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Formation of Influenza Virus Proteins

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Eight virus-specific proteins have been found in chicken embryo fibroblasts infected with fowl plague virus. Among them are two glycoproteins which are the constituents of the hemagglutinin on the virus particle. They are derived from a large precursor glycoprotein by cleavage of a covalent linkage. The reaction can be blocked by the protease inhibitor diisopropylfluorophosphate and the amino acid analogue fluorophenylalanine. This indicates that a peptide bond is cleaved. If infected cells are kept at 25 C, a temperature at which virus maturation is inhibited, the precursor glycoprotein is cleaved at a significantly slower rate than at 37 C. It appears, however, that a reduced synthesis of the carbohydrate-free envelope protein is responsible for the block of virus maturation at 25 C rather than the lower cleavage rate of the precursor.

The protein constituents of influenza virus consist of carbohydrate-free polypeptides and of glycoproteins. One of the carbohydrate-free polypeptides forms the protein subunit of the nucleocapsid (10, 16), another one is a constituent of the viral envelope where it coats the inner side of the lipid layer (2, 20, 21). The glycoproteins are located on the outside of the lipid layer as constituents of the surface projections or spikes (2, 12, 20).

In addition to the constituent proteins of the virion, nonstructural polypeptides have been described in influenza virus-infected cells (4, 13, 15, 22). Some of these nonstructural proteins are precursors of the viral hemagglutinin, and their analysis throws some light on the sequence of events which are involved in the biosynthesis of this envelope constituent. The first identifiable step is the glycosylation of a polypeptide to a large glycoprotein (13). It was suggested first by Lazarowitz et al. (15) that this glycoprotein is cleaved into two smaller glycoproteins. This concept has been confirmed in the present communication by fowl plague virus (FPV) grown in chicken embryo fibroblasts. Experiments will be described which give further information about the appearance of virus-specific material in infected cells, and particularly about the nature of the cleavage reaction.

MATERIALS AND METHODS

Virus and cells. The Rostock strain of FPV was used in all experiments. It was grown in primary cultures of chicken embryo fibroblasts (23). Virus stocks were grown in embryonated eggs.

Virus assay. Virus yields were assayed by hemagglutination titrations (3) and plaque assays (23).

Chemicals and isotopes. Reagents for polyacrylamide gels and diisopropylfluorophosphate were obtained from Serva, Heidelberg, Germany; DL-*p*-fluorophenylalanine from Calbiochem, Los Angeles, Calif. Protein [*U*-^{14}C]hydrolysate, L-[*U*-^{14}C]valine (300 mCi/mmol), L-[4,5-^{3}H]leucine (1.0 Ci/mmol), L-[2,3-^{3}H]valine (1,5 Ci/mmol), L-[3,5-^{3}H]tyrosine (1,0 Ci/mmol), and D-[1-^{3}H]glucosamine hydrochloride (1,1 Ci/mmol) were purchased from Amersham, Buckinghamshire, England.

Labeling of infected cells. Confluent monolayers of infected cells were inoculated at a multiplicity of 10 to 50 PFU/cell. After a 30-min adsorption period, the inoculum was removed and replaced by minimal medium (6). When experiments were carried out at 37 C, the medium was removed and replaced by medium containing radioactive isotopes 4 h after infection. When experiments were carried out at 25 C, the medium was replaced 24 h after infection. Radioactive isotopes were used at the following concentrations: [^{14}C]protein hydrolysate, 5 μCi/ml; [^{14}C]valine, 5 μCi/ml; [^{3}H]amino acids, 50 μCi/ml; [^{3}H]glucosamine hydrochloride, 50 μCi/ml. The medium containing the isotopes was left on the cells for 10 or 60 min. When the experiment was stopped at this point, the monolayer was washed three times with cold phosphate-buffered saline (PBS) (5), scraped off with a rubber policeman in 1 ml of PBS, and stored at −20 C. When the pulse was followed by a chase period, the radioactive medium was removed and the cells were washed three times with medium containing an excess of the cold analogue of the radioactive label (10 μmol of amino acid/ml) and incubated in this medium. At the end of the chase period the cells were washed and scraped off as described above.

Polyacrylamide gel electrophoresis. The cells were homogenized by sonic treatment. Samples of 100 μliters were used for gel electrophoresis. Proteins were dissociated with sodium dodecyl sulfate and mercaptoethanol and separated by electrophoresis on 10%

acrylamide gels as described (1). The slicing and processing of gels for the determination of radioactivity by liquid scintillation has been reported previously (11).

RESULTS

Viral protein synthesis in infected cells at 37 C. We reported previously that polyacrylamide gel electrophoresis reveals eight fowl plague virus-specific polypeptides in chicken embryo fibroblasts (13). Six of these are structural polypeptides of the virion. They comprise two hemagglutinin glycoproteins, HA_1 (mol wt 49,000) and HA_2 (mol wt 32,000), glycoprotein NA (mol wt 45,000) which has been attributed to the neuraminidase, a carbohydrate-free envelope polypeptide M (mol wt 26,500), the nucleocapsid protein NP (mol wt 60,000), and another internal protein P (mol wt 83,500). In addition to these structural polypeptides, infected cells contain two virus-specific proteins which are not present in the mature fowl plague virion: protein NS (mol wt 25,000) is found in large amounts in the nucleus, protein HA (mol wt 76,000) has been suggested to be a precursor of the hemagglutinin glycoproteins HA_1 and HA_2 (15). This pattern obtained 4 h after infection in a 1-h pulse with radioactive amino acids comprises all fowl plague virus-specific proteins which we were able to detect in infected chicken embryo fibroblasts.

The pattern is different if the cells obtain a pulse of only 10 min in length (Fig. 1). If the pulse is not followed by a chase period, the carbohydrate-free polypeptides P, NP, M, and NS are clearly recognizable. The protein showing the highest incorporation of radioactivity under these conditions is protein HA. Proteins HA_1 and HA_2 cannot be detected.

There is a shift in the virus-specific protein pattern if the 10-min pulse is followed by chase periods of increasing length (Fig. 1 and 2). The radioactivity in proteins P, NP, and M remain constant and in the same relative proportions independently of the length of chase. However, after a chase of 5 min, peak HA is decreased and peaks HA_1 and HA_2 are detectable. If the chase period is extended to 60 min peak HA has almost disappeared, whereas the label in peak HA_1 and that in HA_2 have increased concomitantly. These results indicate that protein HA is less stable than the other virus-specific proteins and that proteins HA_1 and HA_2 are the result of the breakdown of protein HA.

Viral protein synthesis at 25 C. In FPV-infected cells which are kept at 25 C, virus-specific components such as RNA polymerase, ribonucleoprotein antigen, hemagglutinin, and neuraminidase are synthesized, even though at a slower rate than at 37 C. However, infectious virions are not released. These data suggested that, at 25 C, virus maturation is inhibited at a late stage (18).

It was of interest which of the virus-specific proteins were formed at 25 C. Cells were kept at this temperature for 24 h after infection and then labeled in a 1-h pulse with radioactive amino acids. At the end of the experiment the cells contained a considerable amount of hemagglutinin and they showed hemadsorption and agglutinability by concanavalin A (17). However, neither hemagglutinin nor infectious particles were released into the medium. Figure 3 shows a comparison of FPV proteins synthesized at 25 C 24 h after infection with those synthesized at 37 C 4 h after infection. In both cases the proteins have been labeled in a 1-h pulse with radioactive amino acids. Several striking differences in the polypeptide profiles at these two temperatures can be seen. At 25 C protein HA shows the highest incorporation of radioactivity. As can be seen from double-label experiments with radioactive amino acids and glucosamine, protein HA is glycosylated at 25 C (Fig. 4). The other FPV-specific glycoproteins, HA_1 and HA_2, are missing at 25 C.

However, if the pulse at 25 C is followed by a chase period at the same temperature, it can be seen that proteins HA_1 and HA_2 are being formed at the expense of protein HA (Fig. 5). Similar results were obtained if the chase was carried out at 37 C (Fig. 6). These experiments indicate that HA is converted into HA_1 and HA_2 at 25 C, but at a slower rate than at 37 C. Therefore, the formation of the hemagglutinin glycoproteins does not seem to be basically altered at 25 C.

A striking difference, however, between the polypeptide profiles at 25 C and at 37 C was the apparent absence of protein M at the lower temperature. Whereas, at 37 C, proteins M and NS can be identified as a double peak or as a peak and a shoulder, at 25 C protein M could not be discriminated in the polypeptide profile. The fastest migrating peak clearly corresponds to proteins NS (Fig. 4). This was a constant finding in many experiments.

However, it is not clear from the data presented here whether protein M is completely missing or whether small amounts are hidden in a relatively large NS peak. Future studies employing other means of distinguishing these polypeptides, such as peptide mapping or immunological assays, should throw more light on this problem.

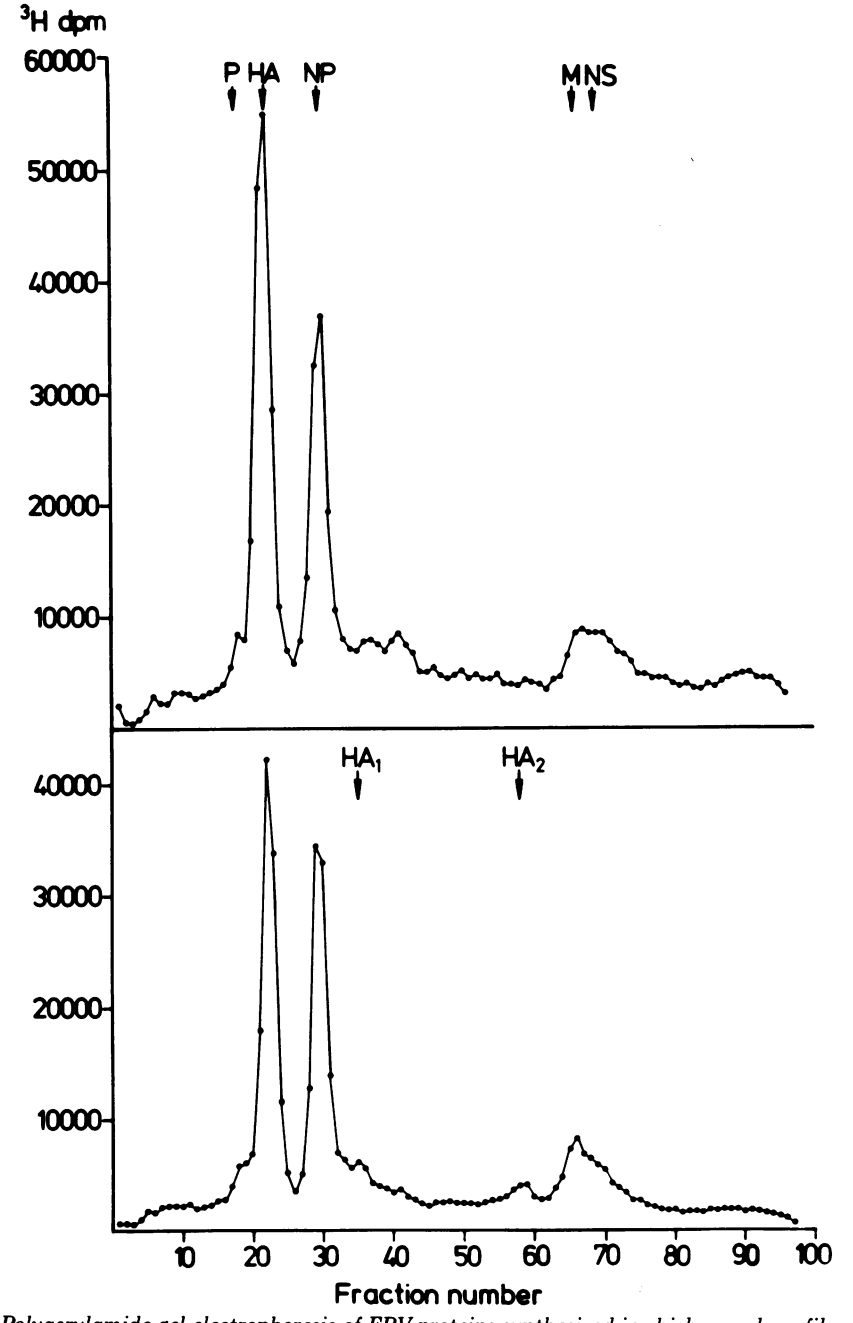

FIG. 1. *Polyacrylamide gel electrophoresis of FPV proteins synthesized in chicken embryo fibroblasts. Cells were labeled 4 h postinfection by a 10-min pulse with [^{3}H]leucine. Top, Cells were scraped off immediately after the pulse; bottom, the pulse was followed by a chase period with cold leucine for 5 min. Migration in this and all subsequent gels is from the left to the right. Arrows indicate the position of polypeptides. The neuraminidase glycoprotein has not been marked on this and most of the other figures in this paper. There is evidence that it migrates in front of the HA$_1$ peak (13).*

Inhibition of the cleavage of glycoprotein HA. The data described above conform with those of Lazarowitz et al. (15). They suggest that glycoproteins HA_1 and HA_2 are derived from glycoprotein HA. Since the sum of the molecular weights of HA_1 and HA_2 is similar to that of HA, a cleavage reaction might be involved. The linkage which is affected by this

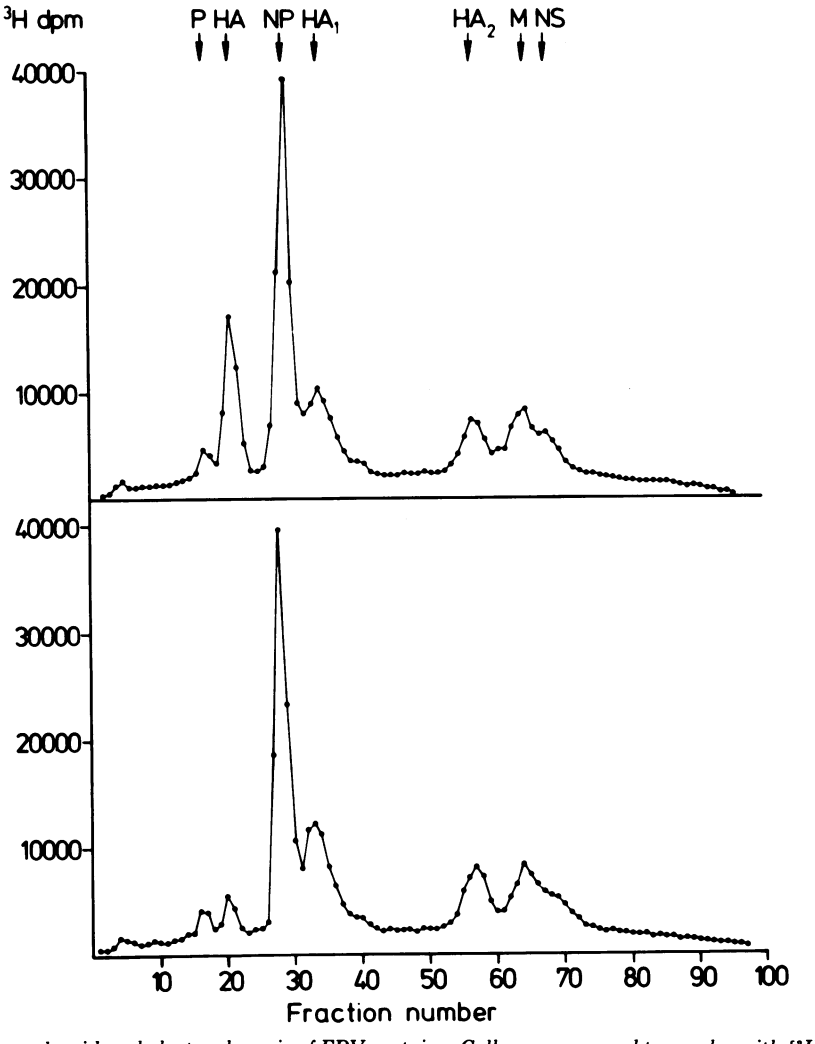

FIG. 2. *Polyacrylamide gel electrophoresis of FPV proteins. Cells were exposed to a pulse with [^{3}H]leucine as described in Fig. 1. Top, The pulse was followed by a 15-min chase period; bottom, the pulse was followed by a 60-min chase period.*

cleavage must be of covalent nature and cannot be a disulfide bond, because protein HA is stable under the conditions of the electrophoresis employed in this study, i.e., in the presence of SDS and a reducing agent. These findings are compatible with the hypothesis that the covalent linkage which is cleaved is a peptide bond and that proteolytic enzymes are involved in this reaction.

To test this hypothesis, proteases have been blocked by the use of the inhibitor diisopropylfluorophosphate (DFP). This substance had been employed successfully in the elucidation of large polypeptides as precursors of poliovirus proteins (9). The effect of DFP on the synthesis of influenza virus proteins is shown in Fig. 6. Infected cells were kept at 25 C for 24 h and then labeled for 1 h with radioactive amino acids. The only glycoprotein labeled under these conditions is protein HA. If the pulse at 25 C is followed by a chase period at 37 C, HA decreases with a concomitant increase of HA_1 and HA_2. However, if DFP is added to the medium during the chase period, the shift of the radioactivity from HA to HA_1 and HA_2 is clearly inhibited. This proves that HA is converted into HA_1 and HA_2 by cleavage of a peptide bond.

Further evidence for such a precursor-product relationship stems from experiments with an amino acid analogue. It has been known for some time that, under appropriate conditions, L-fluorophenylalanine inhibits the formation of

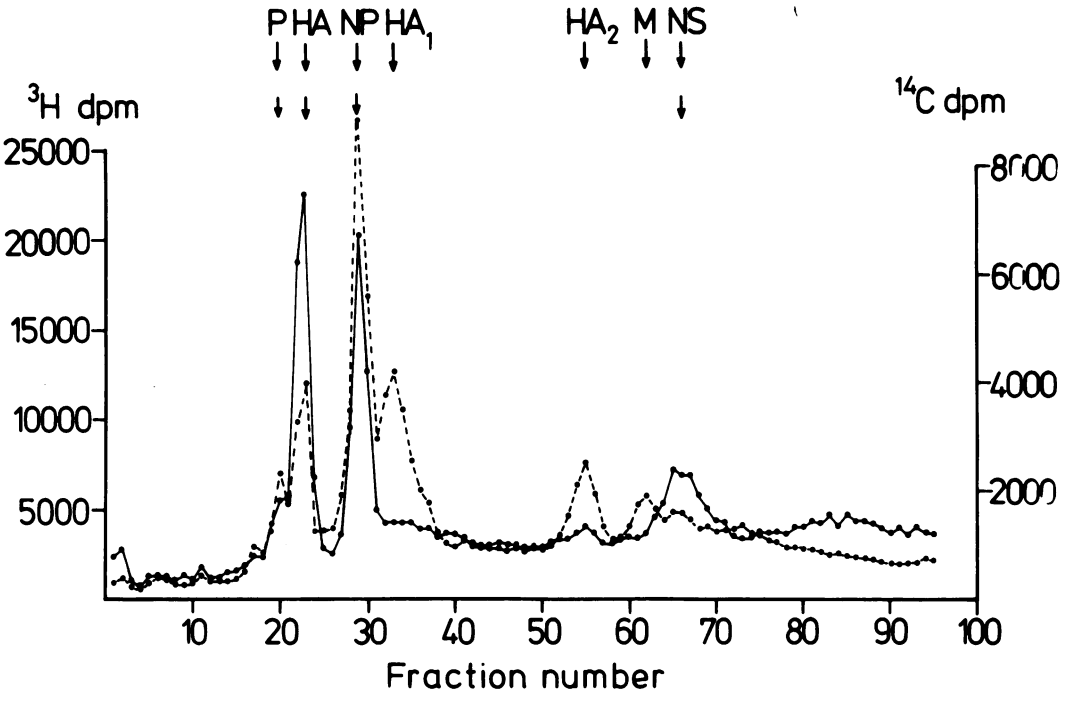

FIG. 3. *Polyacrylamide gel electrophoresis of FPV proteins synthesized at 25 C (●—●). The cells were labeled for 1 h with [³H]leucine, [³H]tyrosine, and [³H]valine. For comparison, virus-specific proteins synthesized at 37 C and labeled for 1 h with [¹⁴C]protein hydrolysate were subjected to coelectrophoresis on the same gel (O- -O).*

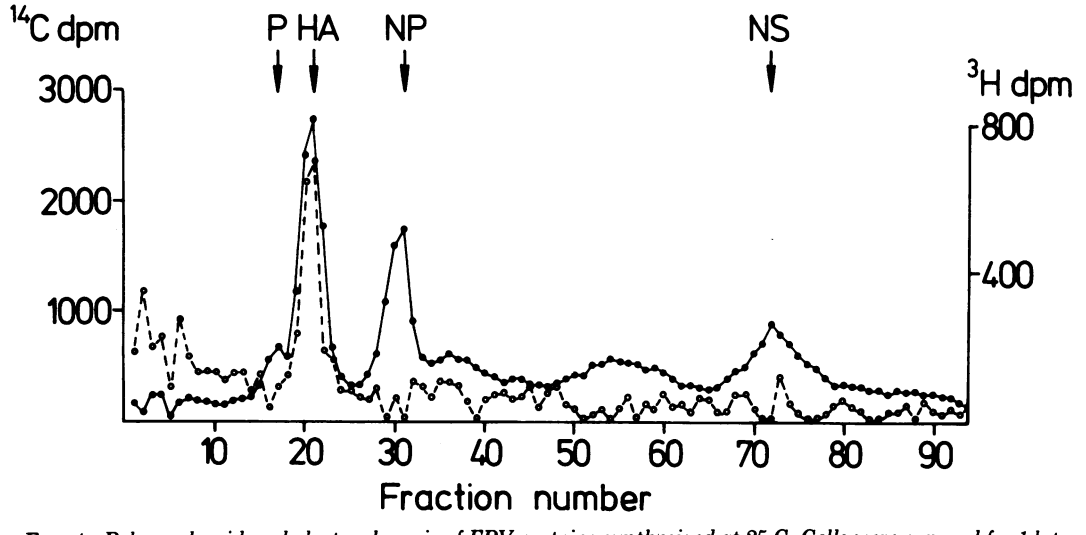

FIG. 4. *Polyacrylamide gel electrophoresis of FPV proteins synthesized at 25 C. Cells were exposed for 1 h to a pulse with [¹⁴C]amino acids (●—●) and [³H]glucosamine hydrochloride (O—O).*

fowl plague virions and biologically active hemagglutinin although nucleocapsid protein is synthesized as detected by immunological methods (19, 23). Polyacrylamide gel electrophoresis reveals that, in the presence of L-fluorophenylalanine (FPA) only proteins P, HA, and NP are labeled (Fig. 7). Proteins HA_1 and HA_2 cannot be detected. On the gel shown in Fig. 6, proteins M and NS cannot be detected either. The absence of these latter proteins, however, was not a constant finding. This experiment indicates that, in the presence of

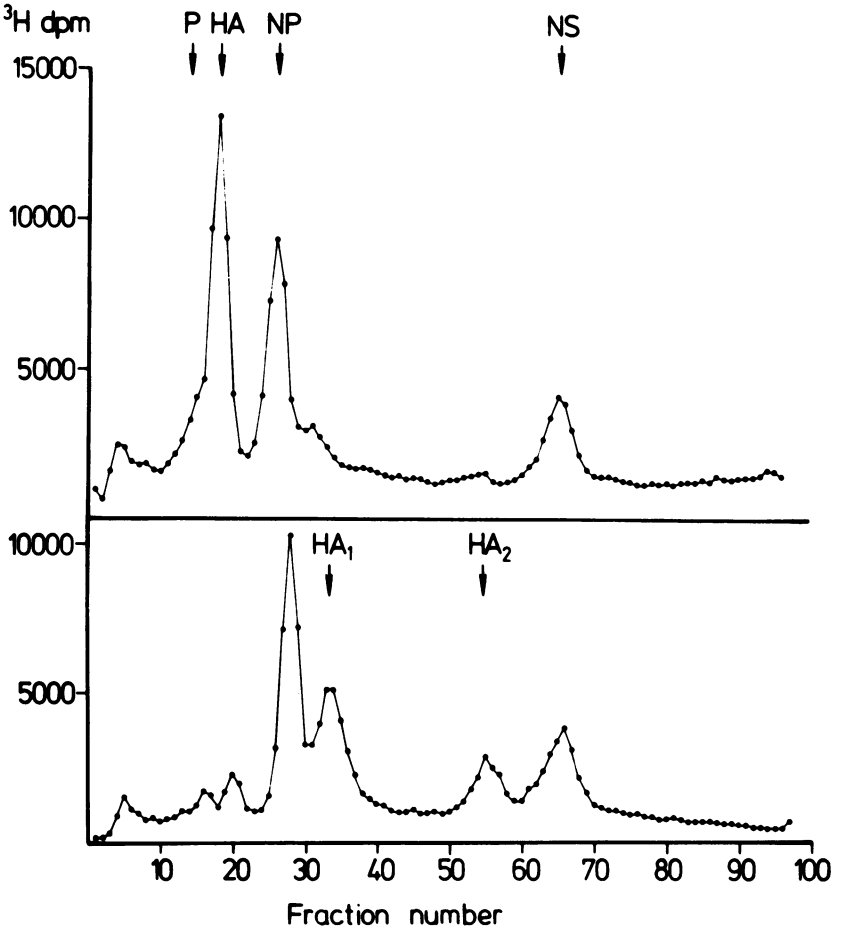

FIG. 5. *Polyacrylamide gel electrophoresis of FPV proteins synthesized at 25 C. Cells were labeled 24 h postinfection by a 1-h pulse with [^{3}H]leucine. Top, Cells were scraped off immediately after the pulse; bottom, the pulse was followed by a 3-h chase at 25 C.*

FPA, cleavage of protein HA is inhibited. The cleavage reaction appears to be completely blocked, not simply retarded, because even after chase periods of several hours HA_1 and HA_2 cannot be detected.

DISCUSSION

The profiles of fowl plague virus-specific proteins reported in this and in a previous communication (13) agree well with the findings of other groups. Lazarowitz et al. (15) found eight virus-specific polypeptides in cells infected with the WSN line of influenza A virus, each of which can be coordinated to a corresponding FPV protein. The polypeptide pattern of FPV reported by Skehel (22) is, in general, similar to our results, although there are some discrepancies in the number as well as in the proportions of the various proteins. Skehel described two additional polypeptides (P1 and 9 according to his nomenclature) which we have not been able to detect. It remains to be seen whether this discrepancy is due to differences in the labeling procedures or in the electrophoresis systems. There are striking differences in the relative amounts of radioactivity incorporated into the various polypeptides between our data and Skehel's. They can be explained by the different labeling techniques. Whereas Skehel used ^{35}S-methionine as protein label in most of his experiments, we employed ^{3}H- or ^{14}C-amino acids. Since methionine is incorporated preferentially into the carbohydrate-free polypeptides (15), this method appears to be less suitable for the analysis of the influenza virus glycoproteins.

Three of the four influenza virus-specific glycoproteins (HA, HA_1, HA_2) were found to be associated with the viral hemagglutinin (8, 14). There is evidence for the presence of another glycoprotein which migrates as a shoulder on

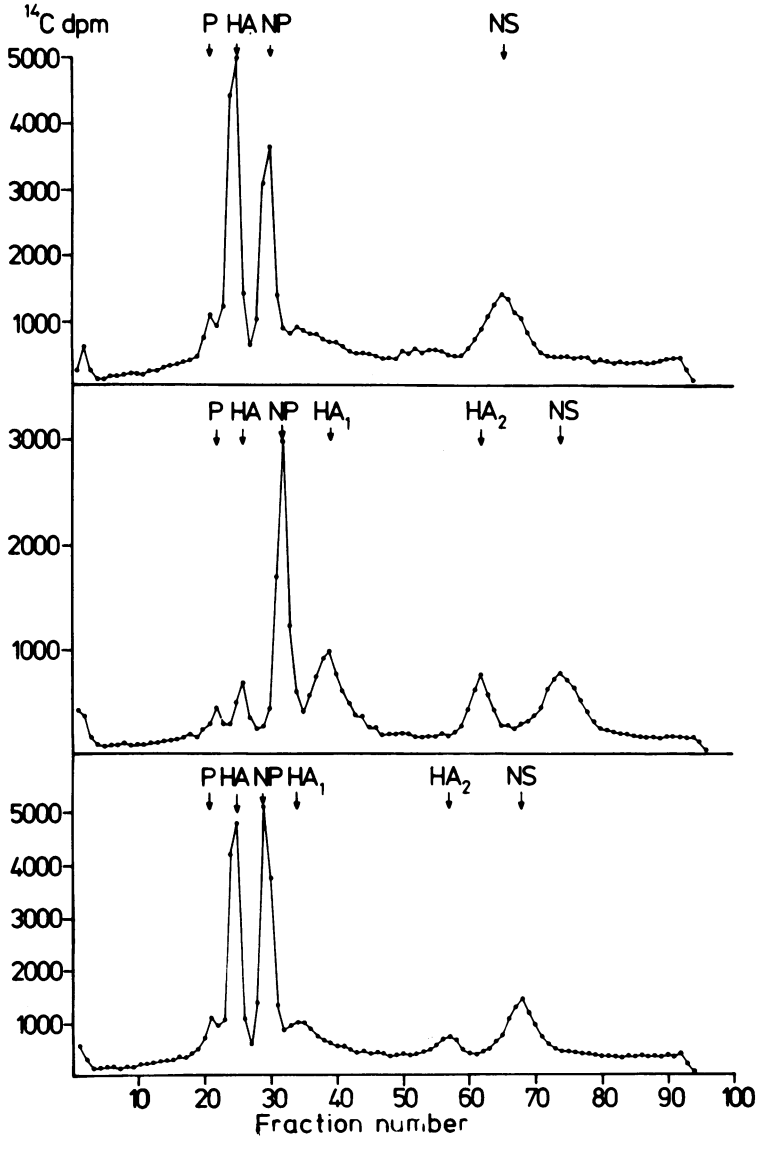

FIG. 6. *Polyacrylamide gel electrophoresis of FPV proteins synthesized at 25 C. Cells were labeled 24 h postinfection by a 1-h pulse with [^{14}C]valine. Top, Cells were scraped off immediately after the pulse; middle, the pulse was followed by a 1-h chase at 37 C; bottom, the pulse was followed by a 1-h chase at 37 C, and the media added to the cells during the chase period contained diisopropylfluorophosphate (20 μmol/ml).*

the front slope of the broad HA_1 peak and which tentatively has been assigned to a subunit of the viral neuraminidase (13). This assignation seems to be justified by comparing the gel patterns of fowl plague virus and other influenza strains (7). However, because of the poor resolution and possibly the small amount of protein NA, little information on the viral neuraminidase can be obtained from the data presented in this publication.

The observations of Lazarowitz et al. (15) suggested that glycoproteins HA_1 and HA_2 are formed by cleavage of the large glycoprotein HA. Their findings are confirmed by the results of our pulse-chase experiments at 25 C as well as at 37 C which also substantiate that a precursor-product relationship exists between HA and HA_1 and HA_2. The block of this reaction by a protease inhibitor proves that it is the cleavage of a peptide bond by which HA is converted into HA_1 and HA_2.

Further evidence for proteolytic cleavage of

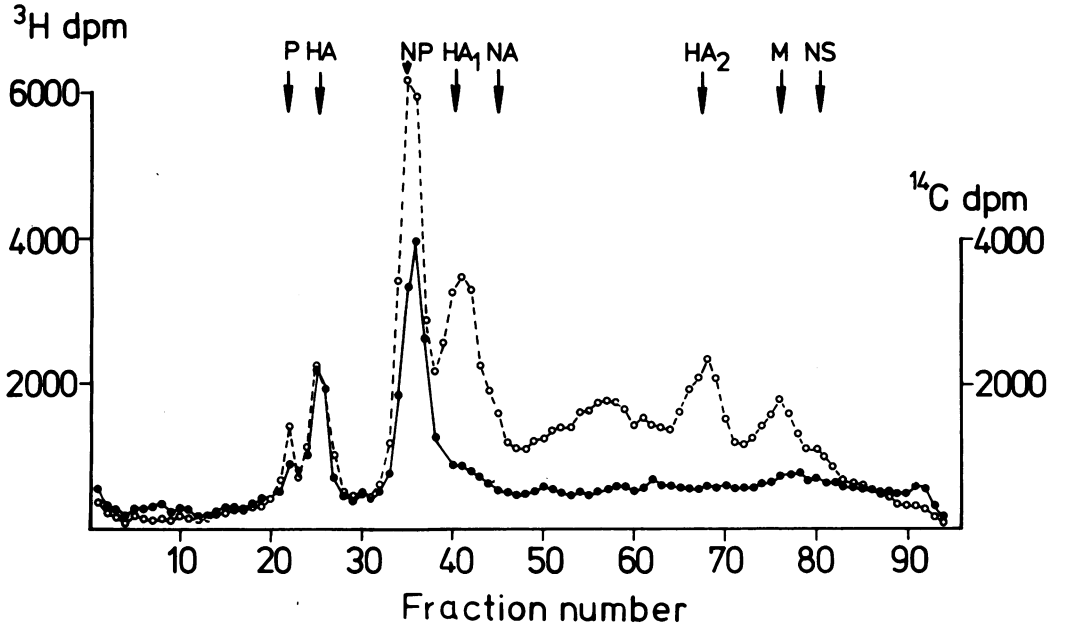

FIG. 7. *Polyacrylamide gel electrophoresis of FPV proteins synthesized in the presence of p-fluorophenylalanine. Two hours postinfection the amino acid analogue (300 μg/ml) was added to the medium. Four hours postinfection the cells were exposed to a pulse with [^{3}H]valine for 1 h (●—●). During the pulse p-fluorophenylalanine was present in the medium. In a control experiment, infected cells were grown in the absence of p-fluorophenylalanine, labeled 4 h postinfection for 1 h with [^{14}C]valine, and subjected to coelectrophoresis on the same gel* (O- -O).

HA stems from the experiments with FPA. It is known that this amino acid analogue is incorporated into poliovirus-specific precursor proteins, thereby blocking their cleavage (9). Accordingly, HA is still being formed in the presence of FPA, but cleavage as indicated by the appearance of HA_1 and HA_2 is inhibited. It appears that, by the incorporation of the amino acid analogue, the configuration of the HA polypeptide chain is altered in such a way that it can no longer be properly cleaved. This concept is also supported by the finding that HA synthesized in the presence of FPA can no longer exert hemagglutination (20). Our findings agree with those of Lazarowitz et al. (Virology, in press), who demonstrated the proteolytic nature of the cleavage with trypsin in vitro. These authors also have good evidence that the enzymes involved are host cell specified.

At 25 C cleavage of glycoprotein HA takes place at a significantly slower rate than at 37 C. This, however, does not explain the block of virus maturation at 25 C, because there is good evidence that the cleavage reaction is not essential for virus assembly. This is suggested by the finding that large amounts of glycoprotein HA are present in mature virions of several influenza strains (2, 8, 12, 20). Furthermore, experiments of Lazarowitz et al. (Virology, in press) show that cleavage of glycoprotein HA is not required for the expression of the biological properties of the virion, including hemagglutination, but that it appears to be a nonessential result of events occurring in infected cells which are undergoing cytopathic effects. At 25 C the hemagglutinin glycoproteins are not only properly formed, but they are also incorporated into the plasma membrane of the cell. This can be shown by a positive hemadsorption reaction (unpublished results) and by agglutinability of infected cells with concanavalin A (17). Therefore, the block of virus maturation at 25 C appears to be neither at the stage of the biosynthesis of the hemagglutinin glycoproteins nor at the stage of their incorporation into the cell surface.

Our data suggest that at 25 C the carbohydrate-free envelope protein M is made in such small amounts that it cannot be detected on polyacrylamide gels as a distinct peak in the polypeptide profile. Even at 37 C this protein is found in the infected cell in relatively small amounts compared to those in the virion, and therefore it has been suggested that the synthesis of protein M might be tightly controlled and

a rate-limiting step in virus replication (13, 15). These findings are compatible with the hypothesis that at 25 C the synthesis of protein M is reduced to a rate which no longer permits proper envelope assembly and, therefore, virus maturation.

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