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Berne Virus Is Not 'Coronavirus-like'

By M. C. HORZINEK,* M. WEISS[1] AND J. EDERVEEN

Institute of Virology, Veterinary Faculty, State University, Utrecht, Yalelaan 1, 3508 TD Utrecht, The Netherlands and [1] Virology Department, Institute of Bacteriology, Veterinary Faculty, University of Berne, Switzerland

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SUMMARY

In infected embryonic mule skin cells, Berne virus directs the synthesis of two main polypeptides (22K, 20K); in addition, virus-specific proteins with apparent molecular weights of >200K, 80K to 120K, 32K and 17K were detected after radioimmune precipitation. The replication of Berne virus was reduced more than 1000-fold by actinomycin D, when the drug (0·1 to 1·0 μg/ml) was added during the first 8 h after infection; alpha-amanitin (25 μg/ml) produced a similar though less pronounced effect. U.v. preirradiation of the cells for ⩾5 s led to a dramatic decrease in the production of extracellular virus. The results presented support our suggestion that Berne virus is a representative of a new family of animal viruses.

Recently, we reported the purification and partial characterization of a new enveloped RNA virus which had been isolated from a horse in Berne, Switzerland. 'Berne virus' measures 120 to 140 nm in its largest diameter and consists of a peplomer-bearing envelope and an elongated core which is bent into an open torus within the membrane. Its curved shape is apparently maintained by the tightly fitting envelope; from thin-section electron microscopic images we concluded that the Berne virion has the unique shape of a (biconcave) disc. The core is tubular in appearance and shows a transverse striation indicative of a helical nucleocapsid symmetry. Berne virus possesses an RNA genome: its growth is not affected by iododeoxyuridine (IUdR) under conditions where equine herpesvirus replication is inhibited by more than 3 $\log_{10}$ units of infectivity (Weiss *et al.*, 1983).

We have shown that Berne virus is serologically unrelated to infectious bronchitis, mouse hepatitis (MHV) and transmissible gastroenteritis viruses which are representatives of three antigenic clusters of coronaviruses (Weiss *et al.*, 1983). It is conceivable, however, that Berne virus could still be 'coronavirus-like' with respect to its particle structure and mode of replication. In the present paper we report first observations on the polypeptides synthesized under the control of the genome of Berne virus. To obtain evidence of whether its replication requires a synthetic function of the host cell we have performed controlled inhibition studies using actinomycin D (AMD), alpha-amanitin (AMA) and u.v. exposure of cell monolayers prior to infection.

Embryonic mule skin (EMS) cells were grown to subconfluence in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% foetal calf serum as described previously (Weiss *et al.*, 1983). Four h after infection of the monolayers with about 1 ID_{50} unit per cell of Berne virus (strain P138/72) [^{35}S]methionine (44 μCi per 35 mm Costar Petri dish) was added in a methionine-free medium supplemented with 2% dialysed foetal calf serum. Cells and medium were harvested separately at 20 h post-infection and samples of an infected and a mock-infected culture were electrophoresed in parallel in an SDS–polyacrylamide gel (17.5%) following a modified Laemmli procedure. Radioiodinated MHV and a ^{14}C-methylated protein molecular weight marker set (Amersham International) were used as internal standards.

Two prominent polypeptides with apparent molecular weights of 22K and 20K, respectively, can be distinguished in cells and medium of the infected cultures (Fig. 1, left). They are distinctly smaller than the E1 glycosylated envelope proteins of MHV (26.5K to 24K; Rottier *et al.*, 1981).

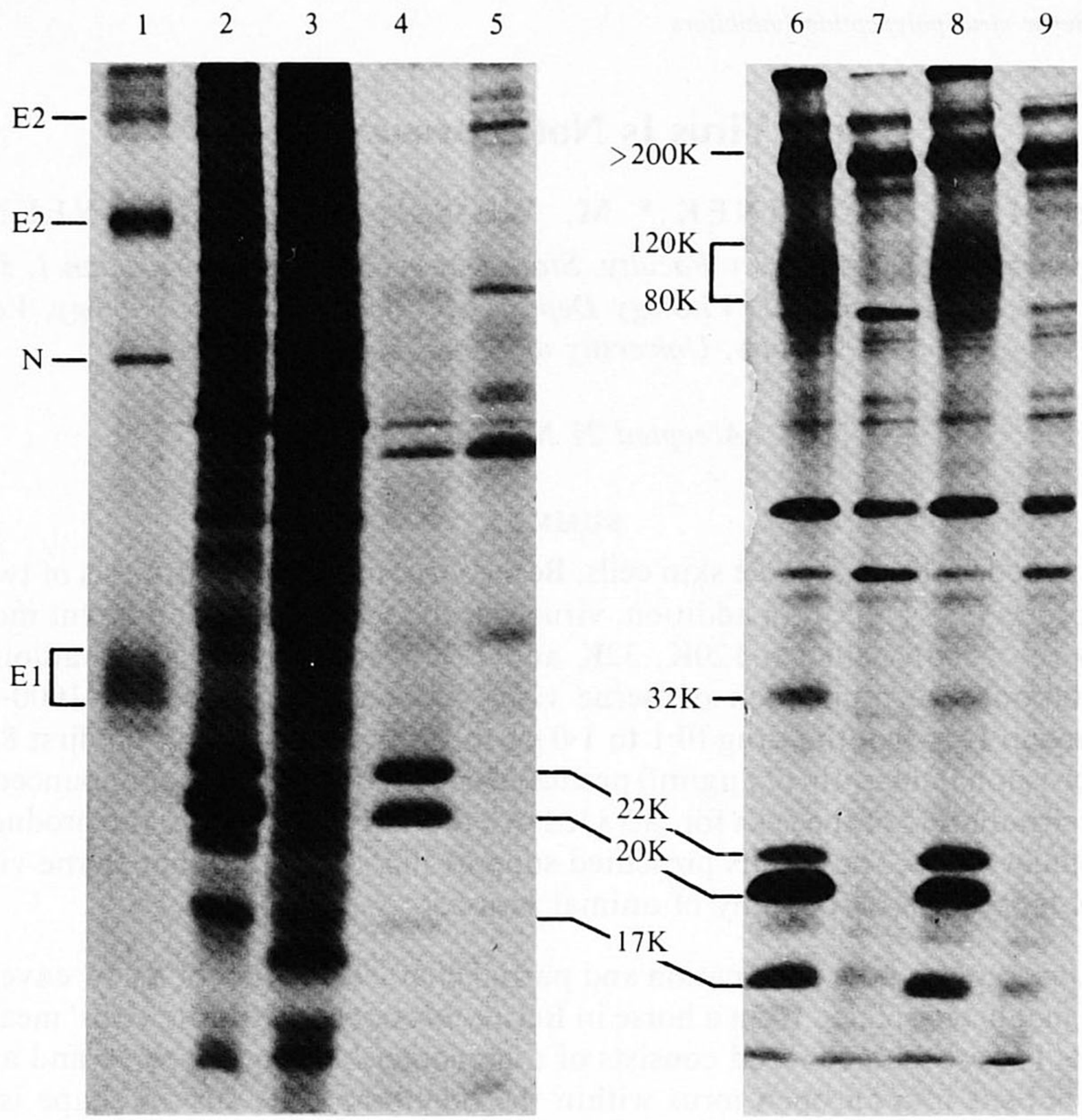

Fig. 1. SDS–polyacrylamide gel electrophoresis of ^{35}S-labelled proteins of Berne virus and radioiodinated MHV (lane 1). Infected (even numbers) and uninfected (odd numbers) EMS cell extracts and culture supernatants (lanes 4 and 5) were analysed directly (left, 17·5% gel) or after immune precipitation using two different horse sera (right, 15% gel). Letters refer to the peplomer (E2, 180K and 90K), nucleocapsid (N, 54K) and envelope proteins (E1, 26·5K to 24K) of MHV.

In order to detect minor viral polypeptides and those of higher molecular weight, radioimmune precipitation followed by electrophoresis in a 15% gel was performed as described previously (Horzinek *et al.*, 1982). Horse sera selected on the basis of their neutralizing activity were employed. Fig. 1 (right) again shows the predominant 22K and 20K species and additional bands for which molecular weights of 17K, 32K, 80K to 120K and >200K were calculated. Experiments using a ^{14}C-labelled amino acid mixture gave similar results: again no label in the size range of coronavirus nucleocapsids (47K to 64K, as listed by Siddell *et al.*, 1982) was found (M. C. Horzinek & J. Ederveen, unpublished observations).

Subconfluent EMS cell monolayers were infected at a low multiplicity (1 to 5 ID_{50} units/cell) with Berne virus or with EMS cell-adapted Semliki Forest virus (SFV), Kumba strain. After 1 h of adsorption, the monolayers were rinsed three times with phosphate-buffered saline (PBS). Actinomycin D (Merck, Sharp & Dohme, Rahway, N.J., U.S.A.) was added at different times after infection and the virus yield per culture was determined at 24 h after infection. Infectivity titrations were performed as described previously (Weiss *et al.*, 1983) or in tissue culture quality Terasaki plates (Greiner & Söhne, Nürtingen, F.R.G.) seeded with 5000 cells in 10 μl per well. Attachment of the cells was allowed to proceed for 2 to 24 h; serial tenfold dilutions of the culture supernatants in Eagle's minimum essential medium with 2% foetal calf serum were added in 10 μl volumes to each well. The plates were incubated for 5 days in a CO_2 atmosphere and infectivity titres were calculated using the Kärber formula.

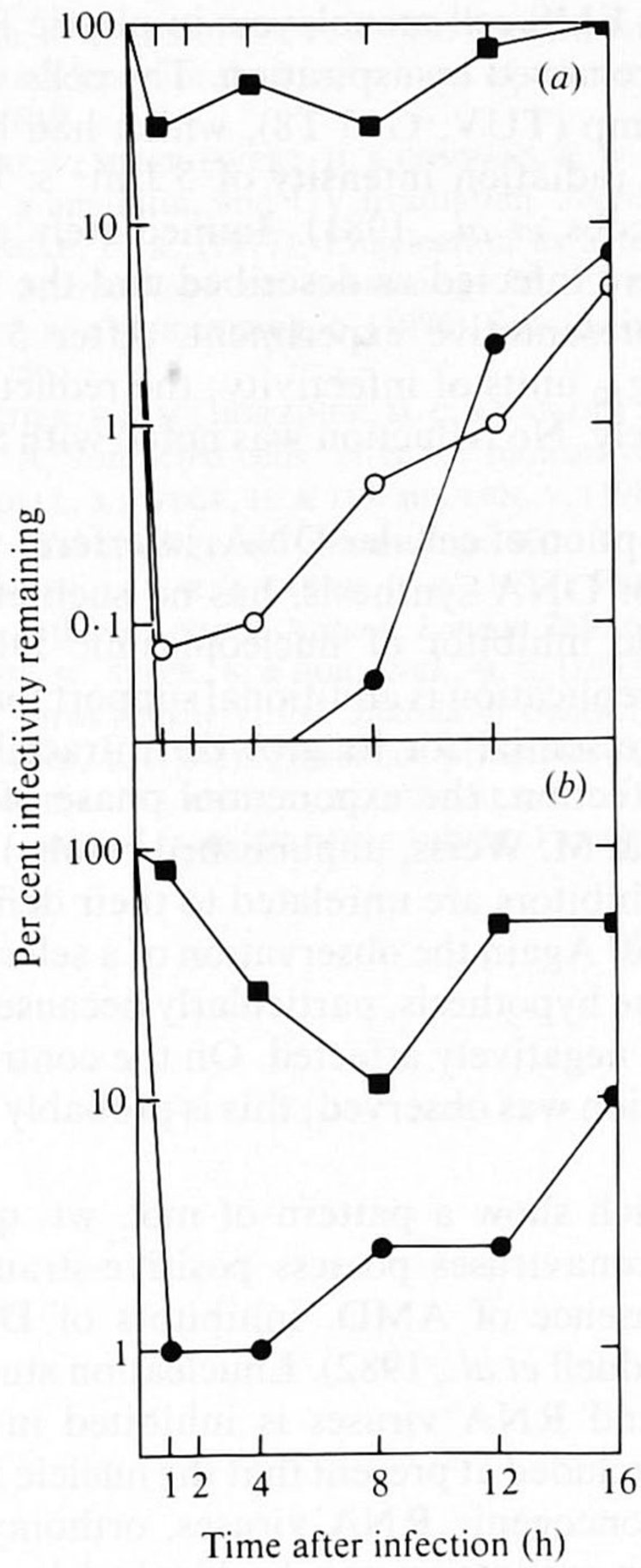

Fig. 2

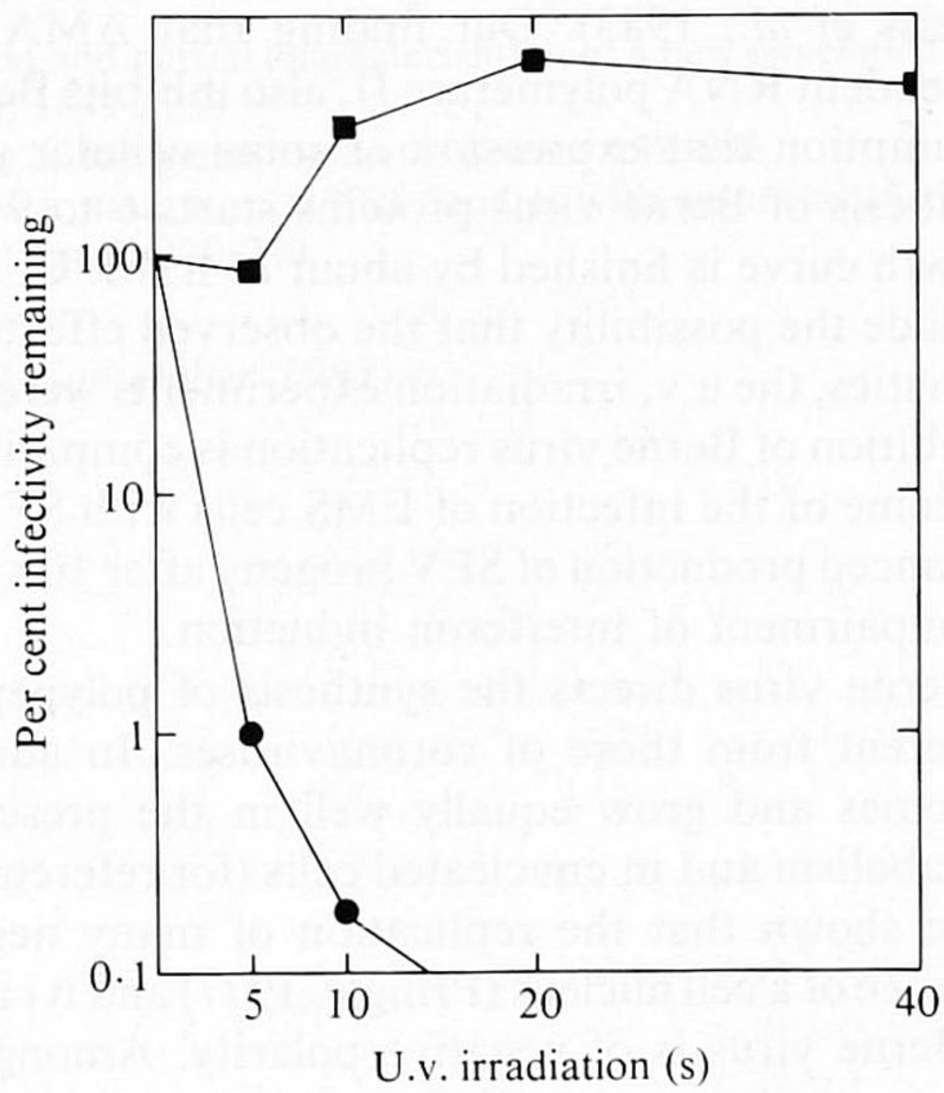

Fig. 3

Fig. 2. Effect of inhibitors on the replication of Berne virus (●, ○) and Semliki Forest virus (■). Actinomycin D (*a*) was added to EMS cell cultures at the indicated times after infection at a concentration of 1 μg/ml (●) or 0·1 μg/ml (○); SFV-infected cultures (■) received 1 μg of the drug. The concentration of alpha-amanitin (*b*) was 25 μg/ml for both viruses. Note that the ordinate scales differ.

Fig. 3. Effect of u.v. pre-irradiation of EMS cells on infectivity yields of Berne virus (●) and Semliki Forest virus (■).

The results of an experiment using two concentrations of AMD (0·1 and 1·0 μg/ml) added at different times after infection are presented in Fig. 2(*a*). Concentrations of the drug as low as 0·1 μg/ml reduced the yields of Berne virus by more than 3 $\log_{10}$ units of infectivity when it had been added during the first 8 h while leaving SFV virtually unaffected (not shown).

Alpha-amanitin, a bicyclic polypeptide from the toadstool *Amanita phalloides*, is known to inhibit specifically the DNA-dependent RNA polymerase II of mammalian cells by interaction with the enzyme rather than with the DNA template (Wigand, 1968). The results of a representative AMA experiment using Berne virus and SFV in parallel are depicted in Fig. 2(*b*). Cell monolayers were infected as described and 25 μg/ml AMA was added at different times after infection. Pronounced decreases in the 24 h yields of Berne virus were observed when the drug had been added 1 to 4 h post-infection; when AMA was added at 8 h or later, a progressive increase in yields was noted.

For the investigation of the effects of u.v. irradiation, EMS cell monolayers in plastic Petri dishes were washed three times with PBS and the fluid removed by aspiration. The cells were then exposed to u.v. from a Philips 15 W germicidal lamp (TUV, G15 T8), which had been positioned at a distance from the culture to result in a radiation intensity of 5 J/m^2 s. U.v. measurements were made as described previously (Jacobs *et al.*, 1981). Immediately after irradiation for different periods of time, the cultures were infected as described and the 24 h yields were titrated. Fig. 3 shows the results of a representative experiment. After 5 s of irradiation, the yield of Berne virus was reduced by 2 $\log_{10}$ units of infectivity; the reductions after 20 and 40 s were 3·5 and $>6{\cdot}5$ $\log_{10}$ units, respectively. No reduction was noted with SFV under identical conditions.

We have shown that AMD, an inhibitor of the transcription of cellular DNA, interferes with the multiplication of Berne virus; IUdR, an inhibitor of DNA synthesis, has no such effect (Weiss *et al.*, 1983). Our finding that AMA, a specific inhibitor of nucleoplasmic DNA-dependent RNA polymerase II, also inhibits Berne virus replication is additional support for the assumption that expression of some cellular gene(s) is essential for its growth. Intracellular synthesis of Berne virus proteins starts 6 to 9 h after infection; the exponential phase of the growth curve is finished by about 16 h (M. C. Horzinek & M. Weiss, unpublished results). To exclude the possibility that the observed effects of the inhibitors are unrelated to their defined activities, the u.v. irradiation experiments were performed. Again the observation of a selective inhibition of Berne virus replication is compatible with the hypothesis, particularly because the outcome of the infection of EMS cells with SFV was not negatively affected. On the contrary, enhanced production of SFV progeny after 10 s of irradiation was observed; this is probably due to impairment of interferon induction.

Berne virus directs the synthesis of polypeptides which show a pattern of mol. wt. quite different from those of coronaviruses. In addition, coronaviruses possess positive-stranded genomes and grow equally well in the presence or absence of AMD, inhibitors of DNA metabolism and in enucleated cells (for references, see Siddell *et al.*, 1982). Enucleation studies have shown that the replication of many negative-strand RNA viruses is inhibited in the absence of a cell nucleus (Pringle, 1977) and it cannot be excluded at present that the nucleic acid of Berne virus is of negative polarity. Among the non-oncogenic RNA viruses, orthomyxoviruses until now occupied a special position since their replication can be blocked by u.v. irradiation of the host cell and by inhibitors, including AMD and AMA (Barry, 1964; Rott & Scholtissek, 1970; Lamb & Choppin, 1977; Spooner & Barry, 1977; Petri *et al.*, 1979). Functioning of the host nuclear RNA polymerase II is required for influenza viral RNA transcription; viral mRNAs synthesized *in vivo* contain a short stretch of nucleotides at their 5′ end, including the cap, that are not virus-coded (Krug *et al.*, 1979). It remains to be shown whether a similar mechanism operates in Berne virus-infected cells.

In negatively stained preparations, Berne virus and the antigenically related Breda isolates (Woode *et al.*, 1982) appear 'coronavirus-like'. The present study shows that this resemblance is only superficial. Experiments are under way to study the location and function of the virion polypeptides and to determine the strandedness of the genome of Berne virus.

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