A New, not Virus Related Reverse Transcriptase in the Chicken System

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Materials and Methods

Labelled deoxyribonucleoside triphosphates were products of Amersham/Buchler and Schwarz Mann. Unlabelled deoxyribonucleoside triphosphates and PolyA $(dT)_{12}$ were obtained from Boehringer, Mannheim. All other templates used were products of P. L. Biochemicals, Milwaukee. Bovine Serum Albumin (A grade) was from Calbiochem.

AMV was generously supplied by Dr. J. Beard.

Purified REV, strain T, was kindly provided by Dr. K. Moelling and Dr. R. Friis, University of Giessen.

Anti-IgG was a generous gift of the laboratories of Dr. Gallo and Dr. Spiegelman.

Most of the experiments were performed with allantoic fluid of eggs from SPF-VALO chickens. The eggs were generously supplied, to the greater part, by Dr. E. Vielitz, Lohmann Tierzucht, Cuxhaven.

Standardpolymerase assay: The concentrations were 10 mM MgAcetat, 20 mM KCl, 50 mM Tris - HCl, pH 8.3, 10 mM DTE, 1 mM ATP. The usual assays of 100 µl volume contained 5 µCi labelled deoxynucleoside triphosphate (8 Ci/mMol) and 1 µg of template-primer complex. For the test of particles the assay contained NP 40 in final concentration of 0.1 %. Tests of purified enzyme contained 50 µg bovine serum albumin in 100 µl reaction mix.

When heteropolymeric nucleic acid was used as template, the unlabelled deoxynucleoside triphosphates were each at a concentration of 0.3 mM.

To get optimal activity with pC(dG), one has to use extremely clean reagents and has to heat the template-primer to 80 °C for 5–10 minutes before using it.

The reaction mix was incubated at 37 °C and the reaction was stopped by adding 10 % TCA, containing 1 % pyrophosphate. The acid-insoluble radioactiv-

ity was collected by filtering the samples through Millipore nitrocellulose filters (pore size of 0.6 μ).

Purification of the polymerases from particles, AMV and REV was as described by Markus et al. (11).

Sedimentation gradients of the enzymes in glycerol was as described by Markus et al. (11).

The IgG-inhibition test of purified polymerases was as described by Watson et al. (15), except that the preincubation of enzyme together with IgG was at 0–4 °C for 12 hours instead of 15 min at 37 °C.

Introduction

In the past years reverse transcription was not only discussed as an unique step in RNA virus-related transformation but also as a possible mechanism involved in transfer of genetic information in normal cells, uninfected with virus. For this reason studies have been performed to detect, in addition to viral reverse transcriptase, corresponding cellular enzymes. However, all enzymes studied could be either related to viruses (1) or do not have the typical template specificity of "true" reverse transcriptase (2, 3, 4): i. e. more efficient utilization of $pA(dT)_{12}$ than of $pdA(dT)_{12}$, high activity with $pC(dG)_{12}$ and the ability to synthesize DNA complementary to natural heteropolymeric RNA (5, 6, 7). As ist was not possible till now, to isolate a cell-specific enzyme with these characteristics from normal cells, the demonstration of reverse transcriptase is often taken as an unmistakable viral footprint. Thus in looking for early diagnostic signals, indicating tumor formation in human tissue, reverse transcriptase has been regarded as a serious candidate. Here we describe the isolation of a reverse transcriptase from particles in the allantoic fluid of embryonated chicken eggs. This enzyme could not be related to Avian Leukosis Viruses/Avian Sarcoma Viruses (ALV/ASV) or Reticuloendotheliosis Viruses (REV), the only chicken viruses known to contain reverse transcriptase, and so is very likely of cellular origin.

Results

Detection and purification of enzyme-containing particles. The allantoic fluid of ten day-old embryonated chicken eggs was collected, cleaned from cells and cell debris and subjected to ultracentrifugation, as for the collection of enveloped RNA viruses. The first pellet was resuspended in buffer and centrifuged through the 20 % sucrose step of a discontinuous gradient onto a cushion, consisting of 50 % sucrose. The material above the 50 % cushion was tested for its ability to use ribohomopolymers and deoxyribohomopolymers as templates for DNA synthesis. The result is shown in Table 1. As can be seen from the table, the ribohomopolymers pC and pA, as well as the deoxyribohomopolymer pdA are quite efficient templates for the isolated activity. As the activity exhibited some of the template characteristics of reverse transcriptase (use of $pC(dG)_{12}$) as well as of cellular DNA polymerase (preference of $pA(dT)_{12}$ over $pdA(dT)_{12}$) we expected to have a mixture of different enzyme activities.

In the next step the material was fractionated on a linear sucrose gradient, and

Table I: Homopolymer-directed DNA synthesis by the crude particle fraction obtained after centrifugation of the allantoic fluid and subsequent discontinous sucrose gradient centrifugation

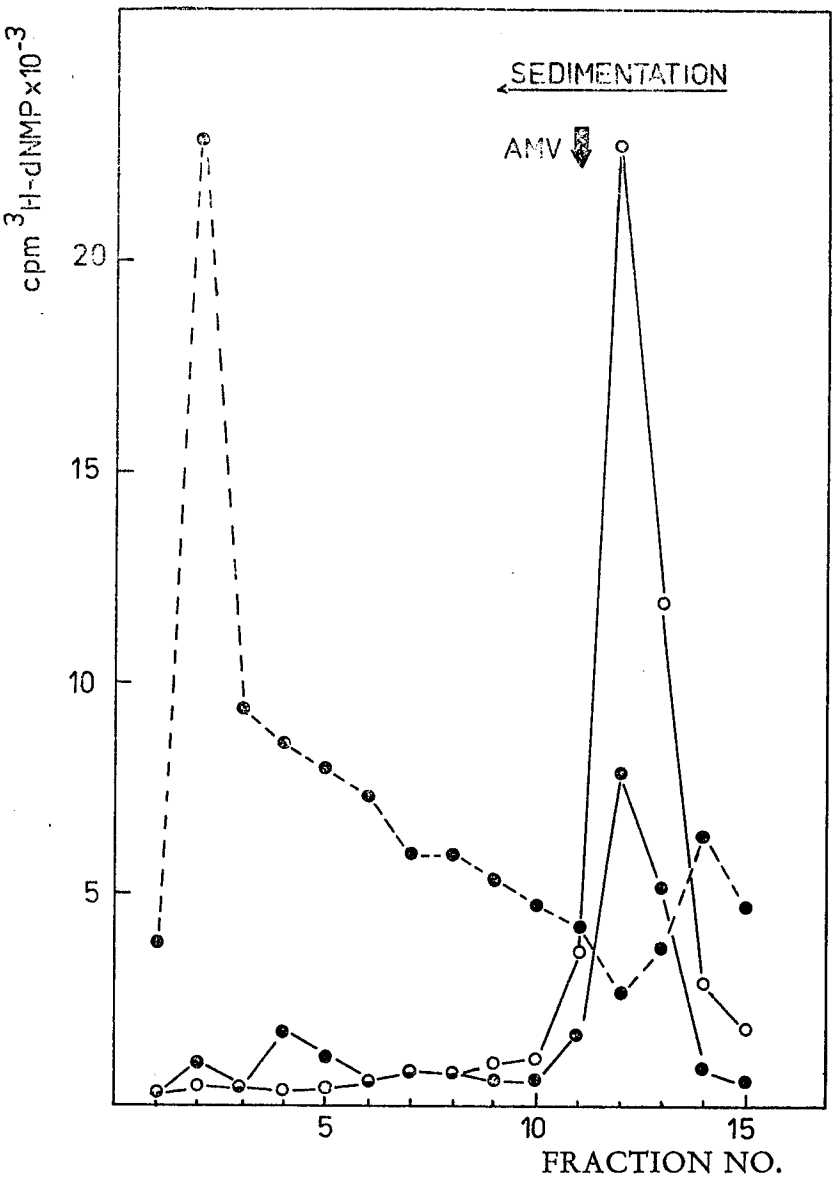
Template-primer complex	labelled dNTP	cpm ^{3}H-dNMP incorporated
pC $(dG)_{12-18}$	^{3}H-dGTP	16 629
pA $(dT)_{12}$	^{3}H-dTTP	8 648
pdA $(dT)_{12}$	^{3}H-dTTP	55 159

Legend to Table 1: The allantoic fluid of leukosis virus-free eggs, embryonated for ten days, was cleaned of cells and cell debris. It was then subjected to ultracentrifugation in a fixed-angle rotor (Rotor 30 Beckman) at 27,000 RPM for 45 minutes at 4 °C. The pellets were resuspended in STE buffer (0.1 M NaCl, 0.01 M Tris – HCl pH 8, 0.001 M EDTA) and layered on a discontinuous sucrose gradient in a Beckman SW 27.1 Rotor. The material was centrifuged through 20 % sucrose onto a 50 % cushion (26,000 RPM, 2.5 hours). The material above the 50 % sucrose was collected and diluted to a protein concentration of 0.9 mg/ml. 9 µg of material were incubated under standard polymerase assay conditions, as described in materials and methods, using different template-primer complexes at a concentration of 1 µg/100 µl. The mixture contained NP 40 to a final concentration of 0.1 % and 5 µCi of the respective ^{3}H-deoxynucleoside triphosphate (8 Ci/mMol) in a total volume of 100 µl. Incubation was at 37 °C for 60 minutes. The reaction was stopped by the addition of TCA and the acidinsoluble radioactivity of the total volumes was determined.

Table II: Homopolymer-directed DNA synthesis by purified particles, obtained after velocity sedimentation and sucrose density gradient centrifugation

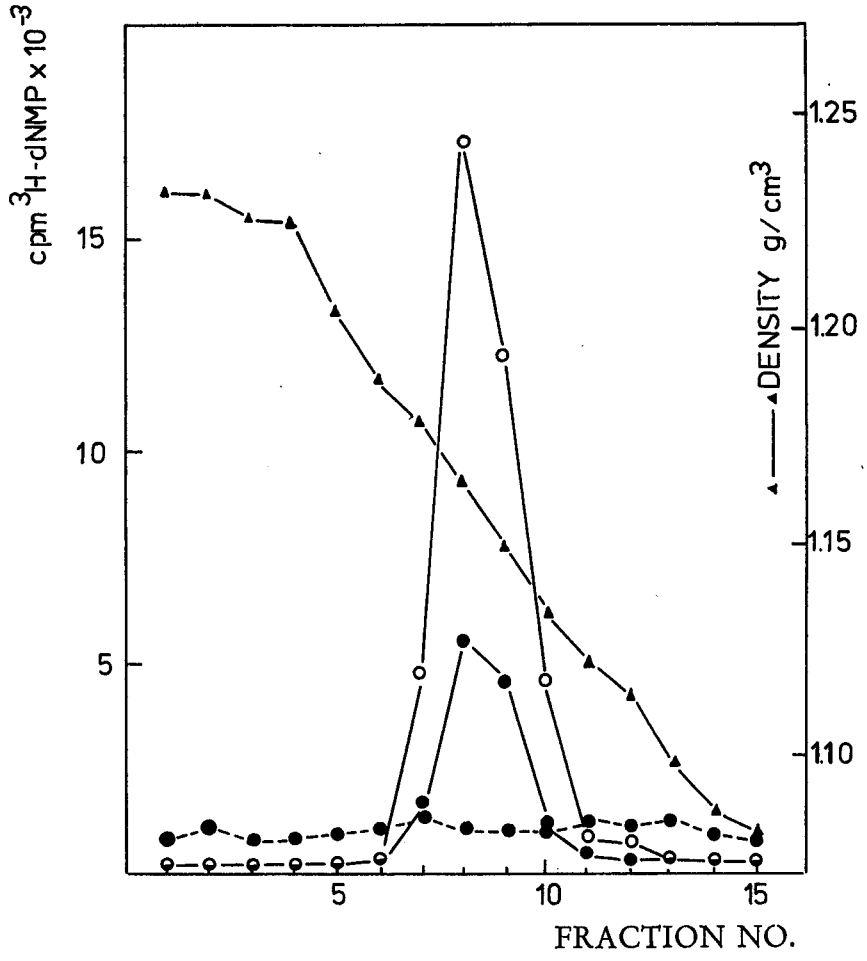
Template-primer	labelled dNTP	reaction conditions	cpm ^{3}H-dNMP incorporated
pC	dGTP	complete	159
$(dG)_{12-18}$	dGTP	complete	0
pC $(dG)_{12-18}$	dGTP	– NP 40	104
pC $(dG)_{12-18}$	dGTP	– particles	0
pC $(dG)_{12-18}$	dGTP	– Mg^{++}	0
pC $(dG)_{12-18}$	dGTP	complete	17 179
pC $(dG)_{12-18}$	dTTP	complete	0
pdC $(dG)_{12-18}$	dGTP	complete	4 100
pA $(dT)_{12}$	dTTP	complete	5 168
pdA $(dT)_{12}$	dTTP	complete	720

Legend to Table 2: 0.4 µg of particles, which had been purified as described in Figure 1 and 2 were incubated under standard polymerase assay conditions as described in Materials and Methods, in the presence of different Template/Primer complexes as indicated. Conditions were as described in the legend to Table 1.



Legend to Fig. 1: Velocity gradient of the sediment from allantoic fluid. The material, after being handled as described in the legend of Table 1, was layered on a continuous sucrose gradient, ranging from 20 to 35 % sucrose w/w (in STE buffer) and centrifuged in a Rotor SW 27.1 in a Spinco ultracentrifuge at 26,000 RPM for 50 minutes. The material was collected from the bottom and the fractions were tested with the template/primers pC (dG) (○—), pA(dT) (●—●), and pdA(dT) (●– – –●) under standard conditions for 1 hour. The acid insoluble radioactivity from the 100 µl reaction mixture was estimated.

the response to the set of templates, as used in the experiment of Table 1, was tested with each fraction. The result is seen in figure 1. Two different enzyme activities can be separated: a pdA-dependent activity, sedimenting to the bottom of the tube, and a slower sedimenting activity, utilizing pA, pC and pdA in the ratio expected for reverse transcriptase. Comparison with AMV, sedimented in a parallel gradient under identical conditions, showed that the material carrying the pC- and pA-directed DNA polymerase sediments slightly slower than AMV. From this and from the fact that the presence of nonionic detergent (NP 40) was requisite for the detection of the pC- and pA-directed DNA polymerase activity, we conclude that the polymerase is particle-bound.



Legend to Fig. 2: Sucrose density gradient of the particles containing polymerase. Fraction 12 and 13 from the gradient described in figure 1 were diluted with STE buffer and layered on a continuous sucrose gradient, ranging from 20 to 50 % sucrose w/w. It was centrifuged in a Rotor SW 41 at 30,000 for 16 hours. The material was collected from the bottom and tested as described in Figure 1. The density of the fractions was calculated by measuring the refractive index. The activity is expressed as cpm of ^{3}H-dNMP incorporated into acid insoluble material within 60 minutes in a volume of 100 µl. (○—○ pC·dG, ●—● pA·dT, ●- - -● pdA·dT dependent activities).

The fractions with the best response to pC and pA (fraction 12 and 13) were further analyzed by sucrose density gradient centrifugation. The fractionated density gradient was again tested with the same set of templates as before. As seen in figure 2, only one activity peak can be observed in response to the ribohomopolymer templates. The weak pdA-directed activity is distributed all over the gradient.

For the further characterization of the activity, the particles from the peak fraction of the density gradient were tested with an extended set of template-primer complexes (Table 2). The enzyme reaction is dependent on the presence of nonionic detergent, Mg^{++}, primer, template and corresponding deoxynucleoside triphosphate. Template or primer alone do not allow the reaction, and therefore terminal deoxynucleotidyltransferase (8) can be excluded. Furthermore, the efficient utilization of $pC(dG)_{12}$ excludes that R-DNA polymerase (polymerase γ) (9, 10) activity is being tested. The high preference of this template-primer complex and of $pA(dT)_{12}$ over $pdA(dT)_{12}$ clearly differentiates the particle-bound enzyme

from the normal cellular DNA polymerases (5, 7) and is typical for reverse transcriptase.

Characterization of the purified enzyme

For final characterization of the enzyme, particles were disrupted by NP 40 in the presence of high salt and the enzyme was further purified by affinity chromatography on polycytidylate-sepharose (11). The adsorbed and then eluted material, which contained too little protein to measure, was tested with homopolymeric (Table 3) and heteropolymeric templates (Table 4). The enzyme exhibits no activity

Table III: Homopolymer-directed DNA synthesis by purified reverse transcriptases from particles and AMV

Template-primer	labelled dNMP	cpm ^{3}H-dNMP incorporated by enzyme isolated from	
		Particles	AMV
$(dG)_{12-18}$	dGTP	0	0
pC $(dG)_{12-18}$	dGTP	28 417	149 651
pdC $(dG)_{12-18}$	dGTP	14 525	80 658
pA $(dT)_{12}$	dTTP	6 679	32 940
pdA $(dT)_{12}$	dTTP	813	639

Legend to Table 3: Reverse transcriptase was purified from concentrated, disrupted particles or AMV by affinity chromatography on polycytidylate-sepharose, as described by Markus et al. (11). After washing the column, the enzymes were eluted by 0.4 M KCl. One of the active fractions was tested under standard polymerase assay conditions, using different template-primer complexes. The activity is expressed as incorporation of radioactivity into acid insoluble material in 100 µl per 90 minutes. In each case the values were obtained from linear kinetics.

with $(dG)_{12}$ alone, which proves that no polycytidylic acid is eluted from the column, i. e. the enzyme is template-free.

The template characteristics of the purified enzyme are identical with those shown for the particle-bound enzyme in Table 2. When the purified enzyme is compared with AMV reverse transcriptase, the template characteristics and the relative utilization of the templates are almost identical. This result clearly distinguishes the particle enzyme from an enzyme, which was purified by Kang and Temin (3) from a fraction from uninfected chicken cells, exhibiting endogenous RNA-directed DNA polymerase activity. When they tested the isolated activity with synthetic templates, they found a preference for pdA over pA, which is typical for normal cellular DNA polymerases. In contrast, the enzyme purified by us shows just the opposite template preference, which is typical for (viral) reverse transcriptase.

To demonstrate that the enzyme could use heteropolymeric RNA, we used globin mRNA as a template for reverse transcription, having added $(dT)_{12}$ as primer (12). To be sure of measuring reverse transcription of the heteropolymeric

Table IV: Response of reverse transcriptases from particles or AMV to natural, heteropolymeric RNA

Template	Primer	Reaction conditions	cpm ^{3}H-dGMP incorporated by particle enzyme	cpm ^{3}H-dGMP incorporated by AMV enzyme
globin mRNA	$(dT)_{12}$	complete	3 299	3 800
globin mRNA	$(dT)_{12}$	complete + Act. D	2 866	
globin mRNA	$(dT)_{12}$	complete + RNAse	0	
globin mRNA	–	complete	866	
globin mRNA	$(dT)_{12}$	– dCTP	880	
–	$(dT)_{12}$	complete	0	
pC	$(dG)_{12-18}$	complete	100 883	96 200

Legend to Table 4: Aliquots of the purified enzymes were incubated in the presence of 2.5 µg rabbit globin mRNA and 1 µg $(dT)_{12}$ per 100 µl in the complete system. The conditions were as described for the standard polymerase assay in materials and methods, with the addition of (unlabelled) dATP, dCTP, dTTP to a final concentration of 0.6 mM respectively. The only labelled deoxynucleoside triphosphate was ^{3}H-dGTP (8 Ci/mMol) and was contained in a final concentration of 0.012 mM. Incubation at 37 °C was for 160 minutes in the case of particle enzyme and 40 minutes in the case of AMV enzyme. The acid insoluble radioactivity was determined from the total volume. For comparison an identical assay were run with $pC(dG)_{12-18}$ instead of globin mRNA as a template.

region of the mRNA, and not of the polyA-strand, ^{3}H-dGTP was used as labelled precursor. Table 4 shows the utilization of oligo dT - globin mRNA by the polymerase purified from particles. The full activity is dependent on the presence of template, primer, all four deoxynucleoside triphosphates, and can be completely inhibited by the presence of ribonuclease. Actinomycin D has no clear effect on the reaction, indicating that it does not proceed significantly further than hybrid synthesis. As can be seen from Table 4 as well, the utilization of globin mRNA in comparison to $pC(dG)_{12}$ is as efficient by the particle enzyme as by the AMV enzyme.

To prove that a faithful transcript had been synthesized by the particle enzyme, the reaction product was analyzed on CS_2SO_4 density gradients after different types of treatment. First the reaction product was purified and subjected to the centrifugation without further treatment. The radioacivity is then found in an intermediate position between RNA and DNA, demonstrating that the product is a RNA·DNA hybrid (figure 3A). After alkaline treatment the radioactivity is shifted to the region of DNA density, indicating that DNA had been synthesized by the enzyme (figure 3B). After incubation of the alkaline treated material under conditions suitable for hybridization and in the presence of excess newly-added globin mRNA the radioactivity is found near the RNA position of the density gradient (figure 3C). This demonstrates that the DNA product is in fact complementary to the messenger RNA originally used as template.

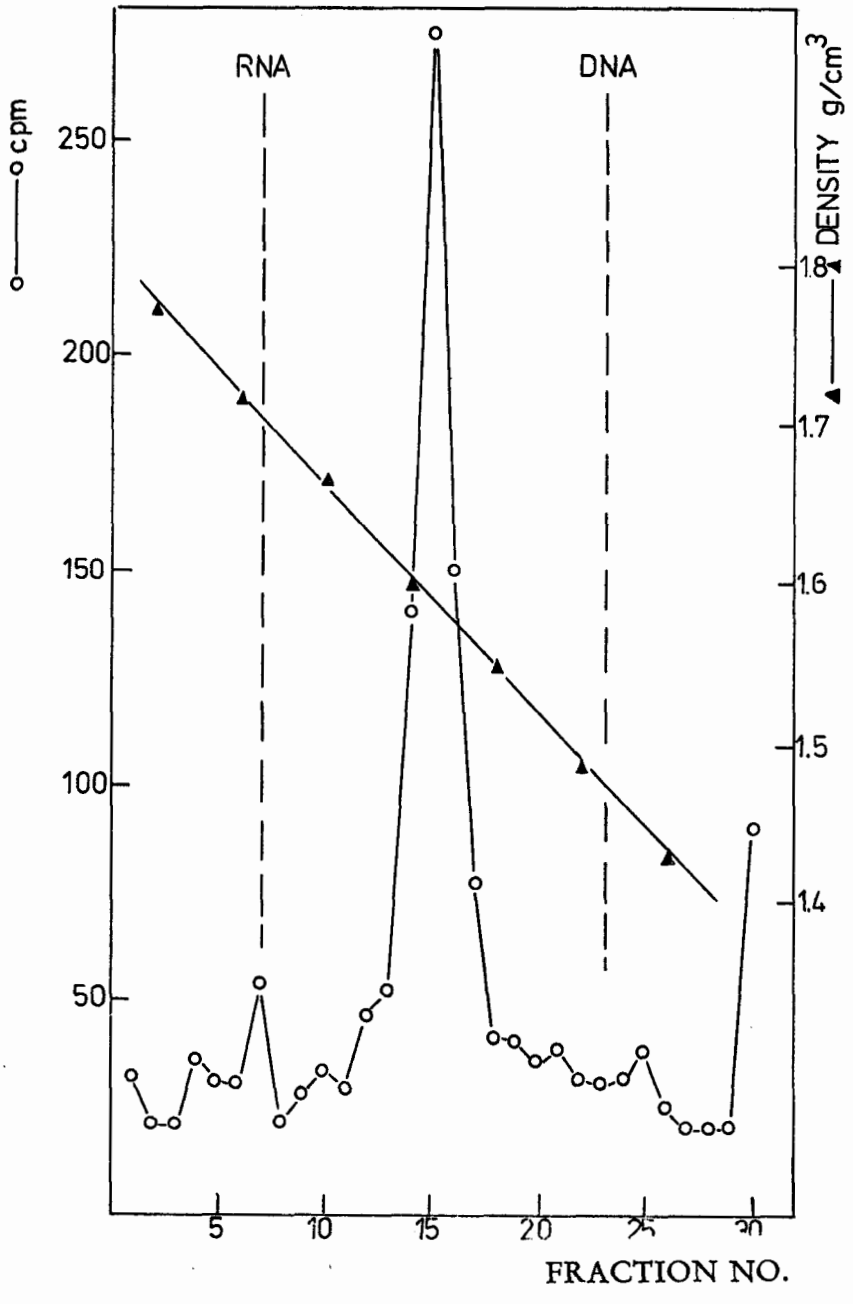


Fig.: 3 A

Legend to Figure 3A–C: Cs_2SO_4 density gradient analysis of ^{3}H-DNA, complementary to globin mRNA, synthesized by purified polymerase from particles. Reaction mixtures as described in the legend to Table 4 were incubated in the presence of oligo dT·globin mRNA, enzyme from particles and Actinomycin D (50 µg/ml) for 2 hours at 37 °C. The product was purified by two-fold phenolisation and subsequent alcohol precipitation. It was resuspended in 2X SSC buffer. The density of the product at this stage of treatment is shown in figure 3A. The product was further treated with 0.25 N KOH for 18 hours at room temperature and then again neutralized. The density of the product at this stage is seen in figure 3B. The alkaline treated product was incubated in a buffer containing 0.4 M NaCl, 2 mM EDTA, 10 mM Tris pH 7.4 and 50 % formamide together with 5 µg of globin messenger RNA at 37 °C for 20 hours. The density of this product is shown in figure 3C.

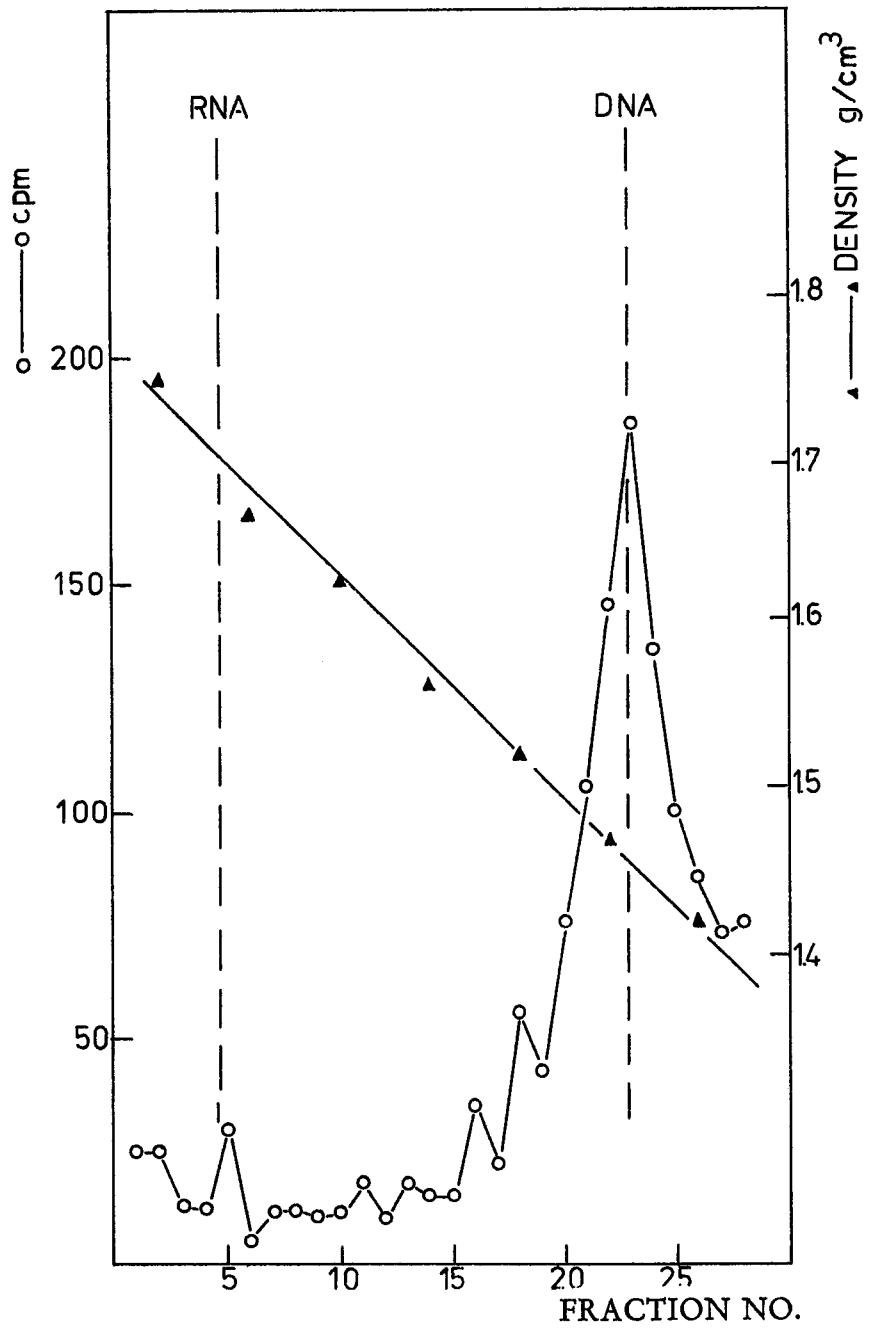


Fig.: 3 B

The density gradients were performed by mixing the products with Cs_2SO_4 at a density of 1.55 g/cm³ and centrifuging this mixture in a Rotor Ti 50 at 43,000 RPM, 15 °C for 60 hours. The gradients were fractionated from the bottom and the density measured via the refractive index. The fraction then were TCA-precipitated and the radioactivity measured.

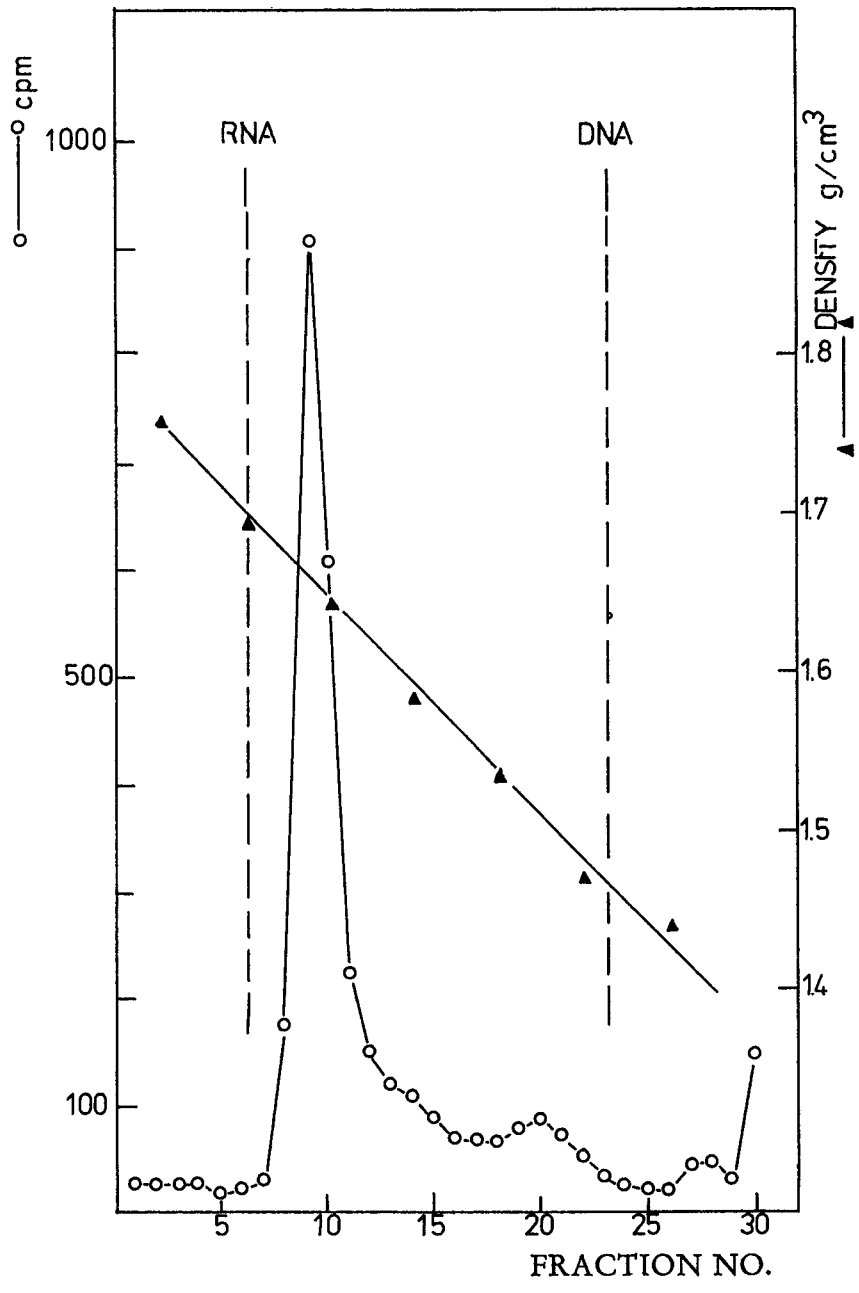
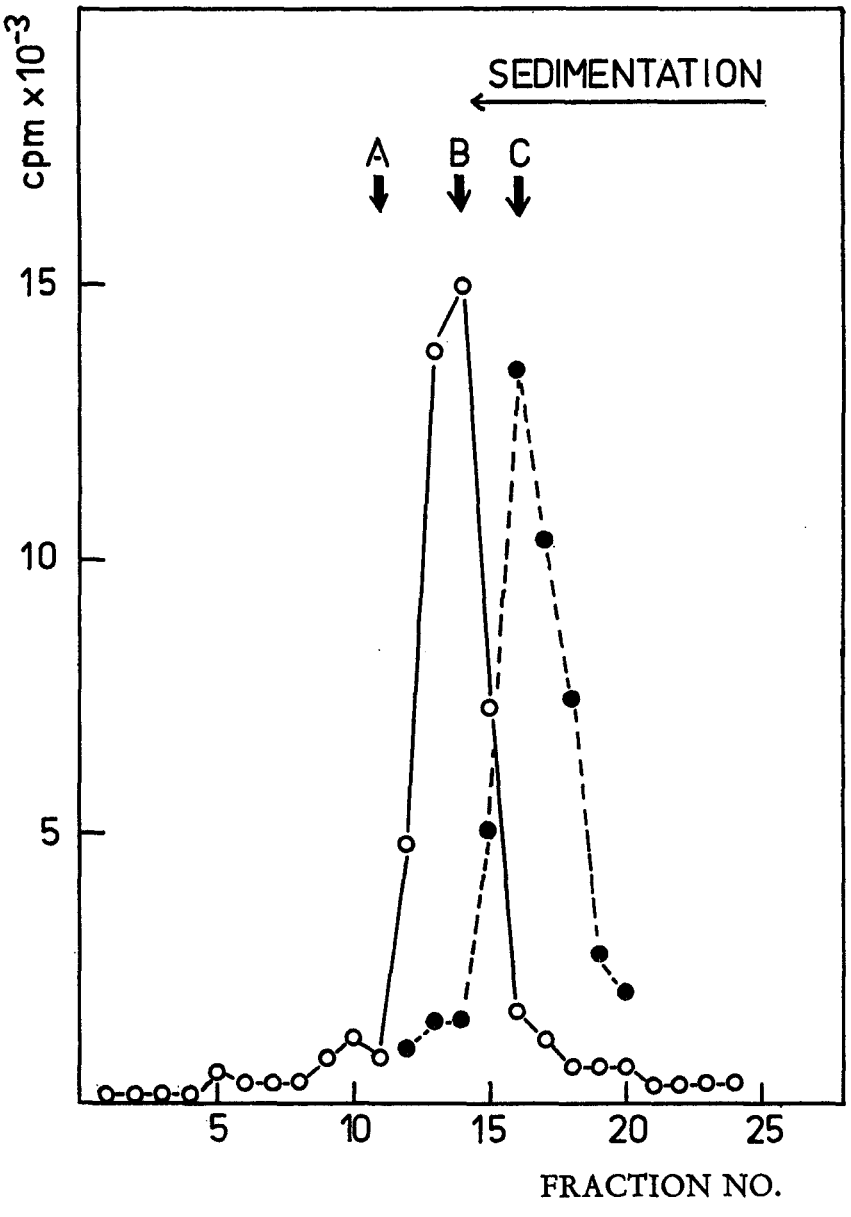

cpm
1000
500
100
RNA
DNA
DENSITY g/cm3
1.8
1.7
1.6
1.5
1.4
5
10
15
20
25
30
FRACTION NO.

Fig.: 3 C



Legend to Fig. 4: Velocity sedimentation of reverse transcriptase from particles, REV and AMV.

Purified polymerases were sedimented in glycerol gradients, in the presence of 0.4 M KCl, as described by Markus et al. (8). The five gradients run under identical conditions contained particle polymerase, REV polymerase, AMV polymerase, aldolase and bovine serum albumin. After the run (17 hours) the gradients were fractionated from the bottom. The positions of the polymerases were found by standard polymerase tests with pC(dG) as template, the position, of the two marker proteins by measuring the protein concentration. The figure show the activity of the fractions, expressed as cpm ^{3}H-dGMP incorporated into acid insoluble material per hour in a reaction volume of 100 µl. ○—○ particle polymerase, ●—● REV polymerase, A = aldolase, B = AMV polymerase and C = bovine serum albumin.

Distinction of the »particle reverse transcriptase« from the reverse transcriptase of REV and the ALV/ASV group

As the enzyme had been shown to be a true reverse transcriptase by its template characteristics and its ability to synthesize a faithful transcript of heteropolymeric RNA, it was interesting and necessary to investigate whether the enzyme was related to or identical with the reverse transcriptase from chicken viruses. We first compared the reverse transcriptases from particles, REV and AMV in respect of their sedimentation constant. The enzymes and two marker proteins were sedimented in parallel gradients under identical conditions. The position of the enzymes was tested by activity tests of each fraction. As can be seen from figure 4, the particle enzyme sediments at the same rate as the AMV enzyme, but can be clearly distinguished from the REV enzyme with respect to sedimentation constant. As can be seen in Table 5, the particle enzyme and the REV enzyme can also be easily

Table V: Comparison of pC-directed DNA synthesis by reverse transcriptase from particles or REV with different divalent cations

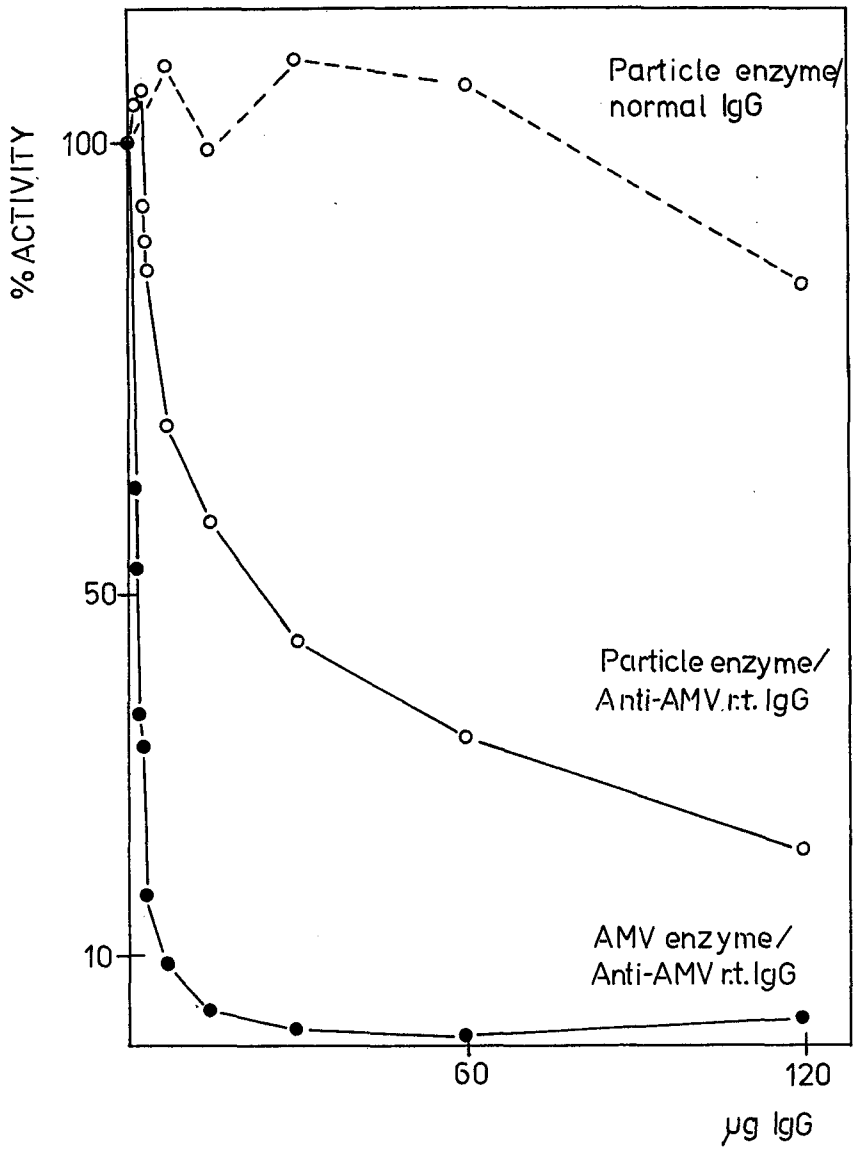
Source of reverse transcriptase	cpm incorporated in the presence of		Relation of activities
	10 mM Mg^{++}	0.4 mM Mn^{++}	Mg^{++} : Mn^{++}
Particles	13 026	1 821	7.15
REV	468 000	1 082 820	0.43

Legend to Table 5: Purified reverse transcriptase was tested under standard conditions in the presence of $pC(dG)_{12-18}$ and ^3H-dGTP. Tests were performed either in the presence of 10 mM Mg^{++} or 0.4 mM Mn^{++}. Incubation was for 60 minutes at 37 °C. The acid insoluble radioactivity was determined in the total volume.

distinguished by their different preferences for divalent cations. Whereas the particle enzyme prefers Mg^{++} over Mn^{++}, the opposite is true for the REV enzyme.

As the antibody against AMV reverse transcriptase has been shown to inhibit the activity of all viruses of the ALV/ASV group, including the inducible ALV (13, 14), we used the antibody inhibition test to elucidate the relationship of the different enzymes. In particular the inhibition of particle and AMV enzyme by IgG against AMV reverse transcriptase was studied in parallel IgG dilution assays. The result is to be seen in figure 5 A and 5 B, where 5 B is just an enlargement of the region near the ordinate of figure 5 A. Whereas the AMV enzyme is inhibited very efficiently by small amounts of IgG, there is only a weak effect on the enzyme purified from the particles. An IgG concentration sufficient to neutralize AMV enzyme to 50 % does not show a significant effect on particle enzyme. The concentration has to be increased to about 70 fold, to obtain a 50 % inhibition. This clearly demonstrates that the particle reverse transcriptase is immunological different from the AMV enzyme.

Still more pronounced data have been obtained recently. Using another batch



Legend to Fig. 5A and 5B: Comparative inhibition of particle and AMV reverse transcriptase by anti-AMV-enzyme IgG.

The inhibitory effect of various amounts of IgG directed against AMV reverse transcriptase and normal IgG on particle reverse transcriptase was compared to the effect on AMV reverse transcriptase. Constant amounts of enzyme were mixed with variable amounts, of IgG, in a reaction mixture of 50 µl, in the presence of 150 mM KCl, 50 µg BSA and 10 mM Tris-HCl, pH 8.2. Incubation was at 0–4 °C for 12 hours. Then each tube was brought to conditions for pC-dependent DNA synthesis and the remaining activity was measured in 1 hour incubation. 100 % activity was obtained from tests without added IgG, 100 % activity was about 15,000 cpm incorporated per hour in 100 µl reaction mix, for particle enzyme as well as for AMV enzyme. Parallel experiments ensured that under the conditions of the test the activity was proportional to the amount of enzyme and was linear within the time of the test.

of IgG, a comparatively much weaker effect on particle enzyme was found. This suggests that the observed inhibition by the antibody against AMV enzyme is due to antibodies against particle enzymes present in the used IgG preparations in small but different amounts. In any case, the particle enzyme is certainly not identical to the reverse transcriptase of the ALV/ASV group, and only weakly, if at all, related to them.

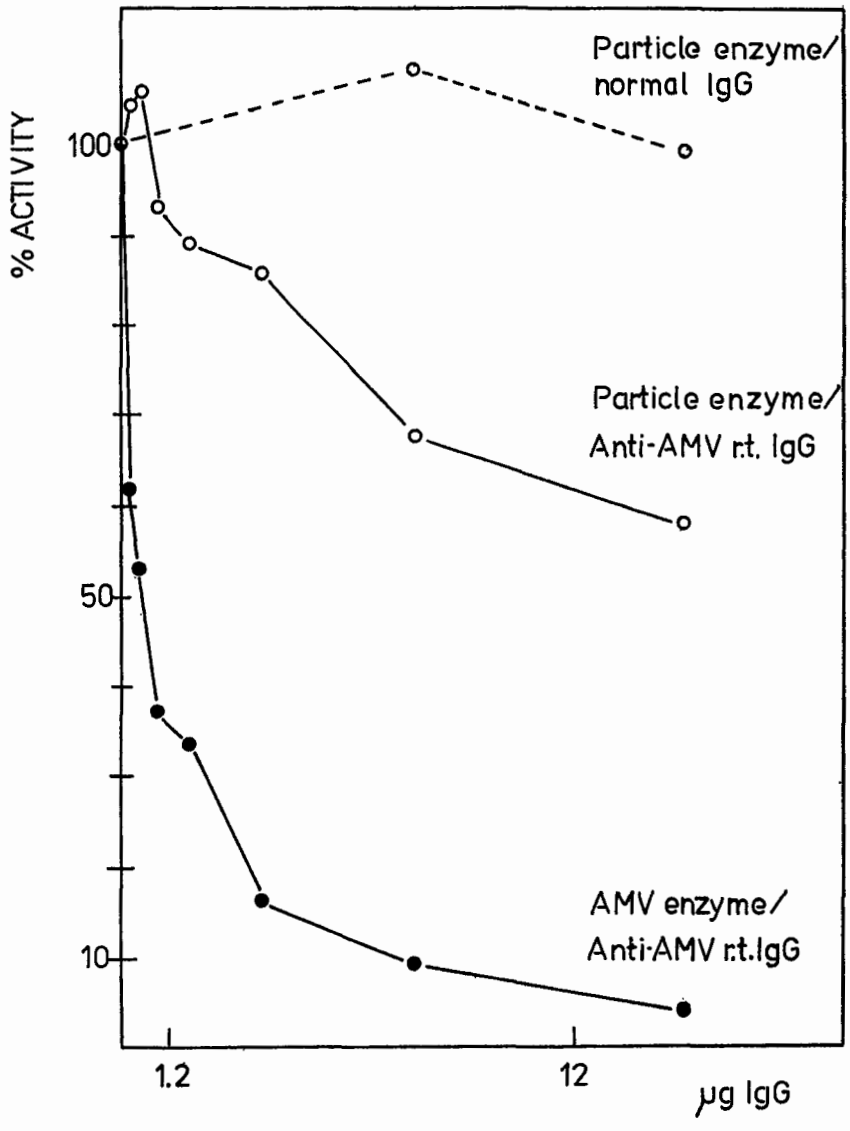


Fig.: 5 B

Conclusion

We were able to purify from particles from the allantoic fluid of ten day-old embryonated eggs an enzyme, which shows the biochemical characteristics of (viral) reverse transcriptase: preference for certain synthetic templates and the ability to synthesize DNA complementary to natural RNA. However the enzyme could be clearly differentiated from the reverse transcriptases of the known chicken RNA Tumor Viruses by several criteria:

1) the enzyme is different from the reverse transcriptase of REV in respect to sedimentation properties and ion-requirements.
2) the enzyme is only weakly, if at all, related to the reverse transcriptase of the viruses from the ALV/ASV group, which is known to be a single immunological species throughout the group.
3) enzymological comparison of particle enzyme to AMV enzyme, in recent experiments (data will be published elsewhere), has shown striking differences in the K_M-values for dGTP and dTTP.

As the enzyme has been proven not to be identical with the reverse transcriptase from the chicken viruses, and is completely different as well from the enzyme purified by Kang and Temin (3), we believe to have isolated a so far unknown chicken enzyme, which seems to be a good candidate for a cellular reverse transcriptase. As the enzyme could be detected in the eggs of all flocks of chicken tested (chicken from the bavarian countryside, from leukosis virus-free SPAFAS Inc., Norwich, Connecticut and from leukosis virus-free SPF-VALO chicken from Lohmann Tierzucht, Cuxhaven, West Germany), and could be isolated by separate purification from each one of 24 single eggs, we conclude that the enzyme might be ubiquitous and therefore has no obvious relation to the induction of malignancy. If this phenomenon is a more generalized one, demonstration of reverse transcriptase alone can not longer be taken as a solid proof for the presence of RNA tumor viruses.

Till now we only isolated the enzyme from particles from the allantoic fluid, and do not know the level of enzyme activity in the embryonic tissue. So it is very interesting and necessary to obtain more information about the nature of these particles. We are presently investigating this problem and know already that in spite of the similar density, particles can be clearly differentiated from Avian RNA tumor viruses in several respects:

1) the particles do not possess the biological properties of ALV/ASV, i.e. helper activity and interfering properties (these tests were kindly performed by Dr. R. Friis, University of Giessen).

2) the protein pattern of purified particles is completely different from the pattern of REV or ALV/ASV. This result could be very much strengthened by tests, kindly performed by Dr. A. Vaheri, University of Helsinki: he could not detect the major group specific antigen of ALV/ASV, the protein P 27 in preparations of particles when he applied a radioimmune assay for this protein.

Since the endogenous reaction is weak and very labile we were not yet able to study the nature of a nucleic acid possibly present as endogenous template. This question has to be answered, to be able to see whether the particles are a new group of endogenous viruses or cellular exclusions.

This problem and the search for intracellular enzyme are presently under investigation.

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