Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 524–528, February 1977
Biochemistry

Bovine papilloma virus: Presence of virus-specific DNA sequences in naturally occurring equine tumors

(equine sarcoid/DNA·DNA hybridization/reassociation kinetics/thermal denaturation)

WAYNE D. LANCASTER*†, CARL OLSON‡, AND WILLIAM MEINKE*

*Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California, 92037; and ‡Department of Veterinary Science, University of Wisconsin, Madison, Wisc. 53706

Communicated by Robert J. Huebner, November 15, 1976

ABSTRACT **Four of five spontaneous benign equine connective tissue tumors of unknown etiology and a bovine papilloma virus (BPV)-induced equine tumor contained BPV-specific DNA sequences as determined by DNA·DNA hybridization of DNA from tumors with BPV DNA labeled *in vitro*. Analysis of the kinetics of reassociation indicated that 20–75% of the BPV genome was present in the various tumors. The number of partial BPV genome equivalents ranged from 60 to 500 copies per diploid quantity of cellular DNA.**

Thermal denaturation profiles of duplexes formed between labeled BPV DNA and DNA from tumor cells indicated two tumors contained viral DNA with base sequences identical to BPV DNA. Three tumors (including DNA from the BPV-induced tumor) contained BPV-related DNA sequences that were less thermally stable. The decrease in thermal denaturation temperature may be due to the presence of (adenine + thymine)-rich regions of the BPV genome in the tumor cells.

The papilloma viruses are a subgroup of the Papovaviridae and produce a self-limiting neoplastic disease in their natural hosts. The bovine papilloma virus (BPV) is the most oncogenic of the papilloma viruses. Intracranial injection of BPV produces meningiomas in calves, while subcutaneous injection of the virus induces fibromas in hamsters and C3H/eB mice (1–3). BPV can transform fetal bovine cells and mouse embryo cells in culture (4, 5). Transformation of fetal bovine skin cells by BPV DNA has also been reported (6).

Intradermal inoculation of BPV into horses and related species can induce a connective tissue tumor that is histologically similar to equine sarcoid, a spontaneous connective tissue tumor (7, 8). Equine sarcoid is one of the most commonly found tumors in horses and does not appear to metastasize, but excision often results in recurrence of the tumor (9, 10). BPV-induced and naturally occurring equine tumors differ in that induced tumors regress while spontaneous forms do not (7, 10). BPV-neutralizing antibodies are not detectable in horses with natural tumors, while neutralizing antibodies are produced in horses with virus-induced disease (11). BPV is equally efficient in inducing sarcoids in normal horses and horses with existing spontaneous tumors (11). Although the epidemiology of equine sarcoid suggests a viral etiology for the disease (12), virus particles have not been detected in or recovered from spontaneous tumors (C. Olson, unpublished observation). However, oncornavirus-like particles have been observed in cell lines derived from spontaneous equine sarcoids (13).

Abbreviations: BPV, bovine papilloma virus; A+T, adenine + thymine; T_m, temperature at which 50% of the hybrid was eluted from hydroxyapatite.

† To whom correspondence should be addressed. Present address: Department of Veterinary Science, University of Wisconsin, Madison, Wisc. 53706.

To determine if BPV may be involved in spontaneous equine sarcoid, we analyzed DNA extracted from tumor tissue as well as normal equine tissue for unlabeled BPV DNA sequences by DNA·DNA reassociation kinetics using BPV DNA labeled *in vitro* as a probe. The results demonstrate the presence of BPV-specific DNA sequences in four of the five naturally occurring tumors and in a BPV-induced equine tumor. There appears to be only a portion of the BPV genome in these tumors and the number of viral genome equivalents per diploid quantity of cellular DNA varies considerably. No BPV genome equivalents were detected in DNA from normal equine tissue.

This report provides direct evidence that a DNA tumor virus may be involved in the production of spontaneously occurring tumors in a genus unrelated to its natural host.

METHODS

Cellular DNA was isolated from equine tumors and normal tissues (stored at −20°) by a urea/phosphate/hydroxyapatite chromatography procedure (14) and further purified as described elsewhere (15). Salmon sperm DNA was purified by the same methods. Purified cellular DNA preparations were mechanically sheared to a uniform piece size at 50,000 pounds/inch^2 (340 MPa) (16).

Virus was extracted from bovine papillomas and partially purified by differential centrifugation and by banding in CsCl equilibrium density gradients (17). Virus was ruptured by treatment with 1% sodium dodecyl sulfate. Supercoiled BPV DNA was phenol extracted and subsequently isolated in CsCl/ethidium bromide equilibrium density gradients (18).

Form I (covalently closed circular, double-stranded) BPV DNA was "nicked" by treatment with pancreatic deoxyribonuclease I and labeled *in vitro* under conditions of repair synthesis with *Escherichia coli* DNA polymerase I (19, 20). The conditions for the reaction have been described (15, 17). ^{125}I-Labeled dCTP (about 100 μCi/μg of DNA), synthesized by the procedure of Shaw *et al.* (21), was used as the radioactive deoxynucleoside triphosphate. This procedure yielded DNA with specific activities of 4.2 to 5.4 × 10^7 cpm/μg. DNA labeled *in vitro* had average single-stranded lengths of 500–600 nucleotides, as determined by velocity sedimentation in alkaline sucrose gradients (17).

DNA·DNA reassociations were performed in 0.48 M sodium phosphate, pH 6.8, 0.05% sodium dodecyl sulfate, 1 mM EDTA. DNA solutions were denatured in screw-capped vials by heating to 109° for 3 min and quickly quenching in an ice bath and the DNA was allowed to reassociate at 68°. At various times, samples were removed and diluted to 2.0 ml in 0.14 M sodium

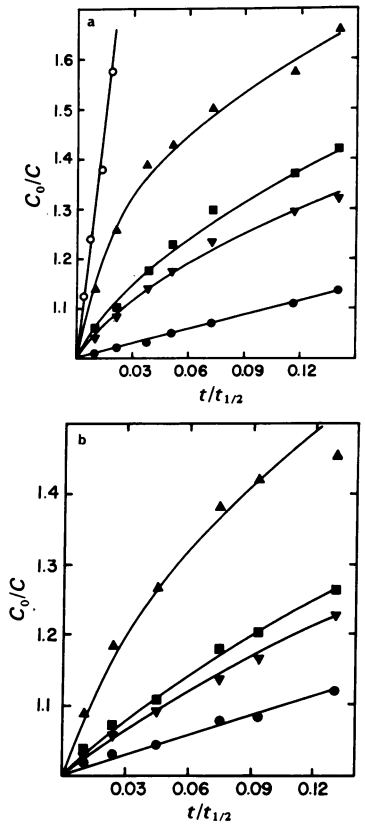

FIG. 1. Reassociation kinetics of ^{125}I-labeled BPV DNA with control DNA and DNA extracted from equine tumors. (a) The reactions contained 0.001 μg/ml of ^{125}I-labeled BPV DNA (5.4 × 10^7 cpm/μg) and 250 μg/ml of salmon sperm DNA (●), horse tumor 65-477 DNA (▼), BPV-induced tumor DNA (■), horse tumor 706931 DNA (▲), or salmon sperm DNA with 0.033 μg/ml of unlabeled BPV DNA (○). DNA solutions in 0.48 M sodium phosphate, pH 6.8, 0.05% sodium dodecyl sulfate, 1 mM EDTA, were heat-denatured and single-stranded DNA was allowed to reassociate at 68°. At various times samples were removed and the amount of BPV DNA reannealed was assayed by hydroxyapatite chromatography. Salmon sperm DNA and salmon sperm DNA plus unlabeled BPV DNA served as control reactions. Each point represents about 3300 cpm. The molar ratio of cellular DNA to ^{125}I-labeled BPV DNA was 0.3125. (b) The reactions contained 0.002 μg/ml of ^{125}I-labeled BPV DNA (5.2 × 10^7 cpm/μg) and 200 μg/ml of salmon sperm DNA (●), horse tumor 65-477 DNA (▼), BPV-induced tumor DNA (■), or horse tumor 706931 DNA (▲). DNA-DNA reassociation was as described above. Each point represents about 8000 cpm. The molar ratio of cellular DNA to ^{125}I-labeled BPV DNA was 0.1250.

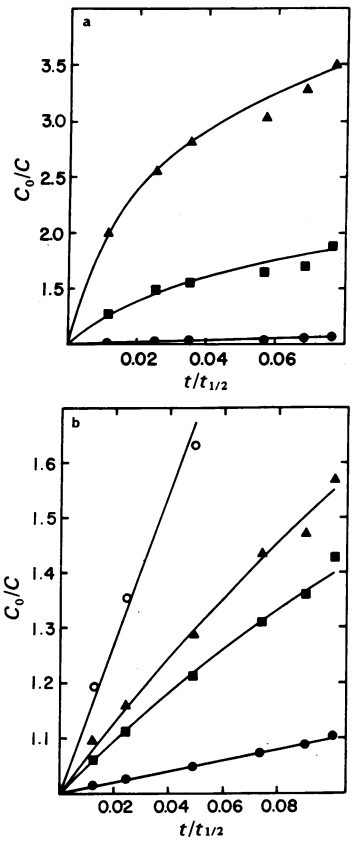

FIG. 2. Reassociation kinetics of ^{125}I-labeled BPV DNA with control DNA and DNA extracted from equine sarcoids. (a) The reactions contained 0.001 μg/ml of ^{125}I-labeled BPV DNA (5.2 × 10^7 cpm/μg) and 250 μg/ml of salmon sperm DNA (●), donkey tumor E29892-75 DNA (■), or horse tumor E36295-75 DNA (▲). DNA-DNA reassociation was as described in Fig. 1. Each point represents about 4200 cpm. The molar ratio of cellular DNA to ^{125}I-labeled BPV DNA was 0.3125. (b) The reactions contained 0.004 μg/ml of ^{125}I-labeled BPV DNA (4.2 × 10^7 cpm/μg) and 200 μg of salmon sperm DNA (●), 50 μg/ml of donkey tumor E29892-75 DNA plus 150 μg/ml of salmon sperm DNA (■), 200 μg/ml of horse tumor E36295-75 DNA (▲), or 200 μg/ml of salmon sperm DNA plus 0.050 μg/ml unlabeled BPV DNA (○). Reassociation conditions were as described in Fig. 1. Salmon sperm DNA and salmon sperm DNA plus unlabeled BPV DNA served as the controls. Each point represents about 3700 cpm. The molar ratio of cellular DNA to labeled BPV DNA was 0.0625 for equine sarcoid E36295-75 and 0.0156 for equine sarcoid E29892-75.

phosphate, pH 6.8, 0.05% sodium dodecyl sulfate at 4°. Reassociated duplex DNA was separated from single-stranded DNA by hydroxyapatite chromatography at 60° (22). Radioactivity was determined by liquid scintillation.

Reassociated DNA was thermally denatured on columns of hydroxyapatite (17). At selected temperature increments, single-stranded DNA was eluted with washes of preheated 0.12 M sodium phosphate, pH 6.8, 0.05% sodium dodecyl sulfate and radioactivity in each wash was determined.

RESULTS

BPV DNA labeled *in vitro* was used as a probe in DNA·DNA reassociation studies to test for the presence of BPV-specific DNA sequences in spontaneous equine sarcoids (23) because intradermal injection of BPV can induce histologically similar tumors in equine species.

The kinetics of DNA·DNA reassociation as described by Britten and Kohne (22) follow the equation

$$C_0/C = 1 + KC_0t \quad [1]$$

in which C_0 is the initial concentration of single-stranded labeled viral DNA, C is the concentration of single-stranded labeled viral DNA at time t, and K is the reassociation constant. A plot of C_0/C versus t results in a straight line with the slope directly proportional to the viral DNA concentration if the reaction follows second-order kinetics.

Reassociation of denatured ^{125}I-labeled BPV DNA in the presence of denatured DNA isolated from four of five spontaneous equine sarcoids and a BPV-induced equine tumor was accelerated over that of control reactions consisting of ^{125}I-labeled BPV DNA reannealing in the presence of salmon sperm DNA (Figs. 1 and 2). ^{125}I-Labeled DNA reassociated with apparent second-order kinetics in the presence of unlabeled BPV DNA (Figs. 1a and 2b) and in the presence of DNA isolated from one of the equine sarcoids that contained no detectable BPV DNA sequences (data not shown) and normal equine

Table 1. Summary of the calculation of values for X, r, and N from the kinetics of DNA·DNA reassociation of ^{125}I-labeled BPV DNA in the presence of equine tumor and normal tissue DNA

Tissue	Source	Molar ratio*	X	r	N†
Tumor					
E29892-75	Donkey	0.3125	0.75	165	528
		0.0156	0.70	7.8	500
E36295-75	Horse	0.3125	0.55	46	147
		0.0625	0.55	7	112
706931	Horse	0.3125	0.35	60	192
		0.1250	0.35	23	184
BPV-induced	Pony	0.3125	0.27	21	67
		0.1250	0.27	6	48
65-477	Horse	0.3125	0.20	22	70
		0.1250	0.20	6	48
E25489-75‡	Horse	0.3125	0	0	<2
Normal§					
Liver	Pony	1.0	0	0	<0.2
Adrenal	Pony	1.0	0	0	<0.2
Lymph node	Pony	1.0	0	0	<0.2
Spleen	Pony	1.0	0	0	<0.2
Kidney	Pony	1.0	0	0	<0.2

* Moles cellular DNA/moles ^{125}I-labeled BPV DNA.
† Number of partial BPV genome equivalents per diploid quantity of cellular DNA.
‡ The level of sensitivity was such that two BPV genome equivalents per diploid quantity of cellular DNA could have been detected.
§ The level of sensitivity was such that 0.2 BPV genome equivalents per diploid quantity of cellular DNA could have been detected. Each tissue was from a different animal.

tissues (Table 1). However, the kinetics of reassociation of ^{125}I-labeled BPV DNA in the presence of DNA from four other spontaneous equine sarcoids or BPV-induced equine tumor DNA were nonlinear (Figs. 1 and 2). These results indicate the presence of BPV-specific DNA in the tumors and suggest that segments of the BPV genome are present at different frequencies in spontaneous equine sarcoids and in the BPV-induced equine tumor.

The kinetics of reassociation of ^{125}I-labeled BPV DNA in the presence of tumor DNA were analyzed by the equations of Sharp *et al.* (24), in which reassociation of labeled viral DNA in the presence of cellular DNA with nonhomologous sequences follows the equation

$$C_0/C = 1 + t/t_{1/2} \qquad [2]$$

in which $t_{1/2}$ is the time required for labeled viral DNA to reach 50% reassociation. Reassociation of labeled viral DNA in the presence of cellular DNA containing homologous sequences follows the equation

$$C_0/C = \left[\frac{X}{1 + (1 + r)t/t_{1/2}} + \frac{1 - X}{1 + t/t_{1/2}}\right]^{-1} \qquad [3]$$

in which X is the fraction of the viral genome present in cellular DNA and r is the molar ratio of viral DNA sequences in cellular DNA to labeled viral DNA (24).

For each set of data points (labeled DNA reassociating in the presence of tumor DNA) values of X and r (Eq. 3) that gave the best fit through the experiment points were determined. The curves drawn through the data points are theoretical curves for given values of X and r. To estimate the number of partial BPV genome equivalents per diploid quantity of cellular DNA (N), a method similar to that of Kraiselburd *et al.* (25) was employed. If one knows the amount of labeled BPV DNA per ml necessary to give one complete viral genome per cell (y), N can be estimated by using the relationship $N = r/(y/a)$, in which a is the amount of labeled BPV DNA per ml in the reassociation mixture. Because the amount of DNA per cell cannot be determined from solid tumors, the value of 3.9×10^{12} was used as the molecular weight of mammalian DNA (26) and 5×10^6 as the molecular weight of BPV DNA (17).

The values of X and r determined for ^{125}I-labeled BPV DNA reassociating in the presence of tumor DNA and the estimation of the number of partial BPV genome equivalents per cell are summarized in Table 1. The kinetics of reassociation of labeled viral DNA with DNA from each tumor were analyzed at different ratios of cellular DNA to labeled viral DNA, and values of X and r determined for each experiment were those that gave best fit to the data. Values of X and r only slightly different from those given in Table 1 will yield curves that do not pass through the data points as shown.

Thermal denaturation profiles of duplexes formed between ^{125}I-labeled BPV DNA and DNA isolated from equine sarcoids are shown in Fig. 3 and the data are summarized in Table 2. DNA duplexes formed between ^{125}I-labeled BPV DNA reassociating in the presence of salmon sperm DNA, E29892-75 DNA, and E36295-75 DNA all have similar 50% elution temperatures (T_ms), which range from 85.2 to 85.8°. Double-stranded DNA formed with labeled viral DNA and DNA from the BPV-induced tumor and tumors 706931 and 65-477 dissociated with a T_m about six degrees lower than ^{125}I-labeled BPV DNA duplexes formed in the presence of unlabeled BPV DNA and salmon sperm DNA, i.e., the control value (Fig. 3, Table 2). The decrease in T_m from the control value could represent 4–8% base pair mismatching, based on 0.7–1.5% mismatch per degree C decrease in T_m (27). Alternatively, the lower T_m could also represent approximately a 12% decrease in guanine + cytosine content of the labeled duplexes being formed (28).

DISCUSSION

In this report we have presented data that demonstrate the presence of BPV-specific DNA sequences in DNA isolated from four of five spontaneous equine connective tissue tumors (equine sarcoid). Also, an equine connective tissue tumor induced by injection of a partially purified BPV suspension

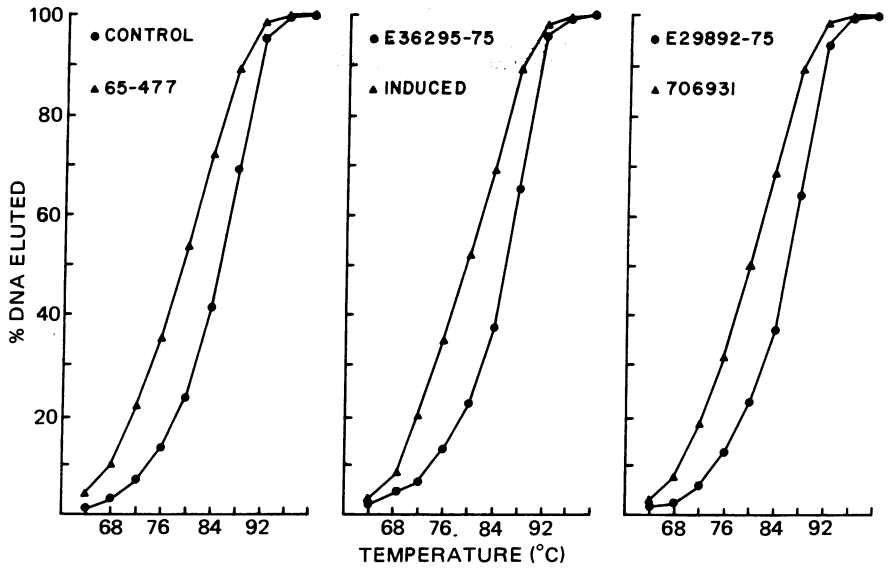

FIG. 3. Thermal denaturation profiles of ^{125}I-labeled BPV DNA·equine tumor DNA hybrids. Labeled BPV DNA was reannealed to the indicated equine tumor DNA as described in Figs. 1 and 2. Hybrids were melted on columns of hydroxyapatite and single-stranded DNA was eluted with washes of preheated 0.12 M sodium phosphate, pH 6.8, 0.05% sodium dodecyl sulfate at various temperature increments. The control was reassociated BPV DNA duplexes formed with ^{125}I-labeled BPV DNA at a 12.5-fold molar excess of unlabeled BPV DNA.

contained BPV-specific DNA sequences. There was about 20–75% of the BPV genome present in the various tumors at frequencies ranging from 60 to 500 partial genome equivalents per diploid quantity of cellular DNA. Thermal denaturation profiles of duplexes formed between labeled viral DNA and virus-specific DNA sequences in two of the spontaneous equine sarcoids (E29892-75 and E36295-75) had T_ms almost identical to labeled viral DNA reassociating in the presence of unlabeled BPV DNA; three of the tumors (two spontaneous and one BPV-induced) contained BPV-related sequences that when denatured had a decrease in T_m from the control. This could suggest that DNA of a virus(es) closely related to BPV is present in the various tumors. However, the possibility that a segment of the BPV genome rich in adenine + thymine (A+T) was present in the tumors seems more likely. The fact that human papilloma virus DNA contains (A+T)-rich regions tends to support this notion (29). Also, simian virus 40 (SV40) and polyoma virus, other members of the Papovaviridae, have (A+T)-rich regions in their genomes (30, 31). Duplexes formed between labeled BPV DNA and DNA from the BPV-induced tumor showed a similar reduction in T_m when thermally denatured. This would further support the presence of (A+T)-rich regions of the BPV genome in the tumor DNA preparations.

Cells transformed by certain oncogenic DNA viruses contain only a portion of the viral genome. Adenovirus-2-transformed rat cells contain only about 14% of the viral DNA (32), while herpes-simplex-virus-2-transformed hamster cells appear to contain variable portions of the herpes virus genome (33). A clone of simian-virus-40-transformed mouse cells, on the other hand, contains a complete viral genome with various portions being represented at different frequencies (34). The calculation of the fraction of BPV genome present in the tumors tested was based on the assumption that a homogenous population of fragments was present in the tumors. The possibility that a few complete copies of the viral genome are present in the tumors cannot be discounted. Experiments using restriction endonuclease cleavage fragments of BPV DNA as probes may resolve this point.

Table 2. Summary of thermal denaturation on hydroxyapatite of duplexes formed between [^{125}I]BPV DNA and cellular DNAs

Duplex of ^{125}I-labeled BPV DNA* and	T_m (°C)	ΔT_m
Unlabeled BPV DNA	85.2	—
E29892-75 DNA	85.8	+0.3
E36295-75 DNA	85.6	+0.1
706931 DNA	79.5	−6.0
BPV-induced tumor DNA	80.0	−5.5
65-477 DNA	79.3	−6.2

* Undenatured duplex ^{125}I-labeled BPV DNA had a T_m of 85.5°.

The mechanism whereby equine cells containing BPV-specific DNA sequences retain only a portion of the viral genome and amplify it to such a degree is unknown. In a previous study, we examined a BPV-induced calf meningioma and hamster fibroma by DNA·DNA hybridization for BPV-related DNA sequences (17). The results indicated there were a large number of copies of BPV-specific DNA in each tumor. However, the calf meningioma contained the whole viral genome, while there appeared to be only a fragment of BPV DNA in the hamster fibroma. It may be that the whole BPV genome cannot exist in cells not of bovine origin.

Orth *et al.* (35) have shown that there is no detectable viral DNA synthesis in proliferating cells of virus-induced rabbit papillomas. Viral DNA synthesis does occur, however, in cells undergoing keratinization. These authors suggested that viral DNA exists in a proviral state as in virus-transformed cells and that viral DNA synthesis and viral maturation do not occur until the cells begin to keratinize. A similar situation seems to exist in virus-induced bovine papillomas. BPV particles can be detected only in the keratinized layers of the tumor (36). Thus, there appears to be a strong association between viral and cellular DNA in proliferating cells of virus-induced papillomas.

It appears that BPV-induced cellular proliferation in equine species may be due to integration of viral DNA sequences, resulting in cell transformation. A cell line (ESM-1) has been established from equine sarcoid E29892-75 that exhibits loss of

contact inhibition and grows under agar. Preliminary experiments with selective extraction of ESM-1 cellular DNA by the method of Hirt (37) indicate that about 75% of the BPV-specific DNA sequences remain with the cellular DNA fraction. Analysis of ESM-1 cellular network DNA, prepared according to Varmus *et al.* (38), by DNA·DNA hybridization, also suggests that BPV DNA sequences are covalently linked to cellular DNA.

We are indebted to Dr. F. R. Robinson and Dr. L. D. Pearson for tumor specimens and to J. P. Sundberg for ESM-1 cultures. This investigation was supported by Public Health Service Research Grant CA-17244 (W.M.) from the National Cancer Institute and in part from the College of Agriculture and Life Sciences, University of Wisconsin. W.D.L. was a recipient of a California Division–American Cancer Society Junior Fellowship, no. J-292.

1. Gordon, D. E. & Olson, C. (1968) *Cancer Res.* **28,** 2423–2431.
2. Robl, M. G. & Olson, C. (1968) *Cancer Res.* **28,** 1596–1604.
3. Boiron, M., Levy, J. P., Thomas, M., Friedman, J. C. & Bernard, J. (1964) *Nature* **201,** 423–424.
4. Black, P. H., Hartley, J. W., Rowe, W. P. & Huebner, R. J. (1963) *Nature* **199,** 1016–1018.
5. Thomas, M., Boiron, M., Tanzer, J., Levy, J. P. & Bernard, J. (1964) *Nature* **202,** 709–710.
6. Boiron, M., Thomas, M. & Chenaille, P. H. (1965) *Virology* **26,** 150–153.
7. Olson, C. & Cook, R. H. (1951) *Proc. Soc. Exp. Biol. Med.* **77,** 281–284.
8. Jackson, C. (1936) *Onderstepoort J. Vet. Sci. Anim. Ind.* **6,** 378–385.
9. Olson, C. (1948) *Am. J. Vet. Res.* **9,** 333–341.
10. Voss, J. L. (1969) *Am. J. Vet. Res.* **30,** 183–191.
11. Ragland, W. L. & Spencer, G. R. (1968) *Am. J. Vet. Res.* **29,** 1363–1366.
12. Ragland, W. L., Keown, G. H. & Gorham, J. R. (1966) *Nature* **210,** 1399.
13. England, J. J., Watson, R. E. & Larson, K. A. (1973) *Am. J. Vet. Res.* **12,** 1601–1603.
14. Meinke, W., Goldstein, D. A. & Hall, M. R. (1974) *Anal. Biochem.* **52,** 82–88.
15. Lancaster, W. D. & Meinke, W. (1975) *Nature* **256,** 434–436.
16. Meinke, W. & Goldstein, D. A. (1974) *J. Mol. Biol.* **86,** 757–773.
17. Lancaster, W. D., Olson, C. & Meinke, W. (1976) *J. Virol.* **17,** 824–831.
18. Radloff, R., Bauer, W. & Vinograd, J. (1967) *Proc. Natl. Acad. Sci. USA* **57,** 1514–1521.
19. Kelly, R. B., Cozzarelli, N. R., Deutscher, M. P., Lehman, I. R. & Kornberg, A. (1970) *J. Biol. Chem.* **245,** 39–45.
20. Dumas, L. B., Darby, G. & Sinsheimer, R. L. (1971) *Biochim. Biophys. Acta* **228,** 407–422.
21. Shaw, J. E., Huang, E.-S. & Pagano, J. S. (1975) *J. Virol.* **16,** 132–140.
22. Britten, R. J. & Kohne, D. E. (1968) *Science* **161,** 529–540.
23. Gelb, L. D., Kohne, D. E. & Martin, M. A. (1971) *J. Mol. Biol.* **57,** 129–145.
24. Sharp, P. A., Pettersson, U. & Sambrook, J. (1974) *J. Mol. Biol.* **86,** 709–726.
25. Kraiselburd, E., Gage, L. P. & Weissbach, A. (1975) *J. Mol. Biol.* **97,** 533–542.
26. Sober, H. (1966) in *Handbook of Biochemistry,* ed. Sober, H. (Chemical Rubber Co., Cleveland, Ohio), pp. 14–58.
27. Bonner, T. I., Brenner, D. J., Neufeld, B. R. & Britten, R. J. (1973) *J. Mol. Biol.* **81,** 123–135.
28. Miyazawa, Y. & Thomas, C. A., Jr. (1965) *J. Mol. Biol.* **11,** 223–237.
29. Follett, E. A. C. & Crawford, L. V. (1967) *J. Mol. Biol.* **28,** 461–467.
30. Yoshiike, K. & Furuno, A. (1972) *J. Mol. Biol.* **70,** 415–423.
31. Lescure, B. & Yaniv, M. (1975) *J. Virol.* **16,** 720–724.
32. Gallimore, P. H., Sharp, P. A. & Sambrook, J. (1974) *J. Mol. Biol.* **89,** 49–72.
33. Frenkel, N., Locker, H., Cox, B., Roizman, B. & Rapp, F. (1976) *J. Virol.* **18,** 885–893.
34. Botchan, M., Ozanne, B., Sugden, B., Sharp, P. A. & Sambrook, J. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4183–4187.
35. Orth, G., Jeanteur, P. & Croissant, O. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1876–1880.
36. Fujimoto, Y. & Olson, C. (1966) *Pathol. Vet.* **3,** 659–684.
37. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369.
38. Varmus, H. E., Vogt, P. K. & Bishop, J. M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3067–3071.