in the presence of the four common ribonucleoside triphosphates, the enzyme incorporated ^{3}H-GMP extensively[12]. At this *p*H, however, in the presence of the four deoxyribonucleoside triphosphates, no ^{3}H-TMP incorporation was demonstrable (Table 4). Furthermore, replacement of even a single ribonucleotide by its homologous deoxyribonucleotide led to no detectable synthesis (my unpublished observation). At *p*H 8·3, the optimum for the R-MLV DNA polymerase, the VSV polymerase catalysed much less ribonucleotide incorporation and no significant deoxyribonucleotide incorporation could be detected.

Table 3. EFFECT OF RIBONUCLEASE ON THE DNA POLYMERASE ACTIVITY OF RAUSCHER MOUSE LEUKAEMIA VIRUS

Conditions	pmoles ^{3}H-TMP incorporation
No preincubation	2·50
Preincubated with no addition	2·20
Preincubated with 20 μg/ml. ribonuclease	0·69
Preincubated with 50 μg/ml. ribonuclease	0·31
Preincubated with 200 μg/ml. ribonuclease	0·08
Preincubated with no addition	3·69
Preincubated with 50 μg/ml. ribonuclease	0·52
Preincubated with 50 μg/ml. lysozyme	3·67
Preincubated with 50 μg/ml. cytochrome *c*	3·97

In experiment 1, for the preincubation, 15 μg of viral protein in 5 μl. of solution was added to 45 μl. of water at 4° C containing the indicated amounts of enzyme. After incubation for 30 min at 22° C, the samples were chilled and 50 μl. of a 2-fold concentrated standard reaction mixture was added. The samples were then incubated at 37° C for 45 min and acid-insoluble radioactivity was measured. In experiment 2, the same procedure was followed, except that the preincubation was for 20 min at 22° C and the 37° C incubation was for 60 min.

Table 4. COMPARISON OF NUCLEOTIDE INCORPORATION BY VESICULAR STOMATITIS VIRUS AND RAUSCHER MOUSE LEUKAEMIA VIRUS

Precursor	*p*H	Incorporation in 45 min (pmoles) Vesicular stomatitis virus	Mouse leukaemia virus
^{3}H-TTP	8·3	< 0·01	2·3
^{3}H-TTP (omit dATP)	8·3	N.D.	0·06
^{3}H-TTP (omit dATP; plus ATP)	8·3	N.D.	0·08
^{3}H-GTP	8·3	0·43	< 0·03
^{3}H-GTP	7·3	3·7	< 0·03

When ^{3}H-TTP was the precursor, standard reaction conditions were used (see Table 1). When ^{3}H-GTP was the precursor, the reaction mixture contained, in 0·1 ml., 5 μmoles Tris-HCl (*p*H as indicated), 0·6 μmoles magnesium acetate, 0·3 μmoles mercaptoethanol, 9 μmoles NaCl, 0·08 μmole each of ATP, CTP, UTP; and 0·001 μmole ^{3}H-GTP (1,040 c.p.m. per pmole). All VSV assays included 0·1 per cent 'Triton N–101' (ref. 12) and 2–5 μg of viral protein. The R-MLV assays contained 15 μg of viral protein.

The R-MLV polymerase incorporated only deoxyribonucleotides. At *p*H 8·3, ^{3}H-TMP incorporation was readily demonstrable but replacement of dATP by ATP completely prevented synthesis (Table 4). Furthermore, no significant incorporation of ^{3}H-GMP could be found in the presence of the four ribonucleotides. At *p*H 7·3, the R-MLV polymerase was also inactive with ribonucleotides. The polymerase in the R-MLV virions is therefore highly specific for deoxyribonucleotides.

DNA Polymerase in Rous Sarcoma Virus

A preparation of the Prague strain of Rous sarcoma virus was assayed for DNA polymerase activity (Table 5). Incorporation of radioactivity from ^{3}H-TTP was demonstrable and the activity was severely reduced by omission of either Mg^{2+} or dATP from the reaction mixture. RNA-dependent DNA polymerase is therefore probably a constituent of all RNA tumour viruses.

These experiments indicate that the virions of Rauscher mouse leukaemia virus and Rous sarcoma virus contain a DNA polymerase. The inhibition of its activity by ribonuclease suggests that the enzyme is an RNA-dependent DNA polymerase. It seems probable that all RNA tumour viruses have such an activity. The existence of this enzyme strongly supports the earlier suggestions[1–7] that genetically specific DNA synthesis is an early event in the replication cycle of the RNA tumour viruses and that DNA is the template for viral RNA synthesis. Whether the viral DNA ("provirus")[2] is integrated into the host genome or remains as a free template for RNA synthesis will require further study. It will also be necessary to determine whether the host DNA-dependent RNA polymerase or a virus-specific enzyme catalyses the synthesis of viral RNA from the DNA.

Table 5. PROPERTIES OF THE ROUS SARCOMA VIRUS DNA POLYMERASE

Reaction system	pmoles ^{3}H-TMP incorporated in 120 min
Complete	2·06
Without magnesium acetate	0·12
Without dATP	0·19

A preparation of the Prague strain (sub-group C) of Rous sarcoma virus[16] having a titre of 5×10^7 focus forming units per ml. was provided by Dr Peter Vogt. The virus was purified from tissue culture fluid by differential centrifugation. Before use the preparation was centrifuged and the pellet dissolved in 1/10 of the initial volume as described for the R-MLV preparation. For each assay 15 μl. of the concentrated Rous sarcoma virus preparation was assayed in a standard reaction mixture by incubation for 2 h. An unincubated control sample had radioactivity corresponding to 0·14 pmole which was subtracted from the experimental values.

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1 Green, M., *Ann. Rev. Biochem.*, **39** (1970, in the press).
2 Temin, H. M., *Virology*, **23**, 486 (1964).
3 Bader, J. P., *Virology*, **22**, 462 (1964).
4 Vigier, P., and Golde, A., *Virology*, **23**, 511 (1964).
5 Duesberg, P. H., and Vogt, P. K., *Proc. US Nat. Acad. Sci.*, **64**, 939 (1969).
6 Temin, H. M., in *Biology of Large RNA Viruses* (edit. by Barry, R., and Mahy, B.) (Academic Press, London, 1970).
7 Temin, H. M., *Virology*, **20**, 577 (1963).
8 Kates, J. R., and McAuslan, B. R., *Proc. US Nat. Acad. Sci.*, **58**, 134 (1967).
9 Munyon, W., Paoletti, E., and Grace, J. T. J., *Proc. US Nat. Acad. Sci.*, **58**, 2280 (1967).
10 Shatkin, A. J., and Sipe, J. D., *Proc. US Nat. Acad. Sci.*, **61**, 1462 (1968).
11 Borsa, J., and Graham, A. F., *Biochem. Biophys. Res. Commun.*, **33**, 895 (1968).
12 Baltimore, D., Huang, A. S., and Stampfer, M., *Proc. US Nat. Acad. Sci.* **66** (1970, in the press).
13 Temin, H. M., and Mizutani, S., *Nature*, **226**, 1211 (1970) (following article).
14 O'Conner, T. E., Rauscher, F. J., and Zeigel, R. F., *Science*, **144**, 1144 (1964).
15 Crawford, L. V., and Crawford, E. M., *Virology*, **13**, 227 (1961).
16 Duesberg, P., and Robinson, W. S., *Proc. US Nat. Acad. Sci.*, **55**, 219 (1966).
17 Duff, R. G., and Vogt, P. K., *Virology*, **39**, 18 (1969).

RNA-dependent DNA Polymerase in Virions of Rous Sarcoma Virus

INFECTION of sensitive cells by RNA sarcoma viruses requires the synthesis of new DNA different from that synthesized in the *S*-phase of the cell cycle (refs. 1, 2 and unpublished results of D. Boettiger and H. M. T.); production of RNA tumour viruses is sensitive to actinomycin D[3,4]; and cells transformed by RNA tumour viruses have new DNA which hybridizes with viral RNA[5,6]. These are the basic observations essential to the DNA provirus hypothesis—replication of RNA tumour viruses takes place through a DNA intermediate, not

through an RNA intermediate as does the replication of other RNA viruses[7].

Formation of the provirus is normal in stationary chicken cells exposed to Rous sarcoma virus (RSV), even in the presence of 0·5 μg/ml. cycloheximide (our unpublished results). This finding, together with the discovery of polymerases in virions of vaccinia virus and of reovirus[8–11], suggested that an enzyme that would synthesize DNA from an RNA template might be present in virions of RSV. We now report data supporting the existence of such an enzyme, and we learn that David Baltimore has independently discovered a similar enzyme in virions of Rauscher leukaemia virus[12].

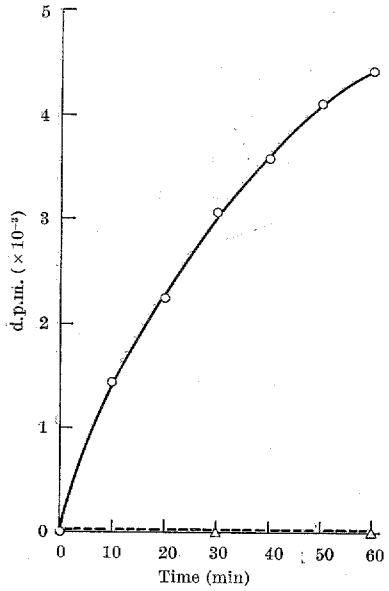

Fig. 1. Kinetics of incorporation. Virus treated with 'Nonidet' and dithiothreitol at 0° C and incubated at 37° C (○—○) or 80° C (△ - - - △) for 10 min was assayed in a standard polymerase assay. ○, Unheated; △, heated.

The sources of virus and methods of concentration have been described[13]. All preparations were carried out in sterile conditions. Concentrated virus was placed on a layer of 15 per cent sucrose and centrifuged at 25,000 r.p.m. for 1 h in the 'SW 25.1' rotor of the Spinco ultracentrifuge on to a cushion of 60 per cent sucrose. The virus band was collected from the interphase and further purified by equilibrium sucrose density gradient centrifugation[14]. Virus further purified by sucrose velocity density gradient centrifugation gave the same results.

Table 1. ACTIVATION OF ENZYME

System	^{3}H-TTP incorporated (d.p.m.)
No virions	0
Non-disrupted virions	255
Virions disrupted with 'Nonidet'	
At 0° + DTT	6,730
At 0° − DTT	4,420
At 40° + DTT	5,000
At 40° − DTT	425

Purified virions untreated or incubated for 5 min at 0° C or 40° C with 0·25 per cent 'Nonidet P–40' (Shell Chemical Co.) with 0 or 1 per cent dithiothreitol (DTT) (Sigma) were assayed in the standard polymerase assay.

The polymerase assay consisted of 0·125 μmoles each of dATP, dCTP, and dGTP (Calbiochem) (in 0·02 M Tris-HCl buffer at *p*H 8·0, containing 0·33 M EDTA and 1·7 mM 2-mercaptoethanol); 1·25 μmoles of $MgCl_2$ and 2·5 μmoles of KCl; 2·5 μg phosphoenolpyruvate (Calbiochem); 10 μg pyruvate kinase (Calbiochem); 2·5 μCi of ^{3}H-TTP (Schwarz) (12 Ci/mmole); and 0·025 ml. of enzyme (10^8 focus forming units of disrupted Schmidt-Ruppin virus, $A_{280\,nm} = 0{\cdot}30$) in a total volume of 0·125 ml. Incubation was at 40° C for 1 h. 0·025 ml. of the reaction mixture was withdrawn and assayed for acid-insoluble counts by the method of Furlong[15].

To observe full activity of the enzyme, it was necessary to treat the virions with a non-ionic detergent (Tables 1 and 4). If the treatment was at 40° C the presence of dithiothreitol (DTT) was necessary to recover activity. In most preparations of virions, however, there was some activity: 5–20 per cent of the disrupted virions, in the absence of detergent treatment, which probably represents disrupted virions in the preparation. It is known that virions of RNA tumour viruses are easily disrupted[16,17], so that the activity is probably present in the nucleoid of the virion.

Table 2. REQUIREMENTS FOR ENZYME ACTIVITY

System	^{3}H-TTP incorporated (d.p.m.)
Complete	5,675
Without $MgCl_2$	186
Without $MgCl_2$, with $MnCl_2$	5,570
Without $MgCl_2$, with $CaCl_2$	18
Without dATP	897
Without dCTP	1,780
Without dGTP	2,190

Virus treated with 'Nonidet' and dithiothreitol at 0° C was incubated in the standard polymerase assay with the substitutions listed.

The kinetics of incorporation with disrupted virions are shown in Fig. 1. Incorporation is rapid for 1 h. Other experiments show that incorporation continues at about the same rate for the second hour. Preheating disrupted virus at 80° C prevents any incorporation, and so does pretreatment of disrupted virus with crystalline trypsin.

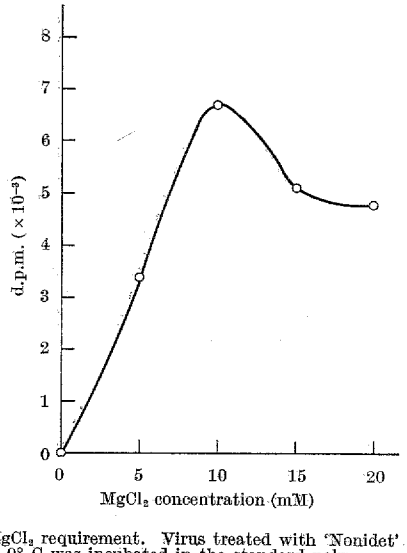

Fig. 2. $MgCl_2$ requirement. Virus treated with 'Nonidet' and dithiothreitol at 0° C was incubated in the standard polymerase assay with different concentrations of $MgCl_2$.

Fig. 2 demonstrates that there is an absolute requirement for $MgCl_2$, 10 mM being the optimum concentration. The data in Table 2 show that $MnCl_2$ can substitute for $MgCl_2$ in the polymerase assay, but $CaCl_2$ cannot. Other experiments show that a monovalent cation is not required for activity, although 20 mM KCl causes a 15 per

cent stimulation. Higher concentrations of KCl are inhibitory: 60 per cent inhibition was observed at 80 mM.

When the amount of disrupted virions present in the polymerase assay was varied, the amount of incorporation varied with second-order kinetics. When incubation was carried out at different temperatures, a broad optimum between 40° C and 50° C was found. (The high temperature of this optimum may relate to the fact that the normal host of the virus is the chicken.) When incubation was carried out at different *p*Hs, a broad optimum at *p*H 8–9·5 was found.

Table 2 demonstrates that all four deoxyribonucleotide triphosphates are required for full activity, but some activity was present when only three deoxyribonucleotide triphosphates were added and 10–20 per cent of full activity was still present with only two deoxyribonucleotide triphosphates. The activity in the presence of three deoxyribonucleotide triphosphates is probably the result of the presence of deoxyribonucleotide triphosphates in the virion. Other host components are known to be incorporated in the virion of RNA tumour viruses[18,19].

Table 3. RNA DEPENDENCE OF POLYMERASE ACTIVITY

Treatment	^{3}H-TTP incorporated (d.p.m.)
Non-treated disrupted virions	9,110
Disrupted virions preincubated with ribonuclease A (50 μg/ml.) at 20° C for 1 h	2,650
Disrupted virions preincubated with ribonuclease A (1 mg/ml.) at 0° C for 1 h	137
Disrupted virions preincubated with lysozyme (50 μg/ml.) at 0° C for 1 h.	9,650

Disrupted virions were incubated with ribonuclease A (Worthington) which was heated at 80° C for 10 min, or with lysozyme at the indicated concentration in the specified conditions, and a standard polymerase assay was performed.

The data in Table 3 demonstrate that incorporation of thymidine triphosphate was more than 99 per cent abolished if the virions were pretreated at 0° with 1 mg ribonuclease per ml. Treatment with 50 μg/ml. ribonuclease at 20° C did not prevent all incorporation of thymidine triphosphate, which suggests that the RNA of the virion may be masked by protein. (Lysozyme was added as a control for non-specific binding of ribonuclease to DNA.) Because the ribonuclease was heated for 10 min at 80° C or 100° C before use to destroy deoxyribonuclease it seems that intact RNA is necessary for incorporation of thymidine triphosphate.

Table 4. SOURCE OF POLYMERASE

Source	^{3}H-TTP incorporated (d.p.m.)
Virions of SRV	1,410
Disrupted virions of SRV	5,675
Virions of AMV	1,875
Disrupted virions of AMV	12,850
Disrupted pellet from supernatant of uninfected cells	0

Virions of Schmidt-Ruppin virus (SRV) were prepared as before (experiment of Table 2). Virions of avian myeloblastosis virus (AMV) and a pellet from uninfected cells were prepared by differential centrifugation. All disrupted preparations were treated with 'Nonidet' and dithiothreitol at 0° C and assayed in a standard polymerase assay. The material used per tube was originally from 45 ml. of culture fluid for SRV, 20 ml. for AMV, and 20 ml. for uninfected cells.

To determine whether the enzyme is present in supernatants of normal cells or in RNA leukaemia viruses, the experiment of Table 4 was performed. Normal cell supernatant did not contain activity even after treatment with 'Nonidet'. Virions of avian myeloblastosis virus (AMV) contained activity that was increased ten-fold by treatment with 'Nonidet'.

The nature of the product of the polymerase assay was investigated by treating portions with deoxyribonuclease, ribonuclease or KOH. About 80 per cent of the product was made acid soluble by treatment with deoxyribonuclease, and the product was resistant to ribonuclease and KOH (Table 5).

Table 5. NATURE OF PRODUCT

Treatment	Residual acid-insoluble ^{3}H-TTP (d.p.m.) Experiment A	Experiment B
Buffer	10,200	8,350
Deoxyribonuclease	697	1,520
Ribonuclease	10,900	7,200
KOH	—	8,250

A standard polymerase assay was performed with 'Nonidet' treated virions. The product was incubated in buffer or 0·3 M KOH at 37° C for 20 h or with (*A*) 1 mg/ml. or (*B*) 50 μg/ml. of deoxyribonuclease I (Worthington), or with 1 mg/ml. of ribonuclease A (Worthington) for 1 h at 37° C, and portions were removed and tested for acid-insoluble counts.

To determine if the polymerase might also make RNA, disrupted virions were incubated with the four ribonucleotide triphosphates, including ^{3}H-UTP (Schwarz, 3·2 Ci/mmole). With either $MgCl_2$ or $MnCl_2$ in the incubation mixture, no incorporation was detected. In a parallel incubation with deoxyribonucleotide triphosphates, 12,200 d.p.m. of ^{3}H-TTP was incorporated.

These results demonstrate that there is a new polymerase inside the virions of RNA tumour viruses. It is not present in supernatants of normal cells but is present in virions of avian sarcoma and leukaemia RNA tumour viruses. The polymerase seems to catalyse the incorporation of deoxyribonucleotide triphosphates into DNA from an RNA template. Work is being performed to characterize further the reaction and the product. If the present results and Baltimore's results[12] with Rauscher leukaemia virus are upheld, they will constitute strong evidence that the DNA provirus hypothesis is correct and that RNA tumour viruses have a DNA genome when they are in cells and an RNA genome when they are in virions. This result would have strong implications for theories of viral carcinogenesis and, possibly, for theories of information transfer in other biological systems[20].

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1 Temin, H. M., *Cancer Res.*, **28**, 1835 (1968).
2 Murray, R. K., and Temin, H. M., *Intern. J. Cancer* (in the press).
3 Temin, H. M., *Virology*, **20**, 577 (1963).
4 Baluda, M. B., and Nayak, D. P., *J. Virol.*, **4**, 554 (1969).
5 Temin, H. M., *Proc. US Nat. Acad. Sci.*, **52**, 323 (1964).
6 Baluda, M. B., and Nayak, D. P., in *Biology of Large RNA Viruses* (edit. by Barry, R., and Mahy, B.) (Academic Press, London, 1970).
7 Temin, H. M., *Nat. Cancer Inst. Monog.*, **17**, 557 (1964).
8 Kates, J. R., and McAuslan, B. R., *Proc. US Nat. Acad. Sci.*, **57**, 314 (1967).
9 Muuyon, W., Paoletti, E., and Grace, J. T., *Proc. US Nat. Acad. Sci.*, **58**, 2280 (1967).
10 Borsa, J., and Graham, A. F., *Biochem. Biophys. Res. Commun.*, **33**, 895 (1968).
11 Shatkin, A. J., and Sipe, J. D., *Proc. US Nat. Acad. Sci.*, **61**, 1462 (1968).
12 Baltimore, D., *Nature*, **226**, 1209 (1970) (preceding article).
13 Altaner, C., and Temin, H. M., *Virology*, **40**, 118 (1970).
14 Robinson, W. S., Pitkanen, A., and Rubin, H., *Proc. US Nat. Acad. Sci.*, **54**, 137 (1965).
15 Furlong, N. B., *Meth. Cancer Res.*, **3**, 27 (1967).
16 Vogt, P. K., *Adv. Virus. Res.*, **11**, 293 (1965).
17 Bauer, H., and Schafer, W., *Virology*, **29**, 494 (1966).
18 Bauer, H., *Z. Naturforsch.*, **21b**, 453 (1966).
19 Erikson, R. L., *Virology*, **37**, 124 (1969).
20 Temin, H. M., *Persp. Biol. Med.* (in the press).