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Replication of Canine Herpesvirus

I. Synthesis of Viral Deoxyribonucleic Acid

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This paper reports the results of two series of experiments. The first series indicated that deoxyribonucleic acid (DNA) extracted from partially purified canine herpesvirus virions is characterized by a high guanine plus cytosine molar base ratio (65 to 67 mole%), similar to the DNA of herpes simplex virus. In the second series of experiments it was estimated, on the basis of uptake of tritiated thymidine, that in dog kidney cells canine herpesvirus-DNA synthesis starts at 4 hr and continues until 16 hr after infection. Treatment of infected cells with puromycin during the first 4 hr of infection blocks the onset of viral DNA synthesis, whereas, after this time the uptake of thymidine is unaffected.

Recently, a virus morphologically similar to the herpesviruses and designated canine herpesvirus (CHV) was isolated from neonatal and fetal pups suffering from a fatal septicemic disease (6, 19, 20). It has been reported that the virus develops, at least in part, in the nucleus of infected cells (19, 20), that it probably contains deoxyribonucleic acid (DNA; 6), and that it is immunologically related to herpes simplex virus (1).

The present studies indicate that CHV has a DNA characterized by a high guanine plus cytosine (GC) molar base ratio, similar to that of herpes simplex DNA (15).

The kinetics of viral DNA synthesis in infected dog kidney (DK) cells were studied by uptake of tritiated thymidine. Since the processes of viral and cellular DNA syntheses overlap in infected DK cells, separation of the two DNA species becomes a requirement for the definitive studies of these events. Toward this end, the nucleic acids were extracted and ultracentrifuged in a CsCl solution.

MATERIALS AND METHODS

Solutions and media. PBS-A consisted of phosphate-buffered-saline with 0.2% bovine albumin (Fraction V, Armour Laboratories, Chicago, Ill.). Medium M-M was maintenance medium consisting of mixture 199 supplemented with 1% fetal calf serum. Medium 199-Mc, a semisolid overlay used for assay of CHV in DK monolayer cultures, consisted of mixture 199 with 1% fetal calf serum and contained 10 g of methyl cellulose (4,000 centipoises) per liter of medium. Tris-saline buffer contained 0.2 M tris-(hydroxymethyl) aminomethane and 0.85% NaCl, *p*H 7.2; Mg^{++} buffer, 10^{-2} M $MgCl_2$ in Tris-saline-buffer, was used as diluent for deoxyribonuclease and for virus suspensions to be treated with deoxyribonuclease. Saline citrate solution (SCS) was prepared as described by Marmur (11).

Chemicals. The following sources provided chemicals: Worthington Biochemical Corp., Freehold, N.J., bovine pancreas 1× crystallized deoxyribonuclease I. Fisher Scientific Co., Fairlawn, N.J., cesium chloride purified; J. T. Baker Chemical Co., Phillipsburg, N.J., sucrose crystal Baker analyzed; New England Nuclear Corp., Boston, Mass., ^{3}H-(methyl)thymidine (specific activity 6.7 c/mM); Nutritional Biochemical Co., Cleveland, Ohio, puromycin dihydrochloride.

Cells. DK cells were maintained in this laboratory since 1963.

Virus. The pertinent properties of the F205 strain of CHV have been described (1, 2, 6, 20). Virus assays were performed by a method previously described (1), and titers are expressed in terms of plaque-forming units (PFU)/ml of fluid.

Virus purification. Virus was partially purified by a method previously described for herpes simplex virus (4). Essentially, 24 hr after infection the cells were scraped, sedimented, washed with Tris-saline, and suspended in 10 ml of Tris-saline buffer. Virus was released from the cells by three cycles of freezing and thawing, and the cellular debris was removed by centrifugation at 2,000 rev/min for 30 min. The resulting supernatant fluid was centrifuged at 20,000 rev/min for 60 min in a Spinco 30° angle rotor. The virus pellet resuspended by sonic oscillation in 2 ml of Mg^{++} buffer was exposed to a solution of deoxyribonuclease (final concentration 30 μg/ml) at 37 C for 1 hr to degrade free DNA. This preparation was immediately layered on preformed gradients of 12 to 52% (w/w) sucrose in cellulose nitrate tubes [3 by 1 inch (7.62 by 2.54 cm)] and centrifuged for 1 hr at 15,000 rev/min in a Spinco SW25 rotor head. Eight fractions were aspirated from the side of each tube by

means of syringe and needle inserted at appropriate levels starting from the top. Individual fractions were dialyzed against Tris-saline buffer for 2 hr, and each fraction was assayed for infectivity and radioactivity. The fractions containing the peak infectivity and radioactivity were centrifuged at 20,000 rev/min for 1 hr. The virus pellets were resuspended in 1 ml of Tris-saline buffer by ultrasonic vibration, mixed with a solution of CsCl of a starting specific gravity of 1.220 g/cm^3, and centrifuged to equilibrium (40 hr). Drops collected from the bottom of the tube were assayed for infectivity and radioactivity. Densities were determined by weighing filled 50-μliter micropipettes of known weight.

Extraction and density determination of DNA. DNA was extracted from ^{3}H-(methyl)thymidine-labeled infected DK cells as follows. Cells from two replicate cultures were pooled, washed twice in SCS, and resuspended in a solution of sodium lauryl sulfate (3%) in SCS. After 30 min of incubation at 37 C, an equal volume of 95% ethyl alcohol was added at room temperature. The precipitate was sedimented, washed twice with cold ethyl alcohol, and resuspended in SCS. DNA was extracted from ^{3}H-(methyl)thymidine-labeled CHV previously partially purified by banding in CsCl density gradients by adding 0.3 ml of 6% sodium lauryl sulfate to 0.9 ml of virus in cellulose nitrate tubes [1 by 3/8 inch (2.5 by 0.9 cm)]. A solution of CsCl of known starting specific gravity was added to each, and the mixtures were centrifuged in the SW39 rotor of a Spinco model L centrifuge at 35,000 rev/min and 10 C for 40 hr. Drops were collected in vials containing filter discs (cellulose esters; Millipore Corp., Bedford, Mass.). The tritium disintegrations were counted in a Nuclear-Chicago liquid spectrometer. The densities were determined by weighing filled micropipettes (50 μliter) of known weight.

RESULTS

Virus purification. Monolayers of DK cells exposed to 100 PFU of CHV per cell for 2 hr were washed with PBS-A, overlayed with M-M containing 20 μc of ^{3}H-(methyl)thymidine per ml, and incubated at 37 C for 24 hr. At this time the cells were scraped, and the virus was released by three cycles of freezing and thawing and partially purified as described. Two visible bands designated B and G appeared consistently after centrifugation on sucrose gradients (Fig. 1). The major zones of infectivity and radioactivity corresponded to the band in fraction G. The large amount of label in fractions A and B can be attributed to degraded cellular DNA. The virus in fraction G was pelleted by centrifugation at 20,000 rev/min for 60 min and centrifuged to equilibrium in a CsCl density gradient. Infectivity and radioactivity peaked in the same fraction at a density of 1.258 to 1.260 g/cm^3 (Fig. 1), a density reported for other herpesviruses (14, 18). The virus obtained by pooling the peak fractions of this gradient was used in DNA extractions.

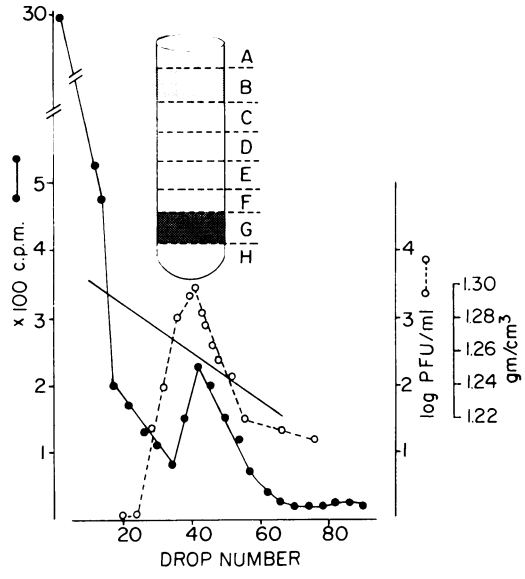

FIG. 1. *Distribution of infectivity and radioactivity in fractions collected from a CsCl gradient (initial specific gravity = 1.220 g/cm^3) after equilibrium sedimentation of the virions that had banded in fraction "G" of a 12 to 52% linear sucrose gradient centrifuged for 1 hr at 15,000 rev/min.*

Differentiation of viral and cellular DNA. The differentiation of viral and cellular DNA is based on their buoyant densities as determined by equilibrium sedimentation in CsCl. The density of DNA extracted from mammalian cells ranges generally from about 1.680 to 1.700 g/cm^3 (3, 16). By contrast, higher densities have been reported for all herpesviruses. A density of 1.720 to 1.725 g/cm^3 has been described for herpes simplex and pseudorabies DNA, on the basis of GC molar base ratios (5) as well as by direct measurements (3, 15, 16). A somewhat lower density (1.715 to 1.717 g/cm^3), also by direct measurement, has been reported for equine abortion virus (17).

To determine the density of CHV-DNA, two extracts were prepared: (i) a *crude extract* from infected DK cells, incubated in medium containing 20 μc of ^{3}H-(methyl)thymidine per ml and consisting of a solution, in SCS and CsCl, of the constituents released from the infected cells with sodium lauryl sulfate and precipitated with ethyl alcohol; and (ii) a *purified extract* from virus labeled with ^{3}H-(methyl)thymidine previously partially purified as described.

The tritium label in the crude extract localized in two bands with apparent densities of 1.725 and 1.700 g/cm^3, respectively (Fig. 2A). The tritium label of the partially purified extract localized in one band with an apparent density of 1.723 g/cm^3 (Fig. 2B). The thymidine-labeled material

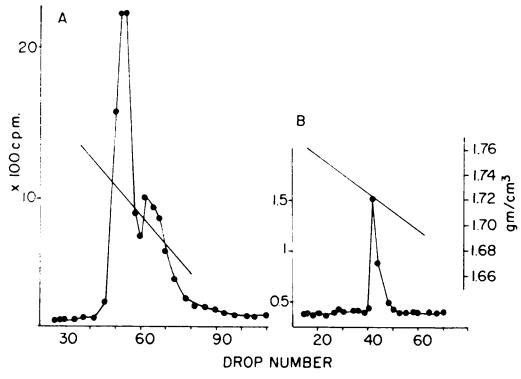

FIG. 2. *Distribution of ^3H label in nucleic acids after equilibrium sedimentation in a CsCl density gradient. (A) Crude extract prepared from infected DK cells; (B) extract prepared from virus concentrated and partially purified by equilibrium sedimentation in CsCl density gradient.*

in the low density fraction of the crude extract is identified with cellular DNA, whereas the label in the fractions with density of 1.723 to 1.725 g/cm³ may be identified with viral DNA.

Identification of DNA made at intervals in the replicative cycle. DK cells exposed for 1 hr to 100 PFU of CHV per cell were washed and overlayed with M-M containing 20 μc of ^3H-(methyl)-thymidine per ml, immediately (0 hr) and at 2, 4, 7, 10, and 13 hr after infection. At 2 to 3 hr after addition of the label, the cells were scraped and washed, and the nucleic acids were extracted and ultracentrifuged in a solution of CsCl. The distribution of tritium label in fractions obtained after equilibrium sedimentation is shown in Fig. 3. During the first 4 hr after infection, viral DNA is not made. This is evident from the fact that the tritium label was found almost exclusively in a fraction with characteristic density of cellular DNA. During 4 and 10 hr, only a small amount of radioactive thymidine became incorporated into the fraction with characteristic density of viral DNA. However, after this time, uptake into viral DNA began to increase and, between 13 and 16 hr, label was preferentially incorporated into viral rather than cellular DNA. The data suggest that the bulk of viral DNA is synthesized between 10 and 16 hr after infection. Thymidine uptake into cellular DNA fraction decreased with time after infection but was not entirely abolished.

Effect of puromycin on viral DNA synthesis. Three infected and one uninfected DK monolayer cultures were overlaid with M-M and incubated at 37 C. At 3 and 6 hr, respectively, one of the infected cultures received 50 μg of puromycin per ml (16, 21). At 15 min after addition of puromycin, each of the four cultures received sufficient ^3H-(methyl)thymidine to yield 20 μc/ml. At 14 to 17 hr after addition of thymidine, the nucleic acids were extracted and centrifuged in CsCl.

The distribution of tritium label in fractions collected after equilibrium sedimentation is shown in Fig. 4. The ^3H-(methyl)thymidine in extracts of uninfected cells (Fig. 4A) localized in one band with a density of approximately 1.700 g/cm³. The tritium label in extracts of infected cells, untreated (Fig. 4B) and treated with puromycin at 6 hr after infection (Fig. 4D), localized in two bands with approximate densities of 1.724 and 1.706 g/cm³, respectively. The label in extracts of infected cells treated with puromycin immediately or at 3 hr after incubation (Fig. 4C) localized in one band with a density similar to that of cellular DNA.

The amount of label recovered from the uninfected cells (Fig. 4A) roughly corresponds to the amount of label in the low density band of the infected untreated cells (Fig. 4B). On the other hand, the amounts of tritium label in the extract of untreated infected cells (Fig. 4B) and of the infected cells treated with puromycin at 6 hr after incubation (Fig. 4D) differ severalfold.

The appearance of the band of heavy DNA made in the presence of puromycin suggests that it consists of DNA molecules varying slightly in density. It could be that we are measuring primarily fragments of viral DNA differing in GC content.

DISCUSSION

The objectives of these experiments were to differentiate between cellular and viral DNA on the basis of their buoyant densities and to measure the time course of synthesis of viral DNA in DK cells treated and untreated with puromycin. The results obtained merit discussion from the standpoint of the GC content and the pattern of synthesis of viral DNA, of whether there is host DNA synthesis in infected cells, and lastly of the dependence of viral DNA synthesis on uninterrupted protein synthesis during the replicative cycle.

Synthesis of viral DNA. The progress recently achieved in the understanding of the synthesis of the DNA of herpesviruses is largely due to the fact that the DNA of herpes simplex and pseudorabies viruses, the two most widely studied exponents of this group of viruses, has a high GC molar base ratio and is readily separated from cellular DNA by isopycnic centrifugation in CsCl density gradients. The most extensive studies were done with pseudorabies DNA synthesis in rabbit kidney cells (8–10). This system is particularly advantageous because only viral DNA is synthesized in rabbit kidney cells arrested prior to infection by contact inhibition; host DNA synthesis along with cell division remain inhibited

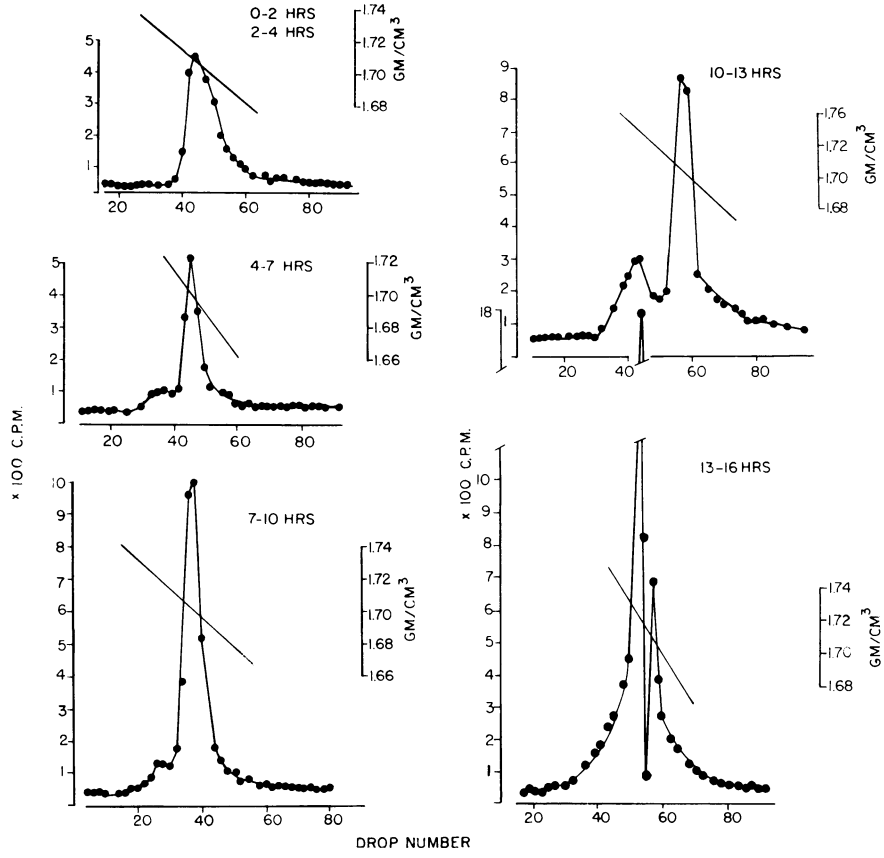

FIG. 3. *Distribution of ^3H label in nucleic acids extracted from infected DK cells after equilibrium sedimentation in a CsCl density gradient. The cells were incubated in a medium containing ^3H-(methyl)thymidine for the intervals shown in the upper right of each curve.*

throughout the reproductive cycle (7). Such is not the case with CHV; optimal yields of this virus are obtained from young rapidly growing DK cells (2) in which host DNA synthesis persists after infection. Therefore, differentiation of viral from cellular DNA becomes essential for the understanding of the temporal relationships of host and viral DNA syntheses.

Whereas there is good agreement that the DNA of all herpesviruses have a GC content greater than that of animal cells, the DNA of some herpesviruses has been shown to contain a lower GC content than that of others (17). The observation that CHV-DNA, like the DNA of herpes simplex and pseudorabies viruses, localizes at a density of 1.723 to 1.725 g/cm^3 upon centrifugation in CsCl density gradients suggests that it contains a high GC molar base ratio (65 to 67 mole%) and therefore can be differentiated from cellular DNA by centrifugation in a CsCl solution.

In general agreement with the results obtained for other herpesviruses (8, 13, 16), synthesis of CHV-DNA starts at 4 hr after infection. Thymidine uptake into DNA with a density characteristic of viral DNA increases with time after infection, and, between 13 and 16 hr, label is preferentially incorporated into viral rather than cellular DNA. At this time viral DNA synthesis levels off as suggested by the following observations. (i) The disparity between cellular and viral DNA does not increase with incubation for intervals longer than 16 hr. (ii) Between 16 and 24 hr after infection tritium label is incorporated only into viral DNA; however, the rate of uptake is very low (Aurelian, *unpublished data*).

Synthesis of host DNA. In cells exposed to CHV, tritium label becomes incorporated into both cellular and viral DNA (Fig. 2 and 3). The question arises whether cellular DNA synthesis continues after infection. At the high multiplicities of infection used in this study, all or most of the cells should have been infected within a relatively

short time. If cellular DNA were made only in the few remaining uninfected cells, it would be expected that the amount of label incorporated into cellular DNA during the 3-hr intervals (4 to 7, 7 to 10, 10 to 13, and 13 to 16 hr) after exposure to virus would remain relatively constant. However, whereas tritium uptake into cellular DNA increases slightly during the first 10 hr after infection, uptake gradually decreases after this time (Fig. 3). These findings suggest that, until 16 hr, CHV infection decreases but does not abolish cellular DNA synthesis. However, at this time, inhibition of cellular DNA synthesis appears to be total. This is evident from the observation that, in infected cells incubated in presence of tritiated thymidine during 16 to 24 hr after infection, label is incorporated only into viral DNA (Aurelian, *unpublished data*). The transient increase in the amount of radioactive thymidine incorporated into cellular DNA is without precedent for herpesviruses.

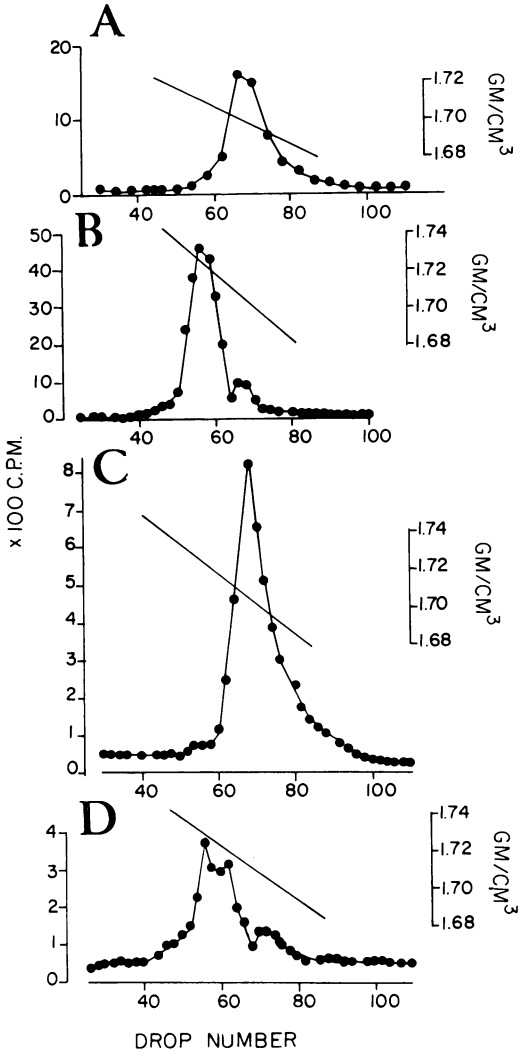

FIG. 4. *Distribution of tritium label in nucleic acids extracted from DK cells incubated in medium containing ^{3}H-(methyl)thymidine. (A) Uninfected cells; (B) CHV-infected cells incubated in medium containing ^{3}H-(methyl)thymidine from 3 hr after infection; (C) CHV-infected cells incubated in medium containing puromycin from 3 hr after infection; (D) CHV-infected cells incubated in medium containing puromycin from 6 hr after infection. ^{3}H-(methyl)thymidine was added 15 min after puromycin.*

Requirements for synthesis of viral DNA. First, the exposure of CHV-infected DK cells to puromycin at any time between 0 and 3 to 4 hr after infection blocks the synthesis of viral DNA. This finding is in agreement with the data reported for herpes simplex virus (16) and indicates that protein synthesis lasting for 3.5 to 4 hr after infection is required to initiate synthesis of viral DNA.

Second, cells exposed to puromycin at 4 and 6 hr after infection, i.e., after onset of viral DNA synthesis, continue to incorporate labeled thymidine into viral DNA, but at a reduced rate.

The observation that puromycin treatment immediately after infection blocks the onset of synthesis of viral DNA indicates that viral DNA synthesis requires the participation of new enzymes made after infection. However, there is no simple explanation for the observation that, in cells treated with puromycin after the onset of viral DNA synthesis, the rate of incorporation of thymidine into viral DNA is reduced. The greatest reduction in rate of incorporation of thymidine into viral DNA occurs within a short period of time after addition of puromycin, and could reflect either a sudden change in the pool size of thymidine or a real change in the rate of synthesis of viral DNA. The first explanation must be considered in view of the possibility raised by Newton et al. (12) that the size of the thymidine pool in the infected cells may change during the reproductive cycle, and the observation made in this study that the rate of uptake of thymidine into cellular DNA increases during 4 and 10 hr after infection. Also, it is of interest that puromycin treatment gives rise to a reduction in the rate of incorporation of thymidine into cellular as well as viral DNA (Fig. 4D). The second hypothesis would indicate that puromycin interferes with the availability of a necessary rate-limiting constituent, such as an enzyme or the primer itself. In this respect it might be pertinent that in several experiments the appearance of the heavy DNA band made in the presence of puro-

mycin suggested that it consists of DNA molecules varying slightly in density, i.e., viral DNA seemed to consist of fragments with varying GC content.

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LITERATURE CITED

1. Aurelian, L. 1968. Demonstration by plaque-reduction technique of immunologic relationship between canine herpesvirus and herpes simplex virus. Proc. Soc. Exp. Biol. Med. 127:485–488.
2. Aurelian, L. 1969. Factors affecting the growth of canine herpesvirus in dog kidney cells. Appl. Microbiol. 17:179–181.
3. Aurelian, L., and B. Roizman. 1964. The host range of herpes simplex virus. Interferon, viral DNA and antigen synthesis in abortive infection of dog kidney cells. Virology 22:452–461.
4. Aurelian, L., and R. R. Wagner. 1966. Two populations of Herpesvirus virions which appear to differ in physical properties and DNA composition. Proc. Nat. Acad. Sci. U.S.A. 56:902–909.
5. Ben-Porat, T., and A. S. Kaplan. 1962. The chemical composition of herpes simplex and pseudorabies viruses. Virology 16:261–266.
6. Carmichael, L. E., J. D. Strandberg, and F. D. Barnes. 1965. Identification of a cytopathogenic agent infectious for puppies as a canine herpesvirus. Proc. Soc. Exp. Biol. Med. 120:644–650.
7. Kaplan, A. S., and T. Ben-Porat. 1960. The incorporation of C^{14}-labeled nucleosides into rabbit kidney cells infected with pseudorabies virus. Virology 11:12–27.
8. Kaplan, A. S., and T. Ben-Porat. 1963. The pattern of viral and cellular DNA synthesis in pseudorabies virus-infected cells in the logarithmic phase of growth. Virology 19:205–214.
9. Kaplan, A. S., and T. Ben-Porat. 1964. Mode of replication of pseudorabies virus DNA. Virology 23:90–95.
10. Kaplan, A. S., T. Ben-Porat, and C. Coto. 1967. Studies in the control of the infective process in cells infected with pseudorabies virus, p. 527–545. *In* T. Colter (ed.), Molecular biology of viruses. Academy Press Inc., New York.
11. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208–218.
12. Newton, A. A., P. P. Dendy, C. L. Smith, and P. Wildy. 1962. A problem associated with the use of tritiated thymidine. Nature 194:886–887.
13. O'Callaghan, J. D., W. P. Cheevers, G. A. Gentry, and C. C. Randall. 1968. Kinetics of cellular and viral DNA synthesis in equine abortion (herpes) virus infection of L-M cells. Virology 36:104–114.
14. Roizman, B., and L. Aurelian. 1965. Abortive infection of canine cells by herpes simplex virus. I. Characterization of viral progeny from cooperative infection with mutants differing in capacity to multiply in canine cells. J. Mol. Biol. 11:528–538.
15. Roizman, B., L. Aurelian, and P. R. Roane, Jr. 1963. The multiplication of herpes simplex virus. I. The programming of viral DNA duplication in HEp-2 cells. Virology 21:482–498.
16. Roizman, B., and P. R. Roane. Jr. 1964. The multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology 22:262–269.
17. Soehner, R. L., G. A. Gentry, and C. C. Randall. 1965. Some physiochemical characteristics of equine abortion virus nucleic acid. Virology 26:394–405.
18. Spear, P. G., and B. Roizman. 1967. Buoyant density of herpes simplex virus in caesium chloride. Nature 214:713–714.
19. Stewart, S. E., J. David-Fereira, E. Lovelace, J. Landon, and N. Stock. 1965. Herpes-like virus isolated from neonatal and fetal dogs. Science 148:1341–1343.
20. Strandberg, J. D., and L. E. Carmichael. 1965. Electron microscopy of a canine herpesvirus. J. Bacteriol. 90:1790–1792.
21. Wheelock, E. F. 1962. The role of protein synthesis in the eclipse period of Newcastle virus multiplication in HeLa cells as studied with puromycin. Proc. Nat. Acad. Sci. U.S.A. 48:1358–1365