Synthesis of Vaccinia Viral Proteins in Cytoplasmic Extracts

I. Incorporation of Radioactively Labeled Amino Acids into Polypeptides

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Received for publication 25 June 1969

Polypeptide synthesis has been studied in cell-free systems prepared from vaccinia virus-infected and uninfected HeLa cells. Cytoplasmic extracts containing endogenous messenger ribonucleic acid were used. Amino acid incorporation into hot trichloroacetic acid-precipitable material was linear for 15 to 20 min at 37 C. The initial rate of protein synthesis was approximately 15% of the rate in intact cells. Optimal conditions for polypeptide synthesis were similar in cell-free systems prepared from infected or uninfected cells. Requirements for an energy source and Mg^{++} were demonstrated. The optimal Mg^{++} concentration was 4 to 5 mM. Ribonuclease, puromycin, and cycloheximide were inhibitory. The molecular weights of the polypeptides labeled in the cell-free systems, as determined by gel filtration in 5 M guanidine hydrochloride, ranged from 16,000 to above 68,000. Polyacrylamide gel electrophoresis indicated that the polypeptides labeled in cell-free extracts of uninfected and infected cells were different. The latter closely corresponded in electrophoretic mobility with the viral polypeptides made in intact, infected cells.

Evidence for the synthesis of viral proteins in cell-free systems derived from animal cells has depended on immunological methods. Attardi and Smith (2) and Summers and Levintow (22), using cell-free systems prepared from poliovirus-infected HeLa cells, found that radioactive amino acids are incorporated into material precipitable with antiserum.

Cell-free systems have been used for studying the regulation of phage protein synthesis. In those studies, viral proteins were identified by immunological analysis, polyacrylamide gel electrophoresis, and peptide mapping (10). We were interested in developing an *in vitro* system for the synthesis of vaccinia viral proteins in order to further study translational regulation in poxvirus-infected cells (15, 16). Amino acid incorporation was compared in cytoplasmic extracts prepared from uninfected and vaccinia virus-infected HeLa cells. The labeled polypeptides were examined by gel filtration and gel electrophoresis.

MATERIALS AND METHODS

Cell culture. HeLa S3 cells were grown in suspension with Eagle modified medium (7) supplemented with 5% horse serum.

Virus. Stock suspensions of vaccinia virus, strain WR, were prepared as previously described (20). The virus titer was determined by plaque assay on

HeLa cell monolayers without the use of an agar overlay (17).

Infection procedure. Virus suspensions containing approximately 2×10^8 plaque-forming units (PFU) per ml were subjected to sonic vibrations at 20 kc for a total of 75 sec and were clarified by centrifugation at $1,000 \times g$ for 5 min. HeLa cells, at a concentration of 4×10^6 cells per ml, were infected with 40 PFU per cell. After 1 hr of adsorption at 37 C, the cells were diluted to a concentration of 4×10^5 per ml.

Cell extract. Infected or uninfected cells were washed twice at 2 C in phosphate-buffered saline (6), and the cell pellet was resuspended in an equal volume of 0.18 M tris(hydroxymethyl)aminomethane (Tris), pH 7.8, containing 4.5 mM dithiothreitol. The cells were broken with a Dounce homogenizer, and any unbroken cells, nuclei, and large particulate material were removed by two centrifugations at $1,000 \times g$ for 5 min. The supernatant fluid, referred to as the cytoplasmic extract, was used for the cell-free incubations. The extract contained from 14 to 18 mg of protein per ml, as determined by the method of Lowry et al. (13).

Reaction mixture. Each 0.5-ml incubation mixture consisted of 0.37 ml of cytoplasmic extract, containing 90 mM Tris (pH 7.8) and 2.25 mM dithiothreitol, and in addition 4 mM $MgCl_2$, 8 mM KCl, 1 mM adenosine triphosphate (ATP), 0.25 mM guanosine triphosphate, 5 mM 2-phosphoenolpyruvate, 25 μg of pyruvate kinase (Calbiochem, Los Angeles, Calif.) and 1 μM of each L-amino acid. Radioactive amino acids (Schwarz BioResearch Inc., Orangeburg, N.Y.) were

added either as ^{14}C -L-phenylalanine (325 mc/mole) or as a mixture of ^{14}C -amino acids. Incorporation was started by placing the ice-cold mixture into a water bath at 37 C. At intervals, 50 μl of reaction mixture were spotted on Whatman no. 3 filter-paper circles 2.3 cm in diameter. Samples were also removed from the incubation mixture prior to incubating at 37 C and were used for the calculation of zero-time values. The filters were washed and boiled in trichloroacetic acid and were extracted with alcohol-ether (1:1) and with ether, similar to the procedure of Mans and Novelli (14). The filters were counted in a toluene-based scintillator fluid (Liquifluor, New England Nuclear Corp., Boston, Mass.)

Gel filtration. Sephadex G-200 beads (Pharmacia, Uppsala, Sweden) were swollen in boiling water for 5 min and at 80 C for 4 hr. After cooling, the gel slurry was made 5% in guanidine hydrochloride; on the following day a column (0.9 by 50 cm) was poured. The column was equilibrated with 5 M guanidine hydrochloride, 0.1 M Tris (pH 7.8), 0.01 M ethylenediaminetetraacetate (EDTA), and 0.02 M dithiothreitol at a hydrostatic pressure of 10 cm. Cytoplasmic extract or protein standards were made 7 M in guanidine hydrochloride, 0.1 M in Tris (pH 7.8), 0.01 M in EDTA, and 0.1 M in dithiothreitol and then were incubated at 37 C for 2 hr in a stoppered vessel and applied to the top of the column. Fractions (0.5 ml) were collected, and 0.2-ml samples were spotted in duplicate on strips of filter paper (2 by 3 cm). One set of strips was counted directly in scintillation fluid and the other set was first washed and boiled in trichloroacetic acid.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed essentially as described by Summers, Maizel, and Darnell (23). Purified virus or cell cytoplasm was solubilized by incubation in 2% sodium dodecyl sulfate (SDS), 1% mercaptoethanol, and 60 mM EDTA in a stoppered vessel at 37 C for 1 hr. The solution was then dialyzed against 0.01 M sodium phosphate (pH 7.1), 0.1% SDS, and 0.1% mercaptoethanol for 18 hr. Gels (10 by 0.6 cm) were prepared of 7.5% acrylamide, 0.2% *N,N'*-methylenebisacrylamide in 0.1 M sodium phosphate (pH 7.1), and 0.1% SDS. Prior to use, excess catalyst was removed from the gels by electrophoresis at 5 ma per gel for 1 hr. The dialyzed samples were made 10% in sucrose, and 50 to 80 μl was applied to each gel. Electrophoresis was carried out at 5 ma per gel for 12 to 18 hr at a constant temperature of 18 C. With the longer time, the separation of the major polypeptides was improved; however, rapidly migrating components were lost. After electrophoresis, the gels were placed in 10% trichloroacetic acid to precipitate the proteins. Aminoacyl transfer ribonucleic acid (RNA) was hydrolyzed by heating the gels in 5% trichloroacetic acid at 80 C for 15 min. The gels were washed with multiple changes of 5% trichloroacetic acid for 3 days to remove unincorporated amino acids. The washed gels were sliced into 1.3 mm sections (4), and dissolved in hydrogen peroxide and then in scintillation fluid as previously described (16). Counting efficiencies were determined by using internal standards. Alternatively, the washed gels were

stained with 0.25% Coomassie Blue in 10% trichloroacetic acid (5) for 5 hr, washed with 7.5% acetic acid for 24 hr, sliced longitudinally, and dried for radioautography (8). The density of the X-ray film was measured by a double beam recording microdensitometer (Joyce-Loebel, England).

RESULTS

Properties of the cell-free amino-acid incorporating system. Amino-acid incorporation was measured in the diluted cytoplasm obtained from mechanically homogenized HeLa cells. Initial experiments were done with uninfected cells to establish that the system had characteristics expected for a cell-free system. Incorporation into hot trichloroacetic-acid insoluble material was linear for 15 to 20 min and then it plateaued. Further dilution of the cytoplasmic extract resulted in a lower initial rate of amino acid incorporation; however, linear kinetics was maintained for a longer period of time. Activity was dependent upon the addition of ATP and was further enhanced by an ATP-generating system (Fig. 1). In the absence of added energy source, the activity was less than 5% of the complete system. Phosphoenolpyruvate-pyruvate kinase and creatine phosphate-creatine kinase functioned equally well in the system. Substantially better incorporation with creatine kinase had been reported for the rabbit reticulocyte lysate system (1).

The ability to incorporate amino acids was lost upon addition of ribonuclease at a concentration

FIG. 1. Requirement for ATP and an energy-generating system. Cytoplasmic extracts were incubated with 5 μC of ^{14}C -phenylalanine per ml. The complete system contained a pyruvate kinase ATP-generating system (▲). A second incubation mixture contained a creatine kinase energy-generating system (■). A third incubation mixture contained ATP and GTP but lacked an energy-generating system (○). Another incubation mixture lacked ATP, GTP, and an energy-generating system (□).

of 5 μg per ml, but a similar concentration of deoxyribonuclease was without effect (Fig. 2).

The effects of inhibitors of protein synthesis were measured. Puromycin at a concentration of 100 μg per ml reduced total amino acid incorporation by approximately 95% (Fig. 2). In contrast, cycloheximide at 400 μg per ml reduced the initial rate of amino acid incorporation by more than 70%, but incorporation did not plateau at 15 to 20 min (Fig. 2). A similar change in kinetics was reported for a cell-free system prepared from rat liver (24).

After determining that the cell-free system prepared from uninfected HeLa cells had the characteristics expected of an *in vitro* system, work was begun with vaccinia-infected cells. Cytoplasmic extracts were prepared from cells 6 hr after infection. These extracts had requirements for amino acid incorporation similar to those of extracts from uninfected cells.

The concentration of Mg^{++} is critical for *in vitro* systems. A comparison of amino acid incorporation in the uninfected and infected systems at different Mg^{++} concentration is shown in Fig. 3. An optimum of 4 mM was found for both systems. The cytoplasmic preparations were not dialyzed and, presumably for this reason, showed

FIG. 2. Effect of ribonuclease, deoxyribonuclease, cycloheximide, and puromycin. Incorporation of ^{14}C -phenylalanine was measured in the complete system (●), or in the presence of 5 μg of deoxyribonuclease per ml (▲), 5 μg of ribonuclease per ml (■), 400 μg of cycloheximide per ml (□), or 100 μg of puromycin per ml (○).

no dependence on added amino acids or K^{+} for amino acid incorporation. Addition of NH_4^{+} up to 40 mM was also without effect. Added hemin was shown to greatly sustain the activity of the reticulocyte lysate system (1, 25), possibly because of its inhibitory effect on reticulocyte ribonuclease (3, 9). Hemin at a concentration of 3×10^{-4} M had no effect on amino acid incorporation in the cell-free system derived from HeLa cells, and higher concentrations were inhibitory.

The half life of polyribosomes containing late vaccinia viral messenger RNA is much shorter than the half-life of polyribosomes in uninfected HeLa cells (18, 21). Despite this difference, similar kinetics of cell-free amino acid incorporation were found in systems derived from infected or uninfected cells (Fig. 4). The initial rates of amino acid incorporation were lower at 27°C than at 37°C; however, the final levels of incorporation were similar.

Gel filtration. The molecular weights of the polypeptides labeled in the cell-free systems were

FIG. 3. Effect of Mg^{++} concentration. Extracts were prepared from uninfected cells and from cells 6 hr after vaccinia infection. Incorporation of ^{14}C -phenylalanine was measured at different concentrations of added Mg^{++} .

FIG. 4. Effect of temperature. Incorporation of ^{14}C -phenylalanine at various temperatures was measured in cell-free systems prepared from uninfected cells and from cells at 6 hr after infection.

estimated by gel filtration. Amino acid incorporation was carried out for 25 min in cell-free systems prepared from infected and uninfected HeLa cells. The entire incubation mixtures were then made 7 M in guanidine hydrochloride and 0.1 M in dithiothreitol to dissociate the proteins into component polypeptide chains. The mixture was filtered through a column of Sephadex G-200 equilibrated with the dissociating and reducing agents. Portions of each fraction were counted directly, and other portions were first washed and boiled in trichloroacetic acid. The acid precipitable material synthesized in both systems was judged, by comparison with known polypeptide markers, to be of high molecular weight extending above 68,000 (Fig. 5). The high molecular weight material that was soluble in hot trichloroacetic acid is presumably amino-acylated transfer RNA. Similar amounts of this acid-soluble material formed in both systems.

Polyacrylamide gel electrophoresis. The combination of SDS and mercaptoethanol has been used to completely solubilize radioactive proteins in cytoplasmic extracts for polyacrylamide gel electrophoresis (23). This procedure was used to observe changes in the species of proteins synthesized in HeLa cells (16) and L cells (11) during the course of vaccinia virus replication. These experiments confirmed previous findings that cell protein synthesis is inhibited during the first 2 hr of vaccinia virus replication. Double-labeling techniques were used to compare the polypeptides made in cell-free extracts with the viral polypeptides made in intact cells 6 hr after infection. To obtain a sufficiently high specific activity, mixtures of ^3H -amino acids or ^{14}C -amino acids were used. The polyacrylamide gel patterns are identical when intact infected cells are incubated with either the ^{14}C or the ^3H mixture of amino acids, despite some differences in the specific activities and the proportions of the individual amino acids (Fig. 6a). Although the pattern is complex, the proteins synthesized in the cell-free extracts of infected cells appeared very similar to the proteins made in intact infected cells (Fig. 6b). In contrast, the proteins made in the cell-free system from uninfected cells appeared different (Fig. 6c).

To compare the polypeptides synthesized in the cell-free system with structural viral proteins, purified vaccinia virus labeled with ^3H -amino acids was prepared. The proteins were extracted from the virions with SDS and mercaptoethanol, mixed with ^{14}C -labeled proteins made in cell-free extracts, and separated by polyacrylamide gel electrophoresis. The correspondence of certain of the ^{14}C -amino acid-labeled proteins made in the cell-free extract with ^3H -amino acid-labeled

FIG. 5. Gel filtration in guanidine hydrochloride. Incorporation of ^{14}C -amino acids (25 $\mu\text{C}/\text{ml}$) was carried out for 25 min in cell-free systems prepared from uninfected cells and from vaccinia-infected cells. The reaction mixture was then dissociated in guanidine hydrochloride and reduced with dithiothreitol. Samples were applied separately to a Sephadex G-200 column equilibrated with buffer containing the reducing and dissociating agents. Fractions (0.5 ml) were collected, and 0.2-ml portions were spotted on duplicate filter-paper strips. One set of strips was counted directly in scintillation vials ($\circ$), and the other was first washed and boiled in trichloroacetic acid ($\bullet$). (a) Uninfected cells; (b) infected cells. (Note the increase in scale at fraction 58.) Protein standards were dissociated in the same manner and applied to the column (c). The positions of the markers were determined from the turbidity measured at 450 nm after adding trichloroacetic acid (solid line). The position of the ^{14}C -amino acids was determined by measurement of radioactivity (broken line).

FIG. 6. Comparison by SDS gel electrophoresis of polypeptides labeled in the cell-free system with viral polypeptides made in intact cells. Incorporation of ^{14}C -amino acids ($150 \mu\text{C}/\text{ml}$) was carried out for 25 min in cell-free systems prepared with cytoplasmic extracts from uninfected cells and cells 6 hr after infection. Intact cells (4×10^6 cells/ml) were incubated from 6 to 6.5 hr after infection with ^3H -amino acids ($16 \mu\text{C}/\text{ml}$) or with ^{14}C -amino acids ($2.5 \mu\text{C}/\text{ml}$) in growth medium containing 10% of the usual amounts of amino acids. Each of the samples was dissociated and reduced with SDS and mercaptoethanol and then dialyzed. Prior to electrophoresis at 5 ma/gel for 18 hr, polypeptides labeled with ^3H -amino acids in intact infected cells (broken line) were mixed with ^{14}C -labeled polypeptides (solid line) synthesized in (a) intact infected cells, (b) cell-free system from infected cells, and (c) cell-free system from uninfected cells. The gels were washed, sliced, and counted as described in Materials and Methods.

proteins from purified virus particles is apparent (Fig. 7). In agreement with previous findings, not all of the polypeptides resolved from the cytoplasmic extracts of infected cells are found in the purified virus particle (11, 16). The additional polypeptides in the cytoplasm are either non-structural or are modified prior to virus assembly.

Although analysis of the radioactivity, by

FIG. 7. Comparison by SDS gel electrophoresis of polypeptides labeled in the cell-free system with polypeptides extracted from purified virions. HeLa cells at a concentration of $4 \times 10^6/\text{ml}$ in media containing 10% of the usual amount of amino acids were infected with 10 PFU of vaccinia virus per cell; at 1 hr post infection, $0.5 \mu\text{C}$ of ^3H -amino acids per ml was added. The cells were harvested after 22 hr and the virus was purified by sucrose density gradient centrifugation. The virus was dissociated and reduced with SDS and mercaptoethanol (broken line) and mixed with the ^{14}C -labeled polypeptides (solid line), synthesized in cell-free extracts of infected cells described in Fig. 6. After SDS-polyacrylamide gel electrophoresis for 18 hr, the gels were sliced and counted.

slicing the gels and counting the individual slices in scintillation vials, is useful for quantitation and double labeling, the resolution is limited by the thickness of the slice. Much greater resolution and, therefore, better identification of polypeptides was obtained by radioautography (Fig. 8). The similarity between the proteins made in the cell-free system and in the intact cells is evident. The higher base line level of radioactivity in this experiment may have resulted from the fact that the polyacrylamide gels were washed, but not boiled with trichloroacetic acid, as was done in the previous experiment.

DISCUSSION

Amino acid incorporation into polypeptide chains occurs in cytoplasmic extracts prepared from uninfected and vaccinia virus-infected HeLa cells. The properties of the system, including the kinetics, requirement for an energy-generating system, narrow Mg^{++} optimum, sensitivity to ribonuclease and protein synthesis inhibitors, are those expected for a cell-free system. No differences in optimal conditions for amino acid incorporation were found between the uninfected and infected systems. Amino acids were incorporated into polypeptide chains ranging above 68,000 in molecular weight, as determined by gel filtration in guanidine hydrochloride.

Although incorporation remained linear at 37 C

FIG. 8. Radioautographs of SDS polyacrylamide gels comparing the polypeptides labeled in the cell-free system with viral proteins labeled in intact cells. Cells were infected as described in Fig. 6. Incorporation was carried out in the cell-free systems with 200 μ C of 14 C-amino acids per ml and in the whole cells with 3.3 μ C/ml. Reduced and dissociated proteins were separated by SDS polyacrylamide gel electrophoresis for 12 hr at 5 ma/gel. After washing in trichloroacetic acid and acetic acid, the gels were sliced longitudinally, dried, and exposed to x-ray film; microdensitometer tracings were made. Marker proteins, bovine serum albumin, and horse globin were applied to separate gels. Their positions, indicated by arrows, were determined by staining with Coomassie Blue.

for only 10 to 20 min, the efficiency of the system during that time was calculated to be 15% of the rate in intact cells. The rate of protein synthesis was calculated by using values for the intracellular pool of phenylalanine and the percentage of phenylalanine in HeLa cell proteins reported by Piez and Eagle (19). This efficiency is similar to that reported for the reticulocyte lysate system of Lamfrom and Knopf (12), but not as high as the hemin-stimulated reticulocyte systems (1, 25).

Both nascent polypeptides and polypeptides released from ribosomes were solubilized with sodium dodecyl sulfate and mercaptoethanol.

These polypeptides were then separated by polyacrylamide gel electrophoresis. The polypeptides labeled in the cell-free extracts of infected cells were different from those made in the system derived from uninfected cells. The former corresponded closely in electrophoretic mobility to the viral polypeptides made in intact infected cells. Some of the polypeptides labeled in the cell-free system appeared similar to structural proteins of the virus.

Experiments involving the immunological identification of completed polypeptide chains synthesized in the cell-free system, and determination of the extent of polypeptide chain initiation, are in progress.

LITERATURE CITED

- Adamson, S. D., E. Herbert, and W. Godchaux. 1968. Factors affecting the rate of protein synthesis in lysate systems from reticulocytes. *Arch. Biochem. Biophys.* 125:671-683.
- Attardi, G., and J. Smith. 1962. Virus specific protein and a ribonucleic acid associated with ribosomes in poliovirus infected HeLa cells. *Cold Spring Harbor Symp. Quant. Biol.* 27:271-292.
- Burka, E. R. 1968. Hemin: an inhibitor of erythroid cell ribonuclease. *Science* 162:1287.
- Chrambach, A. 1966. Device for sectioning of polyacrylamide gel cylinders and its use in determining biological activity in the sections. *Anal. Biochem.* 15:544-548.
- Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Anal. Biochem.* 20:150-154.
- Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167-182.
- Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* 130:432-437.
- Fairbanks, G., Jr., C. Levinthal, and R. H. Reeder. 1965. Analysis of 14 C-labeled proteins by disc electrophoresis. *Biochem. Biophys. Res. Commun.* 20:393-399.
- Farkas, W., and P. A. Marks. 1968. Partial purification and properties of a ribonuclease from rabbit reticulocytes. *J. Biol. Chem.* 243:6464-6473.
- Fraenkel-Conrat, H., and C. Weissmann. 1968. In-vitro synthesis of viral components, p. 209-221. *In* H. Fraenkel-Conrat (ed.), *Molecular basis of virology*. Reinhold Book Corp., New York.
- Holowczak, J. A., and W. K. Joklik. 1967. Studies on the structural proteins of vaccinia virus. II. Kinetics of the synthesis of individual groups of structural proteins. *Virology* 33:726-739.
- Lamfrom, H., and P. M. Knopf. 1964. Initiation of haemoglobin synthesis in cell-free systems. *J. Mol. Biol.* 9:558-575.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mans, R. J., and G. D. Novelli. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Arch. Biochem. Biophys.* 94:48-53.
- Moss, B. 1968. Inhibition of HeLa cell protein synthesis by the vaccinia virion. *J. Virol.* 2:1028-1037.
- Moss, B., and N. P. Salzman. 1968. Sequential protein synthesis following vaccinia virus infection. *J. Virol.* 2:1016-1027.
- Nishimi, M., and R. Keller. 1962. Titration of vaccinia virus and its neutralizing antibody by the plaque technique. *Nature (London)* 193:150-151.

18. Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell. 1963. Polyribosomes in normal and poliovirus infected HeLa cells and their relationship to messenger RNA. *Proc. Nat. Acad. Sci. U.S.A.* 49:654-662.
19. Piez, K. A., and H. Eagle. 1958. The free amino acid pool of cultured human cells. *J. Biol. Chem.* 231:533-545.
20. Salzman, N. P., A. J. Shatkin, and E. D. Sebring. 1963. Viral protein and DNA synthesis in vaccinia virus-infected HeLa cell cultures. *Virology* 19:542-550.
21. Sebring, E. D., and N. P. Salzman. 1967. Metabolic properties of early and late vaccinia virus messenger ribonucleic acid. *J. Virol.* 1:550-558.
22. Summers, D. F., and L. Levintow. 1965. Constitution and function of polyribosomes of poliovirus-infected HeLa cells. *Virology* 27:44-53.
23. Summers, D. F., J. V. Maizel, Jr., and J. E. Darnell, Jr. 1965. Evidence for virus specific noncapsid proteins in poliovirus infected HeLa cells. *Proc. Nat. Acad. Sci. U.S.A.* 54:505-513.
24. Wettstein, F. O., H. Noll, and S. Penman. 1964. Effect of cycloheximide on ribosomal aggregates engaged in protein synthesis in vitro. *Biochim. Biophys. Acta.* 87:525-528.
25. Zucker, W. V., and H. M. Schulman. 1968. Stimulation of globin chain initiation by hemin in the reticulocyte cell-free system. *Proc. Nat. Acad. Sci. U.S.A.* 59:582-589.