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Enzyme from Calf Thymus Degrading the RNA Moiety of DNA-RNA Hybrids: Effect on DNA-Dependent RNA Polymerase

Abstract. *An enzyme present in extracts from calf thymus degrades specifically the RNA moiety of DNA-RNA hybrids. Other nucleic acids, such as single- or double-stranded DNA and single- or double-stranded RNA, are not affected to a comparable degree. If prepared free of the hybrid-degrading enzyme, RNA polymerase from calf thymus shows a fivefold increase in activity on denatured DNA as compared to native DNA.*

In experiments on the purification of RNA polymerase from calf thymus, we observed that, in contrast to other reports (*1–3*), the template activity of denatured DNA for a highly purified polymerase preparation is much higher than that of native DNA. However, if less purified enzyme preparations are used, only native and not denatured DNA stimulates the synthesis of RNA. This finding suggested that a factor is present in the raw extract which specifically influences RNA synthesis on denatured DNA. Further studies on this factor indicated that it is an enzyme which specifically degrades the RNA moiety of DNA-RNA hybrids.

The enzyme is contained in a fraction which occurs as a by-product during the course of RNA polymerase purification. Calf thymus tissue was homogenized in 0.01*M* tris, *p*H 7.8, containing 15 m*M* mercaptoethanol. Then $MnCl_2$ and $(NH_4)_2SO_4$ were added to give a final concentration of 2 m*M* and 0.1*M*, respectively. The resulting precipitate consisting mainly of deoxyribonucleoprotein was sedimented by centrifuging at 20,000*g* for 100 minutes. The supernatant was freed from remnants of deoxyribonucleoprotein by filtration through an asbestos filter plate (Seitz, Bad Kreuznach). To the preparation, one-third of its volume of packed DEAE (diethylaminoethyl) cellulose, previously equilibrated with the same buffer, was added. The suspension was stirred at 4°C for 15 minutes and centrifuged (10 minutes, 3000*g*). The supernatant, from which virtually all RNA polymerase activity had been removed, was dialyzed against 0.01*M* tris, *p*H 8, and retained as fraction A for the following experiments. Fraction A can be kept for several weeks at −70°C without detectable loss of activity.

The influence of 0.05 ml of fraction A (25 μg of protein) on the standard polymerase assay is shown in Table 1. The assays contained in a total volume of 0.5 ml: 0.03*M* tris, *p*H 7.8; 1 m*M* $MnCl_2$; 10 m*M* mercaptoethanol; 0.1*M* $(NH_4)_2SO_4$; 0.2 m*M* each of guanosine, cytidine, and adenosine triphosphates (GTP, CTP, and ATP); 0.1 μc of C^{14}-uridine triphosphate (UTP) (50 μc/μmole; Radiochemical Centre, Amersham) and 15 μg of protein of calf thymus RNA polymerase (*4*). Calf thymus DNA (Sigma) and fraction A were added as indicated in Table 1. The mixtures were incubated for 10 minutes at 37°C. After addition of 150 μg of yeast RNA (as carrier) and five drops of a saturated $Na_4P_2O_7$ solution, the acid-insoluble material was precipitated by 4 percent trichloroacetic acid (TCA). The precipitate was sedimented by centrifugation, resuspended in TCA and collected on Millipore filters for counting in a Packard Tri-Carb liquid scintillation spectrometer.

Two effects of fraction A can be distinguished: (i) Incorporation of UTP is stimulated by a factor of 3 if native DNA is used as template. (ii) This incorporation is inhibited by 90 percent if denatured DNA is used. If fraction A is present in the assay, addition of denatured DNA inhibits the RNA synthesis on native DNA. A similar inhibiting effect of denatured DNA on the RNA polymerase reaction has been described by Furth and Ho (*3*).

For further characterization of the active agents, fraction A was chromatographed on a DEAE-cellulose column, and portions of the individual fractions were added to standard polymerase assays containing either native or heat-denatured DNA. The factor stimulating RNA synthesis on native DNA elutes at about 0.03*M* tris, *p*H 7.8, whereas the factor inhibiting RNA synthesis on denatured DNA elutes at about 0.20*M*. Thus, stimulation and inhibition are the effects of two distinct moieties. The purified stimulating factor was not active if denatured DNA was used in the assay, and conversely no effect of the inhibiting factor was observed if native DNA was used.

The nature of the inhibitory effect of fraction A became obvious by the finding that, upon addition of fraction A, the product of the RNA polymerase reaction, when denatured DNA was used as template, was rapidly degraded. By way of contrast, if native DNA was used as template the product remained almost unaffected. It is known from work on bacterial RNA polymerase (*5*) that, if single-stranded DNA is used as template, the reaction product consists largely of DNA-RNA hybrids. Our findings therefore suggested that the factor present in fraction A specifically degrades RNA when it is hybridized to DNA.

In order to test this hypothesis, the single-stranded DNA of the bacterial phage fd (*6*) was used as template for RNA synthesis, and the product was analyzed in a CsCl density gradient. RNA was synthesized under standard assay conditions (see above) with the exception that 7 μg of phage DNA per milliliter and 0.04 μmole of UTP per milliliter (0.2 μc/ml) were used. After the reaction mixture was incubated for 15 minutes, CsCl was added to give a density of 1.71 g/ml. After addition of ^{3}H-labeled phage DNA as marker, the sample was centrifuged for 60 hours at 35,000 rev/min (SW-39 swinging bucket rotor of the Spinco L ultracentrifuge). Fifty fractions were collected. Portions were precipitated with 4 percent TCA together with 150 μg of yeast RNA as carrier, and the acid-insoluble material was collected on Millipore filters and analyzed for radioactivity.

As expected, the ^{14}C-labeled product bands at a density slightly higher than the single-stranded tritiated DNA from phage fd which was added as a marker (*7*). The fractions containing ^{14}C—the yield amounted to more than 90 percent of the total labeled RNA—were collected and dialyzed against a solution containing 0.03*M* tris, *p*H 7.8;

1 mM $MnCl_2$; 10 mM mercaptoethanol and 0.1M $(NH_4)_2SO_4$. Portions (0.5ml) of this preparation were distributed in test tubes containing 2.5 μg of ribonuclease A (Sigma), or 0.05 ml of fraction A (250 μg of protein), or 0.05 ml of 0.01 mM tris, pH 8. The assay mixtures were incubated at 37°C. At intervals samples were removed, and the acid-insoluble material, precipitated with 4 percent TCA together with 150 μg of yeast RNA as carrier, was analyzed for radioactivity. The hybrid proved largely resistant to ribonuclease A and completely sensitive to fraction A (Fig. 1). Upon heating, it was converted to a form sensitive to ribonuclease A, but at least ten times more resistant to fraction A than the unheated hybrid. The fact that homogeneous DNA as used in this experiment shows rapid renaturation with complementary RNA may explain the observation that the heated preparation becomes increasingly sensitive with time to the action of fraction A. This degradation of the heated preparation was not observed in the analogous experiment when denatured calf thymus was used as template.

In order to show that the transition from sensitivity to resistance to the action of fraction A in fact depends on the "melting" of a double strand, the following experiment was performed. Labeled hybrid consisting of fd DNA and synthetic RNA was isolated from a CsCl density gradient. Portions of this preparation were heated for 5 minutes to different temperatures and then rapidly cooled. One half of these samples was digested with ribonuclease A, and the remainder with fraction A. The radioactivity of the material remaining acid-insoluble was determined (Fig. 2). Both sensitivity to ribonuclease A and stability against fraction A revealed a typical melting curve with a T_m at about 80°C.

Table 1. Effects of fraction A on the incorporation of ^{14}C-UTP into RNA by calf thymus RNA polymerase.

DNA (20 μg)	Fraction A (250 μg protein)	Incorporation of ^{14}C-UTP (pmole/ 10 min)
Native	+	30
Native	—	11.05
Denatured	+	5.0
Denatured	—	60
Denatured and native	+	4.2

As evident from the above results, fraction A contains very little ribonuclease activity acting on single-stranded RNA. The question arises whether the enzyme present in fraction A specifically splits DNA-RNA hybrids or merely double-stranded nucleic acids. Deoxyribonuclease activity was tested with ^{32}P-labeled DNA from *Escherichia coli*. Labeled replicative form of the RNA phage M12 was used to study the effect on double-stranded RNA. The nucleic acids were incubated with fraction A at 37°C. At intervals, samples were removed, and the nucleic acids were precipitated by 4 percent TCA with 100 μg of yeast RNA as carrier. The radioactivity of the nucleotides released into acid-soluble material was determined. The results showed that 100 μg of fraction A protein, which solubilized 100 pmole of ribonucleotide from DNA-RNA hybrid within 10 minutes, released less than 0.15 pmole of deoxyribonucleotide from native DNA, approximately 0.3 pmole of deoxyribonucleotide from denatured DNA, and less than 0.5 pmole of ribonucleotide from double-stranded RNA. The latter result strongly suggests that the enzyme is different from ribonuclease III, which does degrade double-stranded RNA (*8*).

In a further experiment ^{14}C-RNA was synthesized on ^{32}P-labeled fd DNA in order to obtain double-labeled hybrid. The reaction product formed a double-labeled peak in a CsCl density gradient. The radioactive material was collected, dialyzed as described above, and subjected to degradation by fraction A. As calculated from the specific activities of the ^{14}C-UTP used for the synthesis of RNA and of the ^{32}P-DNA, approximately 35 percent of the DNA was initially hybridized with DNA. More than 90 percent of the ^{14}C-RNA was converted to acid-soluble material, but no ^{32}P activity was found in this fraction. Since a degradation of 3 per-

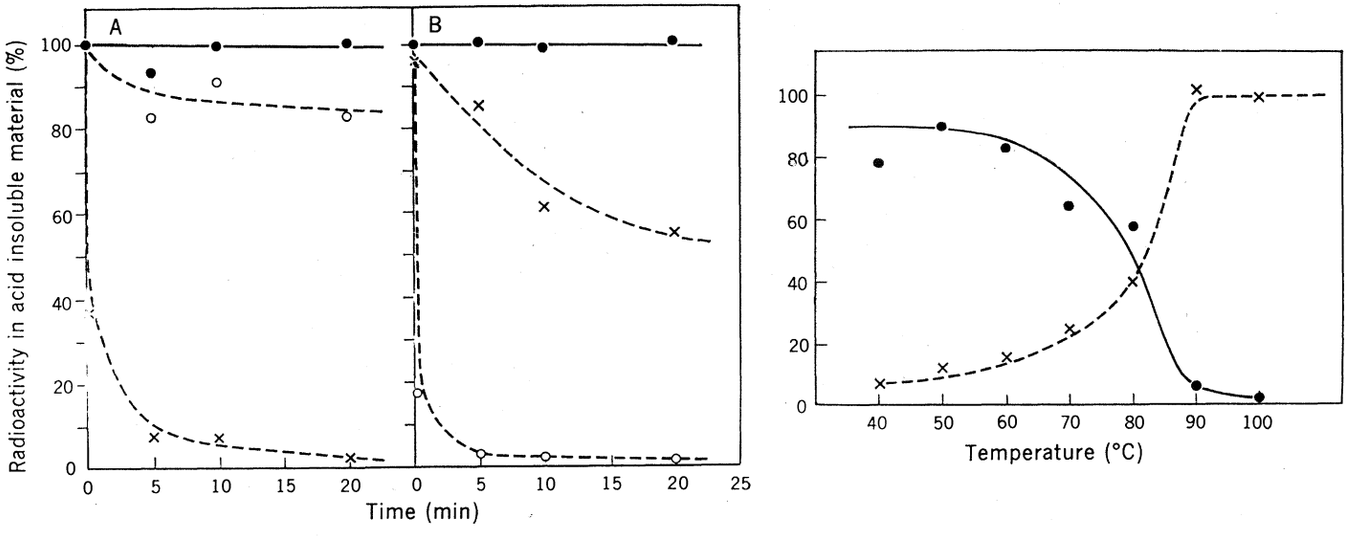

Fig. 1 (left). Sensitivity of the DNA-RNA hybrid obtained from the CsCl density gradient to ribonuclease A and to fraction A. (A) Native hybrid. (B) Hybrid denatured by heating for 5 minutes to 100°C and then rapidly cooled. •—•, Controls. ○---○, Samples containing ribonuclease A. x---x, Samples containing fraction A. The 100 percent value is 30 pmole of uridine monophosphate in acid-insoluble material. Fig. 2 (right). Effect of different temperatures on the sensitivity of DNA-RNA hybrid to ribonuclease A and fraction A. The 100 percent value is 15 pmole of ^{14}C-uridine monophosphate in acid-insoluble material. •—•, Samples treated with ribonuclease A. x---x, Samples treated with fraction A.

cent of the DNA would have been detected, this experiment indicates that, in the DNA-RNA hybrid, it is specifically the RNA moiety which is degraded by the enzyme present in fraction A.

Several of the degradation experiments described have been repeated with DNA-RNA hybrids synthesized with RNA polymerase from *E. coli* instead of from calf thymus. These hybrids showed essentially the same sensitivity to the action of fraction A. The enzyme has been purified some 50-fold by column chromatography and $(NH_4)_2SO_4$ precipitation. Of this preparation, 0.25 μg of protein solubilizes 100 pmole of RNA nucleotide from a DNA-RNA hybrid within 10 minutes at 37°C.

A variable degree of contamination of RNA polymerase preparations with the enzyme that splits DNA-RNA hybrids may explain the conflicting results described by others regarding the template activity of denatured DNA for mammalian RNA polymerase. Thus, depending on the purity of the polymerase preparations used, denatured DNA was not active as template at all (*3*), or it exhibited activity similar to that of native DNA (*2*). In contrast to that, our preparations show a fivefold higher template activity of denatured DNA (see Table 1).

The biological function of the hybrid-degrading enzyme is obscure. A function of DNA-RNA hybrids in the regulation of RNA synthesis has been suggested by Bekhor *et al.* (*9*). Such a mechanism might require an enzyme with the characteristics of the one described here.

Note added in proof: Further recent experiments have shown that the enzyme described above degrades polyuridylic acid if it is hybridized to polydeoxyadenylic acid but not if it is hybridized to polyadenylic acid. This further demonstrates the specificity of the enzyme.

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Salt Toleration by Plants: Enhancement with Calcium

Abstract. *Bean plants subjected to a sodium chloride concentration about one-tenth that of seawater for 1 week suffered no damage if the calcium concentration of the nutrient solution was 1 millimole per liter or higher, but at lower calcium concentrations damage was severe and apparently due to a massive breakthrough of sodium into the leaves.*

Of all the inhibitory substances that plants may encounter in their natural chemical environment, none impairs or prevents their growth on so large a scale as salt (*1*). About a quarter of the irrigated land in our own West is said to be affected by salinity (*2*). Saline conditions prevail in areas otherwise particularly well suited to crop production if water for irrigation is available; the unleached soils of these regions are inherently fertile, growing seasons are long, and temperatures and light intensities are high. Vast areas in developing countries belong in this category.

Calcium ions play a crucial role in the regulation of the salt economy of plants and specifically in the selective transport or exclusion of Na and other mineral ions by plant cell membranes (*3*, *4*). Despite this evidence, there seems to be little recognition of the role of Ca in affecting the responses of plants to saline conditions. The only three comprehensive compendia on the subject of salinity (*5*) make no mention of it. Hyder and Greenway (*6*) were concerned solely with the effect of NaCl at low levels of nutrients.

Brittle wax bush bean plants, *Phaseolus vulgaris* L., were first grown in a tenfold dilution of the nutrient solution described by Johnson *et al.* (*7*). After 14 days, they were transferred to fresh Johnson solution (1/10) lacking $Ca(NO_3)_2$ but containing NaCl at 50 m*M* and graded additions of $CaSO_4$ as indicated in Fig. 1. Control plants were grown in cultures otherwise identical with these but containing no NaCl.

The plants grew in these solutions for 7 days. The experiment was then discontinued, and fresh and dry weights of the roots, stems, and leaves were taken. Both Na and K were determined by flame spectrophotometry, and Ca and Mg were determined by atomic absorption spectrophotometry.

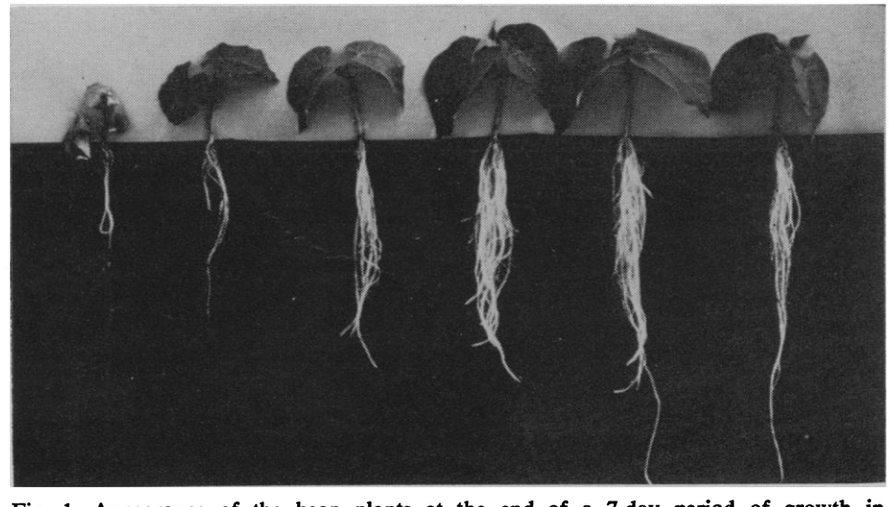

Fig. 1. Appearance of the bean plants at the end of a 7-day period of growth in aerated nutrient solutions in the greenhouse. The concentration of NaCl was 50 m*M*, and that of $CaSO_4$ was (left to right) zero, 0.1, 0.3, 1.0, 3.0, and 10 m*M*.