INFECTION AND IMMUNITY, July 1979, p. 396-402
0019-9567/79/07-0396/07$02.00/0

Vol. 25, No. 1

Infection of Chicken Erythrocytes with Influenza and Other Viruses

R. F. COOK, R. J. AVERY, AND N. J. DIMMOCK*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, England

Received for publication 30 March 1979

Chicken erythrocytes can be infected by the fowl plague (Rostock) strain (FP/R) of influenza type A, Newcastle disease virus (NDV), and Semliki Forest virus (SFV). Only NDV and SFV produced infectious progeny, albeit at low levels. Infection by FP/R was monitored by de novo synthesis of viral proteins, and the proteins synthesized could be identified by comparison with infected chicken fibroblast cells. FP/R synthesized far greater amounts of viral protein than did NDV or SFV.

In our earlier work (18, 29) we investigated chicken erythrocyte nuclei which are metabolically dormant (2, 13, 14, 27), to see if they could provide those nuclear functions which are necessary for the intracellular synthesis of influenza virus-coded components (4, 12, 17, 24, 28, 30). We introduced the erythrocyte nuclei into cultured cells which had been enucleated functionally with actinomycin D (18) or physically (29) and found that such reconstituted cells could support some viral macromolecular synthesis when infected with an influenza virus. This raised the possibility that viruses might be able to multiply in erythrocytes themselves; evidence to support this idea is provided by observations that erythrocytes are capable of pinocytosis of influenza viruses (5).

The association of viruses and erythrocytes during infections of animals has been reported for members of a number of different virus groups (see reference 11), and Colorado tick fever virus has been one of the most thoroughly studied (11, 33). However, these authors conclude that Colorado tick fever virus infects erythropoietic cells, which results in the presence of virions in erythrocytes. In this report we describe the infection of chicken erythrocytes in vitro by influenza and other viruses and the subsequent synthesis of virus-coded components or infectious virus. We understand that this is the first instance of virus multiplication in erythrocytes infected in vitro, and we believe that this will be of interest to those investigating both pathogenesis and virus-cell interactions.

MATERIALS AND METHODS

Cells. Chicken embryos used in these experiments were from a hybrid flock derived from a Light Sussex female and a C020 male. Eggs were obtained from a commercial breeder (Locksley Ltd., Nuneaton, England).

Avian erythrocytes were obtained from 13-day-old chicken embryos by cutting the allantoic blood vessels and allowing blood to drain into the allantoic fluid for 10 min. This fluid was removed from the egg, and erythrocytes were collected by centrifugation at 500 × *g* for 10 min and washed twice in Alsever solution.

Monolayers of primary chicken embryo fibroblast (CEF) cells were prepared in 5-cm petri dishes (32).

Viruses. We used influenza virus A/FPV/Rostock/34 (Hav1 N1) (FP/R), Newcastle disease virus (NDV) strain Texas, and Semliki Forest virus (SFV). FP/R stocks were grown in 11-day embryonated chicken eggs by injection into the allantoic cavities of approximately 10^5 plaque-forming units (PFU) in 0.1 ml of phosphate-buffered saline (PBS). After incubation for 18 h at 37°C, the eggs were chilled and the allantoic fluid was collected. This was used as stock virus. NDV stocks were prepared similarly except that infected eggs were incubated for 48 h at 37°C. Infectivity was determined by plaque assay in CEF monolayers, and the hemagglutinin (HA) was titrated as previously described (11). Neuraminidase (NA) was assayed by the liberation of *N*-acetyl neuraminic acid from fetuin (37). SFV was grown in CEF cells in suspension (19).

Purification of erythrocytes by centrifugation through 10% Ficoll. A 10% solution of Ficoll (Sigma Chemicals Ltd., Poole, Dorset, England) was prepared in PBS and sterilized by autoclaving. Blood cells (2 ml) in Alsever solution were diluted with ice-cold 10% Ficoll (1 ml) to a final concentration of 4×10^8 cells/ml. This mixture (1.5 ml) was layered onto 10 ml of 10% Ficoll and centrifuged at 100 × *g* for 10 min at 4°C. Erythrocytes sedimented to the bottom of the tube leaving a mixture of leukocytes and erythrocytes at the interface (see Results).

Infection of cells. Both CEF cell monolayers and the purified suspension of erythrocytes were infected at a multiplicity of 30 PFU/cell. Virus was adsorbed to cells for 1 h at 37°C. As expected, this caused erythrocytes to aggregate and, where necessary, aggregates were dispersed, as described in Results. Cells were incubated in medium 199 containing 5% newborn calf serum (Flow Laboratories Ltd., Irvine, Scotland) buffered to pH 7.4 with 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)-

NaOH. Erythrocytes were kept in suspension on a Rolamix mixing wheel (Luckham & Co. Ltd., Burgess Hill, Sussex, England). Zero time of infection is taken as the time of addition of virus.

Radiolabeling of cells. Before labeling, cells were washed twice with PBS. Labeling was carried out in Earles saline buffered with 20 mM HEPES-NaOH to pH 7.4 and containing 100 μCi of [^{35}S]methionine (specific activity, 800 μCi/mmol, Radiochemical Centre, Amersham) per ml.

PAGE. Gradient slab gels for polyacrylamide gel electrophoresis (PAGE) containing sodium dodecyl sulfate (SDS) (20 by 20 cm) were prepared by using the tris(hydroxymethyl)aminomethane (Tris)-glycine buffer system (23). The polyacrylamide gradient was from 10 to 30% (wt/vol) and contained 0 to 8% glycerol. The ratio of bisacrylamide (Eastman-Kodak, Rochester, N.Y.) to acrylamide was 1:212.

Samples were prepared by the addition of 1/10 of a volume of 1% β-mercaptoethanol together with 1.4% (wt/vol) SDS followed by heating in a boiling water bath for 2 min. After electrophoresis at 20 mA for 13 h the gels were dried under suction onto a filter paper and exposed to Kodirex film (Kodak Ltd., Hemel Hempstead, Herts, England) for 5 to 8 days.

Fluorescent antibody staining. After infection with FP/R for 1 h at 37°C, cells were pelleted and suspended in Earles saline containing 25 mM Tris-hydrochloride (pH 7.7) and 10 mg of trypsin (type III, Sigma Chemicals Ltd., Poole, Dorset, England) per ml to disperse aggregates. Incubation was continued for 30 min at 37°C and the cells were rinsed and resuspended in PBS adjusted to pH 3 for 1 min at 4°C to inactivate residual infectious virus (36). This treatment did not affect the ability of the erythrocytes to synthesize labeled viral proteins as determined by PAGE. After the required periods of incubation at 37°C a smear of infected and noninfected cells in 20% calf serum was made on microscope slides. These were dried with a fan and subsequently fixed in acetone at 4°C. Cells were stained by the indirect technique, firstly with anti-NP serum (18) for 30 min at 37°C and then with fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin G (Wellcome Reagents Ltd., Beckenham, England) for the same period. After incubation with serum, preparations were soaked in PBS for 40 min with six changes of solution to reduce the background level of fluorescence to a minimum. Fluorescence was observed with a Reichart Binolux II microscope, and all photographic procedures were kept constant to allow direct comparison of the prints.

RESULTS

Preparation of purified erythrocytes. Preliminary experiments showed that newly synthesized radiolabeled viral protein could be detected in washed but unfractionated chicken blood cells infected with FP/R. However, 13-day chicken embryo blood contains a small proportion (0.01%) of leukocytes (35) and it was necessary to separate erythrocytes and leukocytes to show which were responsible for viral protein synthesis.

Cells were separated as outlined in Materials and Methods. The leukocyte fraction was further processed by a second centrifugation through 10% Ficoll. In this way, two populations of cells were obtained: purified erythrocytes and an enriched population of leukocytes in which there was an approximately equal proportion of leukocytes to erythrocytes (i.e., a 10^4-fold enrichment of leukocytes). Blood from 60 embryos yielded approximately 4×10^6 leukocytes. The purity of these cell populations was monitored by phase-contrast microscopy and by the use of a Coulter Channelyzer C-1000 (Coulter Electronics Ltd., Harpenden, Herts, England) which measures the distribution of suspended particles as a function of their volume. Figure 1 is a trace obtained from the Coulter Channelyzer showing that erythrocytes and leukocytes from the interface of the Ficoll separation consist of two size classes. By applying the appropriate formula (16) to the relative volumes, the actual volumes were calculated as approximately 79 and 84 μm^3, respectively. Cells which pelleted through the 10% Ficoll were distributed in a single peak corresponding to a volume of 84 μm^3. These data are consistent with the microscopic observation showing that interface material consists of leukocytes and erythrocytes, whereas pelleted material comprised only erythrocytes. The volumes

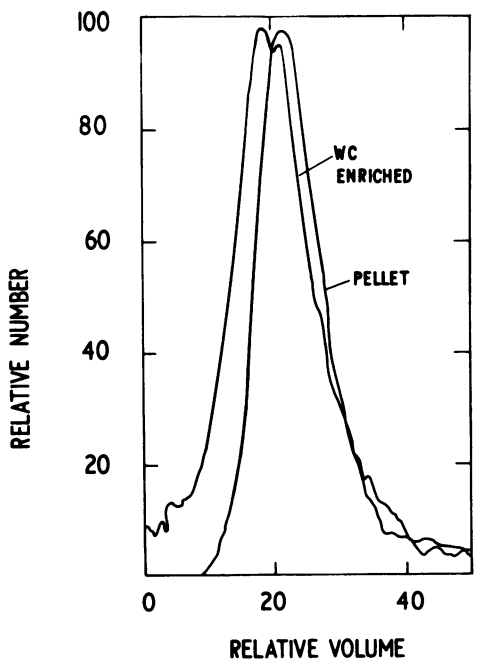

FIG. 1. *Size distribution of purified erythrocytes and of a preparation enriched for leukocytes (WC) made by centrifugation in 10% Ficoll, in which cell volume is plotted against relative cell number. The graph is the direct trace from a Coulter Channelyzer.*

given above are in agreement with values calculated from published figures (35). We conclude that centrifugation through 10% Ficoll offers a procedure for purifying chicken erythrocytes.

Infection of erythrocytes and leukocytes with FP/R. A total of 10^7 unfractionated blood cells, 10^7 purified erythrocytes, and 2×10^6 cells from the enriched leukocyte population were infected with FP/R. Incorporation of trichloroacetic acid-precipitable radioactivity into blood cells or purified erythrocytes was not stimulated on infection. However, newly synthesized radiolabeled viral proteins were detected on analysis by PAGE; Fig. 2 shows that both preparations synthesize viral proteins in approximately equal amounts. No protein synthesis was detected in leukocytes on PAGE even on exposure of the autoradiogram for 8 weeks. Leukocyte viability was confirmed by the exclusion of trypan blue by around 50% of the population. Since the enriched white cell population contained about 10^4-fold more leukocytes than whole blood we conclude that leukocytes are not contributing significantly to the synthesis of viral proteins observed in infected blood. Thus, although it was not necessary to purify erythrocytes, we continued to use the procedure as an added precaution.

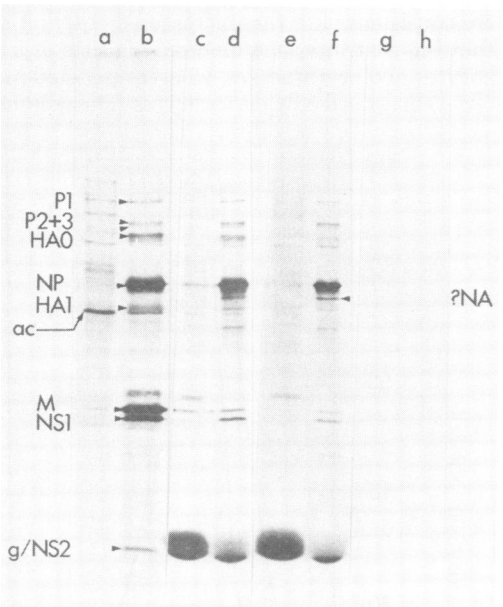

FIG. 2. *PAGE of proteins synthesized in chicken cells infected with FP/R: (a), (b) CEF cells; (c), (d) unfractionated blood cells (10^7/ml); (e), (f) purified erythrocytes (10^7/ml); (g), (h) enriched white cells (2×10^6/ml). (a), (c), (e), and (g) were not infected and (b), (d), (f), and (h) were infected with 30 PFU per cell. Cells were labeled with [^{35}S]methionine from 5 to 6 h postinfection. Host proteins actin (ac) and globin (g) are also indicated. Nomenclature of FP/R proteins follows the established convention (15, 21, 24). Tracks c to f were loaded with approximately equal radioactivity (80,000 cpm) and g and h each were loaded with 8,000 cpm, the maximum available.*

By comparison with the proteins known to be synthesized in CEF cells it is apparent that erythrocytes synthesize P_1, P_2, and P_3, the uncleaved HA precursor which may or may not be glycosylated, NP, M, and NS_1. HA_1 but not HA_2 was detected; in addition, there was a protein migrating in advance of NP which appeared in neither noninfected erythrocytes or CEF cells. This protein is in the position expected of nonglycosylated NA, which in its glycosylated form co-migrates with NP. The smallest viral protein NS_2 was obscured by globin in infected erythrocytes and blood.

Fluorescent antibody staining of FP/R-infected erythrocytes. It was important to determine whether viral proteins were synthesized in all or possibly in only a few particularly productive erythrocytes. Infected erythrocytes stained with anti-NP serum fluoresced more brightly than noninfected cells. However, the level of fluorescence was low at all times. Positive fluorescence was found at 3 h postinfection and this increased up to 8 h postinfection (Fig. 3) showing that NP antigen is synthesized de novo. It is not due to the progressive accumulation of inoculum virus, since cells were disaggregated after infection with trypsin and residual virus was removed with pH 3 buffer solution. As shown in Fig. 3, all erythrocytes fluoresced with the same intensity, indicating that infection and synthesis were uniform with respect to time and quantity of antigen synthesized. Fluorescence was too faint to be sure of its location, although it seemed to be brightest in the region of the nucleus, as described originally by Breitenfeld and Schafer (6).

Comparison of the amounts of FP/R protein synthesized in purified erythrocytes and CEF cells. Equal amounts of total protein from infected erythrocytes and infected CEF cells were loaded onto a polyacrylamide gel. Both cell types were infected at 30 PFU/cell and labeled at 5 to 6 h postinfection with [^{35}S]methionine (100 μCi/10^7 cells). A comparison made at this time was justified since the time courses of viral protein synthesis in erythrocytes and CEF are very similar (unpublished data). The resulting autoradiogram was scanned with a Joyce-Loebl densitometer, and the area under the viral NP peak produced by each cell type was calculated. When normalized for total protein, erythrocytes synthesized 5% of the amount of NP found in CEF cells. However, if the calculations are made on the amount of NP syn-

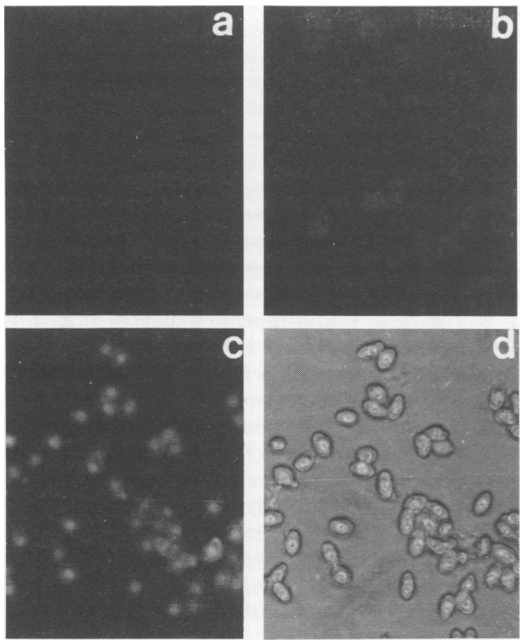

FIG. 3. *Fluorescent antibody staining of (a) noninfected erythrocytes, (b) infected erythrocytes at 3.5 h postinfection, and (c) infected erythrocytes at 8 h postinfection with FP/R; (d) shows the same field as (c) viewed under phase-contrast optics. All aspects of photography of the stained cells were kept constant.*

thesized per cell, then erythrocytes make 0.65% NP compared with CEF cells.

Multiplication of NDV and SFV in erythrocytes. Before looking for the production of infectious FP/R, we infected erythrocytes with viruses which do not need nuclei for their multiplication. NDV and SFV both have RNA genomes and are natural pathogens of birds. After infection, cells were washed extensively and treated with trypsin as before. Residual NDV inoculum was further reduced by treatment with neutralizing antiserum for 1 h at 37°C and residual SFV was further reduced by washing cells with pH 3 buffer solution. Samples of cells and culture fluids were disrupted at intervals after infection, and infectivity was measured by plaque assay on CEF monolayers. There was an increase in infectivity of about 100-fold of NDV and SFV during the incubation time (Fig. 4), showing that erythrocytes are able to sustain a productive infection by both viruses.

Failure of FP/R to multiply in erythrocytes. Erythrocytes were infected as described previously, and after 1 h at 37°C, cells were washed, disaggregated with trypsin, and treated with pH 3 buffer solution as described above.

Cells were disrupted in culture fluids at intervals after infection and assayed on CEF monolayers. No rise in the amount of infectious virus present was detected (Fig. 4). Titrations were repeated in suspension plaque assays (26) in the presence of trypsin to enhance infectivity (1, 10). Again the level of infectious virus present did not rise above the initial level of 900 PFU/10^7 cells even though A/PR/8/34, which requires trypsin for plaquing, was assayed successfully (Table 1). By calculating the ratio of NP synthesized in CEF cells to NP synthesized in erythrocytes, and by knowing the number of plaque-forming units synthesized in CEF cells, we estimated that the infectivity in erythrocytes was over 400-fold less than the expected value (Table 2). The expected-observed ratios for HA and NA are discussed in the next section.

Hemagglutination by and NA activity in FP/R-infected erythrocytes. Erythrocytes were infected, and residual inoculum was reduced as described for the preparation of cells for fluorescent antibody staining. Samples of cells were removed at intervals and disrupted by ultrasonication on ice. No hemagglutinating activity was detected. Standard NA assays incubated for 1 h at 37°C failed to give a positive result so incubation was continued for 30 h. This revealed NA activity which increased with the duration of infection (Fig. 5) and which was

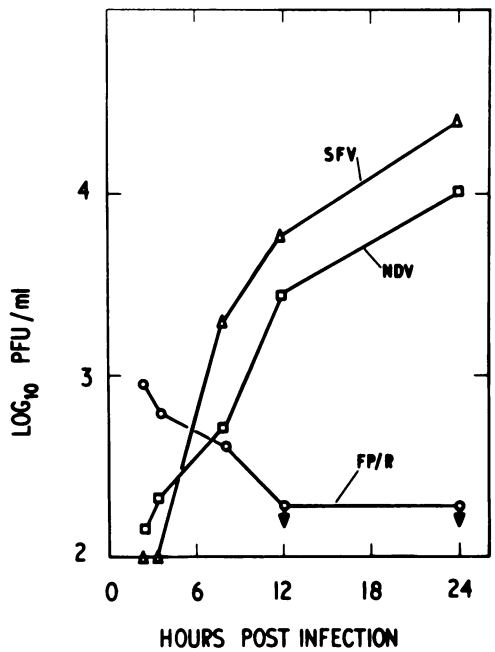

FIG. 4. *Extent of multiplication in erythrocytes of NDV, SFV, and FP/R. Cells (10^7/ml) were infected at 30 PFU/cell and then treated to remove inoculum as described in Materials and Methods. Samples were taken at intervals, and production of infectious virus was measured by plaque assay.*

TABLE 1. *Cell suspension plaque assay for the multiplication of FP/R in erythrocytes*[a]

Virus	+ Trypsin	− Trypsin
FP/R in erythrocytes postinfection (hours)		
2.5	900[b]	NT[c]
3.5	600	NT
8.0	400	NT
12.0	<150	NT
24.0	<150	<150
A/FP/Rostock[d]	8.9×10^8	8.0×10^8
A/PR/8/34[d]	3.1×10^7	$<10^2$

[a] Samples of erythrocytes were mixed with 2.5 ml of 3×10^7 CEF cells in suspension and dispersed in an equal volume of double-strength overlay medium containing 20 μg of diethylaminoethyl-dextran and 100 μg of trypsin per ml. FP/R and PR/8 were inoculated directly into the CEF suspension.

[b] Number of plaque-forming units per 10^7 erythrocytes.

[c] NT, Not tested.

[d] Number of plaque-forming units per milliliter of virus suspension.

TABLE 2. *Synthesis of PFU, HA, and NA activities in FP/R-infected erythrocytes*[a]

Cells infected	PFU	NA	HA
Erythrocytes			
Expected	6×10^4	0.11	13.0
Observed	1.5×10^2	0.14[b]	<0.6
Expected-observed	400	0.8	≥21.7
CEF	8.5×10^{6c}	17.0[d]	2,000

[a] Expected values were calculated from the ratio of ND synthesized in CEF cells and erythrocytes, i.e., 100:0.65 arbitrary units (see text).

[b] Optical density at 549 nm per hour calculated from a 30-h incubation.

[c] All figures are for 10^7 cells plus culture fluids.

[d] Optical density at 549 nm per hour.

serologically identical with the FP/R virion enzyme (data not shown). It was calculated that the ratio of NP to NA activity synthesized in erythrocytes is similar to that in CEF cells (Table 2). However, the relative HA titer was more than 6.5-fold lower than expected.

Viral proteins synthesized by erythrocytes infected with NDV and SFV. Since these viruses multiplied in erythrocytes we investigated the synthesis of viral proteins. Figure 6 shows PAGE autoradiograms of cells infected by the standard procedure with NDV or SFV. In the former there was very little detectable viral protein synthesis and in the latter there was none (compare with FP/R in Fig. 2), even at times when infectious virus production had increased by 100-fold. The major NDV polypeptide appeared in the 55,000 to 65,000 molecular weight region, which agrees with a previous study (8). In another experiment, a faint band was seen in SFV-infected erythrocytes (data not shown), corresponding to the envelope protein.

DISCUSSION

We have shown that purified erythrocytes can be infected with FP/R and will synthesize viral proteins. Confirmation that leukocytes were not responsible for viral synthesis was obtained when a leukocyte preparation containing approximately 10^4-fold more leukocytes than unfractionated blood failed to synthesize any detectable viral proteins. The question of whether all erythrocytes were equally capable of being infected or whether the observed viral protein was synthesized by a few highly productive erythrocytes was resolved by fluorescent antibody staining; NP antigen was detected at a uniform level in all erythrocytes.

To our knowledge this is the first account of de novo infection and synthesis of viral components in erythrocytes. However, no infectious FP/R was produced by the infected erythrocytes. This is not due to an inability of these cells to form progeny virus particles per se since two other enveloped viruses (NDV and SFV) were able to productively infect erythrocytes. The amounts of viral components or infectious virus produced by erythrocytes are low compared with CEF cells, but since the numbers of erythrocytes per individual bird are very large, there is considerable potential for virus produc-

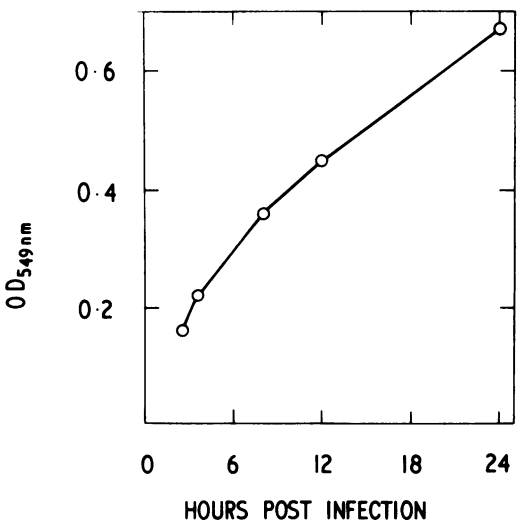

FIG. 5. *Synthesis of active NA in FP/R-infected erythrocytes. Samples of 10^7 erythrocytes were assayed for the presence of NA by incubation with fetuin for 30 h at 37°C. The initial value is thought to represent residual inoculum.*

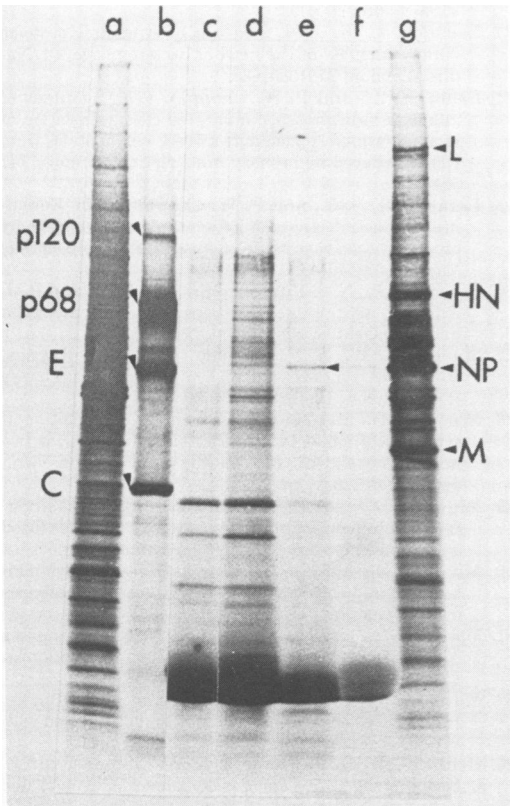

FIG. 6. *PAGE of proteins synthesized by erythrocytes and CEF cells infected with NDV or SFV under the same conditions as those used in Fig. 2 for FP/R. Viral proteins are arrowed. (a) Noninfected CEF cells; (b) SFV-infected CEF cells both a and b pulsed with [^{35}S]methionine from 6 to 6.5 h postinfection; (c) SFV-infected erythrocytes pulsed from 9 to 9.5 h postinfection; (d) noninfected erythrocytes; (e) (f) NDV-infected erythrocytes pulsed 3 to 3.5 and 9 to 9.5 h postinfection respectively; (g) NDV-infected CEF cells. The experiment with (c), (e), and (f) was done in the presence of 3 μg of actinomycin D per ml to reduce endogenous protein synthesis and enhance the detection of viral proteins. Nomenclature of SFV and NDV proteins follows the conventions (7, 8).*

tion. However, we have no experimental knowledge of the significance of our findings to pathogenesis.

The production of infectious progeny virus was not related to the amount of newly synthesized viral protein accumulating inside the cell, since the proteins specified by SFV and NDV were barely detectable, whereas the FP/R-directed synthesis was considerable. The reason why no FP/R progeny virions were formed did not emerge. We eliminated the possibility that it was due to the failure of HA_0 to be cleaved, by plaquing in the presence of trypsin which cleaves HA_0 and activates infectivity (22, 25). Another reason for this lack of infectious FP/R could be associated with the levels of functional HA found in infected cells (Table 2). Since the relative amount of HA_0 polypeptide was normal, the absence of functional protein may be due to a defect in posttranslational modification (evidenced perhaps by the absence of HA_2), although the erythrocytes evidently carry out analogous processing on NDV and SFV. However, there are many other possible defects in virion formation which might account for the lack of production of infectious virus observed. Even so, we have not ruled out that erythrocytes are making very low levels of infectious virus. This possibility is relevant to the "recycling" hypothesis of antigenic shift in the antigenicity of influenza viruses, which proposes that strains lie dormant and reemerge later to infect humans when herd immunity falls to an ineffective level (20). As yet no source of persistent or chronic infection of influenza has been identified.

While this work was in progress a parallel arose between the FP/R-erythrocyte system and the coupled transcription-translation in vitro system (9, 31). The erythrocyte nucleus is dormant (compared with a normal dividing cell) and the in vitro system does not require nuclei for the synthesis of viral proteins. This possibility was tested by the use of actinomycin D, which does not inhibit the in vitro system (31), but which abolished the synthesis of FP/R proteins in erythrocytes, showing that nuclear function(s) were indeed operating (Cook, Avery, and Dimmock, manuscript in preparation). The failure of rabbit erythrocytes, which are enucleate, to synthesize FP/R proteins is consistent with this involvement of the nucleus (unpublished data).

ACKNOWLEDGMENTS

We are grateful for financial support from the Agricultural Research Council and to P. D. Minor for advice on the polyacrylamide gel linear gradient.

LITERATURE CITED

1. **Appleyard, G., and H. B. Maber.** 1974. Plaque formation by influenza viruses in the presence of trypsin. J. Gen. Virol. **25**:351-357.
2. **Auer, G., G. P. M. Moore, N. R. Ringertz, and A. Zetterberg.** 1973. DNA-dependent RNA synthesis in nuclear chromatin of fixed cells. Relationship between dye binding properties, nuclear protein content and RNA polymerase activity. Exp. Cell Res. **76**:229-233.
3. **Barry, R. D.** 1964. The effects of actinomycin D and ultraviolet light on the production of fowl plaque virus. Virology **24**:563-569.
4. **Barry, R. D., D. R. Ives, and J. G. Cruickshank.** 1962. Participation of deoxyribonucleic acid in the multiplication of influenza virus. Nature (London) **194**:1139-1141.
5. **Bossart, W., J. Meyer, and K. Bienz.** 1973. Electron microscopic study on influenza virus haemagglutina-

tion: pinocytosis of virions by red cells. Virology **55**: 295-298.

6. **Breitenfeld, P. M., and W. Schafer.** 1957. The formation of fowl plaque virus antigens in infected cells, as studied with fluorescent antibodies. Virology **55**:295-298.
7. **Bruton, C., A. Porter, and S. I. T. Kennedy.** 1976. Defective-interfering particles of Semliki Forest virus: intracellular events during interference. J. Gen. Virol. **31**:397-416.
8. **Clinkscales, C. W., and M. A. Bratt.** 1978. Initiation of protein synthesis by Newcastle disease virus, p. 617-627. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses and the host cell. Academic Press Inc., London.
9. **Content, J., L. De Wit, and H. Horisberger.** 1977. Cell-free coupling of influenza virus RNA transcription and translation. J. Virol. **22**:247-255.
10. **Cooper, P. D.** 1955. A method for producing plaques in a suspension of animal cells. Virology **1**:397-401.
11. **Emmons, R. W., L. S. Oshiro, H. N. Johnson, and E. H. Lennette.** 1972. Intra-erythrocytic location of Colorado tick fever virus. J. Gen. Virol. **17**:185-195.
12. **Follett, E. A. C., C. R. Pringle, W. H. Wunner, and J. J. Skehel.** 1974. Virus replication in enucleate cells: vesicular stomatatis and influenza virus. J. Virol. **13**: 394-399.
13. **Harris, H.** 1967. The reactivation of the red cell nucleus. J. Cell Sci. **2**:23-32.
14. **Henschel, C. C., and J. R. Tata.** 1978. Template-engaged and free RNA polymerase during Xenopus erythroid cell maturation. Dev. Biol. **65**:496-507.
15. **Inglis, S. C., A. C. Carroll, R. A. Lamb, and B. W. J. Mahy.** 1976. Polypeptides specified by the influenza virus genome. Virology **74**:489-503.
16. **Instruction Manual for Coulter Channelyzer C-1000.** 4th edition. 1975. Coulter Electronics Ltd., Harpenden, England.
17. **Kelly, D. C., R. J. Avery, and N. J. Dimmock.** 1974. Failure of an influenza virus to initiate infection in enucleate BHK cells. J. Virol. **13**:1155-1161.
18. **Kelly, D. C., and N. J. Dimmock.** 1974. Fowl plague virus replication in mammalian cell-avian erythrocyte heterokaryons: studies concerning the actinomycin D and ultraviolet light sensitive phase in influenza virus replication. Virology **61**:210-222.
19. **Kennedy, S. I. T., and D. C. Burke.** 1972. Studies on the structural proteins of Semliki forest virus. J. Gen. Virol. **14**:87-98.
20. **Kilbourne, E. D.** 1975. Epidemiology of influenza, p. 483-538. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press Inc., New York.
21. **Kilbourne, E. D., P. W. Choppin, I. T. Schultze, C. Scholtissek, and D. Bucher.** 1972. Influenza virus polypeptides and antigens—summary of influenza virus workshop I. J. Infect. Dis. **125**:447-455.
22. **Klenk, H.-D., R. Rott, M. Orlich, and J. Blödorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68**:426-439.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
24. **Lamb, R. A., and P. W. Choppin.** 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides from RNA produced by primary transcription. Virology **74**: 504-519.
25. **Lazarowitz, S. G., and P. W. Choppin.** 1975. Enrichment of the infectivity of influenza A and B viruses by proteolytic cleavage of the haemagglutinin polypeptide. Virology **68**:440-454.
26. **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.
27. **Madgwick, W. J., N. Maclean, and Y. A. Baynes.** 1972. RNA synthesis in chicken erythrocytes. Nature (London) New Biol. **238**:137-139.
28. **Minor, P. D., and N. J. Dimmock.** 1975. Inhibition of synthesis of influenza virus proteins: evidence for two host cell-dependent events during multiplication. Virology **67**:114-123.
29. **Minor, P. D., and N. J. Dimmock.** 1976. The multiplication of influenza virus in enucleated BHK cells fused with chicken erythrocytes. Virology **69**:336-338.
30. **Minor, P. D., and N. J. Dimmock.** 1977. Selective inhibition of influenza virus protein synthesis by inhibitors of DNA function. Virology **78**:393-406.
31. **Minor, P. D., and N. J. Dimmock.** 1979. The coupling of transcription of influenza virions to translation *in vitro*. Arch. Virol. **59**:201-212.
32. **Morser, M. J., S. I. T. Kennedy, and D. C. Burke.** 1972. Virus-specified polypeptides in cells infected with Semliki forest virus. J. Gen. Virol. **21**:19-29.
33. **Oshiro, L. S., D. V. Dondero, R. W. Emmons, and E. H. Lennette.** 1978. The development of Colorado tick fever virus within cells of the haemopoietic system. J. Gen. Virol. **39**:73-79.
34. **Palese, P., M. B. Ritchey, and J. L. Schulman.** 1977. Mapping of the influenza virus genome. II. Identification of the P1, P2 and P3 genes. Virology **76**:114-121.
35. **Romanoff, A. L.** 1960. The avian embryo. The Macmillan Co., New York.
36. **Stephenson, J. R., J. B. Hudson, and N. J. Dimmock.** 1978. Early events in influenza virus multiplication. II. Penetration of virus into cells at 4°C. Virology **86**:264-271.
37. **Webster, R. G., and W. G. Laver.** 1967. Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. J. Immunol. **99**: 49-55.
38. **Tobita, K., and E. D. Kilbourne.** 1974. Genetic recombination for antigenic markers of antigenically different strains of influenza B virus. J. Virol. **13**:347-352.