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Inhibition of reverse transcriptases by seminalplasmin

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Seminalplasmin, an antibacterial protein present in bovine seminal plasma, is shown to be a potent inhibitor of reverse transcriptases (RNA-dependent DNA nucleotidyltransferases). Seminalplasmin inhibits RNA-directed, hybrid-directed, and DNA-directed DNA-polymerizing activities of purified reverse transcriptase from avian myeloblastosis virus and from crude viral lysates of several retroviruses by binding to the enzyme, at least in the case of avian myeloblastosis virus. Seminalplasmin does not inhibit significantly DNA synthesis either by *Escherichia coli* DNA polymerase I, or a mammalian α-DNA polymerase. The presence of seminalplasmin in the seminal fluid could provide protection to the male and/or the female reproductive tract against retroviruses.

It has been recently shown that seminalplasmin, an antimicrobial protein present in bovine seminal plasma (Reddy & Bhargava, 1979), is a potent inhibitor of transcription by *Escherichia coli* RNA polymerase *in vitro*, and that it acts by binding to the enzyme with a high affinity (Scheit *et al.*, 1979). In this report we show that seminalplasmin is a powerful inhibitor of reverse transcriptase (EC 2.7.7.49) as well; it, again, appears to act by binding strongly to the enzyme.

Experimental

Materials

Cold deoxynucleoside triphosphates and the synthetic templates were obtained from P.L. Biochemicals, [^{3}H]dTTP, [^{3}H]dGTP and [^{3}H]dCTP from New England Nuclear and Amersham Searle, acrylamide and bisacrylamide from Serva and calf thymus DNA from Sigma.

Feline leukaemia virus and Raucher leukaemia virus were obtained through the Office of Resources and Logistics of the Virus Cancer Program. AMV and purified AMV reverse transcriptase (specific activity 40000–45000 units/mg of protein) were either gifts from Dr. J. Beard or were prepared by us according to methods already described (Kacian & Spiegelman, 1974). The virus suspension in TNE buffer [0.01 M-Tris/HCl (pH 8.3)/0.15 M-NaCl/0.002 M-EDTA] was purified by rebanding twice in a 10–40% sucrose gradient in TNE buffer before use. MCF-7 α-polymerase isolated from the human breast cancer cell line, MCF-7, was prepared as described earlier (Furmanski *et al.*, 1980). *E. coli* DNA polymerase I was a gift from Dr. L. Loeb. Activated calf thymus DNA was prepared as described earlier (Furmanski *et al.*, 1980; Schalabach *et al.*, 1971). Seminalplasmin was prepared as described by Reddy & Bhargava (1979); it was free of ribonuclease activity (Scheit *et al.*, 1979) and homogeneous according to criteria already described (Reddy & Bhargava, 1979).

Abbreviation used: AMV, avian myeloblastosis virus.

Reverse transcriptase assays

Experiments with purified AMV reverse transcriptase. The reverse transcriptase assays were carried out at 37°C for the desired time in 100 μl of Tris buffer [50 mM-Tris/HCl (pH 8.3)/40 mM-KCl/10 mM-$MgCl_2$/20 mM-dithiothreitol] containing the enzyme (1.57 μg/ml, unless otherwise mentioned), the template [120 μg/ml for activated calf thymus DNA; 6 μg/ml for globin mRNA with 25 μg/ml of $(dT)_{12-18}$ as primer; 8–10 μg/ml in all other cases], and [^{3}H]dTTP, [^{3}H]dGTP or [^{3}H]dCTP (all 0.4 mM, 400 c.p.m./pmol, unless otherwise mentioned), as stated in the legends to the Figures. When globin mRNA or activated calf thymus DNA was used as the template, the reaction mixture contained unlabelled dATP, dCTP and dGTP (400 μM each),

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in addition to [^{3}H]dTTP. The reaction was started by addition of the template and the substrates. DNA synthesis at the end of the desired time was measured following trichloroacetic acid precipitation (Furmanski *et al.*, 1980) or chromatography on paper strips (Scheit *et al.*, 1979). Radioactivity was measured in a liquid scintillation counter with a toluene-based scintillation mixture.

Experiments with viral lysates. The virus suspension in 0.001M-Tris/HCl (pH 8.3) was lysed with Triton X-100 (0.3%) containing dithiothreitol (5mM). Reverse transcriptase assays with virus lysates using the endogenous template (500μg of viral protein/ml of final incubation mixture) were carried out as for calf thymus DNA above, with the addition of actinomycin D (80μg/ml). Reverse transcriptase assays with virus lysates using synthetic templates were carried out as described for the templates above, with the following changes: 45mM-glycine/HCl buffer (pH 8.3), containing NaCl (0.3M) and dithiothreitol (0.02M), was used in place of the Tris buffer; and the reaction mixture also contained 0.1mM-Mg^{2+} for feline leukaemia virus and Raucher leukaemia virus, and 1.0mM-Mg^{2+} for AMV.

DNA polymerase assay

This assay was carried out in 100μl as for the purified reverse transcriptase assay except that *E. coli* DNA polymerase (12μg/ml) or the MCF-7 α-polymerase (24μg/ml) was used in place of the reverse transcriptase; calf thymus DNA was used as the template, and the reaction mixture contained unlabelled dATP, dCTP, dGTP (400μM each) and [^{3}H]dTTP (43.6μM, 5045c.p.m./pmol). The reaction was started by addition of the template and the substrates, and was carried out for 20min. In the absence of seminalplasmin, 113000 and 1100c.p.m. of [^{3}H]dTTP were incorporated into DNA in 30μl of the reaction mixture for the *E. coli* polymerase and the MCF-7 polymerase respectively.

Experiments with seminalplasmin

When it was desired to study the effect of seminalplasmin on reverse transcriptase or on DNA polymerase, unless specified otherwise the enzyme or the virus lysate was preincubated with the required amount of seminalplasmin at 37°C for 15min, in the same incubation medium as used for the enzyme assay in the absence of seminalplasmin. The reaction was started by the addition of the template and the nucleoside triphosphates. In the controls, the enzyme alone was preincubated under conditions identical with those used for preincubation of the enzyme with seminalplasmin.

Gel electrophoresis

Polyacrylamide gel electrophoresis of reverse transcriptase, seminalplasmin and the preincubated mixture of these two proteins was carried out using a 6% gel in 0.05M-Tris/glycine buffer (pH 8.4), at 5mA/gel for 3h. The gels were stained with Coomassie Blue G-250 (0.08%) (Blakesley & Boezi, 1977).

Results

Inhibition by seminalplasmin of DNA synthesis catalysed by purified AMV reverse transcriptase, using various templates

Seminalplasmin, at concentrations above 15μg/ml, inhibited strongly the incorporation of [^{3}H]-dTTP into DNA by AMV reverse transcriptase (1μg/ml), using poly(rA)·$(dT)_{12-18}$ as the template (Fig. 1), provided the enzyme was preincubated with

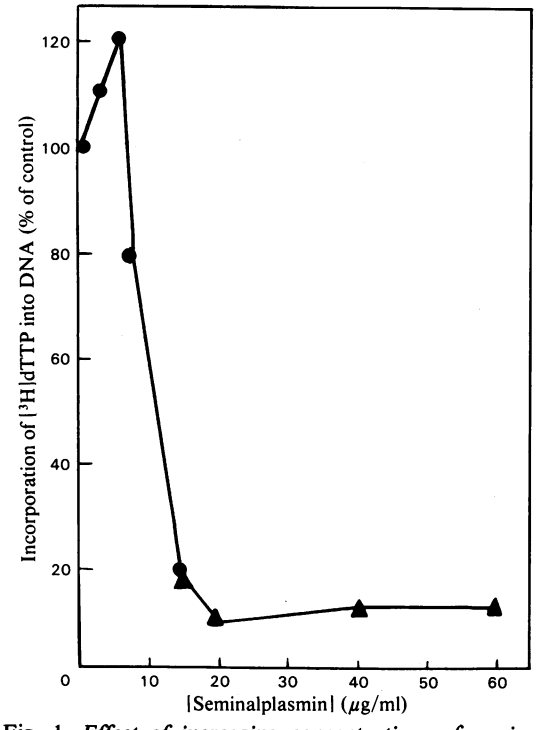

Fig. 1. *Effect of increasing concentrations of seminalplasmin on the reverse transcription of poly-$(rA)\cdot(dT)_{12-18}$ by AMV reverse transcriptase*

The reverse transcriptase was preincubated with or without seminalplasmin at 37°C and the reverse transcriptase assay was carried out for 20min, using [^{3}H]dTTP as the labelled precursor, as described in the Experimental section. The incorporation of the labelled precursor into DNA was measured after paper-strip chromatography. The radioactivity in 30μl of the control incubation mixture was 105900c.p.m. The concentration of seminalplasmin shown is the final concentration in the reaction mixture. ●, experiment 1; ▲, experiment 2.

seminalplasmin for 15min at 37°C before the template and the substrates were added to the reaction mixture. About 90% inhibition of the reverse transcriptase was obtained with 15–20μg of seminalplasmin/ml, at a seminalplasmin/reverse transcriptase molar ratio of 75–100; higher concentrations of seminalplasmin (200μg/ml, the reverse transcriptase concentration being the same as above) gave virtually complete inhibition (Fig. 2). Seminalplasmin (200μg/ml), when preincubated for 15min at 37°C with the enzyme, also inhibited, to an extent of 94–99%, DNA synthesis by AMV reverse transcriptase on all the other synthetic RNA or DNA templates tried, i.e. poly(rC)·$(dG)_{12-18}$ or poly(dA)·$(dT)_{12-18}$ (Fig. 2*a*); over 80% inhibition was obtained with a seminalplasmin concentration of 15μg/ml wherever tried.

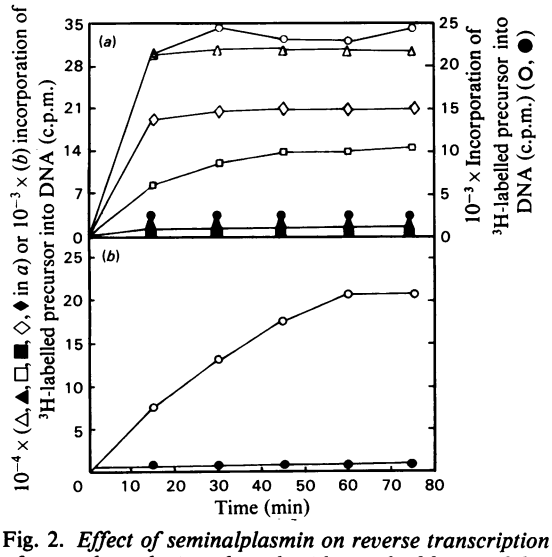

Fig. 2. *Effect of seminalplasmin on reverse transcription of several synthetic polynucleotides and of haemoglobin mRNA by AMV reverse transcriptase*

The reverse transcriptase was preincubated with or without seminalplasmin at 37°C and the reverse transcriptase assay was carried out for the stated time, using (*a*) poly(dC)·$(dG)_{12-18}$ (○, ●; 8.4μg/ml), poly(rC)·$(dG)_{12-18}$ (△, ▲; 8.4μg/ml), poly(rA)·$(dT)_{12-18}$ (◇, ♦; 8.4μg/ml) or poly(dA)·$(dT)_{12-18}$ (□, ■; 8.4μg/ml) or (*b*) haemoglobin mRNA (○, ●; 6μg/ml) as the template, and [^{3}H]dGTP (for the first two templates), [^{3}H]dTTP (for the next two templates) or [^{3}H]dCTP (for the mRNA) as the labelled precursor. The incorporation of the precursor into DNA was measured after precipitation with trichloroacetic acid as described in the Experimental section. The concentration of AMV reverse transcriptase in the final reaction mixture was 1.57μg/ml. Open symbols, without seminalplasmin; filled symbols, with seminalplasmin (200μg/ml final concentration).

In the absence of preincubation of AMV reverse transcriptase (1μg/ml) with seminalplasmin (15μg/ml) or on preincubation of the template [poly(rA)·$(dT)_{12-18}$ or poly(rC)·$(dG)_{12-18}$, instead of the enzyme] with seminalplasmin at 37°C, no inhibition whatsoever of the reverse transcription reaction was observed. At concentrations below 8μg/ml (that is, at a seminalplasmin/reverse transcriptase molar ratio below 40), seminalplasmin incubated with AMV reverse transcriptase at 37°C for 15min stimulated slightly (range 12–20%, average 15%) the reverse transcription of poly(rA)·$(dT)_{12-18}$ (Fig. 1). A 20% stimulation of the enzyme activity was also obtained with poly(rA)·$(dT)_{12-18}$ [but not with poly(rC)·$(dG)_{12-18}$] as the template, when seminalplasmin was incubated at a higher concentration than above (i.e. 15μg/ml), with the reverse transcriptase (1μg/ml) for 15min at 0°C. No stimulation was observed if seminalplasmin was replaced by bovine serum albumin.

The inhibitory activity of seminalplasmin towards AMV reverse transcriptase was retained on heating seminalplasmin at 80°C for 10min, as with its antimicrobial activity (Reddy & Bhargava, 1979).

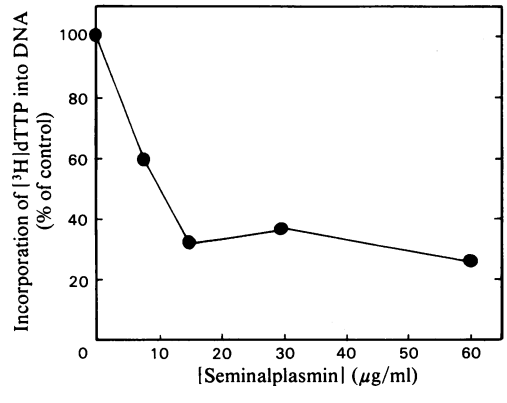

Fig. 3. *Effect of seminalplasmin on DNA synthesis by AMV reverse transcriptase using calf thymus DNA as the template*

The reverse transcriptase was preincubated with or without seminalplasmin at 37°C and the reverse transcriptase assay was carried out for 20min using calf thymus DNA as the template and [^{3}H]dTTP as the labelled precursor, and the incorporation of the precursor into DNA measured after paper-strip chromatography, as described in the Experimental section. The radioactivity in 30μl of the control incubation mixture (without seminalplasmin) was 10600c.p.m. The concentration of seminalplasmin shown is the final concentration in the reaction mixture; the final concentration of AMV reverse transcriptase was 1μg/ml.

As in the case of reactions with synthetic templates, seminalplasmin inhibited strongly the synthesis of DNA from heteropolymeric RNA and DNA templates [e.g. the haemoglobin mRNA (Fig. 2*b*) or activated calf thymus DNA (Fig. 3)] by AMV reverse transcriptase, when the enzyme was preincubated with seminalplasmin for 15 min at 37°C before commencement of the reaction.

Inhibition by seminalplasmin of DNA synthesis catalysed by reverse transcriptase in retrovirus lysates

Fig. 4 shows that seminalplasmin inhibited strongly reverse transcriptase-catalysed DNA synthesis in lysates of three different retroviruses, Raucher leukaemia virus, feline leukaemia virus and AMV, irrespective of whether the resident viral RNA, poly(rA)·$(dT)_{12-18}$ or poly(dA)·$(dT)_{12-18}$ was used as the template. In the experiment of Fig. 4, the lysates were preincubated with seminalplasmin; in this system, in fact, seminalplasmin inhibited reverse transcriptase-catalysed synthesis of DNA even in the absence of preincubation.

Mechanism of inhibition of seminalplasmin of DNA synthesis by reverse transcriptases

The inhibition of AMV reverse transcriptase by seminalplasmin described above was relieved by increasing the concentration of the reverse transcriptase (but not of the template) in the final incubation mixture (Fig. 5). This observation, coupled with the requirement for preincubation of the reverse transcriptase with seminalplasmin for inhibition of the enzyme activity, suggested binding of the reverse transcriptase with seminalplasmin as a likely mechanism for the inhibitory action of the latter on the former. It is already known that seminalplasmin inhibits *E. coli* RNA polymerase by binding strongly to the enzyme (Scheit *et al.*, 1979). We, therefore, looked for more direct evidence for the binding of reverse transcriptase to seminalplasmin.

We compared the gel electrophoresis mobilities at pH 8.3 of AMV reverse transcriptase, seminalplasmin and a mixture of the two proteins after a 15 min preincubation at 37°C (Fig. 6). Under the conditions used, seminalplasmin, a basic protein (Reddy & Bhargava, 1979), moves in the direction opposite to that in which the reverse transcriptase moves, and is thus lost from the gel (Fig. 6*a*). Binding of seminalplasmin to the reverse transcriptase could be expected to neutralize some of the negative charge of the latter protein, which would reduce its mobility. A substantial reduction in the mobility of the reverse transcriptase was obtained on its preincubation with seminalplasmin (compare Fig. 6*b* with Fig. 6*c*), suggesting the formation of a stable complex between the two proteins.

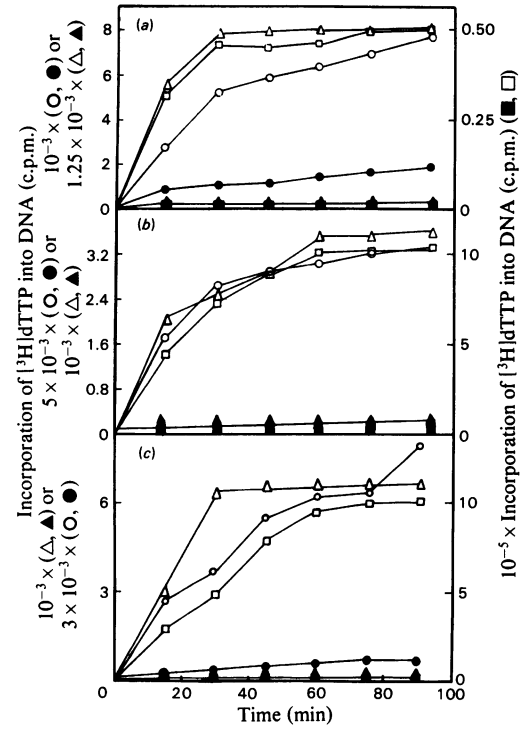

Fig. 4. *Effect of seminalplasmin on reverse transcription of endogenous and exogenous templates in retrovirus lysates*

The virus lysate was prepared and preincubated with (filled symbols) or without (open symbols) seminalplasmin at 37°C and the reverse transcriptase assay was carried out for the stated time using endogenous viral RNA (○, ●), poly(rA)·$(dT)_{12-18}$ (□, ■) or poly(dA)·$(dT)_{12-18}$ (△, ▲) as the template and [³H]dCTP (for endogenous viral RNA) or [³H]dTTP (for synthetic templates) as the labelled precursor. The incorporation of the precursor into DNA was measured after precipitation with trichloroacetic acid, as described in the Experimental section. The concentration of seminalplasmin in the final reaction mixture was 50 μg/ml. (*a*), AMV; (*b*), Raucher leukaemia virus; (*c*), feline leukaemia virus.

If, after preincubation of the reverse transcriptase with seminalplasmin, the mixture was incubated for a further period of 15 min in the presence of 1 M-NaCl and then cooled quickly to 0°C, the enzyme was still inactive in synthesizing DNA from poly(rA)·$(dT)_{12-18}$ as the template, in contrast to enzyme which was similarly treated but without seminalplasmin. It would, therefore, appear that the reverse transcriptase–seminalplasmin complex is not disassociated by salt concentrations up to 1 M.

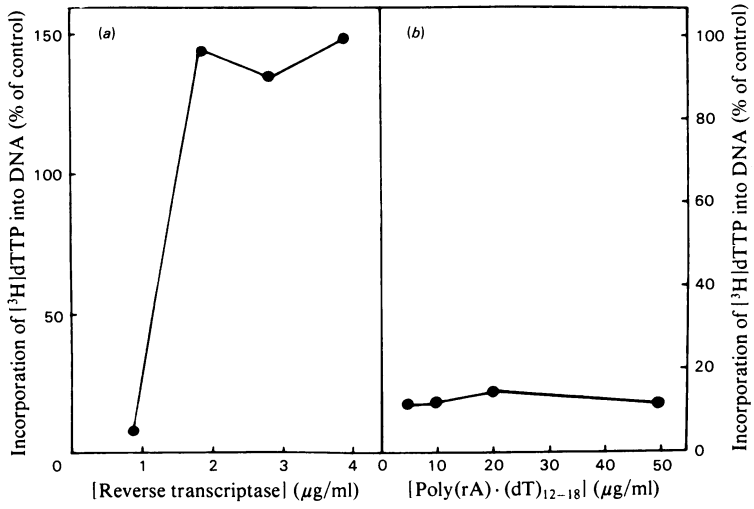

Fig. 5. *Effect of increasing the concentration of the reverse transcriptase (a) or of the template (b) on the inhibition by seminalplasmin of reverse transcription of poly(rA)·$(dT)_{12-18}$ by AMV reverse transcriptase*

The reverse transcriptase was preincubated with or without seminalplasmin at 37°C, and the reverse transcriptase assay was carried out for 20min, using poly(rA)·$(dT)_{12-18}$ as the template and [^{3}H]dTTP as the labelled precursor, as in Fig. 1, except that the final concentration of the enzyme (in *a*) or the template (in *b*) was varied as stated. The concentration of seminalplasmin in the final reaction mixture in both (*a*) and (*b*) was 15μg/ml, of the template in (*a*) 10μg/ml, and of the reverse transcriptase in (*b*) 1μg/ml. The incorporation in 30μl of the control incubation mixture (without seminalplasmin) was: in (*a*) 22000, 21000, 44000 and 61020c.p.m. for 0.93, 1.86, 2.79 and 3.72μg of the reverse transcriptase/ml, respectively, and in (*b*) 84800, 92460, 111900 and 106300c.p.m. for 5, 10, 20 and 50μg of template/ml, respectively.

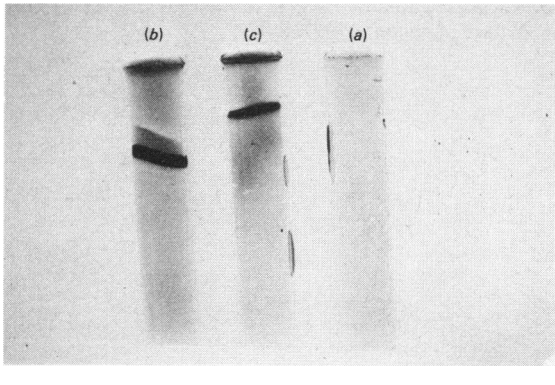

Fig. 6. *Polyacrylamide-gel electrophoresis of seminalplasmin, reverse transcriptase and the complex of seminalplasmin and reverse transcriptase*

The electrophoresis was carried out as described in the Experimental section. (*a*), Seminalplasmin (40μg loaded on the gel in 0.1ml); (*b*) reverse transcriptase (2.6μg loaded on the gel in 0.1ml); (*c*) reverse transcriptase (2.6μg) and seminalplasmin (40μg) incubated in 0.1ml for 15min at 37°C and then loaded on the gel.

Effect of seminalplasmin on DNA-dependent DNA polymerases

The synthesis of DNA by MCF-7 α-polymerase was not inhibited at all by seminalplasmin at a concentration of 65μg/ml, and by not more than 10% by 125μg of seminalplasmin/ml. The *E. coli* DNA polymerase was inhibited at most by 10–25% by 125–250μg of seminalplasmin/ml.

Discussion

Seminalplasmin, an antimicrobial protein present in bovine seminal plasma, which has already been shown to be a potent inhibitor of *E. coli* RNA polymerase, is now shown to be a potent inhibitor of several reverse transcriptases, purified or in viral lysates (Figs. 1–4). All the three activities of the reverse transcriptases (the RNA-instructed, the hybrid-instructed and the DNA-instructed polymerizing activities) were inhibited about equally. On the other hand, the two DNA polymerases investigated (α-polymerase from a human breast cancer cell line, MCF-7, and *E. coli* DNA polymerase I) were not inhibited appreciably.

The ability of seminalplasmin to inhibit RNA-directed, hybrid-directed and DNA-directed activities of reverse transcriptases about equally, and the fact that the inhibition is not reversed by an increase in the concentration of the template, would suggest that seminalplasmin does not act by binding to the template. The inhibition of AMV reverse transcriptase by seminalplasmin, in fact, appears to be due to the binding of the latter to the former (Figs. 5 and 6). A molar ratio of seminalplasmin to reverse transcriptase of 75–100 in the incubation mixture gave optimal inhibition of the enzyme activity. When this ratio was smaller (below, say, 40), or the preincubation of reverse transcriptase with seminalplasmin was done at 0°C at a higher ratio (say, 100) at which seminalplasmin strongly inhibits reverse transcriptase, there appeared to be a slight stimulation by seminalplasmin of DNA synthesis on at least some templates such as poly(rA)·oligo(dT) (Fig. 1).

The above observations are compatible with the existence of two types of binding sites for seminalplasmin on AMV reverse transcriptase, one with high affinity and the other with low affinity. When the concentration of seminalplasmin is low, it may bind only to the high-affinity sites. Such a binding could result in an allosteric modification of the reverse transcriptase, resulting in the observed small enhancement of enzyme activity. At high concentrations of seminalplasmin, the low-affinity as well as the high-affinity sites on the enzyme could be occupied by seminalplasmin. Under these conditions, the inhibition of enzyme activity could result either from a physical blocking of the substrate or template-binding sites, or by further allosteric changes in the catalytic site of the enzyme. Seminalplasmin has no effect (Scheit *et al.*, 1979) on other enzymes like β-galactosidase and polynucleotide phosphorylase, ruling out a non-specific protein–protein interaction as a possible cause for its inhibitory effect on reverse transcriptases.

Seminalplasmin appears to be the first purified protein shown to be a potent inhibitor of reverse transcriptases. It is possible that seminalplasmin may play a role in the protection of the male or the female reproductive tract, or the fertilized ovum, against retroviruses. As α-polymerase is the major enzyme responsible for chromosomal DNA replication in mammalian cells, it is possible that seminalplasmin may selectively inhibit retroviral DNA polymerizing activity in cells infected with retroviruses without affecting cellular DNA synthesis, provided, of course, that seminalplasmin can enter the animal cell as it does bacterial cells, and inhibit transcription of proviral DNA in the host cell.

References

Blakesley, R. W. & Boezi, J. A. (1977) *Anal. Biochem.* **82**, 580–582

Furmanski, P., Bourguignon, G. J., Bolles, C. S., Corombas, J. D. & Das, M. R. (1980) *Cancer Lett.* **8**, 307–315

Kacian, D. L. & Spiegelman, S. (1974) *Methods Enzymol.* **29**, 150–173

Reddy, E. S. P. & Bhargava, P. M. (1979) *Nature (London)* **279**, 725–728

Schalabach, A., Fridlender, B., Bolden, A. & Weissbach, A. (1971) *Biochem. Biophys. Res. Commun.* **44**, 879–885

Scheit, K. H., Reddy, E. S. P. & Bhargava, P. M. (1979) *Nature (London)* **279**, 728–731