is altered and several new forms of lower mobility are produced. β-Mercaptoethanol and dithiothreitol exaggerate the effects of heating.

INTRODUCTION

Coronaviruses are morphologically distinctive enveloped RNA viruses which bud from cytoplasmic membranes such as endoplasmic reticulum, cytoplasmic vesicles and the Golgi apparatus (McIntosh, 1974). While coronaviruses have been studied extensively morphologically, they have not been well-characterized chemically. Using the murine coronavirus A59, an analysis of coronavirus proteins was undertaken in this laboratory to identify the structural and functional roles of each component of the virion. Evidence will be presented that one of the structural proteins is located entirely within the core of the virion, while the others are associated with the viral envelope. Although six species are consistently resolved under the usual conditions of gel electrophoresis, our studies suggest that several of these species are different forms of the same molecule. There appear to be four size classes of structural proteins which have been designated GP180, GP90, VP50, and GP23. GP23 will be shown to exhibit anomalous behavior in SDS-polyacrylamide gels which gives rise to the appearance of additional species. The effect of the conditions of preparation, heating and reducing agents, on the electrophoretic migration of this protein in SDS-polyacrylamide gels will be analyzed.

[1] A portion of this work was presented at the 74th Annual Meeting of the American Society for Microbiology (Abstracts, p. 219, 1974).

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MATERIALS AND METHODS

Virus

The A59 strain of murine coronavirus, originally obtained from Dr. J. Parker, was used in the study. The virus was

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grown, assayed and purified as described elsewhere (Sturman and Takemoto, 1972; Sturman and Holmes, manuscript in preparation). Virus was radiolabeled by the addition of radioisotope(s) to the growth medium, complete Eagle's minimum essential medium containing 10% fetal calf serum, 50 ml/roller bottle, at the completion of the 1-hr adsorption period. The medium containing label was not removed until the virus was harvested.

Polyacrylamide Gel Electrophoresis

(a) Discontinuous buffer-high pH system: Initially the high pH-SDS discontinuous buffer system was employed as described by Laemmli (1970) and Maizel (1971). Samples were solubilized in a buffer which contained, in final concentrations, 0.060 *M* Tris-phosphate (pH 6.7), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol (β-MSH), and 0.001% bromphenol blue (BPB), and the mixtures were heated for 1.5–2 min at 100°. In some cases boiled samples were stored at 4° or −20° for several days and reboiled for 1.5 min before being placed on gels. Subsequently the amount and type of reducing agent and the conditions of heating were varied as indicated. The volume of the spacer gel was 1.2–2 times that of the sample; the 10% resolving gel was 20 cm long. Electrophoresis was carried out at 50 V for 22–26 hr. For estimation of molecular weights, gels containing reference proteins were fixed and stained at room temperature for 1 hr in a solution containing 2.50 g of Coomassie brilliant blue G250 (Diezel *et al.*, 1972), 454 ml of methanol, and 95 ml of glacial acetic acid per liter. Diffusion destaining was accomplished by incubating stained gels at 37° in 7.5% acetic acid–5% methanol with AG501-X8 Bio-Rad ion exchange resin, 4 g/20 cm of gel. Apparent molecular weights were determined according to Weber and Osborn (1975). The reference proteins used were β-galactosidase (130,000), ovalbumen (43,000), trypsin (23,000), cytochrome *c* (11,700), and *E. coli* RNA polymerase (165,000, 89,000, and 39,000). For liquid scintillation spectrometry, the gels were crushed in 1- or 2-mm portions on the Gilson automatic linear gel fractionator (Gilson *et al.*, 1972) and collected in scintillation vials in a stream of water. In some cases the gel was fractionated from top to bottom, in the reverse direction as it is usually performed with this apparatus. When fractionation began at the top, the spacer gel was placed in the first vial. The water was evaporated at 60° and each vial was filled with 10 ml of scintillation mixture composed of 102 ml of NCS-water (5.5:1), 160 ml of Liquifluor, and 3683 ml (1 gal minus 102 ml) of toluene (Kiehn and Holland, 1970). The filled vials were incubated at 37° overnight and counted in a Beckman LS 250 liquid scintillation spectrometer.

(b) Neutral pH-SDS phosphate gel system: The procedure described by Weber and Osborn (1975) was employed. Preparation of the samples is described in the figure legend.

(c) Neutral pH-SDS phosphate, urea gel system: The procedure given by Uemura and Mizishima (1975) was employed. The concentration of urea in the gel was 7 *M*. The details of sample preparation are given in the figure legend.

Protease Treatment of Virus

Purified virus which had been sedimented to equilibrium in sucrose gradients in a buffer containing 0.05 *M* Tris-maleate, 0.001 *M* EDTA, 0.1 *M* NaCl, pH 6.0 at 4° (TMEN, pH 6) was diluted two- to fivefold with TMEN (pH 6) and incubated at 37° with pronase (Sigma, Protease VI) or bromelain (see below). Pronase was preincubated for 1 hr at 37° before use. After specified intervals, pronase treated virus was resedimented in an SW 27.1 rotor at 25,000 rpm overnight in a linear 20–50% sucrose gradient at 4°. This rebanded treated virus was either diluted to 17.5 ml with TMEN (pH 6) and pelleted in an SW 27.1 rotor at 25,000 rpm for 50 min, or diluted to 5.5 ml and sedimented in an SW 50.1 rotor at 45,000 rpm for 3 hr. In some cases the pellet was resuspended in complete Laemmli buffer, 0.060 *M* Tris-phosphate (pH 6.7), 2% SDS, 10% glycerol, 5% β-MSH, and 0.001% BPB, and the sample was heated at 100° for 2 min. In other experiments the pellet was resuspended in

0.060 *M* Tris-phosphate and treated further as indicated in the text.

Bromelain treatment was carried out in the presence of 0.1 m*M* β-MSH. The rebanded treated virus was diluted to 17.5 ml with TMEN (pH 6) containing 1 m*M* *p*-chloromercuribenzoate (PCMB) (Calbiochem) and pelleted in an SW 27.1 rotor at 25,000 rpm for 50 min. The pellet was resuspended in 0.060 *M* Tris-phosphate, pH 6.7, and treated as described in the figure legend.

Chemicals and Isotopes

Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylenediamine were purchased from Eastman Organic Chemicals, Rochester, N.Y.; ammonium persulfate, bromphenol blue, and urea from Fisher Chemical Co., Fairlawn, N.Y.; sodium dodecyl sulfate from Matheson, Coleman & Bell, Norwood, Ohio; β-mercaptoethanol from Eastman and Matheson, Coleman & Bell; Coomassie brilliant blue G-250 from Gallard-Schlesinger Chemical Manufacturing, Carle Place, N.Y.; β-galactosidase, trypsin, and Tris-maleate from Sigma Chemical Co., St. Louis, Mo.; Tris (ultrapure) from Schwarz/Mann, Orangeburg, N.Y.; cytochrome *c* and dithiothreitol from Calbiochem, La Jolla, Calif.; ovalbumen from Worthington Biochemical Corp., Freehold, N.J.; NCS solubilizer from Amersham-Searle Corp., Arlington Heights, Ill.; mixed bed resin AG-501-X8, 20–50 mesh, analytical grade, from Bio-Rad, Richmond, Calif.; and Liquifluor from New England Nuclear Corp., Boston. *E. coli* RNA polymerase was a gift from Dr. R. Trimble. Purified bromelain was a gift from Dr. A. Tarentino.

L-[2,3-^{3}H]Valine, 16–23 Ci/mmol, L-[*carboxyl*-^{14}C]valine, 33 mCi/mmol, L-[U-^{14}C]glutamic acid, 260 mCi/mmol, and L-[*carboxyl*-^{14}C]leucine, 50 mCi/mmol were obtained from Schwarz/Mann. L-[*guanido*-^{14}C]arginine, 26 mCi/mmol, L-[3-^{3}H]glutamic acid, 24 Ci/mmol, L-[^{3}H]amino acid mixture, L-[U-^{14}C]amino acid mixture, and L-[^{35}S]methionine, 157 Ci/mmol, were purchased from New England Nuclear. L-[^{35}S]methionine, 230–250 Ci/mmol, was also purchased from Amersham-Searle.

RESULTS

Identification of Coronavirus Polypeptides and Characterization Based on the Ratios of Incorporation of Different Radiolabeled Precursors

Six major polypeptide species were detected on SDS-polyacrylamide gels by electrophoretic analysis of purified radiolabeled coronavirus A59, performed according to the procedure described by Laemmli. The profile of radioactivity in polypeptides labeled with a mixture of [^{3}H]amino acids is shown in Fig. 1. The two smallest polypeptides which had apparent molecular weights of 23,000 and 38,000 have been designated GP23 and GP38. In the following report (Sturman and Holmes, 1977) it will be shown that the species designated GP contain carbohydrate. A polypeptide with a molecular weight of 50,000 (VP50) was present in the largest amount. A fourth polypeptide with

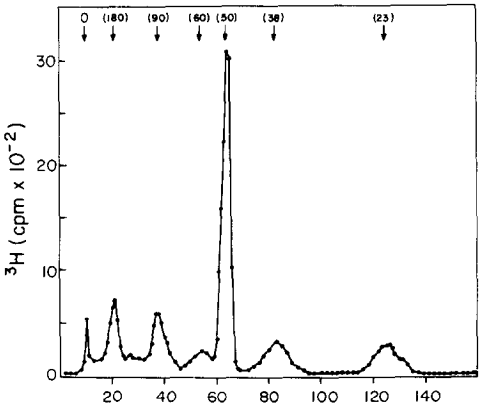

FIG. 1. SDS-polyacrylamide gel electrophoresis of the polypeptides of the coronavirus A59 virion separated by the high pH-discontinuous buffer system of Laemmli. The virus was labeled for 25 hr with a mixture of ^{3}H-labeled acids, 2 μCi/ml. 5% β-MSH was present in the sample which was heated at 100° for 1.5 min. Apparent molecular weights are given in parentheses. In this and the following figures, arrows indicate the position of the peak fractions of the virion polypeptides and in some cases the origin (O) of the resolving gel. The anode is to the right.

an apparent molecular weight of 60,000 (GP60) can be distinguished and also two larger polypeptides with apparent molecular weights of 90,000 (GP90) and 180,000 (GP180). In some cases a moderate amount of aggregated protein is found at the origin (Fig. 1, see discussion).

As a means of classifying the structural proteins according to the extent of labeling with hydrophobic and hydrophilic amino acids, and to determine whether particular amino acids can be used to label individual proteins preferentially, A59 virus was labeled with combinations of ^{3}H-, ^{14}C-, and ^{35}S-labeled amino acids, and viral polypeptides were characterized on the basis of the ratios of incorporation of these labeled precursors.

The ratio of incorporation of valine and a mixture of amino acids was found to be the same for all of the polypeptide species (data not shown). Valine was used therefore as the standard for comparison with other individual amino acids. Based on the ratios of incorporation of five amino acids, valine, arginine, glutamic acid, leucine, and methionine, coronavirus polypeptides can be separated into three classes: (1) VP50, (2) GP23 and GP38, and (3) GP90 and GP180. The polypeptide present in largest amount, VP50, was found to be the most highly labeled with arginine and glutamic acid and least labeled by leucine and methionine, relative to valine (Figs. 2A–D). The two smallest polypeptides, GP23 and GP38, were the most highly labeled by methionine and poorly labeled with glutamic acid relative to valine (Figs. 2D, B). GP90 and GP180 were intermediate in methionine and glutamic acid-labeling. GP60 could not be distinguished well in these experiments. In terms of carbohydrate labeling, GP60 is similar to GP23 and GP38 (Sturman and Holmes, 1977).

The ratios of labels in single peak fractions are summarized in Table 1. The ratio of arginine:valine in VP50 is more than twice as high as in any other protein. VP50 is also labeled significantly more by glutamic acid than the other proteins, particularly GP23 and GP38. With leucine, less pronounced differences were found. The ratio of leucine:valine in VP50 is about 20% less than in the other proteins. The most striking differences were obtained with methionine. The ratio of methionine:valine in GP23 and GP38 is almost threefold higher than in VP50 and almost twice as great as in GP90 and GP180.

Localization of Coronavirus Proteins in the Virion Based on Susceptibility to Proteases

Intact virus was treated with proteolytic enzymes to identify those proteins which are exposed on the surface of the virus envelope. Similar results were obtained with pronase and bromelain. As shown in Fig. 3, only VP50 is unaffected by pronase treatment and several new polypeptide species are formed. The derivation of these new species, p*18, p*31, and p*48, was investigated by analysis of pronase-treated virions doubly labeled with methionine and glutamic acid. Methionine-labeling is greatest in GP23, GP38, and GP60, while glutamic acid label is found primarily in VP50 (Table 1).

At a concentration of pronase of 1.0 mg/ml, treatment for 3 hr at 37° produced a complete loss of all of the original species except VP50, accompanied by the appearance of p*18, p*31, and p*48. This treatment reduces virus infectivity by 4.5 log. VP50 was highly labeled with glutamic acid, but this label was virtually absent in p*18, p*31, and p*48 (data not shown). Therefore it is unlikely that these species have been derived from VP50. The level of methionine-label in p*18 and p*31 was about 70% of that in GP23 and GP38 in the control (data not shown), whereas the quantities of methionine-label in GP90 and GP180 were insufficient to account for the amounts in p*18 and p*31. Therefore p*18, p*31, and p*48 appear to be derived from GP23, GP38, and GP60. The relationship between GP23 and p*18 has been corroborated by tryptic peptide analysis (unpublished data). The origin of p*31 and p*48 is discussed further below.

The resistance of those polypeptides which are insusceptible to pronase in the intact virion was tested under conditions in which the virus envelope was destroyed. Incubation with 1 mg/ml of pronase for 2

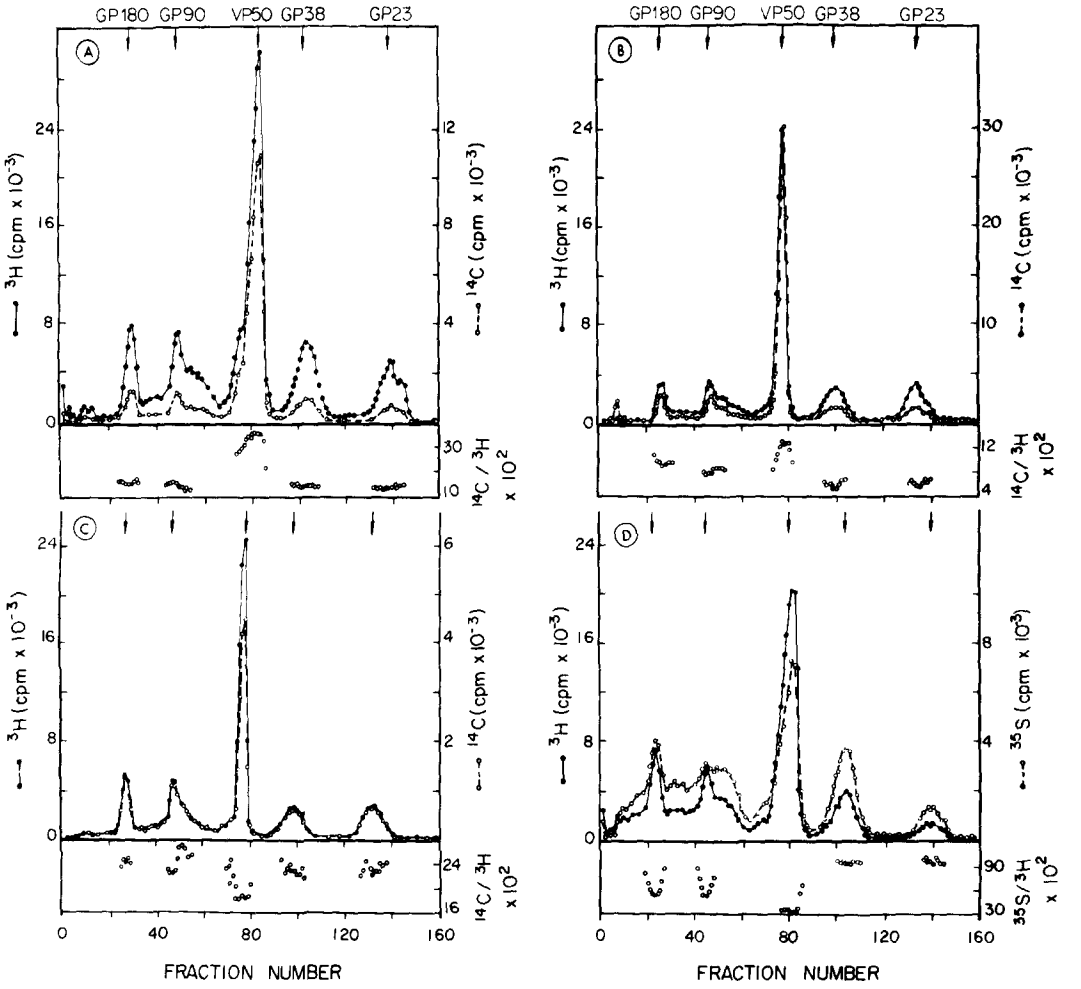

FIG. 2. SDS-polyacrylamide gel electrophoresis (Laemmli system) of the polypeptides of the coronavirus A59 virion labeled with different combinations of amino acids: (A) [^{3}H]valine (●——●) and [^{14}C]arginine (O- -O); (B) [^{3}H]valine (●——●) and [^{14}C]glutamic acid (O- -O); (C) [^{3}H]valine (●——●) and [^{14}C]leucine (O- -O); and (D) [^{3}H]valine (●——●) and [^{35}S]methionine (O- -O). Virus was grown for 25-38 hr in the presence of 4 μCi/ml of [^{3}H]valine and either 0.2 μCi/ml of [^{14}C]arginine, 0.5 μCi/ml of [^{14}C]glutamic acid, 0.5 μCi/ml of [^{14}C]leucine, or 1 μCi/ml of [^{35}S]methionine. 5% β-MSH was present in each sample mixture which was heated at 100° for 1.5-3.0 min. The lower panels show the ratios of ^{14}C:^{3}H or ^{35}S:^{3}H. The ratios in the peak fractions are designated by filled circles (●).

hr in the presence of 0.1% SDS produced complete degradation of all viral proteins (data not shown), although treatment of intact virus with as much as 2 mg/ml of pronase for 16 hr at 37° in the absence of SDS did not result in the destruction of VP50 or of p*18, p*31, and p*48.

From the results of these experiments five of the six polypeptide species are identified as membrane proteins. GP90 and GP180 are completely accessible to pronase and bromelain and therefore appear to reside outside the viral membrane, while GP23, GP38, and GP60 possess large domains which are protected from these proteases, and these portions presumably lie within the viral envelope. VP50, a major species and the only polypeptide localized wholly within the virion, can be considered a core protein.

Effects of Conditions of Preparation (Heat and Reducing Agents) on the Migration of Coronavirus Protein in Polyacrylamide Gels

In order to eliminate the possibility of aggregate formation during sample preparation, a series of experiments was performed analyzing coronavirus A59 polypeptides after alkylation in 6-8 *M* guanidine. Alkylation under these conditions resulted in the formation of aggregates of GP90 and GP180 (unpublished data, see discussion). Therefore another approach was taken: the conditions of preparation of the sample for SDS-PAGE were varied, and the effects were analyzed. As a result, the electrophoretic profile of coronavirus A59 viral proteins was found to be highly dependent upon the conditions employed for preparation.

TABLE 1

RATIOS OF INCORPORATION OF VARIOUS AMINO ACIDS INTO DOUBLY LABELED A59 VIRION POLYPEPTIDES

Polypeptide species	$^{14}C/^{3}H$ and $^{35}S/^{3}H$ ratios × 10^2 in peak fractions			
	Arginine/valine	Glutamic acid/valine	Leucine/valine	Methionine/valine
GP180	16	9	24	54
GP90	16	8	23	53
VP50	37	13	18	33
GP38	15	5	23	93
GP23	14	5	22	93

The effects of heat and reducing agents on the PAGE pattern of coronavirus proteins are shown in the following experiments. If the viral proteins were solubilized in SDS at 25°, without β-MSH or dithiothreitol (DTT), only four polypeptide species were seen on PAGE: GP23, VP50, GP90, and GP180 (Fig. 4A). The same pattern was obtained when the sample was dissolved in SDS at 37° in the presence of 5% (749 m*M*) β-MSH (data not shown). Heating the sample at 100° in the absence of β-MSH or DTT resulted in the appearance of GP38 and GP60, and a concomitant reduction in the amount of GP23 (Fig. 4B). A diffuse increase in the amount of label retained in the upper portion of the gel was also apparent. In this experiment the polypeptides were labeled with [^{35}S]methionine to accentuate GP23. GP23 is threefold more highly labeled by methionine than VP50.

If the sample was heated at 100° in the presence of β-MSH or DTT, there was a further decrease in the amount of the polypeptide migrating as GP23 and a greater proportion was found near the top of the gel. The individual pattern obtained depended on the duration for which the sam-

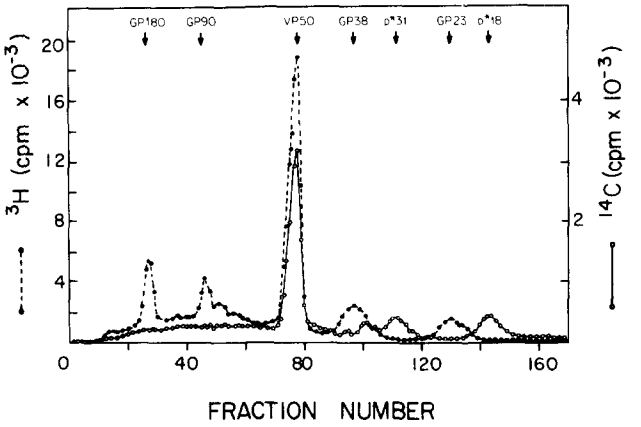

FIG. 3. Coelectrophoresis of the polypeptides of [^{3}H]valine (control, ●- - -●) and [^{14}C]valine (pronase treated, O——O) labeled coronavirus A59 virions. Virus was grown for 34 hr in the presence of 4 μCi/ml of [^{3}H]valine or 1 μCi/ml of [^{14}C]valine. Purified [^{14}C]valine-labeled virus was treated with pronase, 2 mg/ml, for 2 hr at 37° and repurified (see Materials and Methods for details). Pelleted pronase-treated virions resuspended in complete Laemmli buffer were heated to 100° for 2 min and mixed with [^{3}H]valine-labeled control virus which had been boiled for 2 min.

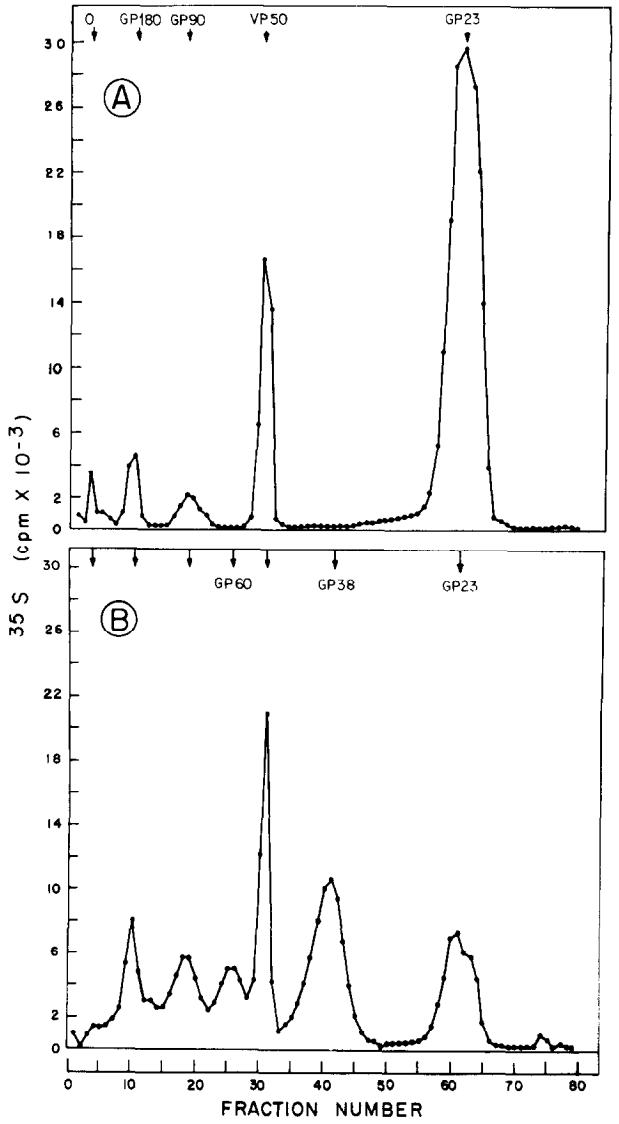

FIG. 4. Effects of boiling on the electrophoretic pattern of coronavirus virion polypeptides. A59 virus was grown for 24 hr in the presence of [^{35}S]methionine, 3 μCi/ml. Purified pelleted virus was resuspended in 0.060 *M* Tris-phosphate (pH 7.0), 2% SDS, 10% glycerol, and 0.001% BPB at 25° and divided into two parts. One portion was analyzed directly (without heating or addition of β-MSH) while the other was first heated at 100° for 30 min without β-MSH. (A) sample unheated, no β-MSH; (B) sample heated at 100° for 30 min, no β-MSH.

ple was boiled and on the concentration of β-MSH or DTT. The effects of boiling and two concentrations of β-MSH and DTT are shown in Fig. 5 (A–E). All samples were boiled for 2 min. The control pattern from an aliquot boiled without β-MSH or DTT (Fig. 5A) was similar to the pattern obtained previously (Fig. 4B). The presence of 5% (749 m*M*) β-MSH or 500 m*M* DTT resulted in a substantial decrease in the GP23 peak with a corresponding increase in the amount of label migrating in the region from the origin to GP90 (Fig. 5B,D). A twofold higher concentration of β-MSH or DTT in the boiled sample resulted in an almost complete shift in GP23 to the top of the gel (Fig. 5C,E).

None of the proteins which were em-

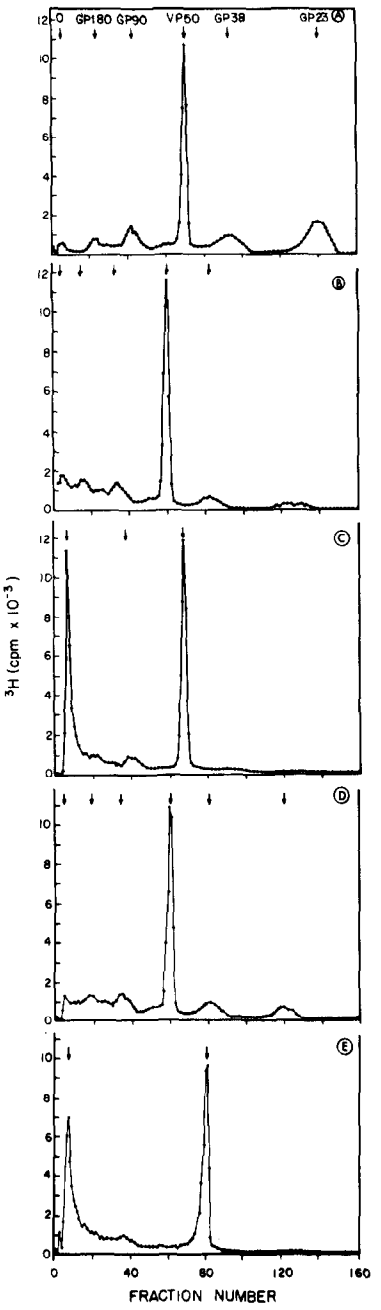

FIG. 5. Effect of reducing agents on the electrophoretic pattern of coronavirus virion polypeptides. A59 virus was grown for 24 hr in the presence of 4 μCi/ml of [^{3}H]-labeled amino acid mixture. Purified virus was resuspended in 0.060 *M* Tris–phosphate (pH 7.0), 2% SDS, 10% glycerol, and 0.001% BPB and divided into five portions. β-MSH or DTT was added to some samples before boiling. All samples were heated at 100° for 2 min before analysis. (A) no β-MSH or DTT, (B) 0.75 *M* β-MSH (5%), (C) 1.49 *M* β-MSH (10%), (D) 0.50 *M* DTT, and (E) 1.0 *M* DTT.

ployed as molecular weight markers nor the viral proteins, VP50, GP90, and GP180, exhibited such behavior upon heating in SDS either with or without β-MSH or DTT.

The SDS–gel electrophoresis experiments described thus far were performed in the high pH–discontinuous buffer system popularized by Laemmli. The procedure which Laemmli employed involved heating the sample at 100° for 1.5 min in the presence of 5% β-MSH before placing it on the gel. The effects of boiling and β-MSH upon the migration of GP23 were also investigated in neutral pH–continuous buffer phosphate gels and phosphate gels with urea. In both of these systems, as in the high pH–discontinuous buffer system, only four polypeptide species were found as long as the samples were prepared at 25° (Fig. 6A,C). Heating at 100° and addition of β-MSH both in the presence and absence of urea resulted in a reduction in the amount of GP23 and the appearance of slower migrating species (Fig. 6B,D). Thus, GP23 may give rise to several species of apparently larger size on SDS–polyacrylamide gels, including GP38 and GP60, depending upon the conditions employed in the preparation of the sample, regardless of the pH (7.2 or 9.5), buffer ion (phosphate or glycine), or presence of urea.

Since p*31 and p*48 were considered earlier to be derived from GP38 and GP60 and these in turn have been shown to arise from GP23, the effects of heating and β-MSH on the appearance of p*31 and p*48 in bromelain-treated virions was examined. Residual bromelain activity was blocked by addition of 1 m*M* PCMB before the final pelleting. By this means it was possible to prevent degradation of the surviving polypeptides after solubilization in SDS, without boiling. If the sample was prepared for PAGE without boiling, bromelain treatment of the virions resulted in the disappearance of GP180, GP90, and GP23, and the appearance of a single new polypeptide product, p*18 (Fig. 7A). VP50 was unaffected by bromelain treatment.

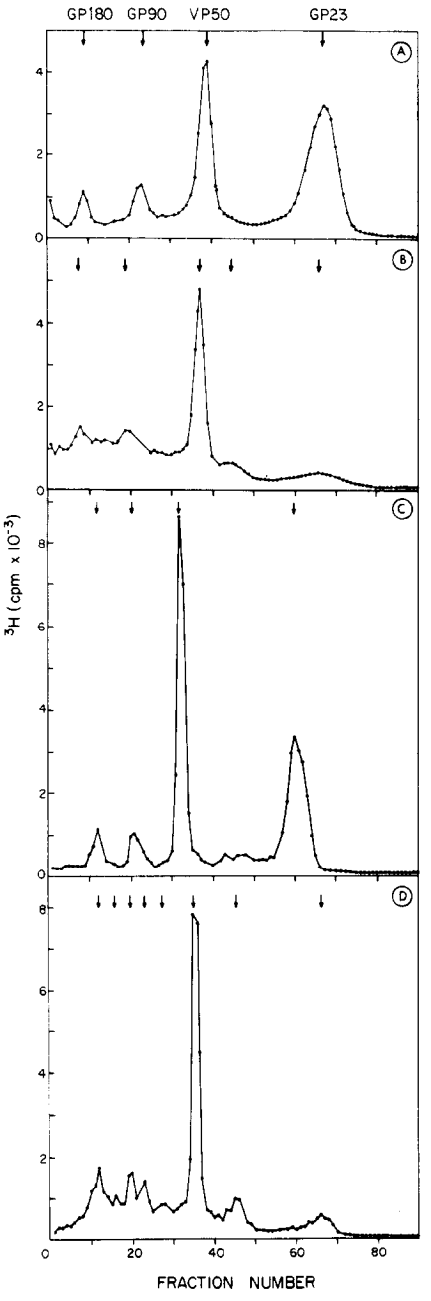

FIG. 6. Effects of heating at 100° and β-MSH on the pattern of coronavirus virion polypeptides separated by PAGE in neutral pH-SDS phosphate gel systems with and without urea. A59 virus was grown for 24 hr in the presence of 4 μCi/ml of [^{3}H]valine. Purified virus was suspended in 0.01 *M* phosphate buffer (pH 7.2), 1% SDS, 0.001% BPB, and 10% glycerol. This mixture was divided into two parts, one receiving 8 *M* urea. Each half was further divided into two portions. One portion of each was analyzed directly while 10% β-MSH was added to the other and this sample was heated to 100° for 5 min before analysis. (A) and (B) are 7.5% neutral pH-SDS phosphate gels; (C) and (D) are 8% neutral pH-SDS phosphate gels containing 7 *M* urea. (A) and (C) no heating, no β-MSH; (B) and (D) 100° for 5 min, 10% β-MSH.

After bromelain treatment there is an absence of label accumulated at the origin. Both the control and enzyme-treated samples contain a small amount of GP23 in the form of GP38.

After boiling with β-MSH, the polypeptide pattern from such [^{14}C]valine-labeled virions exhibited the appearance of p*31, a broad shoulder on the leading edge of VP50 (p*48), and the accumulation of a substantial amount of [^{14}C]valine-labeled polypeptide in the upper third of the gel. [^{14}C]Valine-labeled p*18 was decreased proportionately (Fig. 7B). Thus p*31, p*48, and other slower migrating species were derived from p*18 as a result of boiling in an analogous fashion to the generation of GP38 and GP60 from GP23. The effects of boiling and reducing agents on the electrophoretic mobility of GP23 and p*18 are summarized in Fig. 8.

DISCUSSION

Based on the results described in this and in the following paper (Sturman and Holmes, 1977), coronavirus A59 possesses four size classes of structural proteins representing three different species.

(1) VP50 is the most highly labeled by polar amino acids such as glutamic acid and arginine and least highly labeled with apolar, hydrophobic amino acids such as methionine and leucine. VP50 is unaffected by pronase or bromelain treatment of the intact virion and probably represents an inner core protein. VP50 is approximately the same size as the major core proteins found in several other enveloped RNA viruses. Myxoviruses, paramyxoviruses, and rhabdoviruses, all of which have nucleocapsids with helical symmetry, contain a core polypeptide species 50,000 to 60,000 in molecular weight (Lenard and Compans, 1974).

(2) The remaining coronavirus proteins

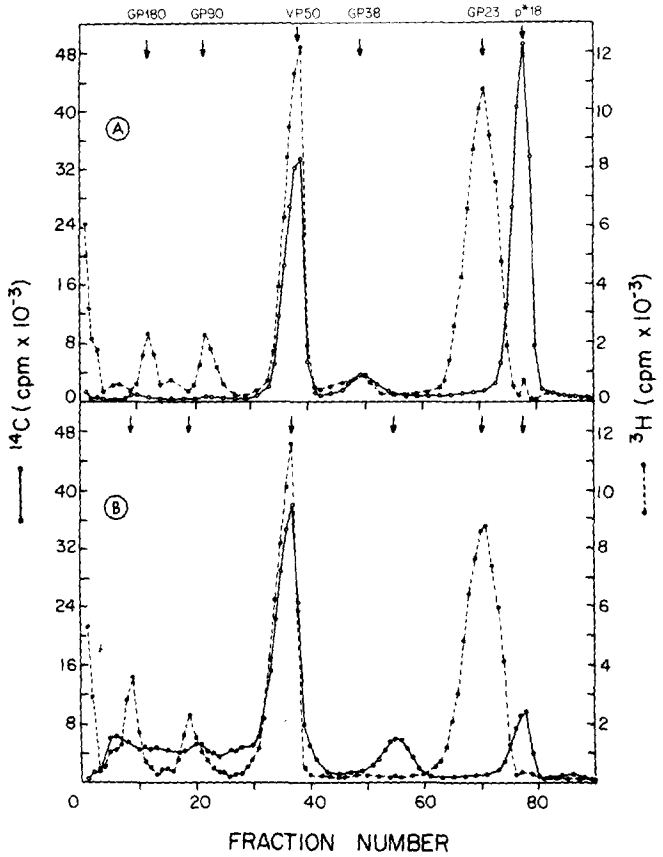

FIG. 7. Effects of boiling and β-MSH on the electrophoretic pattern of bromelain-treated A59 virion polypeptides; coelectrophoresis of the polypeptides of [^{3}H]valine-labeled (control, ●- - -●) and [^{14}C]valine-labeled (bromelain-treated, ○——○) virions. A59 virus was grown for 24 hr in the presence of 4 μCi/ml of [^{3}H]valine or 1 μCi/ml of [^{14}C]valine. Purified [^{14}C]valine-labeled virions were incubated at 37° for 3 hr in TMEN (pH 6) with 1 mg/ml of bromelain plus 0.1 m*M* β-MSH; [^{3}H]valine-labeled virus was kept at 4° in TMEN (pH 6). Both preparations were repurified (see Materials and Methods) and treated as follows: to one half of the bromelain-treated [^{14}C]-labeled virus, SDS, glycerol, and BPB were added to final concentrations of 2%, 10%, and 0.001%, respectively. This sample was mixed with one half of the [^{3}H]valine-labeled untreated virus similarly diluted at room temperature, and the mixture was placed immediately on a gel. The remainder of the bromelain-treated [^{14}C]valine-labeled virus received 2% SDS, 10% glycerol, 0.001% BPB, and 5% β-MSH and was boiled for 5 min. After cooling to room temperature, the sample was mixed with the remainder of the [^{3}H]valine-labeled untreated virus which was resuspended in SDS with glycerol and BPB at 25°, and these were coelectrophoresed on a second gel. (A) bromelain-treated and untreated virus, both samples unheated, no β-MSH; (B) bromelain-treated virus heated at 100° for 5 min with 5% β-MSH; untreated virus, unheated, no added β-MSH before mixing with the bromelain-treated sample.

are membrane-associated. GP90 and GP180 appear to comprise the peplomers on the external surface of the viral envelope which give a characteristic appearance to these viruses by negative contrast electron microscopy. The apparent molecular weight of these proteins as estimated from SDS-PAGE is substantially greater than the species which comprise the surface projections of other enveloped viruses such as togaviruses, myxoviruses, paramyxoviruses, and rhabdoviruses (Lenard and Compans, 1974). In the following report, it will be shown that GP90 and GP180 produce identical tryptic peptide patterns, and GP180 can be converted to GP90 *in vitro* by treatment of virions with trypsin (Sturman and Holmes, 1977).

GP90 and GP180 have been found to undergo aggregation under a variety of con-

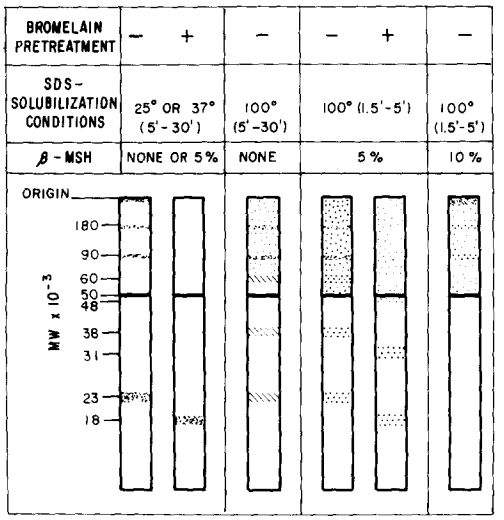

FIG. 8. Summary schematic illustration of the effects of boiling and reducing agents on the migration of coronavirus A59 polypeptides analyzed by SDS-PAGE. Heating the SDS-solubilized sample at 100° in the absence of β-MSH (or DTT) results in the appearance of species with apparent molecular weights of 38,000 and 60,000 and reduction in the amount of GP23. Heating in the presence of 5% β-MSH (or 0.5 *M* DTT) gives rise to more species with lower mobilities. After heating with 10% β-MSH (or 1.0 *M* DTT), almost all of the GP23 may be found at the top of the gel. p*18, which is derived from GP23 by bromelain treatment of virions, similarly gives rise to species with apparent molecular weights of 31,000, 48,000, and others with lower mobilities.

ditions including gel filtration and alkylation in 6–8 *M* guanidine (unpublished data) and in SDS-PAGE (Sturman and Holmes, 1977; Sturman and Holmes, manuscript in preparation). These aggregates comprise the material which is found at the origin of some polyacrylamide gels, especially after preparation of samples without boiling. In contrast, GP23 is found at the origin only after boiling in the presence of very high levels of reducing agents (Fig. 5).

(3) GP23 is partially exposed and partially concealed within the viral envelope. About 20% of the GP23 molecule is lost upon pronase or bromelain digestion of the intact virus. The protease resistant portion may remain entirely within the lipid bilayer, or it may span the viral membrane and extend into the region of the nucleocapsid, or penetrate and occupy the inner surface of the viral envelope.

Studies on the proteins of three coronaviruses, OC 43 virus, transmissible gastroenteritis virus, and infectious bronchitis virus have been reported (Hierholzer *et al.*, 1972; Garwes and Popcock, 1975; Bingham, 1975; Collins *et al.*, 1976). From six to sixteen polypeptides were found by SDS-PAGE. In three studies, the coronaviruses were produced in mice or in eggs and the polypeptide patterns were analyzed spectrophotometrically. In all of the investigations reported previously, coronavirus polypeptides were solubilized in SDS at 100° with β-MSH. While there are many differences among the results reported in these studies, some of the findings are in agreement. In all of the investigations a major polypeptide, approximately 50,000 in weight, was identified and was unaffected by bromelain treatment of intact virions. Also in three cases, a very large species, 180,000–200,000 in weight, was found. In addition each laboratory identified from two to six species intermediate in size and from two to eight polypeptides smaller than 50,000. It is highly doubtful that the great variation in numbers and sizes of coronavirus proteins reported previously is due to dissimilarities between coronavirus strains or host-related differences. Two of the factors producing erroneous results may have been contamination by host proteins, where polypeptides were analyzed by staining alone, and artifacts produced as a result of heating with β-MSH in sample preparation.

GP23 and p*18, the product from the pronase or bromelain treatment of the virions, exhibit anomalous behavior on SDS-PAGE. The results are summarized in Fig. 8. The author is not aware of any other reported example of similar behavior by a virus structural protein. However, the electrophoretic mobility in SDS of one of the outer membrane proteins from *E. coli*, as well as *flagellin*, and some proteins of the human erythrocyte membrane are also reduced after boiling (Bragg and Hou, 1972; Fairbanks *et al.*, 1972; Garten *et al.*,

1975; Inouye and Yee, 1973; Kondoh and Hotani, 1974; Koplow and Goldfine, 1974; Reithmeier and Bragg, 1974; Schnaitman, 1973a,b, 1974). Decreased mobility of the *E. coli* membrane protein after heating is believed to be due to a change in the charge density of the SDS–protein complex. The precise alteration in this species which affects SDS binding has not been described. Heat-induced modifications of the *E. coli* membrane protein is not known to be affected by β-MSH or DTT.

Glycophorin A also exhibits a change in electrophoretic mobility after heating (Marton and Garvin, 1973; Tuech and Morrison, 1974; Slutzky and Ji, 1974). This erythrocyte membrane sialoglycoprotein forms dimers in SDS at 37° via interactions between hydrophobic domains of the chains (Furthmayr and Marchesi, 1976). Pretreatment of the sample at 100° results in a significant increase in the proportion of the monomeric form. Carboxymethylation of a single methionine residue situated in the hydrophobic domain of glycophorin A leads to a substantial reduction in dimer formation (Silverberg *et al.*, 1976).

The effect of boiling on the electrophoretic mobility of the coronavirus envelope protein, GP23, may be due to the formation of aggregates, changes in SDS binding, or alteration of the hydrodynamic behavior of GP23. Boiling may promote unfolding of a region of GP23 which is incompletely denatured in SDS at lower temperatures. Heating may also increase the frequency of collisions of GP23 and facilitate hydrophobic interactions between domains with hydrophobic surfaces of two or more GP23 chains. A hydrophobic domain may also act as a nucleus for mixed micelle formation with SDS.

Boiling per se is not required to produce changes in the electrophoretic mobility of GP23. Prolonged dialysis of virions in 7 *M* guanidine followed by dialysis in 9 *M* urea and then 1% SDS, all at 25° in the presence of 1 m*M* β-MSH, produced the same change in the electrophoretic mobility of GP23 as boiling in the presence of a high concentration of a reducing agent (unpublished data). This suggests that GP23 is incompletely denatured by SDS at 25° or 37°.

The role of β-MSH and DTT at elevated temperatures is unclear. More vigorous conditions may result in formation of stable mixed disulfides. DTT is a bifunctional reagent and β-MSH may contain impurities (e.g. butyl mercaptan) and at the high levels used (0.7–1.5 *M*) a chemical modification beyond simple denaturation is a reasonable alternative. Further investigations are being undertaken to elucidate the mechanism responsible for the variation in electrophoretic mobility in SDS of the coronavirus envelope protein, GP23.

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