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DNA-dependent RNA polymerase from Agrobacterium tumefaciens and its involvement in crown gall disease.

Judith A. Cigna
University of Massachusetts Amherst

DNA-DEPENDENT RNA POLYMERASE FROM *AGROBACTERIUM TUMEFACIENS* AND ITS INVOLVEMENT IN CROWN GALL DISEASE

A Thesis Presented

by

Judith A. Cigna

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Approved as to style and content by:

Mark S. Mount, Chairman

Richard A. Rohde, Member

Peter L. Webster, Member

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ABSTRACT

DNA-dependent RNA polymerases of virulent and avirulent strains of Agrobacterium tumefaciens were purified by ammonium sulfate preciptiation. In these crude preparations, enzyme from the avirulent strain showed a single peak of activity at pH 8.5 while the same preparation from the virulent strain showed two distinct peaks of activity, at pH 8.5 and 9.5. When fractionated by DEAE- and DNA-cellulose, both strains exhibited dual enzyme activity peaks. These purified enzymes were Mn^{+2} dependent and inhibited by Mg^{+2} at pH 9.5. An apparent inhibitor of RNA polymerase activity at pH 9.5 was fractioned from avirulent preparations.

RNA polymerase was isolated from plant callus cultures of both crown gall and normal tissue, and tested for inhibition of enzyme activity with rifampicin, a specific inhibitor of bacterial RNA polymerase. Enzyme activity was inhibited in crude chromatin fractions from the gall tissue to a significantly greater degree than from the normal tissue, suggesting the possibility of the presence of the bacterial enzyme in gall tissue.

INTRODUCTION

Crown gall is a non-self-limiting tumorous disease of many dicotyledonous plants which is incited by virulent strains of the bacterium Agrobacterium tumefaciens (34). The plants must previously be conditioned by wounding, and transformation to the neoplastic state occurs within a few days after infection by the bacteria, after which time the bacteria are no longer required (3, 56). In fact, bacteria-free sections of the tumors can be isolated and cultured indefinitely on minimal media lacking the growth hormones which are required by normal plant cells in culture (6).

There is great interest in the study of this disease because of the parallels which have been drawn between these plant tumors and some cancerous diseases of animals (8). Crown gall thus provides a handy model for studying tumigenesis in general.

Though the search for a "tumor inducing principal" has been underway for more than 25 years (17), the actual mechanism of tumor induction has not yet been determined. Much evidence has accumulated suggesting that bacterial nucleic acids are involved. Some investigators have claimed induction of gall-like growths on plants using sterile bacterial DNA preparations (5,29,40), others with RNA (4,49). Yet none of these studies have shown unequivocal proof that what they have produced is in fact a crown gall (17). Others have shown evidence of a transfer of DNA from A. tumefaciens to plant cells (2), yet hybridization studies have consistently failed to detect the

presence of complete bacterial DNA in tumor tissue; they do not rule out the possibility of the presence of less than the entire bacterial genome (13,16,18,43). Strong evidence for some sort of genetic transfer is the finding that crown gall tumors contain the unusual amino acids, octopine, lysopine, and nopaline, which are not detectable in normal tissue (33), and that the presence or absence of these amino acids depends on the strain of bacteria used to induce the tumor. Often the bacteria used to induce the tumor can specifically synthesize the same amino acid derivative found in the tumor, and the ability to catabolize octopine or nopaline often parallels the ability of the bacterial strain to induce tumors (17). Also, crown gall tumors have been found to contain bacteria-specific antigens which the normal host tissue does not contain (11,14,15).

Currently, there is much research being conducted concerning the possibility that a DNA plasmid from the bacteria is a key factor in tumor induction. It has recently been shown that many strains of A. tumefaciens contain a DNA plasmid of relatively high molecular weight. So far, all virulent strains of the bacteria examined have been found to contain this plasmid, while most non-tumorogenic strains do not (57). Furthermore, strain C58 of A. tumefaciens is cured of its plasmid when grown at 37°C, and the strain loses its ability to cause tumors at the same rate that it loses its plasmid (53,54). When the plasmids from virulent strains C58 or K27 are transferred to the avirulent strain A136, virulence also is transferred (54). Also,

sensitivity to agrocin, a bacteriocin produced by A. radiobacter, has been shown to be directly correlated with both tumorogenic ability and the presence of the plasmid (30,42), and in strain C58 both sensitivity to the bacteriocin and utilization of nopaline have been found to be coded for on the plasmid (54).

Thus, much evidence points to the involvement of bacterial nucleic acids, particularly the DNA plasmids, in the induction of crown gall tumors. Certainly there is some sort of alteration in the genetic code itself, or the transcription of this code, and, in either case, this change in genetic expression would require transcription by DNA-dependent RNA polymerases. Indeed, there is evidence that DNA-dependent RNA polymerase of bacterial origin may be necessary for tumor induction. Rifamycin, a specific inhibitor of bacterial DNA-dependent RNA polymerase (50,55), has been shown to prevent the initiation of tumors by the bacteria, to a degree which cannot be accounted for by simple killing of the bacteria (1,46). Again, workers in general have been unable to unequivocally prove induction of tumors in plants using isolated bacterial DNA or RNA, despite the mounting evidence that bacterial nucleic acids are involved; Stroun (45) has suggested that this is because DNA dependent RNA polymerase of bacterial origin must accompany the DNA, and that it does accompany the DNA when the bacteria induce a tumor. This might be analogous to the situation found in viral infection. It is known that some viruses must carry their own RNA replicases in order to be infectious (24), and

that some viruses alter the specificity of DNA transcription in the host cell by coding for a virus-specific RNA polymerase σ-factor (44,48,51,52).

Based on this information, this study was undertaken; first, to compare DNA dependent RNA polymerases from virulent and avirulent strains of the bacteria, to see if there were differences in the enzyme which might shed light on the mechanism of tumor induction, and, second, to examine plant galls for the presence of the bacterial enzyme.

EXPERIMENTAL PROCEDURES

Materials

Acrylamide: Eastman Kodak Co., Rochester, New York

Actinomycin D: Sigma Chemical Co., Saint Louis, Missouri

Agar: Bacto-agar; Difco Laboratories, Detriot Michigan

Agarose: Bio Rad Laboratories, Richmond, California

Agrobacterium tumefaciens: strain 806 - Dr. R. Beardsley, Manhattan College, New York, New York; strain B6 - Dr. John Kemp, University of Wisconisn, Madison

∝ Amanitin: Sigma Chemical Co.

Ammonium persulfate: Eastman Chemical Co.

BA 85 nitrocellulose filters, 0.45μ: Schleicher & Schuell, Keene, New Hampshire

Bovine serum albumin: Sigma Chemical Co.

Bromphenol blue: Sigma Chemical Co.

Calf thymus deoxyribonucleic acid: Sigma Chemical Co.

Cellex 410 anion exchange cellulose: Bio Rad Laboratories

Coomassie brilliant blue R-250: Sigma Chemical Co.

Diethyl amino ethyl cellulose: Sigma Chemical Co.

DL-dithiothreitol: Sigma Chemical Co.

Ethylenediamine tetraacetic acid: Sigma Chemical Co.

Freund's Adjuvant, complete and incomplete: Difco Laboratories

2-Mercaptoethanol: Sigma Chemical Co.

N',N' - Methylenebisacrylamide: Eastman Kodak

Miracloth: Calbiochem, San Diego, California

Nucleotides: cytidine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate: Sigma Chemical Co.; adenosine-8-^{14}C 5'-triphosphate: New England Nuclear, Worcester, Mass.

Nutrient broth: Difco Laboratories

Phenylmethyl sulfonyl flouride: Sigma Chemical Co.

Rifampicin: Sigma Chemical Co.

RNAase A: Sigma Chemical Co.

Salmon testes deoxyribonucleic acid: Worthington Biochemical Corp, Freehold, New Jersey

Sodium Dodecl Sulfate: Bio Rad Laboratories

N,N,N',N'-Tetramethylethylenediamine: Eastman Kodak

Thiamine HCl: Sigma Chemical Co.

Tissue cultures, sunflower: Dr. John Kemp, University of Wisconsin, Madison

Trichloroacetic Acid: Fisher Scientific Co., Pittsburg, Pennsylvania

Trizma-HCl: Sigma

Whatman Filter Paper: W&R Balston, Ltd., England

Buffers*

Buffer A: 0.01M Tris-HCl (pH 7.9); 0.01M $MgCl_2$; 0.1mM EDTA, 0.1mM DTT, and 5% glycerol. Solid DTT was added to all buffers immediately before use.

Buffer G: 0.05M Tris-HCl (pH 7.5); 0.01M $MgCl_2$; 0.2M KCl; 0.1M DTT; 0.1mM EDTA (pH 7.0); and 5% glycerol.

Buffer C: 0.05M Tris-HCl (pH 7.9); 1mM DTT; 1.0mM $MgCl_2$; 0.25M sucrose; 0.1mM EDTA; 0.5mM phenylmethyl sulfonyl flouride; and 1% DMSO. Phenylmethyl sulfonyl flouride was dissolved in 100% DMSO and added just prior to use.

Buffer D: 0.05M Tris-HCl (pH 7.9); 1mM DTT; 5mM $MgCl_2$; 25% glycerol; 0.1mM EDTA; 0.5mM phenylmethyl sulfonyl flouride; and 1% DMSO.

Polymin P: 10% polymin P, adjusted to pH 7.9 with concentrated HCl.

Electrophoresis Buffer: 0.04M Tris-Base; 0.02M sodium acetate; and 1.0mM EDTA; adjusted to pH 8.6 with glacial acetic acid.

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*Abbreviations: Tris-HCl [Tris(hydroxymethyl)amino methane, the HCl salt]; EDTA (ethylenediamine tetraacetic acid); DTT(dithiothreitol); DMSO(dimethyl sulfoxide); TCA(trichoroacetic acid); SDS(sodium dodecl sulfate); ATP(adenosine 5'-triphosphate); CTP(cytosine 5'-triphosphate); GTP(guanosine 5'-triphosphate); UTP(uridine 5'-triphosphate)

Growth of Bacteria

A. tumefaciens cultures were maintained on slants containing Nutrient Broth in 1% agar. For polymerase isolations, inoculations were made with a loop from these slants into 200 ml of liquid Nutrient Broth plus 0.5% glucose. Flasks were maintained on a shaker for 3 days at 25°, after which the bacteria were harvested by centrifugation at 10,000xG for 20 minutes.

Growth of Tissue Cultures

Plant tissue cultures are sunflower tissue (Helianthus annuus, cv. Mammoth); they were maintained at 28°C on Linsmaier and Scoog medium (32) from which the plant growth regulators, auxin and cytokinin, were omitted. "Normal" tissue is habituated sunflower - i.e. normal tissue which after a period of time in culture has spontaneously developed the ability to grow without exogenously applied growth hormones. Tumor tissue is sunflower crown gall incited by A. tumefaciens, strain B6. Tissue cultures were used for two reasons: 1.) they are aseptic, and thus any bacterial enzyme found would be from the plant tissue rather than from contaminating bacteria; 2.) RNA polymerase in fully grown, differentiated tissue is present in very small quantities and is extremely difficult to isolate. We were able to isolate more polymerase from tissue culture, which contains more dividing cells.

Purification of A. tumefaciens RNA Polymerase

Bacterial DNA dependent RNA polymerase (ribonucleoside triphosphate: RNA nucleotidyl transferase, EC2.7.7.6) was isolated according

to a modified method of Burgess (9). Approximately 20 grams of cells of A. tumefaciens, strain 806, were suspended in 80 ml of Buffer G and the suspensions were sonicated for 10 minutes with a sonicator cell disruptor (Heat Systems - Ultrasonics, Inc.) at a voltage output of 4 for virulent bacteria, 6 for avirulent; the solution was kept cool in an ethanol-ice water bath. The solution was centrifuged at 68,000xG in a Type 65 Spinco rotor for 2 hours; then the supernatant was collected and brought to 33% ammonium sulfate saturation with solid ammonium sulfate. To prevent the pH from dropping below 7.0, 0.05 ml of 1M NaOH/10g $(NH_4)_2SO_4$ was added. The solution was then stirred for 30 minutes and the precipitate removed by centrifugation at 13,000xG for 30 minutes. The supernatant was brought to 50% $(NH_4)_2SO_4$ saturation, stirred for 30 minutes, centrifuged as before, and the protein pellet was resuspended in 5 ml Buffer A.

A DEAE-cellulose column (1.5x16 cm) was prepared according to Burgess (9) and equilibrated with Buffer A. Two milliliters of the ammonium sulfate purified enzyme solution were applied to the column and eluted with a linear gradient of from 0-0.5M ammonium sulfate. Five milliliter fractions were collected.

DNA cellulose columns were run according to the method of Litman (35). The DNA cellulose was prepared using Cellex 410 cellulose and salmon testes DNA, and packed into columns 0.9 cm x 15 cm. One milliliter of the ammonium-sulfate-purified RNA polymerase preparation was applied, and eluted by a stepwise gradient of 0, 0.3 and

0.8M NaCl: 0.5 ml fractions were collected. Purity of the enzymes was demonstrated by SDS disc gel electrophoresis (Fig. 1).

Purification of Plant RNA Polymerase

Chromatin-bound RNA polymerase was isolated from the tissue cultures according to the method of Guilfoyle et al (23), or Hardin and Cherry (25), and soluble polymerase was extracted according to Jendrisak and Burgess (28), or Guilfoyle et al (23). All procedures were carried out at 4°C.

Tissue (100 gm) was homogenized with an equal amount (weight/volume) of Buffer C in a Virtis Homogenizer; glass beads were added to insure cell disruption. Extracts were filtered through six layers of cheesecloth, then one layer of miracloth. The filtrate was centrifuged for 30 minutes at 13,000xG and the resulting pellet constituted the "chromatin", and the supernatant the "soluble" fraction.

The chromatin was further treated as follows: using a glass homogenizer, the pellet was resuspended in 10 ml Buffer C + 1% Triton X-100. The suspension was again centrifuged at 13,000xG for 30 minutes, the supernatant was discarded or assayed for activity, and the pellet was resuspended in 10 ml Buffer C and centrifuged as before. The pellet was washed an additional time, and the "chromatin-bound" polymerase solubilized as follows (chromatin pellets without the Triton X-100 wash treatment were also used): 1.) Pellets were suspended in Buffer D containing 0.2-0.8M ammonium sulfate and stirred for 5 hr. at 4° C. 2.) Pellets were suspended in Buffer D and sonicated at

Fig. 1. Polyacrylamide, SDS disc gel electrophoresis of DNA-cellulose purified RNA polymerase from A. tumefaciens. Bands represent the β, β', α and σ subunits of the enzyme.

voltage 3 for 4 cycles of 30 seconds on/30 seconds off (in an ice bath), then stirred for one hour in Buffer D + 0.5M ammonium sulfate. 3.) Pellets were resuspended in Buffer D + 0.5M ammonium sulfate and incubated for 30 minutes at 37°C.

Following any of these treatments, the chromatin suspension was centrifuged for 30 minutes at 13,000xG, and the pellet was saved for assay of polymerase activity, or discarded. To precipitate the solubilized enzyme from the supernatant, $(NH_4)_2SO_4$ was added to bring the concentration to 67% $(NH_4)_2SO_4$ saturation (including any ammonium sulfate used for solubilization); the solution was then centrifuged at 13,000xG for 30 minutes. It was found that there was too little protein in these solutions to form a pellet on centrifugation, so bovine serum albumin was added to facilitate pelleting of the enzyme. The supernatant from this centrifugation was saved for enzyme assay, or discarded. The protein pellet was resuspended in 1 ml of Buffer D.

The supernatant fraction was treated as follows: Polymin P (10%, pH 7.9) was added, dropwise with stirring, to the supernatant from the first centrifugation. Various amounts of polymin P were used from 20 µl polymin P/ml supernatant to 200 µl/ml and the resulting solutions were centrifuged at 13,000xG for 15 minutes. To solubilize the enzyme from the resulting polymin P pellet, the pellet was resuspended in Buffer D + 0-0.8M $(NH_4)_2SO_4$. Solutions were stirred at 4°C for 30 minutes, then centrifuged at 13,000xG for 15 minutes. It was found

that the polymerase activity of the pellet could not be measured because the polymin P contained in the pellet precipitated the available [^{14}C]-ATP, added in the reaction mixture, with or without polymerase activity. To precipitate the polymerase from the supernatant, ammonium sulfate was added to bring the total concentration of ammonium sulfate to 67% saturation, and the solution was stirred for 30 minutes in the cold, then centrifuged at 13,000xG for 30 minutes. It was found that, again, there was not enough protein in the supernatant to form a pellet, so BSA was added to facilitate pelleting of the enzyme. The precipitated protein was resuspended in 1 ml of Buffer D.

Alternately, the supernatant from the first centrifugation was stirred with 0.5M ammonium sulfate for 2 hours, followed by precipitation of the protein by the addition of 0.38 g/ml solid ammonium sulfate. The solution was stirred for 30 minutes, then the protein pellet was recovered by centrifugation at 13,000xG for 30 minutes, and resuspended in Buffer D.

RNA Polymerase Assay

Bacterial RNA polymerase activity was measured by adding 20 µl of the enzyme preparation to a reaction mixture with a total volume of 250 µl and containing as a final concentration: 28mM Tris-HCl (pH 7.0-9.0; for normal activity tests a pH of 8.5 was used) or 28mM NaOH-glycine (pH 9.5-10); 10mM $MgCl_2$; 10mM $MnCl_2$; 0.1M KCl; 4mM DTT; 0.15mM CTP and GTP, 0.3mM UTP, 0.016mM [^{14}C]-ATP (50mC/ml); 37.5µg calf thymus DNA; and 2.8mM EDTA. Reactions

were carried out at 37°C for 20 minutes and terminated by the addition of 3 ml of cold 10% trichloroacetic acid with 0.1M sodium pyrophosphate. The reaction mixture plus TCA was kept at 4°C for 1 to 4 hours, then precipitates were collected on BA 85 nitrocellulose filters, dried and counted in a liquid scintillation counter (Nuclear Chicago - Unilux).

To confirm that the product of the reaction mixtures was, in fact, RNA, 150µg/ml rifampicin (a rifamycin derivative), 200µg/ml Actinomycin D, or 200µg/ml of RNAase A were added to the reaction mixtures. To detect possible polynucleotide phosphorylase activity, enzyme assays were run with RNA in place of DNA as the primer, or all the nucleotides except [^{14}C] ATP were deleted, or K_2HPO_4 was added to the reaction mixtures (to a final concentration of 0.4mM) (9).

Plant RNA polymerase activity was measured by adding 50µl of the enzyme preparation to a reaction mixture with a final volume of 250µl and containing as a final concentration: 50mM Tris-HCl (pH 8.0): 10mM DTT; 5mM $MgCl_2$; 1mM $MnCl_2$; 50mM NH_4Cl; 37.5µg calf thymus DNA; 0.15mM CTP and GTP; 0.3mM UTP; and 0.016mM [^{14}C]-ATP (50mC/ml). Assays were carried out at 28°C for 20 minutes and stopped with 3ml of cold 10% TCA plus 0.1M sodium pyrophosphate. Mixtures were filtered, dried, and counted as in the bacterial assay.

Assays with Inhibitors

In tests of rifampicin inhibition the bacterial polymerase assay mixture was used (at 28°C for crude enzyme preparations) and the enzyme preparations were incubated with 30µg/ml (final concentration)

rifampicin at 4°C for 30 minutes before addition of the remainder of the reaction mixture.

In tests of ∝amanitin inhibition, the ∝amanitin was added along with the rest of the reaction mixture ingredients at a concentration of 0.4μg/ml.

Gel Electrophoresis

SDS polyacrylamide gels were run as described by Laemmli (31), with modifications as described by Jendrisak and Burgess (28). Acrylamide was used at a concentration of 7.5% and gels were stained for 12 hours with 0.05% Coomassie Brilliant Blue R-250 in 25% isopropyl alcohol plus 10% acetic acid.

Antibodies*

DNA-cellulose purified RNA polymerase from A. tumefaciens strain B6 was used for the production of antibodies. The animals used were 3-4 lb male Dutch Belted rabbits which had previously been tested for reactivity to the antigen - only rabbits which tested negative were used. Enzyme preparations containing .3-.4 mg of protein in 1 ml of solution were mixed with an equal volume of either Freund's complete or incomplete adjuvant and injected either intra-muscularly or subcutaneously into the rabbits. A booster injection containing the RNA polymerase, without the adjuvant, was given 30 days later. After an additional 10 days, rabbits were bled by cardiac puncture. Serum

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*Antibodies were prepared by Joseph Barbieri, research assistant in the Department of Microbiology at the University of Massachusetts.

was collected by allowing the whole blood to sit for 1 hr. at room temperature, after which it was centrifuged at 30,000xG for 30 minutes, to remove the clotted cells, leaving the antiserum.

Electroimmuno Precipitation Assay

Electroimmuno precipitation assays were performed according to the method of Dr. Joseph Kunkel of the University of Massachusetts (personal communication).

Glass slides were precoated with 0.2% agarose and thoroughly dried. Agarose was dissolved in electrophoresis buffer, and when the agarose had cooled to 55°C, antiserum to the A. tumefaciens RNA polymerase was added. The final concentration of agarose was 0.8%, and in this work it was necessary to use the antiserum at a concentration of 10% of the buffer. The agarose-antiserum solution was carefully layered on warm, levelled slides (2ml/1x3" slide), and after gelling, wells were cut 1 cm from one end with a 16 gauge needle. The agarose plugs were removed from the wells with a pasteur pipette connected to an aspirator, and a capillary tube was used to fill the wells (3.2 µl) with the solution to be tested for similarity to the bacterial RNA polymerase. The slides were then placed in a Helena Titan Junior Electrophoresis apparatus, with the filled wells towards the cathode; electrophoresis buffer was used as the running buffer and Whatman #4 filter paper was used as wick. The slides were run at 2mA/1x3" slide for 4 hours at room temperature, allowed to sit overnight, and then washed for 24 hours in 0.15N NaCl.

The agarose was dried by covering the slides with wet filter paper followed by several layers of paper toweling. Weights were placed on the slide for ten minutes, after which the weight and paper toweling were removed and the slides were left in the air until the filter paper had dried. Slides were stained in 0.05% Coomassie Blue in 7% acetic acid overnight and destained with 7% acetic acid.

RESULTS AND DISCUSSION

A. tumefaciens RNA Polymerase

It was found that the optimal activity for the crude $(NH_4)_2SO_4$ precipitated RNA polymerase of the avirulent bacteria occurred as a single peak at pH 8.5, whereas the same preparations from the virulent bacteria showed two pH optima, at pH 8.5 and 9.5, with a significant drop at pH 9.0 (Fig. 2). DEAE- and DNA-cellulose purified enzymes from both virulent and avirulent bacteria showed the same property of two pH optima. This was true when reaction mixtures were run in the presence of Mn^{+2} or both Mn^{+2} and Mg^{+2}. When the crude enzymes from the virulent strain, and further purified enzymes from either strain were run in the presence of Mg^{+2} alone, a broad activity peak from pH 8.5-9.0 was observed (Fig. 3). In ion requirement studies, 10mM Mg^{+2} or Mn^{+2} gave optimal enzyme activity. At pH 8.5 either ion is utilized, while at pH 9.5 the enzyme activity is definitely Mn^{+2} dependent.

Enzyme fractions from the virulent bacteria consistently eluted from DEAE-cellulose at a salt concentration of 0.32-0.36M, whereas

Fig. 2. RNA polymerase activity at various pH values of crude ammonium sulfate precipitated enzymes from virulent and avirulent A. tumefaciens. (△) represents activity of the enzyme from the virulent bacteria, and (o), from the avirulent.

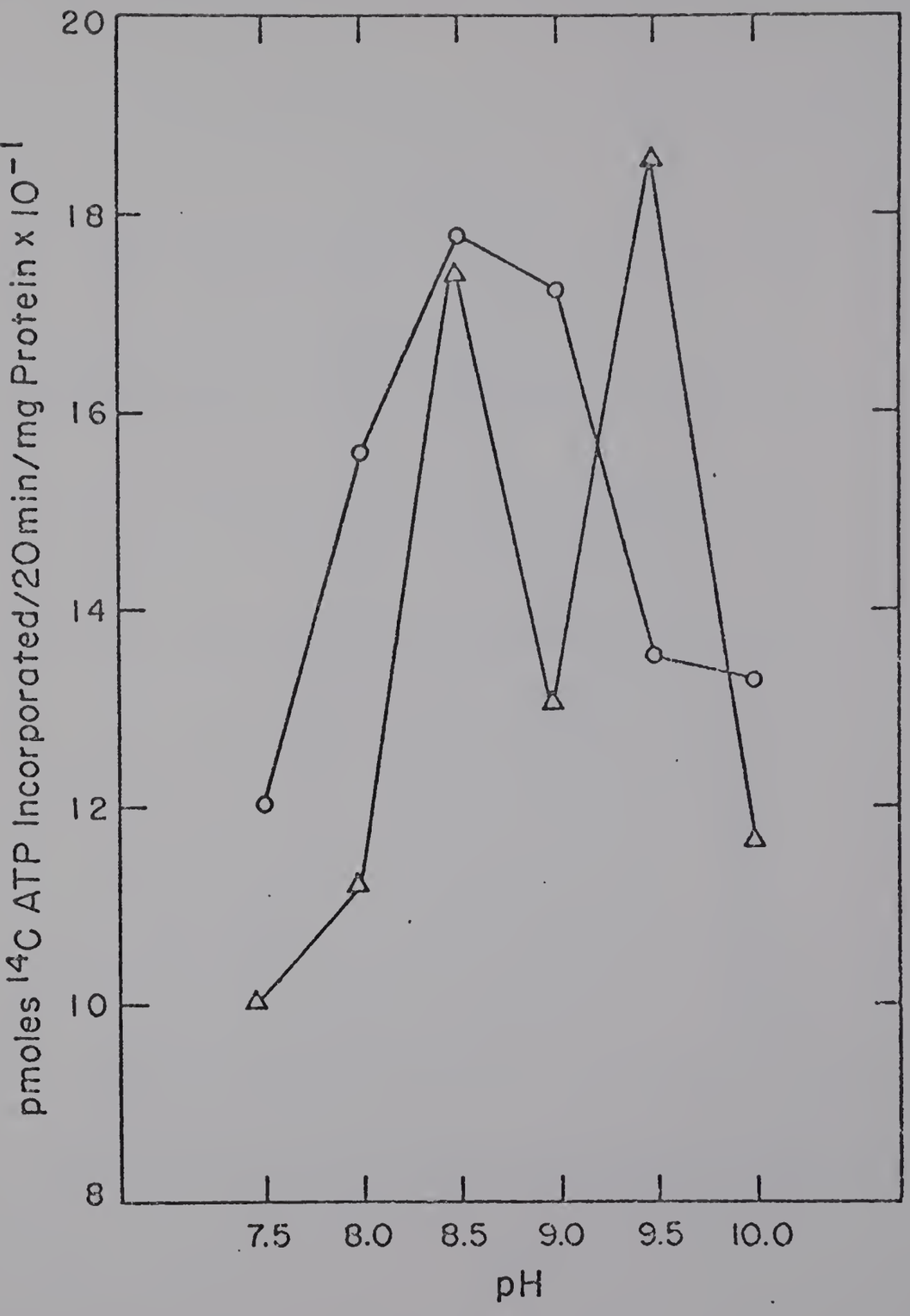
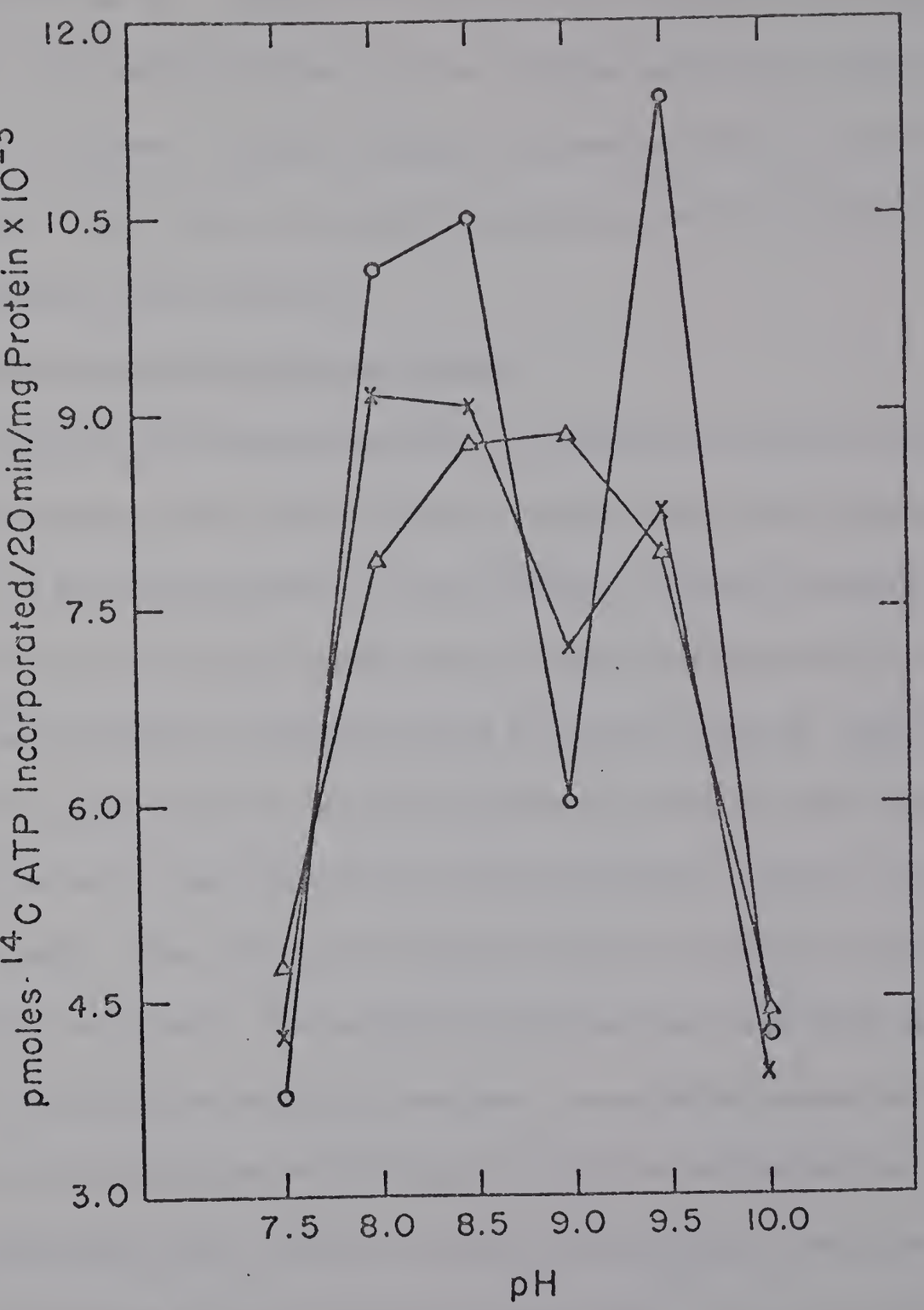

20
18
16
14
12
10
8
pmoles ^{14}C ATP Incorporated/20min/mg Protein x 10^{-1}
7.5
8.0
8.5
9.0
9.5
10.0
pH

Fig. 3. RNA polymerase activity, at various pH values, of DEAE-cellulose purified enzyme from avirulent A. tumefaciens. Reactions were run in the presence of 10mM Mg^{+2} only (△), or 10mM Mn^{+2} only (o), or the two combined (x). Similar results were obtained using enzyme preparations from virulent bacteria under the same conditions.

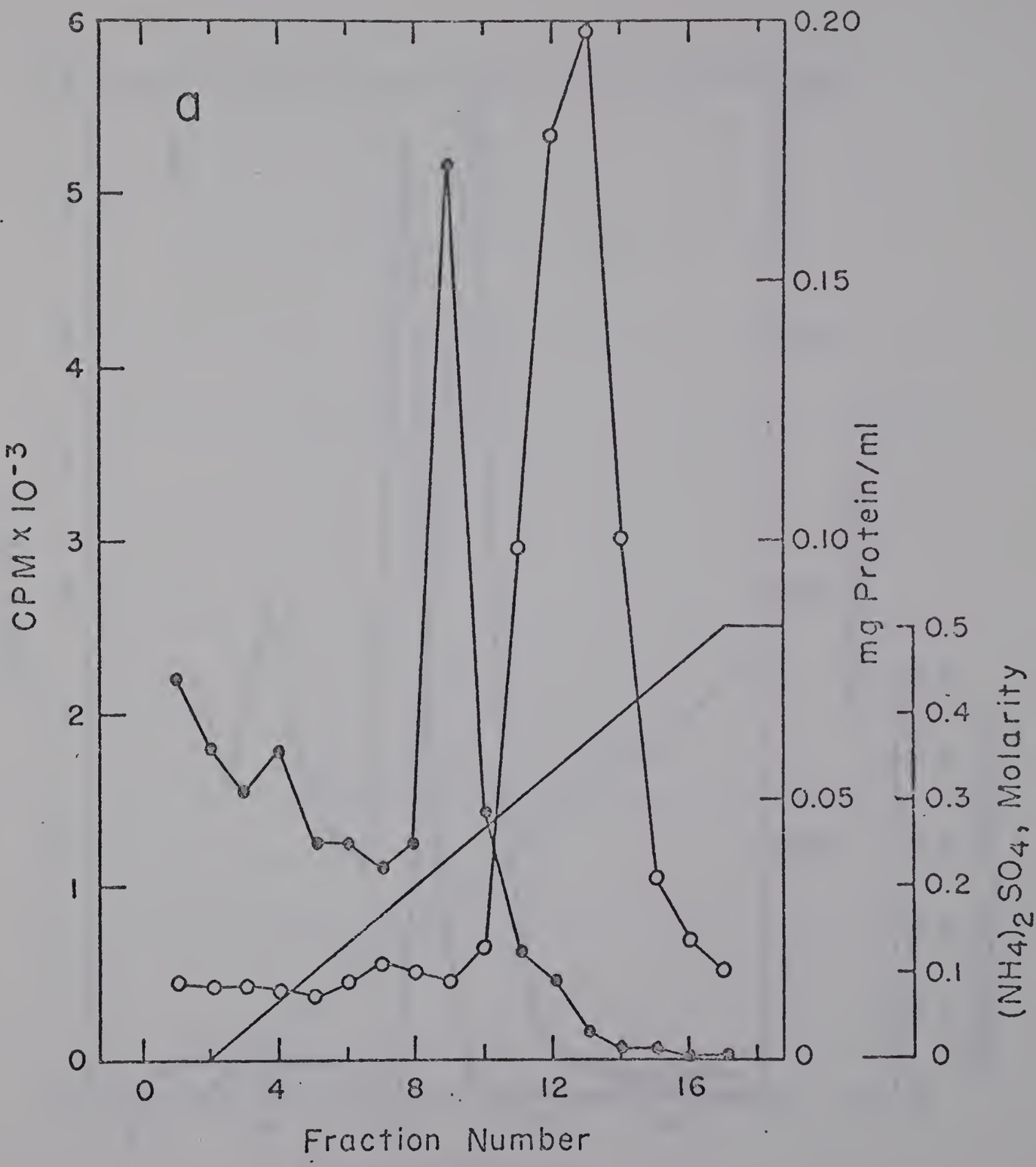

12.0
10.5
9.0
7.5
6.0
4.5
3.0
pmoles ^{14}C ATP Incorporated/20 min/mg Protein x 10^{-3}
7.5
8.0
8.5
9.0
9.5
10.0
pH

the enzyme from the avirulent strain eluted at 0.25-0.29M. In both cases, the majority of the protein eluted just prior to the enzyme fractions (Fig. 4). Inhibition of activity was observed when 20 µl of this peak protein fraction from the avirulent column was added to reaction mixtures. Optimal inhibition occurred at pH 9.5, and this "inhibitor" was much more active or present in greater amounts in the avirulent strain (Table 1).

Polymerases from Plant Tissue Cultures

Using all the methods described for isolation of the DNA dependent RNA polymerases from callus tissue cultures, it was found that isolation of the RNA polymerase is very difficult. Original attempts toward this end had been made with sterilized sunflower stems and with galls incited on sunflower stems by injection with A. tumefaciens. In these experiments the only RNA polymerase activity which could be measured was a small amount of "chromatin-bound" activity in the crown galls. After changing to callus cultures, somewhat the same situation was found. No "soluble" polymerase was recovered using any of the isolation methods; there was a measurable amount of "chromatin-bound" polymerase activity found in both the habituated tissue and the crown gall tissue, but this activity was unstable to isolation. This may be because of the nature of the tissue used. In all the studies encountered by this investigator, tissues used for isolation of DNA dependent RNA polymerases were very young, small, embryonic, meristematic, or rapidly enlarging cells; e.g., cauliflower inflorescences

Fig. 4. Elution profile from DEAE-cellulose chromatography of RNA polymerase isolated from virulent (a) and avirulent (b) A. tumefaciens. Five ml fractions were collected and all activity assays were performed at pH 9.5. (o) represents enzyme activity and (x) represents mg of protein/ml of the fractions.


a
CPM x 10⁻³
mg Protein/ml
(NH4)2 SO4, Molarity
Fraction Number
0
1
2
3
4
5
6
0.05
0.10
0.15
0.20
0.1
0.2
0.3
0.4
0.5
4
8
12
16

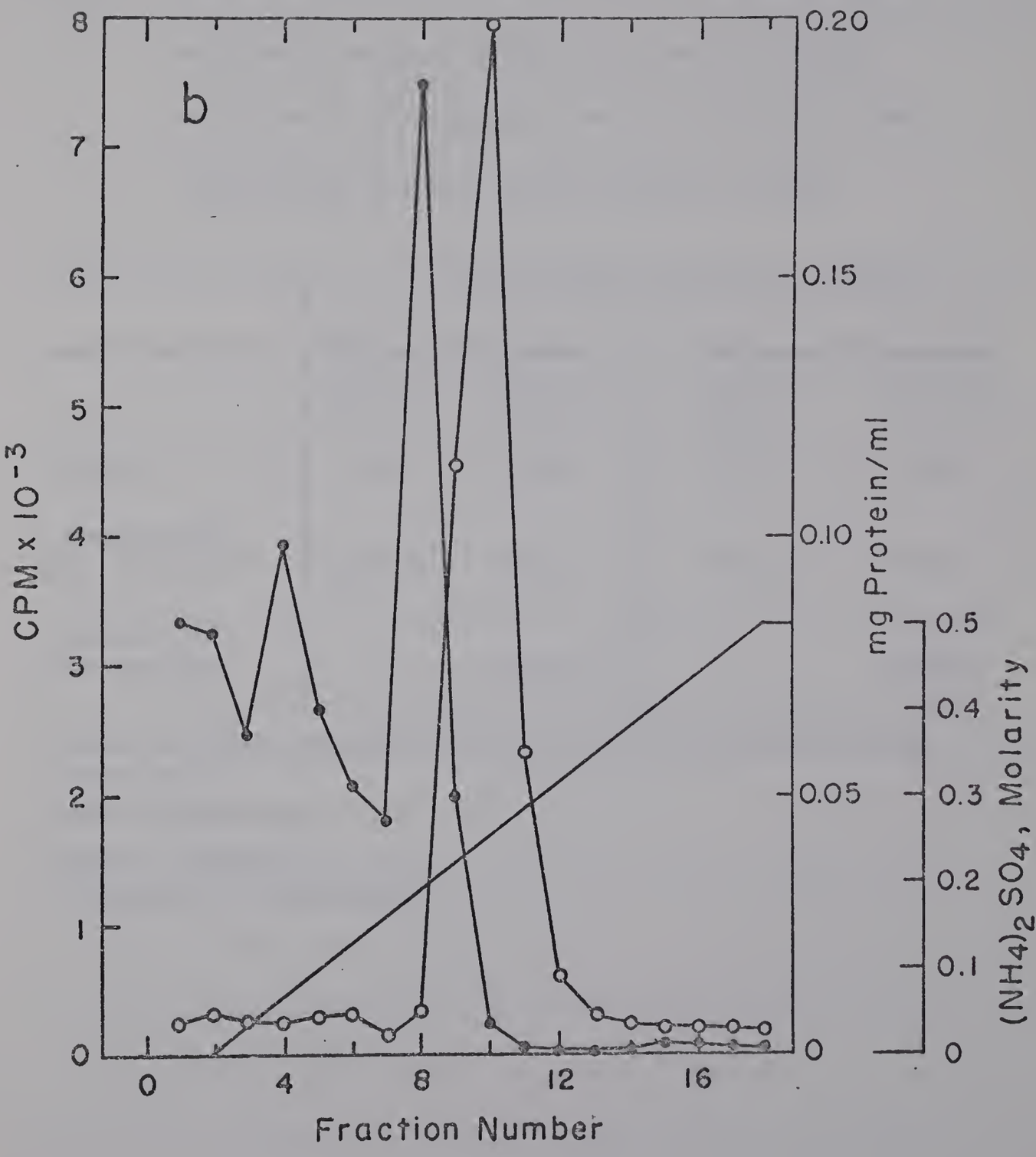

b
CPM x 10⁻³
mg Protein/ml
(NH4)2SO4, Molarity
Fraction Number
0
1
2
3
4
5
6
7
8
0.05
0.10
0.15
0.20
0.1
0.2
0.3
0.4
0.5
0
4
8
12
16

Table 1. Decrease of RNA polymerase activity caused by the addition of maximum protein fractions from DEAE-cellulose chromatography to reaction mixtures. The RNA polymerases were DEAE-cellulose purified.

Assay Conditions	Percent RNA Polymerase Activity[a]			
	Virulent Polymerase		Avirulent Polymerase	
	pH 8.5	pH 9.5	pH 8.5	pH 9.5
Control	100	100	100	100
+ fraction #8 from avirulent[b]	86-100	25-60	70-100	9-44
+ fraction #9 from virulent[c]	--	75-100	--	76-100

[a]Range of RNA polymerase activity from 3-5 replicates for each experiment.

[b]Refer to Figure 4b

[c]Refer to Figure 4a

--Experiment not performed

(19,21), pea embryos at the beginning of germination (20), germinating soybean hypocotyls (23,25,39) or wheat germ (27,28). Unfortunately, one cannot grow a gall on embryonic tissue, since it takes a few to several weeks to develop the gall. And, unlike these small cells with very little cytoplasm and the tendency and potential to grow and differentiate, cells in tissue culture are generally quiescent and very large and highly vacuolated, with very little cytoplasm and only a faintly visible nucleus, interspersed with unorganized vascular elements and centers of smaller dividing cells (10,36). It would seem that these large, highly vacuolated cells may contain too little RNA polymerase in proportion to their volume to measure polymerase activity; this may be because the levels of polymerase acitivity change with the stage of growth and hormone levels (22,23,26,47). Possibly the large cells have reached a certain degree of "differentiation" as indicated by their size, degree of vacuolation, and the presence of vessel elements, and as a result the amount of RNA polymerase in these cells has dropped down to some "subsistence" level. Alternately, or in addition, the large amount of large sized vacuoles might prevent isolation of active polymerase by releasing hydrolytic enzymes or by causing drastic internal pH changes in the cells upon disruption.

As previously stated, there was measurable "chromatin-bound" polymerase activity in both the habituated and crown gall callus cultures. It is not unexpected that this activity would be high in callus cultures, as investigators (22,23,26,47) have found that auxin treatment

of plant cells, which causes the cells to become "swollen and proliferate abnormally" (23) (i.e, form callus tissue) occurs with a concomittent increase of activity of RNA polymerase I, nucleolar or "chromatin-bound" polymerase. This may be what happens in crown gall tumors on normally grown plants, since the tumors really do resemble callus cultures and contain high levels of auxin (7). In our work it was found possible to isolate chromatin-bound polymerase activity from stem grown gall tissue but not from normal stem tissue. Unfortunately, this chromatin-bound polymerase activity did not appear to be stable to solubilization. With the milder treatments the activity remained with the chromatin pellet, and on harsher treatment the activity disappeared from the pellet but could not be recovered in the treatment supernatant. This may not be too atypical of plant tissues, for as recently as 1974 (12) it was stated that "Because of its extreme instability, plant nuclear enzyme I has not yet been purified." It appears that in some embryonic or germinating tissue it is possible to solubilize the enzyme, but it may not be possible from sunflower (or tobacco) tissue cultures, using the methods mentioned.

So, for the inhibitor studies, the crude chromatin pellet was used. It was found that the RNA polymerase activity was ∝amanitin insensitive, indicating that the plant polymerase present was polymerase I, the nucleolar polymerase believed to be involved in transcribing ribosomal RNA (38).

Interestingly, though the chromatin polymerase activity was sensitive to rifampicin inhibition in both types of tissue culture to a certain degree, the gall tissue was inhibited to a greater degree than the habituated tissue. In the habituated tissue the rifampicin-treated chromatin showed an average of 91.5% of the RNA polymerase activity of the control, whereas the gall chromatin treated with rifampicin showed 78.74% of the activity of the control (Fig. 5).

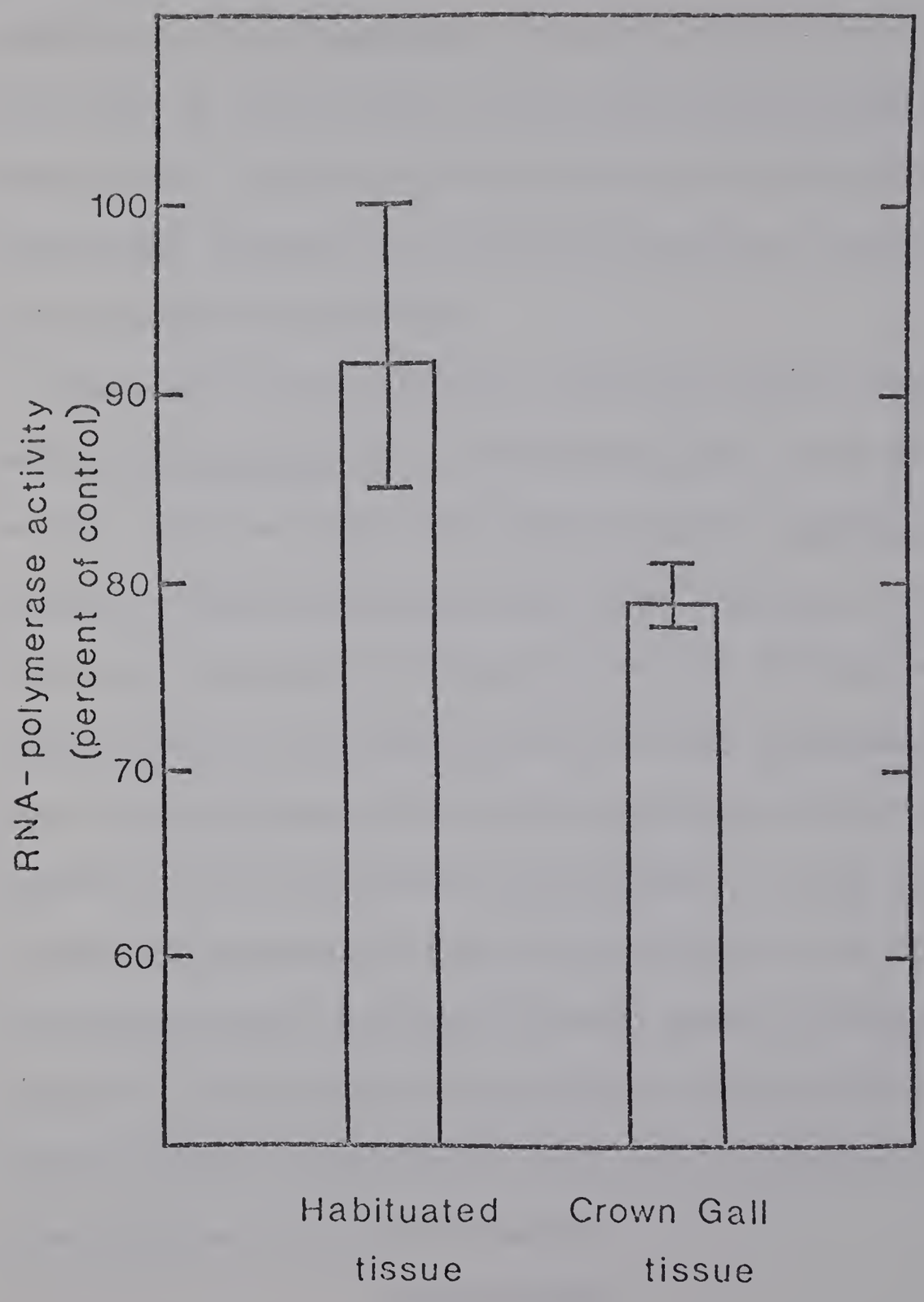
This greater degree of inhibition of RNA polymerase activity in the gall than in the habituated tissue with the inhibitor rifampicin, which is specific for bacterial RNA polymerase, would seem to indicate that there is indeed bacterial RNA polymerase in the gall tissue which is not present in the normal tissue.

Electroimmuno Assay

As stated before, it was not possible to recover RNA polymerase activity from the soluble fraction of the cell and not possible to solubilize the RNA polymerase from the chromatin pellet in active form. However, it was felt that the activity assay might be too insensitive to detect the bacterial polymerase if it were present in small amounts in the soluble fraction. Also, although no RNA polymerase activity could be recovered after attempts to "solubilize" it from the chromatin, there also was no activity retained on the chromatin pellet. Possibly the enzyme was being removed from the pellet, but was inactivated in the process.

If this were the case, then this "solubilized" enzyme might be

Fig. 5. RNA polymerase activity of rifampicin treated chromatin. Enzyme from habituated tissue showed an average of 91.50% of the activity of untreated controls, with a range of 85.02% to 100%. Enzyme from crown gall tissue showed an average of 78.74% of the activity of the control, with a range of 77.51% to 80.34%.


RNA-polymerase activity
(percent of control)
100
90
80
70
60
Habituated tissue
Crown Gall tissue

immunologically similar to the active enzyme, and if A. tumefaciens RNA polymerase were present it might react positively in a precipitin test with antibodies to the bacterial polymerase. (Interestingly, the antibodies to the A. tumefaciens polymerase, although immunologically active (Fig. 6), did not inhibit activity of the bacterial enzyme in polymerase assays, and thus could not be used, as the rifampicin was, to show specific inhibition of the bacterial polymerase in assays of chroatin-bound polymerase activity).

Electroimmuno assays were run, using the antibodies made to purified A. tumefaciens (strain 806) RNA polymerase incorporated in the agar. Wells were filled with either purified A. tumefaciens RNA polymerase or protein preparations from either crown gall or habituated tissue, both the soluble and chromatin fractions. Although the expected "rockets" were formed with the purified A. tumefaciens enzyme, (Fig. 6) no immunoprecipitates were formed with any of the plant preparations. This could mean one of three things: 1.) there was no bacterial RNA polymerase in the plant preparations; 2.) the test was not sensitive enough to measure very small amounts of the bacterial enzyme; 3.) chromatin-bound enzyme was not solubilized from the chromatin pellet, or was altered in such a way on solubilization that it was no longer immunologically active.

CONCLUSIONS

The finding that there are differences in the RNA polymerases from virulent and avirulent isolates of A. tumefaciens strain 806 would seem

Fig. 6. Electroimmuno assay of A. tumefaciens RNA polymerase. The assay was run in two dimensions; the first was through plain agarose, while the second was through agarose containing antibodies to the polymerase.

to indicate that the polymerase might be significant in tumor induction - indeed, the "inhibitor" of the bacterial RNA polymerase might be the inhibitor of tumor induction in the avirulent strain. However, different strains of the bacteria were studied and comparisons made of the RNA polymerases from virulent and avirulent isolates, and the patterns and differences found in the 806 strain did not seem to occur in the other strains. Therefore our current hypothesis is that there are different mechanisms by which the different strains are rendered avirulent. This stands to reason, as anything which could interfere anywhere in the process of infection and tumor induction could render a strain avirulent. However, since in strain 806 a difference between virulent and avirulent isolates involves a difference in the DNA-dependent RNA polymerase, this could indicate that the DNA-dependent RNA polymerases are important in the process of tumor induction. Based on the information on the RNA polymerase of strain 806, and the information summarized in the introduction on the indications that an RNA polymerase was involved and that it might be necessary that it be carried with the genetic information (inability to induce tumors with isolated DNA, inhibition of tumor formation by addition of rifampicin, bacterial specific antigens in galls), a study of the actual plant tumor tissue was undertaken, to see if there was evidence of the presence of the bacterial RNA polymerase.

The observation that rifampicin, a specific inhibitor of bacterial DNA-dependent RNA polymerase, inhibits RNA polymerase activity in sterile plant tumor tissue, to a significantly greater degree than in uninfected

plant tissue, would seem to be strong evidence that there is bacterial RNA polymerase in the gall tissue, and that it does not occur in normal plants. The fact that this inhibition was found in the "chromatin-bound" fraction of the plant polymerase activity (where plant polymerase I, or nucleolar, polymerase is located) is interesting in light of the finding that when auxin is applied to plants to induce the abnormal proliferation of callus tissue, which is histologically and cytologically similar to tumor tissue (7), there is also a change in the plant polymerase I. This difference involves a change in the enzyme, rather than the template, and has been shown to be a change in the activity of the molecules themselves (22). If bacterial RNA polymerase were important in induction of abnormal proliferation of plant cells in the development of crown gall tumors, then it would seem logical that it might be involved in a similar sort of change of plant polymerase activity. And indeed, we have found that we can measure a "bacteria-like" response in polymerase I activity in sterile crown gall tissue.

However, our further identification and description of the "bacteria-like" polymerase activity has been hindered by the inability to solubilize the polymerase from the chromatin pellet. If and when this problem is solved it may be possible to determine more specifically whether or not the A. tumefaciens is present and actually transcribing genetic information.

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