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Sequential Protein Synthesis Following Vaccinia Virus Infection

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Inhibition of HeLa cell protein synthesis and the sequential synthesis of viral proteins were followed by pulse-labeling infected cells with ^{14}C-phenylalanine. Proteins were resolved by polyacrylamide gel electrophoresis. The viral origin of native proteins was confirmed by immunodiffusion. The inhibition of host protein synthesis and the synthesis of early viral proteins occur 1 to 3 hr after infection. This early sequence of events also occurs in the presence of 5-fluorodeoxyuridine, an inhibitor of deoxyribonucleic acid synthesis. Other viral proteins are synthesized at a later time. Those proteins which are not made in the absence of viral deoxyribonucleic acid synthesis can be further subdivided into intermediate and late classes. The intermediate protein is synthesized before the late proteins but does not appear to be a precursor of them. Many more viral polypeptides were resolved by polyacrylamide gel electrophoresis after solubilization of the entire cytoplasmic fraction with sodium dodecyl sulfate. Virion and nonvirion proteins were identified. Kinetic experiments suggested that certain structural proteins as well as certain nonstructural proteins are made early, whereas others of both classes are made primarily at later times.

During the replication of vaccinia virus, enzymes involved in nucleic acid metabolism (9, 14, 19) and certain structural proteins (11, 25, 28, 32) are made prior to the synthesis of viral deoxyribonucleic acid (DNA). Most of the proteins incorporated into the virion are made during or after the period of viral DNA synthesis (11, 17, 25, 32). It has not been established whether synthesis of the majority of viral proteins, within the two time categories, are initiated simultaneously. However, if both virus-induced thymidine kinase and DNA polymerase are virus-coded proteins, it is necessary to postulate at least two discrete periods of gene expression prior to poxvirus DNA replication (15).

We have examined the time of synthesis of viral proteins by pulse-labeling infected cells at intervals with radioactive amino acids. Advantage was taken of the inhibition of HeLa cell protein synthesis following vaccinia infection (25, 29). Recent experiments (21) established that inhibition begins within 20 min after adding virus. The time required for a complete block in HeLa cell protein synthesis is inversely related to the amount of virus used for infection. The present experiments demonstrate a rapid change in the species of proteins synthesized by HeLa cells after infection with a high multiplicity of vaccinia virus. The induced proteins, resolved by polyacrylamide gel electrophoresis, were further identified by immunodiffusion with specific antiserum and by comparison with polypeptides dissociated from the purified vaccinia virion. Information was obtained regarding the times of synthesis of structural and nonstructural proteins.

MATERIALS AND METHODS

Cell culture. Human HeLa S3-1 and mouse L-929 cells were grown in suspension culture in Eagle's medium (7) supplemented with 5% horse or fetal bovine serum, respectively.

Virus. The preparation of vaccinia virus stain WR stock suspensions and the titration of infectious virus on primary chick embryo monolayers have been previously described (26).

Infection procedure. Suspension cells, at a concentration of 4×10^6 cells per ml in Eagle's medium modified to contain 0.01 mM L-phenylalanine and 5% dialyzed horse serum, were infected with vaccinia virus by incubation with 2×10^8 to 4×10^8 plaque-forming units (PFU) per ml at 37 C for 30 min. The cells were washed with fresh medium and resuspended at a concentration of 4×10^5 cells per ml. Under these conditions, an increase in intracellular virus was first detected between 4 and 6 hr postinfection, and a yield of 100 to 200 PFU per cell was obtained by 15 hr after infection.

Amino acid incorporation. Portions of infected or similarly treated uninfected cells were resuspended at the same concentration in fresh, warm media 15 min

prior to labeling with uniformly labeled ^{14}C-L-phenylalanine (350 to 355 mc/mM, Schwarz BioResearch, Inc., Orangeburg, N.Y.). In most experiments 10 μc of ^{14}C-phenylalanine was added to 50 ml of cells. Incorporation was terminated by pouring the cell suspension into two volumes of cold or frozen crushed phosphate-buffered saline (6) containing 0.4 mM ^{12}C-phenylalanine. The cells were collected by centrifugation, washed two more times, and resuspended at a 50-fold concentration in 46 mM tris(hydroxymethyl)aminomethane (Tris), 64 mM H_3PO_4, *p*H 6.74. The cooled cell suspension was subjected to sonic vibrations of 20 kc for five intervals of 15 sec. Many experiments were repeated with cytoplasmic extracts prepared by Dounce homogenization of cells in 10 mM Tris, 10 mM KCl, 1.5 mM $MgCl_2$, *p*H 7.8 (RSB). Particulate material was removed by low-speed centrifugation followed by centrifugation at 100,000 $\times$ *g* for 2 hr. The protein concentrations of the postribosomal supernatant fluids were determined by the method of Lowry (18) or by a microbiuret procedure (12) using crystalline bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. Polyacrylamide gels were prepared essentially as described by Davis (5). The concentration of the acrylamide monomer was varied in different experiments from 5.5 to 10%. The upper and lower gels were, respectively, 1.5 and 8.5 cm in length and 0.6 cm in diameter. The postribosomal supernatant fluid was made 10% in sucrose, and 0.2 ml, containing approximately 200 μg of protein, was applied to each gel. Electrophoresis was carried out at 1 ma per tube until the bromophenol blue dye entered the upper gel. At this time, the current was raised to 2 to 3 ma per tube. The gels were usually removed from the tubes after the dye moved 8 cm into the lower gel. The position of the dye band, important for determining the relative mobility of the proteins, was marked by slicing off the remaining 0.5 cm of gel. The proteins were fixed and stained by immersion for 18 hr in 1% amido black in 7.5% acetic acid. Unbound dye and unincorporated ^{14}C-amino acids were removed by electrophoresis in 7.5% acetic acid at 5 ma per gel.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out essentially as described by Summers, Maizel, and Darnell (30). Purified virus or cytoplasm, prepared by Dounce homogenization in RSB and low-speed centrifugation to remove nuclei, was solubilized by incubation in 2% SDS, 1% mercaptoethanol, 0.5 M urea, 0.01 M sodium phosphate, *p*H 7.2, for 1 hr at 37 C in a tightly stoppered vessel. The clear solution was then dialyzed for 24 hr against two changes of a similar solution containing 0.1% SDS, and 10% polyacrylamide gels, (10 $\times$ 0.6 cm) were prepared containing 0.1% SDS, 2 mM ethylenediaminetetraacetate, and 0.1 M sodium phosphate, *p*H 7.2. Immediately prior to use, excess catalyst was removed by electrophoresis at 4 ma gel for 4 to 5 hr. The protein sample was made 10% in sucrose, and 50 μliters was applied to each gel. Fresh buffer was added and electrophoresis was carried out at 4 ma gel for 16 hr. The proteins within the gel were fixed by immersion in 20% trichloroacetic acid for 24 hr, followed by staining with 0.1% Coomassie blue in 10% trichloroacetic acid for 24 hr (4). The trichloroacetic acid was displaced with 7.5% acetic acid.

The gels were sliced longitudinally, dried, and placed in contact with X-ray film (8). The X-ray films were developed and the optical density was measured with a double beam recording microdensitometer (Joyce, Loebl and Co., Ltd., England). A more quantitative technique for determining the radioactivity in polyacrylamide gels was adapted from previously described methods (33, 22). Gel slices, 1.3 mm thick (3), were placed into individual scintillation vials and solubilized with 50 μliters of 30% hydrogen peroxide at 55 C for 15 hr. The material was completely miscible with 0.5 ml of hydroxide of hyamine (Rohm and Haas, Philadelphia, Pa.) and 10 ml of Bray's scintillator fluid (2) or 1 ml of solubilizer (Nuclear Chicago Corp., Des Plaines, Ill.) and 10 ml of a toluene-based scintillator (31). Radioactivity was measured in a scintillation counter, after the vials were allowed to stand overnight to reduce chemiluminescence. Quenching, determined with an external standard, did not reveal significant differences in the counting efficiencies of samples prepared at the same time.

Immunodiffusion. Immediately after electrophoresis, polyacrylamide gels were placed on 3.5 $\times$ 4 inch (8.69 $\times$ 10.16 cm) glass plates and partially imbedded in 15 ml of 1.2% (w/v) agar containing phosphate-buffered saline. The plates were placed in a humid chamber at room temperature. After 5 hr, a channel was cut parallel to and 1 cm from the long axis of the polyacrylamide gel and was filled with antiserum. This antiserum, prepared by infecting rabbits with purified virus, has previously been used for immunodiffusion (25). The plates were returned to the humid chamber and immunoprecipitin lines were allowed to form for 5 days. At the end of this time, the polyacrylamide gels were removed from the agar and the plates were washed by immersion in multiple changes of phosphate-buffered saline for 3 days. The precipitin lines were stained with Ponceau S in 3% trichloroacetic acid for 20 min. Excess stain was removed by washing with 7.5% acetic acid. The agar was covered with moistened Whatman no. 50 filter paper and dried at 37 C for 24 hr. Autoradiographs were made by exposing the dried agar plates to X-ray film for periods of 1 to 3 weeks.

RESULTS

Gel electrophoresis of nonparticulate proteins. The proteins present in vaccinia-infected HeLa cells may be divided into particulate and nonparticulate fractions by high-speed centrifugation. Powerful dissociating agents are required for the complete dissolution of the particulate material. However, the postribosomal supernatant fluid containing 25 to 30% of the total proteins may be examined directly by polyacrylamide gel electrophoresis under conditions which allow retention of immunological reactivity and possibly

other native properties. Specific immunological methods of detection are particularly useful when large amounts of cellular protein are present. Electrophoresis of nonparticulate proteins from uninfected cells revealed a large number of fine bands becoming almost confluent in certain areas. No change in this pattern was detected during the first 3 hr of infection. Two new bands, superimposed on the background of host proteins, were visible in gels containing extracts from cells 4 hr after vaccinia virus infection (Fig. 1). The mobilities of the new proteins relative to the bromophenol blue marker were 0.15 and 0.17 on 7.5% polyacrylamide gels and 0.25 and 0.35 on 5.5% polyacrylamide gels. Although these two protein bands stained with a similar intensity at 3 to 4 hr postinfection, the protein of lower electrophoretic mobility became more prominent during the next 2 hr and eventually became the darkest band in the gel.

Pulse-labeling experiments. The background of host cell proteins limits the ability to detect viral proteins by staining procedures. Advantage was taken of the rapid inhibition of HeLa cell protein synthesis by high multiplicities of vaccinia (21) to preferentially label viral proteins. Cells were pulse-labeled with ^{14}C-amino acids at intervals following infection. The proteins were separated by gel electrophoresis, as before, but now autoradiographs of the gels revealed proteins synthesized only during the period of pulse labeling. A single radioactive amino acid, uniformly labeled ^{14}C-L-phenylalanine, was used. Care was taken to use a concentration of phenylalanine (0.01 mM) which was not limiting for either the final yield of virus or the kinetics of virus formation. With the viral input multiplicity used in the present experiments (50 to 100 PFU per cell), a progressive decline in phenylalanine incorporation occurred (Fig. 2). The change in the percentage of radioactivity found in the nonparticulate fraction at 3 to 4 hr after infection is a constant finding. It is apparent from examination of the polyacrylamide gel autoradiographs that, as early as 1 hr postinfection, large alterations in protein synthesis occurred (Fig. 3). A group of proteins (early or E_1, E_2, and E_3) characterized by high electrophoretic mobility (0.51, 0.64, and 0.90 relative to bromophenol blue on 7.5% polyacrylamide gels) are synthesized maximally at 1 to 3 hr postinfection. Longer autoradiographic exposure times (as in Fig. 4) revealed these proteins more clearly. The early proteins, particularly E_1, presumably because of their low molecular weight, appear as more compact bands when electrophoresis is carried out with 10% polyacrylamide gels (*unpublished data*). An additional protein (intermediate or I) appears soon after the early proteins and achieves a maximal rate of synthesis at the time (3 to 5 hr) that early protein synthesis is being shut off (Fig. 3). The protein is synthesized, although at a continually reduced rate, for the remainder of the growth cycle. The mobility of the intermediate protein relative to bromophenol blue is 0.31 on 7.5% polyacrylamide gels and 0.47 on 5.5% polyacryla-

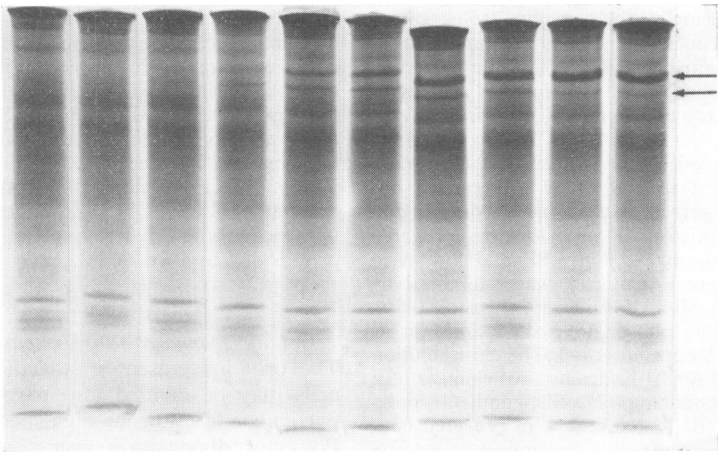

FIG. 1. *Amido black-stained polyacrylamide gels after electrophoresis of extracts prepared from HeLa cells infected with vaccinia virus. Extracts were prepared as described and consisted of the postribosomal supernatant fluids of infected or uninfected cells. In this experiment, and in the majority of subsequent experiments, attempts were made to preserve the native states of the proteins; unless specifically noted, reducing or dissociating agents were not used. From left to right, the gels contained extracts from cells infected for 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 hr. The direction of electrophoretic migration is from top (cathode) to bottom (anode). The positions of the two prominent viral proteins are indicated by arrows.*

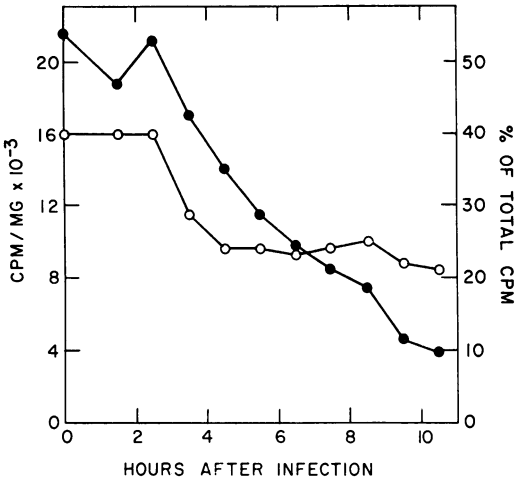

FIG. 2. *Incorporation of ^{14}C-phenylalanine by Hela cells following infection with vaccinia virus. Infected cells were pulse-labeled for successive 1-hr intervals as described. The washed cells were sonically treated and a postribosomal supernatant fraction was obtained by centrifugation. Portions of the total extracts and the postribosomal supernatant fluids were precipitated with trichloroacetic acid. Protein was determined by a microbiuret procedure. Symbols: ●, supernatant fraction, counts per min (CPM) per mg; ○, percentage of total counts per min in the supernatant fraction.*

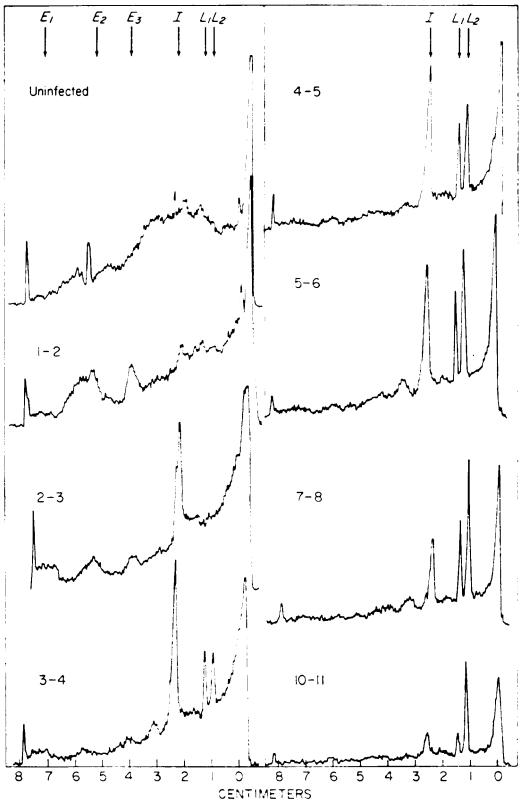

FIG. 3. *Microdensitometer tracings of polyacrylamide gel autoradiographs showing sequential protein synthesis following vaccinia virus infection. Uninfected and vaccinia virus-infected Hela cells were labeled with ^{14}C-phenylalanine for successive 1-hr intervals. Gel electrophoresis was carried out as in Fig. 1. The stained gels were dried and placed in contact with X-ray film. Continuous optical density tracings of the developed X-ray films were obtained with a Joyce-Loebl microdensitometer. The numbers above each tracing refer to the hours postinfection of pulse-labeling. Direction of migration is from right to left. Positions of the virus-induced proteins are indicated by vertical arrows. Early proteins are designated E_1, E_2, and E_3, an intermediate protein as I, late proteins as L_1 and L_2. The peak at 0 cm represents material unable to penetrate the gel; the small extreme left peak represents material moving with the discontinuous electrophoretic boundary.*

mide gels. A small shoulder is apparent primarily when labeling is done at 3 or 4 hr. Immunodiffusion (*see below*) suggests that the contaminating protein (E_4) is an additional early protein. A group of late proteins is initially synthesized at 3 to 4 hr postinfection and corresponds in mobility to the two new bands seen on stained polyacrylamide gels. The progressive alteration in the relative rates of synthesis and accumulation of these two late proteins was seen in both the autoradiographs (Fig. 3) and the stained gels (Fig. 1).

Pulse-labeling experiments in the presence of fluorodeoxyuridine (FUDR). Virus-induced proteins may be divided into two groups based on their time of synthesis relative to viral DNA. It was of interest to determine whether the intermediate protein was an "early" or "late" protein based on this classification. FUDR, a reversible inhibitor of DNA synthesis, has previously been used to block the synthesis of vaccinia DNA (24). A cell suspension was made 10^{-6} M in FUDR and divided into two portions. One portion was infected with vaccinia virus as previously described. After adsorption, the cells were washed and both infected and uninfected cells were resuspended in media containing 10^{-6} M FUDR. The cultures were then divided and one portion received 10^{-6} M thymidine to reverse the effects of the inhibitor. The cultures were pulse-labeled and polyacrylamide gel autoradiographs were made as previously described. The inhibition of host protein synthesis and the synthesis of early proteins proceeded similarly in the presence and absence of DNA synthesis (Fig. 4). Little or no intermediate protein was synthesized in the presence of FUDR. As expected, the late proteins

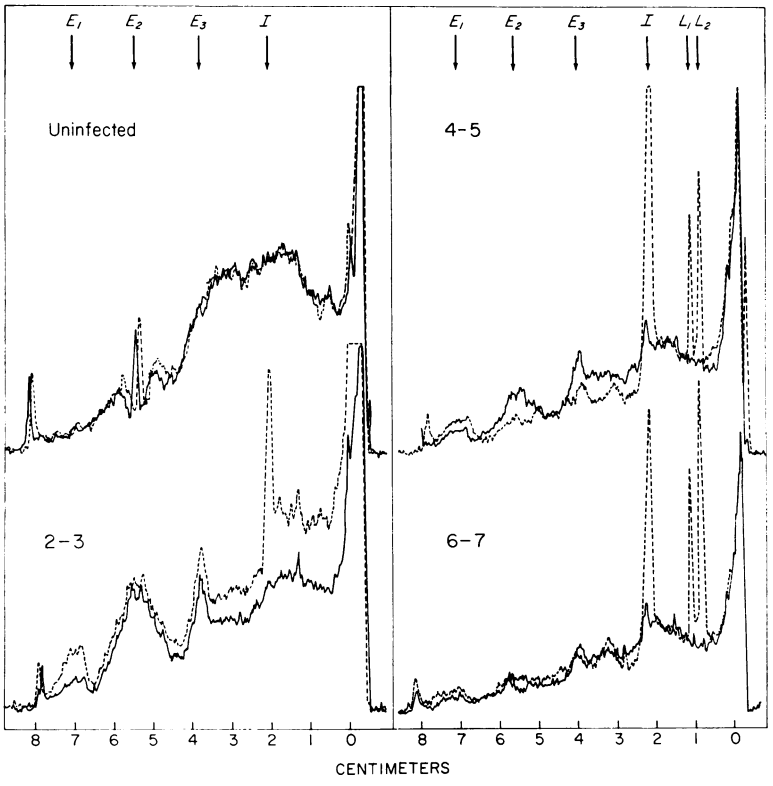

FIG. 4. *Viral protein synthesis in the presence of FUDR. Pulse-labeling experiments were carried out as in Fig. 3, except that cells were infected in the presence of 10^{-6} M FUDR. After 30 min, the cells were washed and resuspended in fresh medium containing FUDR; 10^{-6} M thymidine was added to half the cells. Uninfected cells were treated in a similar manner. Solid lines, microdensitometer tracings of polyacrylamide gel autoradiographs corresponding to FUDR-treated cells; dashed lines correspond to the cells that have received thymidine to reverse the effects of FUDR. Numbers above each tracing correspond to the hours postinfection of pulse-labeling. Arrows indicate the position of early, intermediate, and late proteins. Direction of electrophoresis is from right to left.*

were not synthesized in detectable amounts in the absence of DNA synthesis.

In a separate experiment, HeLa cells were infected with vaccinia virus in the presence of FUDR. After 5 hr, the inhibition was reversed with thymidine and the cells were pulse-labeled for successive 30-min intervals. After reversal, the synthesis of intermediate and late proteins occurred (Fig. 5). However, synthesis of the intermediate protein still preceded that of the late proteins.

Kinetic analysis of intermediate and late protein synthesis. The time course of synthesis of the intermediate and late proteins (Fig. 3–5) suggested that a precursor-product relationship might exist. Kinetic experiments were performed to test this hypothesis. A more quantitative method for determining radioactivity was needed. The proteins were resolved in 5.5% polyacrylamide gels for greater separation of the intermediate and late proteins. To obtain more quantitative data, the stained gels were sectioned at right angles to the long axis into 1.3-mm slices which were then placed in scintillation vials and counted after dissolution. As expected, the resolution (Fig. 6) was not as good as that obtained by a continuous optical density tracing of autoradiographs. The major portion of each protein was contained within a single slice, and in most cases L_1 and L_2 were incompletely resolved.

Infected cells were pulse-labeled with ^{14}C-L-phenylalanine 3 to 4 hr after infection, and then, after washing, were chased with an excess of unlabeled phenylalanine. No evidence for conversion of the intermediate into the late proteins was found (Fig. 7). Both groups of proteins appeared to be stable, within the error limits of this experiment, throughout the 22-hr chase.

In a separate experiment, uniformly labeled ^{14}C-L-phenylalanine was added to cells at 4 hr

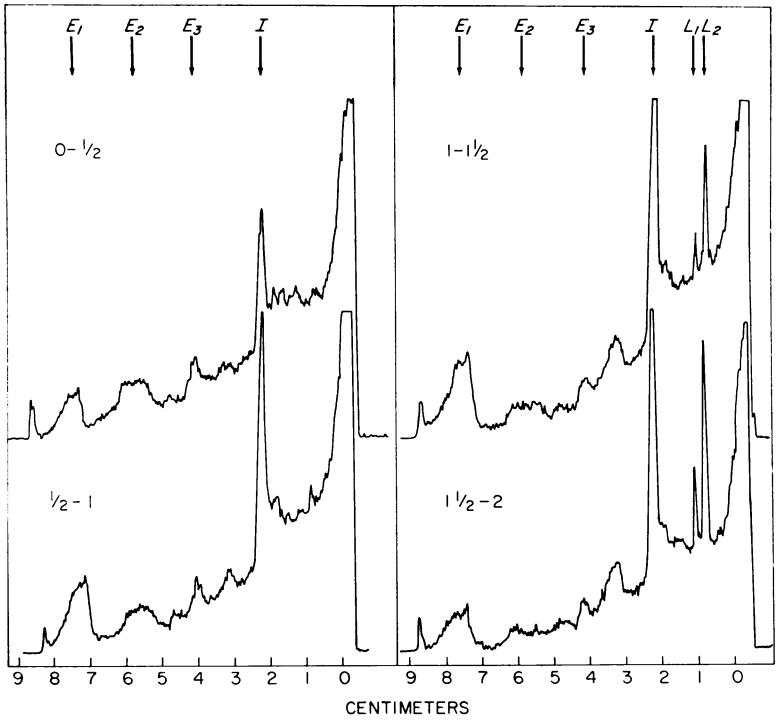

FIG. 5. *Viral protein synthesis following reversal of inhibition of DNA synthesis. HeLa cells were infected with vaccinia virus in the presence of 10^{-6} M FUDR. At 5 hr postinfection, the effect of FUDR was reversed with 10^{-6} M thymidine and the cells were pulse-labeled with ^{14}C-phenylalanine for successive 30-min intervals. Polyacrylamide gel electrophoresis and autoradiography were performed as described previously. Numbers above each microdensitometer tracing refer to hours following reversal of the inhibition. Arrows indicate the positions of early intermediate, and late proteins. Direction of electrophoresis is from right to left.*

after infection. Portions of the cell suspension were removed at 10-min intervals. Again, the proteins were separated on 5.5% polyacrylamide gels and the sections were counted in scintillation vials. The kinetics of synthesis do not suggest a precursor product relationship (Fig. 8).

Synthesis of intermediate and late proteins in L cells. We do not know whether messenger ribonucleic acid (mRNA) for the intermediate and late proteins is synthesized simultaneously or sequentially. It was of interest to compare the synthesis of the intermediate and late proteins in HeLa and L cells, since differences in the transcription of vaccinia mRNA in these two cell lines has been reported (23). In L cells, large amounts of early viral mRNA are made, but late mRNA can barely be detected. In contrast, HeLa cells made significant quantities of late mRNA (23, 27). Despite these differences, the intermediate and late proteins were induced in a similar fashion in both cell lines (Fig. 9). Thus, there did not appear to be a good correlation between the quantities of late mRNA and the rates of synthesis of intermediate and late proteins in these two cell lines. This is consistent with the previous finding that the large decrease in the quantity of mRNA in polyribosomes observed late in the infectious cycle is not associated with a similar decrease in the rate of viral protein synthesis (26, 27, 28).

Immunodiffusion. The viral origin of the three classes of proteins was demonstrated by immunodiffusion experiments using specific antiserum prepared by infecting rabbits with live virus. The antiserum has been previously described (25) and gives no immunoprecipitin lines with HeLa cell proteins. Immunodiffusion was carried out after electrophoresis by imbedding the polyacrylamide gels in agar and adding antivirus serum to channels cut the length of the polyacrylamide gel. Viral antigens were revealed by the formation of precipitin arcs in the agar between the polyacrylamide gel and the antiserum trough. Since the cells were pulse-labeled, autoradiography was performed with the dried agar plates. Photographs of X-ray film autoradio-

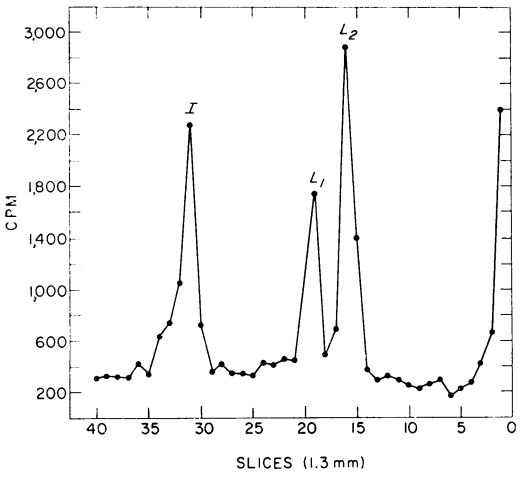

FIG. 6. *Quantitation of intermediate and late protein synthesis by the scintillation counting of polyacrylamide gels. HeLa cells were labeled with ^{14}C-phenylalanine from 4 to 5 hr postinfection and the postribosomal supernatant fluids were fractionated by electrophoresis in 5.5% polyacrylamide gels. The gels were fixed and stained as described previously, then sliced into serial 1.3-mm sections. Each slice was dissolved in 50 μliters of 30% H_2O_2 in scintillation vials and counted as described. Direction of electrophoresis is from right to left. Only a portion of the gel is shown.*

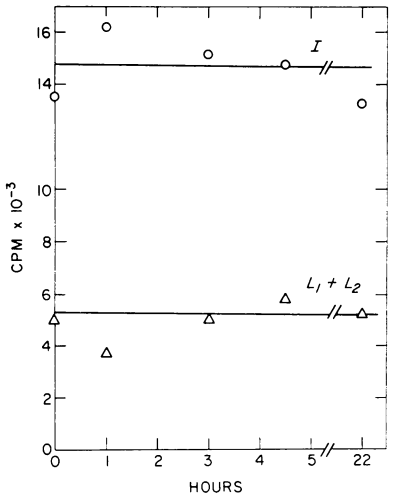

FIG. 7. *Pulse-chase. Vaccinia virus-infected HeLa cells were pulse-labeled with ^{14}C-phenylalanine from 3 to 4 hr after infection. The cells were washed and resuspended in growth medium containing excess ^{12}C-phenylalanine. Portions of the cell suspension were removed at intervals and nonparticulate proteins were separated by polyacrylamide gel electrophoresis as described in Fig. 6. Each set of points was obtained by counting serial sections from an entire gel, adding separately the counts contained in the intermediate (I) and late ($L_1 + L_2$) protein peaks, and subtracting the base line radioactivity. Values were then divided by the number of micrograms of protein applied to the gel.*

graphs are presented in Fig. 10. The periods of pulse-labeling were 1 to 3 hr and 4 to 6 hr postinfection. In this experiment, early proteins were labeled in the first pulse and both intermediate and late proteins were labeled in the second pulse. Electrophoresis was carried out for approximately half the usual time, so that the protein bands would be close enough to each other for the precipitin arcs to intersect. Five immunoprecipitin lines are present in the autoradiographs made from the 1- to 3-hr pulse (Fig. 10a). The mobilities, in addition to the time of labeling, identify them as early proteins. Figure 10b is a composite drawing made from autoradiographs exposed for short and long times. The labels above the arcs represent an attempt to correlate the arcs with the radioactive bands previously resolved by one-dimensional polyacrylamide gel electrophoresis. E_2 cannot be seen in Fig. 10a, but it was visible upon long autoradiographic exposure. One arc was not labeled, because it was not resolved on polyacrylamide gels. The distinct natures of the proteins are indicated by the intersection of immunoprecipitin lines. Three new arcs appeared in the autoradiographs from the 4- to 6-hr pulse. The positions of the arcs suggest that they were formed from the intermediate and late proteins. The intersection of the immunoprecipitin lines indicates that the intermediate and late proteins

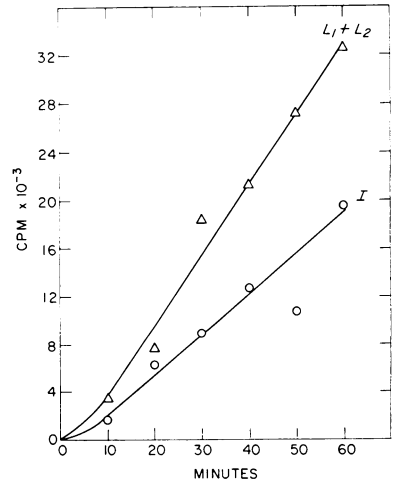

FIG. 8. *Incorporation of ^{14}C-phenylalanine into intermediate and late proteins. ^{14}C-phenylalanine (60 μc) was added to 320 ml of HeLa cells 4 hr after infection. At successive 10-min intervals, 50-ml samples were poured over frozen crushed phosphate-buffered saline containing ^{12}C-phenylalanine. The nonparticulate proteins were separated as described in Fig. 6, and the incorporation values for the intermediate (I) and late (L) proteins were determined as described in Fig. 7.*

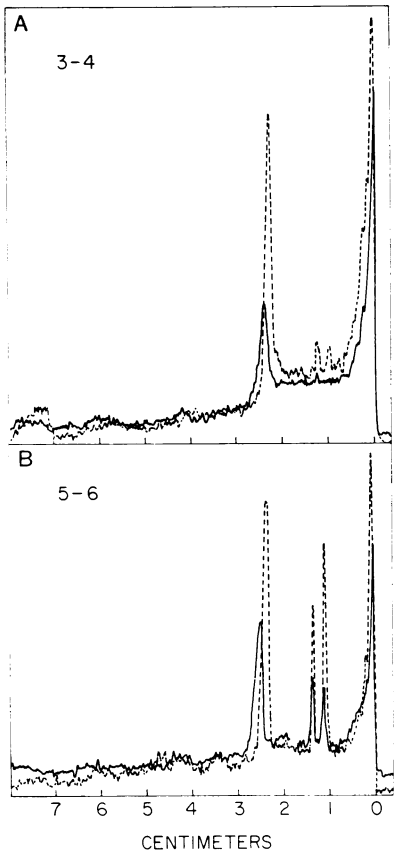

FIG. 9. *Viral protein synthesis in HeLa and L cells. Pulse-labeling, gel electrophoresis, autoradiography, and microdensitometry were carried out as in Fig. 3. Protein from equivalent numbers of HeLa and L cells were used for electrophoresis. Electrophoresis was carried out in separate gels and the microdensitometer tracings were superimposed. Dashed lines, HeLa cells; solid lines, L cells. The slightly higher mobility of the I protein from L cells seen in this figure is not a constant finding and results from the difficulty in reproducing exactly the same mobility in duplicate gels. Pulse-labeling was from 3 to 4 hr in A and from 5 to 6 hr in B.*

are antigenically distinct. Faint lines corresponding to the early proteins can be detected on the autoradiograph from the 4- to 6-hr pulse, but lines corresponding to the intermediate and late proteins cannot be seen at all in the autoradiographs from the 1- to 3-hr pulse.

In contrast to the autoradiographs, which reveal only the proteins synthesized during the pulse period, the stained immunodiffusion patterns (not shown because of the light staining) reveal the accumulated synthesis of viral proteins. Accordingly, the stained counterparts of the autoradiographs in Fig. 10 show the viral antigen synthesized from 0 to 3 hr and from 0 to 6 hr postinfection.

Electrophoresis of total cytoplasmic extract. Attempts were made to extend the observations of sequential viral protein synthesis by examination of the total cytoplasmic extract solubilized with mercaptoethanol and SDS. We presumed, however, that SDS treatment would result in loss of specific immunological reactivity. First, the protein components of the virion were examined. Virus was labeled by the addition of ^{14}C-phenylalanine to HeLa cells 2 hr after infection (multiplicity of 10 PFU/cell); 22 hr later the cells were harvested and the virus was purified to constant specific activity by repeated zonal density gradient centrifugation. The virus was solubilized with mercaptoethanol and SDS, and electrophoresis was performed in 10% polyacrylamide gels with only slight modification of the method described by Summers et al. (30). After electrophoresis, the proteins were fixed within the gel with 20% trichloroacetic acid, stained with Coomassie blue, and dried, and autoradiographs were prepared. Correspondence of the more than 20 bands visible on the stained gels with the autoradiographic bands was found. Well-separated bands appeared as discrete peaks; others appeared as shoulders on the microdensitometer tracings of the X-ray film (Fig. 11). Essentially all of the ^{14}C-material applied to the polyacrylamide gels was recovered after electrophoresis as determined by solubilization of the gel and scintillation counting (22). The pattern appeared unaltered by alkylation of the dissociated and reduced protein with iodoacetamide. The protein separation was similar to that recently reported by Holowczak and Joklik (10).

Sequential protein synthesis was studied by pulse-labeling infected cells with ^{14}C-phenylalanine for 1-hr intervals. Stained polyacrylamide gels following electrophoresis of the total cytoplasmic extract did not reveal any definite new bands because of the background of host proteins. Gels were nevertheless always stained, since the host proteins served as internal markers to establish the reproducibility of the electrophoresis. As expected, the autoradiographs revealed more information (Fig. 11). Changes in the species of proteins synthesized occurred promptly after vaccinia infection. Many more newly synthesized polypeptides were resolved from the total cytoplasmic extract under these conditions of electrophoresis than were found by our prior analysis of the native, nonparticulate proteins. The large number of polypeptides is consistent with the size of the viral genome and complexity of the virion. The cytoplasmic proteins synthesized during each pulse-period were compared with structural proteins dissociated from purified virions. Identification of the cytoplasmic proteins is based entirely on the electrophoretic mobility.

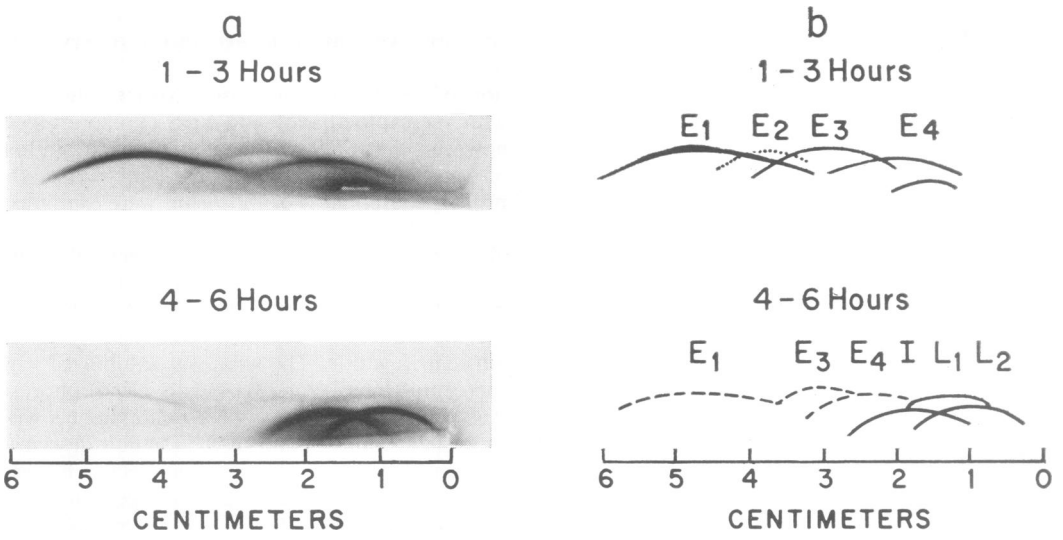

FIG. 10. *Autoradiographs of precipitin lines obtained after immunodiffusion of proteins from polyacrylamide gels. HeLa cells were pulse-labeled for 1 to 3 hr and 4 to 6 hr postinfection. Polyacrylamide gel electrophoresis of the postribosomal supernatant fractions was carried out and the cylindrical gels were immediately imbedded in 1.2% agar. Immune serum was added to channels cut the length of the polyacrylamide gels and diffusion was allowed to proceed for 5 days. The polyacrylamide gels were removed and the agar was washed, stained, dried, and exposed to X-ray film as described. Direction of electrophoretic migration was from right to left. (a) Photograph of autoradiograph; (b) tracings of the X-ray film.*

More positive identification of virion proteins found in the cytoplasm will require their isolation. We have determined, however, that mixing unlabeled cytoplasm with radioactively labeled virus prior to dissociation does not alter the mobilities of the proteins. The data presented in Fig. 11 support or suggest the following. (i) The synthesis of HeLa cell proteins is inhibited after vaccinia infection. (ii) Many of the newly synthesized polypeptides are structural proteins found in the purified virion. (iii) Polypeptides not found or found in only small amounts in the virion are also synthesized. Prominent examples of the latter are the polypeptides of lower electrophoretic mobility and presumably higher molecular weight than the A virion proteins. These polypeptides are synthesized even during the very late stages of virus replication. (iv) At no time does the infected cell make structural proteins in the proportions found within the virion. (v) Certain polypeptides are synthesized at their maximal rates during the early stages and others during the late stage of replication. For example, the changing pattern of the complex corresponding to the B structural proteins indicates changes in the relative rates of synthesis of the component polypeptides. A polypeptide corresponding in electrophoretic position with the structural polypeptide E is made between 2 and 5 hr, whereas the polypeptide corresponding to D is made primarily between 4 and 11 hr. (vi) Some polypeptides, for example F, may be made continuously throughout the replication cycle. Alternatively, the radioactive peak may be composed of several polypeptides synthesized at different times.

In preliminary experiments, we have compared the particulate and nonparticulate cytoplasmic fractions by SDS gel electrophoresis. Major differences in the electrophoretic pattern were obtained (*unpublished data*). However, because of the large number of viral proteins involved, isolation will be required for their definitive identification.

DISCUSSION

Other studies (13, 21, 25, 29) have established that cell protein synthesis is inhibited after vaccinia infection. This conclusion was reached by measuring ^{14}C-amino acid incorporation under conditions where the synthesis of viral proteins was prevented (13, 21, 29) or by using immunoprecipitation to distinguish viral proteins (25, 28). The present experiments, utilizing electrophoretic mobility to characterize HeLa cell proteins, demonstrate more directly the specific inhibition of host cell protein synthesis. The first ^{14}C-amino acid pulse, 1 to 2 hr after infection, showed marked changes in the species of proteins synthesized. This was particularly evident on

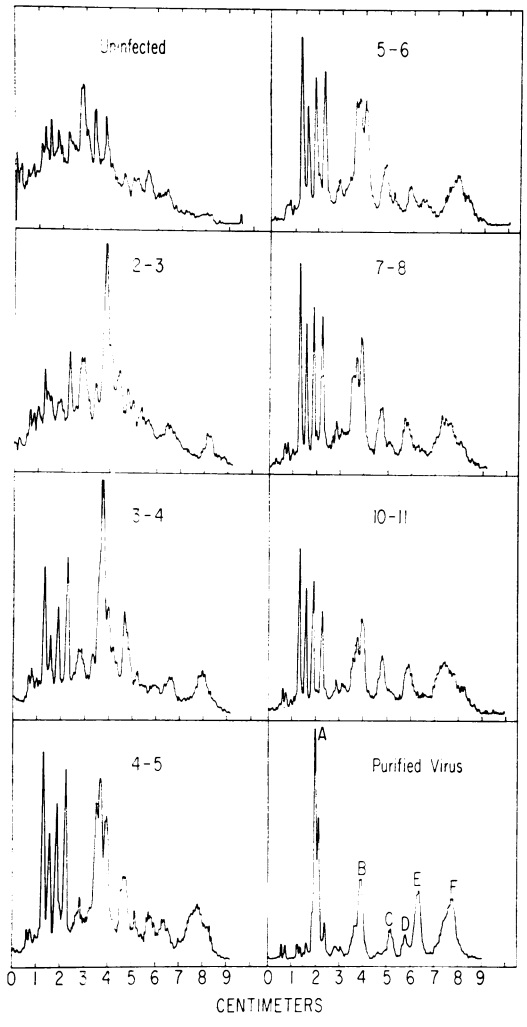

FIG. 11. *Time course of viral protein synthesis analyzed by polyacrylamide gel electrophoresis of cytoplasmic proteins solubilized with SDS and mercaptoethanol. HeLa cells were infected with 50 PFU/cell of vaccinia virus and pulse-labeled with ^{14}C-amino acids for 1-hr intervals. The cells were broken, the cytoplasm was solubilized with SDS, and mercaptoethanol and the proteins were separated in 10% polyacrylamide gels containing SDS. Purified virus, labeled with ^{14}C-phenylalanine, was mixed with an equivalent amount of unlabeled cytoplasm and treated in an identical manner. Autoradiographs were prepared from the gels and microdensitometer tracings were made. Numbers above the tracings refer to the hours postinfection of pulse labeling.*

examination of the nonparticulate proteins because of the smaller number of early viral proteins and their rapid mobility relative to the bulk of host proteins. Quantitative measurements of the extent or the rate of decline of HeLa cell protein synthesis could not be obtained by these methods because of the appearance of new radioactively labeled proteins induced by vaccinia. It is difficult to conclusively prove that the virus-induced proteins are coded for by the viral genome. However, when electrophoresis of the native proteins was carried out, reaction of the induced proteins with specific viral antiserum was shown. In addition, proteins with similar mobilities were induced after infection of two different cell lines.

Pulse labeling experiments demonstrated sequential protein synthesis. However, all proteins could not be simply grouped into early and late categories. A protein designated "intermediate" is synthesized prior to that of other late proteins but does not appear to be a precursor to the latter. It is of interest to determine whether this asynchronous protein synthesis results from transcriptional or translational control. Kates and McAuslan (15) suggest that the mRNA for thymidine kinase and DNA polymerase, two early enzymes induced after poxvirus infection, are synthesized at different periods. Asynchronous synthesis of early viral proteins following T-even infection of *Escherichia coli* has been described (16). In the phage system, transcription of early mRNA is also asynchronous (1).

The nonparticulate proteins we have identified by pulse-labeling and gel electrophoresis may be identical to the low molecular weight and high molecular weight antigens previously identified by immunological methods (32). The latter antigens react with antisera made against dissociated virus and are presumably part of the virion. We have not yet performed the necessary experiments to conclusively establish whether the three classes of proteins described here are also part of the virion. Kinetic experiments suggest, however, that the major portion of these soluble proteins are not chased into the particulate fractions. This observation by no means excludes the possibility that the soluble proteins are identical to polypeptides contained in the virion. Among the several possibilities are: (i) only a small fraction of the structural protein synthesized is incorporated into the virion, (ii) the synthesis and assembly of structural polypeptides is coordinated and the soluble proteins represent polypeptides that have escaped from the route leading to formation of the virion, (iii) assembly of structural proteins is coordinated so that only individual structural proteins synthesized at very specific times are utilized, and (iv) the soluble proteins are not structural proteins.

Our separation of nonparticulate viral proteins does not involve the use of denaturing or dissociating agents, and it may therefore be possible to

determine their function. Since the intermediate protein is synthesized at its greatest rate as early protein synthesis declines, it is tempting to suggest a regulatory function for the protein. A protein repressor for early enzyme synthesis has been postulated by McAuslan (20).

Previous experiments demonstrated that the bulk of the proteins that are incorporated into the mature virus are synthesized at 8 hr postinfection and thereafter (25). The same studies showed that only 15% of the protein found in the mature virus is synthesized early in the infectious cycle. Holowczak and Joklik have recently confirmed these results (11). They have also examined the time of synthesis and assembly of structural proteins by labeling infected cells with radioactive amino acids. The labeling periods were terminated and replication was allowed to proceed in the presence of excess unlabeled amino acids. Those proteins synthesized during the pulse and incorporated into the virion during or after the pulse were separated by polyacrylamide gel electrophoresis. Maximal amounts of a few proteins were incorporated into virions when the pulse-labeling was done at 5 to 6 hr after infection. Only small amounts were incorporated into virions when labeling was done at earlier times, and the majority of proteins were incorporated into the virion when labeling was done at later times. However, the quantity of "early viral protein" that is not incorporated into mature virus is almost as large as the total quantity of viral protein that appears in the mature virus (25). Most of the early proteins are either not structural proteins or they are structural proteins which are not incorporated into the virion. In the present study, we have solubilized the entire cytoplasmic extracts of infected cells with SDS and separated the pulse-labeled proteins by polyacrylamide gel electrophoresis. Many of the polypeptides synthesized during the first 5 hr of infection have the same or similar electrophoretic mobilities as components of the virion. Isolation of these polypeptides will now be required to establish their identity.

It is also of interest that certain of the nonstructural proteins are made even at very late stages of virus replication. Possibly some are precursors of smaller polypeptides found in the virion or are polypeptides required for virus assembly.

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