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Isolation & Characterization of DNA Polymerase Alpha and Gamma from Turnips (Brassica rapa) and Etiolated Soybeans (Glycine max)

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Isolation and Characterization of DNA Polymerase Alpha and Gamma from Turnips (*Brassica rapa*) and Etiolated Soybeans (*Glycine max*)

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Master of Science

by

Donna Rene' Hill

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Isolation and Characterization of DNA Polymerase Alpha and Gamma from Turnip (Brassica rapa) and Etiolated Soybean (Glycine max)

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I would also like to thank Dr. Martin Houston and Dr. Frank Toman for reviewing this thesis and serving on my graduate committee.

I dedicate this thesis to Erin and Amanda, who, I hope, will someday understand why I wasn't always there.

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Isolation and Characterization of DNA Polymerase Alpha and Gamma from Turnips (Brassica rapa) and Etiolated Soybeans (Glycine max)

Donna Rene' Hill December, 1988. 66 pages

Directed by: Valgene L. Dunham, Martin R. Houston and Frank R. Toman

Department of Biology Western Kentucky University

DNA polymerase alpha, the enzyme involved in nuclear DNA replication, and DNA polymerase gamma, the enzyme involved in organellular DNA replication, were isolated and purified from soybean and turnip. The enzymes were characterized following ammonium sulfate precipitation, DEAE-cellulose, phosphocellulose and hydroxylapatite chromatography, and by non-denaturing polyacrylamide gel electrophoresis. Protein bands were electro-eluted and the enzymes characterized using kinetic studies and sensitivity to divalent cations and inhibitors. Molecular weight and subunit composition studies indicated a molecular weight for the catalytic subunit of DNA polymerase alpha in soybean and turnip to be 46kDa. DNA polymerase gamma was composed of a catalytic subunit with a molecular weight of 66kDa. Although the two enzymes appear to share common subunits, characterization of their genetic origin remains to be determined before alpha and gamma can be classified as isoenzymes.

INTRODUCTION

In recent years it has become acceptable to refer to multiple forms of the same enzymatic activity as isoenzymes. Isoenzymes are proteins that are the product of distinct structural genes. Therefore, in addition to the differences in amino acid sequence, differences in the secondary, tertiary and quaternary structure of the proteins also exist (Moss, 1982).

Major problems confront enzymologists in the determination of whether proteins are isoenzymes. These include verification that (1) the proteins have identical mechanisms in vitro and in vivo, (2) the proteins have similar but not identical amino acid sequences, and (3) the proteins are coded for by different genes. Therefore, in addition to a study of the characteristics of the punitive isoenzymes, the genetic origin of the proteins must be determined. The structure of an isoenzyme may be determined by several mechanisms: the existence of multiple gene loci, multiple alleles or post-translational modification of gene expression.

A substantial proportion of enzymes are coded by several structurally different gene loci, and therefore exist in isoenzymatic forms. A survey of evidence relating to 66 human enzymes showed no fewer than 24 to be the products of more than one gene locus, with three loci being involved in determining the structures of nine enzymes (Moss, 1982). Among the numerous examples of isoenzymes determined by multiple gene loci are those forms of enzymes which are characterized by their specific intracellular locations, such as the cytoplasmic and mitochondrial forms of aspartate

aminotransferase or NAD-dependent malate dehydrogenase, as well as isoenzyme systems with similar intracellular localizations but with tissue-specific distributions.

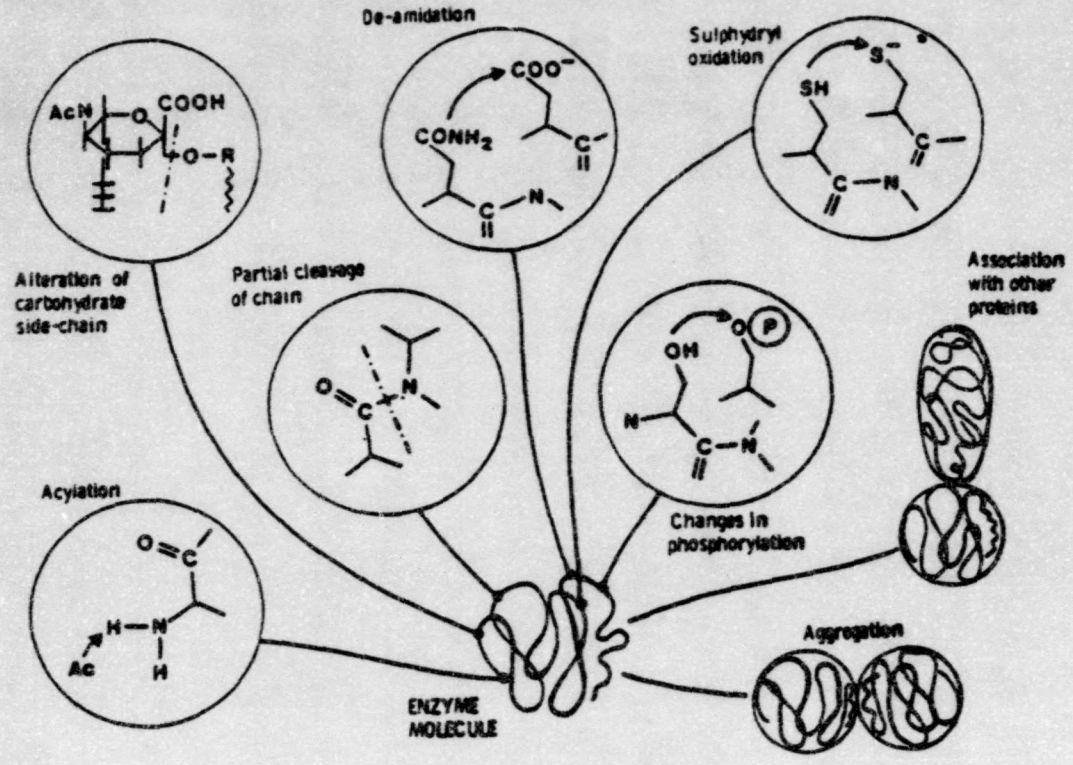
The classic example of isoenzymes which arises by the association of protein subunits that are themselves products of distinct structural genes is lactate dehydrogenase. In human tissue there are two genes determining the structure of M and H subunits of human lactate dehydrogenase which are respectively located on chromosomes 11 and 12. These two genes for lactate dehydrogenase are present in most vertebrates and make similar but non-identical polypeptides refered to as M (muscle) and H (heart). Both genes are equally active in embryonic tissue resulting in equal amounts of the two gene products and an array of possible tetramers (M_4, M_3H_1, M_2H_2, H_3M_1, and H_4) which are considered to be isoenzymes. The relative amounts of M and H forms changes as the embryonic tissue differentiates. In heart tissue, considered to be under conditions of aerobic metabolism, the H_4 tetramer predominates. Under conditions of anaerobic metabolism, such as stressed skeletal muscle tissue, the M_4 isoenzyme predominates, perhaps indicating that the M and H forms have evolved to serve different functions. The purified M_4 and H_4 isoenzymes of lactate dehydrogenase differ in catalytic center activity, the M_4 form being almost twice as active as the H_4 isoenzyme with respect to lactate as the substrate (Moss, 1982).

Isoenzymes determined by multiple gene loci typically differ in their Michaelis constant. The value for the tetramer composed of H-subunits of human lactate dehydrogenase is approximately one tenth of that for the M_4 homopolymer with pyruvate as a substrate, and less than half in the reverse reaction in which the substrate is lactate. Heteropolymers made up of both

H and M subunits have intermediate Michaelis characteristics. The H_4 isoenzyme (LD_1) is inhibited by excess pyruvate to a greater extent than the M_4 isoenzyme (LD_5) when measured under appropriate conditions (Moss, 1982)

Enzymes which have similar catalytic activities yet arise from the expression of the same gene are referred to as multiple forms. Multiple forms of enzymes may arise by several mechanisms including post-translational modification such as amino acid modification, deamination, alteration of carbohydrate side-chain, association with other proteins, and aggregation (Figure 1). Variant forms of enzymes which originate by post-translational modification of a single polypeptide chain, as in the conversion of inactive precursors of proteolytic enzymes to their active forms (zymogens), are not regarded as isoenzymes. In addition, the covalently modified or conformationally different forms in which certain enzymes may exist, and through which their regulation is effected are not considered to be isoenzymes. The five forms of rabbit-muscle aldolase, which can be separated by isoelectric focusing, are due to the intracellular conversion of a single asparagine residue near the carboxyl terminal of the polypeptide chain into an aspartyl residue (Moss, 1982). Deamination has also been identified as the cause of part of the molecular heterogeneity exhibited by various other enzymes, notably amylase (Moss, 1982). Removal of arginine residues from first one and then the other amino terminus of the polypeptide chains of the dimeric alkaline phosphatase molecule of E. coli gives rise to two additional forms of this enzyme. This results in a difference in the electrophoretic mobilities compared to that of the parent molecule (Schlesinger et al., 1975).

Figure 1: Diagrammatic summary of post translational modifications which may give rise to multiple forms of enzymes.


De-amidation
Sulphydryl oxidation
Alteration of carbohydrate side-chain
Partial cleavage of chain
Association with other proteins
Acylation
Changes in phosphorylation
Aggregation
ENZYME MOLECULE

Addition of carbohydrate residues to the side-chains of glycoproteins is a function of specific glycosyl-transferring enzymes. The potential exists for differential genetic control of the structures of the carbohydrate components of glycoprotein enzymes as well as non-enzymatic glycoproteins. Genetic control of this type is well recognized in the case of water-soluble, blood group specific glycoproteins. Glycosyltransferases, determined by A or B alleles at the ABO gene locus, catalyze the addition of either N-acetyl-D-galactosamine residues or D-galactose residues, respectively, to the terminal positions of the polysaccharide chains of these glycoproteins (Moss, 1982). Structural variations in the carbohydrate components of multiple forms of enzymes may similarly be manifestations of the differential expression of multiple gene loci or alleles which control glycosyltransferases.

Multiple forms, which result from either covalent or non-covalent modifications of the enzyme structure ,may be referred to as secondary isoenzymes (Moss, 1982). Covalent attachment of small molecules or radicals to enzyme molecules may alter their properties so much as to generate different molecular forms. Probably the most important examples of this are those multiple forms of enzymes which are due to differences in phosphate content. Two major forms of phenylalanine hydroxylase prepared from rat liver have been shown to differ with respect to their phosphate content (Donlon and Kaufman, 1980). These forms may represent an additional example of the regulation of enzyme activity by phosphorylation and dephosphorylation which was first demonstrated for enzymes of glycogen metabolism (Cohen, 1976).

Aggregation of enzyme molecules with each other or with non-enzymatic proteins may give rise to multiple molecular forms which can be separated

by differences in molecular size. Complex associations between enzymatic and non-enzymatic proteins or other constituents are characteristic of enzymes which are associated with cell membranes or organelles. The nature of the interactions which give rise to multiple forms of enzymes in this way can sometimes be inferred from the results of experiments in which the physical properties of the enzymes are modified without loss of the characteristic catalytic activity. For example, aggregation of the asymmetric forms of acetylcholinesterase is abolished by partial cleavage of its tails with collagenase (Moss, 1982). Analogous effects are produced by treating gamma-glutamyl transferases from various tissues with papain or trypsin, which abolish the tendency of the native enzyme to aggregate. Treatment of preparations of this and other membrane-derived enzymes with detergents or organic solvents disassociates them from lipids or lipoproteins, the complexes being recognized as multiple forms (Moss, 1982).

The determination as to whether a group of enzymes which share catalytic activity characteristics are multiple forms or isoenzymes of each other is not an easy task. To address such a question, the molecular weights, catalytic subunits, kinetic activity with respect to catalytic function and the identity of the gene which codes for the protein moiety must be determined.

With new techniques and improved purification methods, research concerning enzymes involved with DNA replication and their concerted functions has progressed rapidly. In 1956, a protein extract from E. coli was shown to incorporate thymidine into DNA, in the prescence of ATP and Mg^{+2} (Kornberg et al., 1956;1961). Kornberg received the Nobel prize for

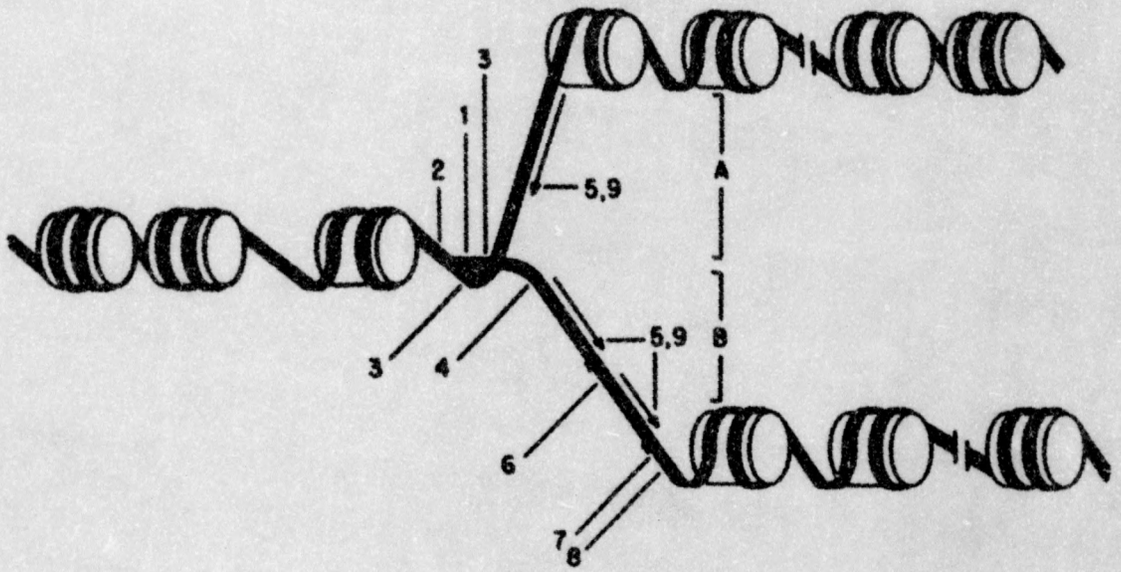
this discovery of DNA polymerase, an enzyme thought to be essential for *in vivo* DNA replication.

Evidence against Kornberg's enzyme being the enzyme of DNA replication came early in the 1970's when Moses and Richardson (1970), using a mutant of *E. coli* isolated by de Lucia and Cairns (1969), showed that even in the absence of the Kornberg enzyme *in vivo* DNA replication could occur. This led to their discovery of DNA polymerase II. The Kornberg enzyme became known as DNA polymerase I.

Utilizing the same mutant, Kornberg discovered DNA polymerase III (Kornberg, 1974). The three enzymes were later found to be encoded by three different gene loci even though, in general, they expressed the same enzymatic activity, replication of DNA *in vivo*. However, only when the three enzymes acted in concert did optimum *in vivo* DNA replication occur. Therefore, DNA polymerase I, II, and III were considered to be multiple forms of the same enzymatic activity, but since they are coded for by three different genes they qualify as isoenzymes. In eukaryotes, DNA polymerases are found in nuclear, mitochondrial, and in the case of plants, in the chloroplastic fractions. The most studied enzymes are DNA polymerases purified and characterized from animal cells. The biochemical events involved in the replication of DNA are caused by a variety of enzymes acting on the replication fork which progresses along the DNA molecule. The concerted activity of the major proteins involved in eukaryotic DNA synthesis is illustrated in Figure 2.

DNA polymerases catalyze the formation of the phosphodiester bond between the 3'-OH group at the growing end of the DNA chain (the primer) and the 5'-phosphate group of the incoming deoxyribonucleoside triphosphate. Growth is in the 5' to 3' direction, and the order in which

Figure 2: Replicating fork of DNA with its associated enzymes.



1 Helicase
2 Topoisomerase I
3 DNA-binding protein
4 Primase
5 DNA polymerase
6 Ribonuclease H
7 DNA ligase
8 Topoisomerase II
9 Exonuclease

A Leading strand
B Lagging strand

the deoxyribonucleotides are added is dictated by base-pairing to a template chain. In addition to the requirement of the four triphosphonucleotides and Mg^{+2} ions, the enzyme requires a primer and a template. No DNA polymerase has been found which can initiate DNA chains. Therefore, the in vitro catalytic activities of the DNA polymerases are identical.

The DNA-dependent DNA polymerases of eukaryotes are referred to as alpha, beta, gamma and delta, the intracellular location and properties of which are summarized in Table 1. Evidence indicates that DNA polymerase alpha is involved in the replication of nuclear DNA, while DNA polymerase beta is active in DNA repair. DNA polymerase gamma is the only polymerase found in the mitochondria where it replicates the organelle genome. Polymerase delta shares many of the properties of alpha but has a 3'-5' exonuclease associated on the same peptide carrying the polymerase catalytic subunit (Campbell, 1986). Therefore, while their in vitro catalytic activities may be similar, the cellular functions of each of the different DNA polymerases in vivo may be different. At this time the question remains unanswered as to whether the DNA polymerase of eukaryotic organisms are multiple forms or isoenzymes. Research at present is focusing on the isolation, purification to homogeneity and characterization of the protein subunits. Once this task has been accomplished the identity of the gene or genes which code for the enzymes can be identified. The focus of this research is the isolation of two of the DNA polymerases in plants, DNA polymerase alpha and gamma, and characterization of their subunits.

Table 1: DNA Polymerases of Animals

Properties	Alpha	Beta	Gamma	Delta
Subcellular localization	Nuclear	Nuclear	Nuclear Mitochondrial	Nuclear
Proposed function	Nuclear DNA replic.	DNA repair	Mit. DNA replication	DNA repair
M.W.holoenzyme (kDa)	150-1000	45	>110	250-290
M.W.catalytic subunit (kDa)	130-170	45		122
Templates				
Act. DNA	Y	Y	Y	Y
DNA template-RNA primer	Y	N	Y	N
Synthetic RNA template-deoxy primer	N	Y	Y*	N
Inhibitors				
Aphidicolin	Y	N	N	Y

*preferred template with Mn^{+2} as divalent cation

MATERIALS AND METHODS

Plant Material

Soybean seeds (Glycine max variety SB 4000) were purchased from Stewart Seed Company, Greensburg, Indiana. Seeds (a 250ml beaker full) were spread on the surface of extremely moist vermiculite at a depth of 2 inches (Terra-Lite, coarse grade) in a 12" X 24" plastic non-drain flat and lightly covered with a thin layer of vermiculite.

Seeds were germinated in the dark at 31°C for 4 to 5 days in a temperature controlled incubator under conditions of high humidity.

Turnip seeds (Brassica rapa variety Purple Top 55014/R5630) were purchased from Arco Seed Company, El Centro, California. Approximately 1 tablespoon of seeds was scattered evenly on the surface of a dampened topsoil/sand mixture 2 inches deep in a 12" X 24" plastic non-drain flat and covered with a thin layer of topsoil.

Turnip seeds were germinated under conditions of constant light provided by Westinghouse 40W Agro-Lite fluorescent light bulbs at 27°C for 3 weeks and were watered daily. After 1 week, the roots of the seedlings were watered with a 1:1 mixture of Miracle Grow solution (1 tablespoon/5 liters of water) and tap water.

Materials

Unless otherwise specified, all chemical materials were purchased from Sigma Chemical Company, St. Louis, Missouri.

Activated calf thymus DNA was either purchased from Sigma Chemical Company or produced as follows. Native calf thymus DNA (250ug) was combined with crystalline pancreatic DNAase I ($5x10^{-4}$ug) by adding 10ul of a solution containing 5ug of enzyme per 10ml of distilled water. The reaction mixture also contained bovine serum albumin (500ug), $MgCl_2$ (5umoles), and Tris-HCl (50umoles), pH 7.25 in a total volume of 25ml. This assay mixture was incubated at 37°C for 15 minutes in a shaking water bath and terminated by heating to 77°C for 5 minutes followed by rapid cooling in ice.

DNA Polymerase Alpha Assay Procedure

DNA polymerase alpha activity was determined using the assay procedure as described by Dunham & Bryant (1986). The assay mixture consisted of 3.0M Tris-HCl, 2.2mM 2-mercaptoethanol, pH 7.8 (10ul); 300mM $MgCl_2$ (2.5ul); dNTP's [50uM dATP, 50uM dCTP, 50uM dGTP & 25uM dTTP] (40ul); activated calf thymus DNA [1mg/600ul] (12ul); 2.5uCi ^3H dTTP (2.5ul); and distilled water (33ul). After the above reactants were combined in the described order, 50ul of enzyme preparation were added to initiate the *in vitro* polymerase reaction to give a final assay volume of 150ul. The reaction was carried out at 37°C for 30 minutes in a shaking water bath. A solution consisting of 20mM tetrasodium pyrophosphate in 100% trichloroacetic acid (0.7ml) and a 1mg/ml bovine serum albumin solution (0.2ml) were added to terminate the reaction and precipitate the reaction product. The reaction tubes were then chilled in ice for at least 45 minutes prior to filtering.

The DNA polymerase alpha assay mixtures were then filtered onto Whatman GF/A filters (2.5cm) which were presoaked in the following mixture to avoid non-specific binding: 5% cold trichloroacetic acid, 40mM pyrophosphate tetrasodium, and 25uM non-radiolabeled dTTP in a total volume of 20ml with distilled water. Reaction tubes were rinsed with twice their volume of cold 5% trichloroacetic acid onto the GF/A filters using a 10 port Selectron vacuum filtration apparatus purchased from Schleicher & Schuell. Filters were then dried in scintillation vials under the hood using an infrared heat lamp for approximately 45 minutes. Prior to the monitoring of radioactivity with a Packard 1500 Tri-Carb Scintillation Counter, 10ml of 2,5-bis [5'-tert-butyl-benz-oxazolyl-(2')] thiophene (BBOT) scintillation cocktail (6mg/liter scintillation grade tolulene) was added to each scintillation vial [Note: filters are not thoroughly dry if they do not appear translucent with addition of scintillation cocktail]. An internal quench curve was produced using purchased standards allowing DPM data to be collected for each assay vial using 10ml of BBOT scintillation cocktail as a background.

Controls for kinetic and inhibition assays were carried out as described above except for the omission of a DNA template. Zero time controls were performed as per protocol with the reaction being stopped immediately by the addition of the above stated terminating agents.

DNA Polymerase Gamma Assay Procedure

DNA polymerase gamma activity was determined using an assay mixture consisting of 50mM Tris-HCl, 150mM KCl, pH 7.8 (40ul); 15mM $MnCl_2$ (5ul); 5mM di-thiothreotol (10ul); poly(rA)oligo$(dT)_{12-18}$ stock of 3mg/500ul $d.H_2O$ purchased from Boringher Manneheim (3ul); 1mg/ml stock of bovine

serum albumin (15ul); 3uCi ^{3}H dTTP (3ul); and 24ul distilled water. The above reactants were combined in order as listed and then 50ul of isolated enzyme preparation was added to initiate the in vitro polymerase reaction to give a final assay volume of 150ul. The reaction was carried out at 37°C for 30 minutes in a shaking water bath. The assay reaction was stopped and filtered in the same manner as described for DNA polymerase alpha.

Controls for kinetic and inhibition assay studies were as outlined above with the exception of a template. Zero time controls were mixed as above with immediate termination by addition of previously described terminating agents.

Specific Activity Calculation

One unit of DNA polymerase activity was defined as the amount of enzyme catalyzing the incorporation of 1 pmol dTTP in 30 minutes at 37°C per mg of protein. The following equation was utilized in the calculation of specific activity: [1pmol of dTMP incorporated/1.737×10^{-3}dpm] X [dpm counts/mg protein in assay] = pmol of dTMP incorporated/mg of protein which represents the specific activities of the isolated enzyme preparations.

Protein Determination

All protein determinations were by the method of Lowry and co-workers (1951) except during fractionation on G-50 Sephadex, DEAE-cellulose, Phosphocellulose, or Hydroxylapatite column profiles when the more rapid 280/260 spectrophotometric analyses were performed. When reading column profiles at 280nm, cuvette offsets were activiated on the

spectrophotometer. In addition, during protein determination by the Lowry method cuvette offsets were activated at a wavelength of 576nm.

Enzyme Isolation

Isolation of soybean and turnip nuclei was by the method described by Dunham & Bryant (1986). Hypocotyl sections (1-2cm), i.e. the hook region, from 4 to 5 day-old etiolated soybean seedlings were harvested and placed in cold homogenization buffer consisting of 50mM Tris-HCl, pH 8.0; 600mM sucrose; 5mM $MgCl_2$; 10mM 2-mercaptoethanol; and 0.2mM phenylmethyl-sulfonyl fluoride. In each preparation approximately 150 to 250 grams of hypocotyl tissue were harvested. Primary leaves, i.e. the first true leaves formed, were harvested from 3-week old, light-grown turnip seedlings and placed in cold homogenization buffer. Approximately 150 to 200 grams of leaf tissue was harvested for each turnip preparation. For both soybean and turnip, the volume of homogenization buffer used was 3X volume/weight of tissue sample. Three drops of antifoam agent were added prior to homogenization with a Sorval Omni-mixer (setting 6 for 45 seconds) at $4^{o}C$. Tissue homogenates were then filtered through two layers of prewetted and chilled miracloth (Calbiochem) which were placed so that the fibers of cloth were perpendicular to each other. Filtrates were then centrifuged at 2,500g (4,500 rpm with a Beckman JA 20 rotor) for 30 minutes at $4^{o}C$. Proteins present in the supernatents were then precipitated with ammonium sulfate (0 to 70%; 43.6gm/100ml). Ammonium sulfate was added slowly over a 15 minute period with constant stirring at $4^{o}C$. The pH was maintained at 8.0 by titration with 1N sodium hydroxide. After an additional 15 minutes of stirring, solutions were centrifuged at 30,000g (15,600 rpm with JA 20) for

30 minutes at 4°C. Pellets were resuspended in 5 to 10 ml of equilibration buffer consisting of 100mM Tris-HCl, pH 8.0; 5mM 2-mercaptoethanol; 25% (v/v) glycerol; and 0.2mM phenylmethyl-sulfonyl fluoride. Resuspended samples were then desalted on a 50ml G-50 Sephadex column at 4°C. The fractions with the highest protein content, as determined by reading at 280nm on a Gilford Response Spectrophotometer, were combined for further purification.

Anion Exchange Chromatography

Preparation of DEAE-cellulose resin followed a standard protocol which included exposure to 0.5N NaOH and 0.5N HCl as follows. The dry DEAE-cellulose resin was allowed to stand for 5 minutes in 5X the volume to weight of 0.5N NaOH. When the decanted liquid from repeated washings with distilled water reached a pH below 10, 5X the volume to weight of 0.5N HCl was added and allowed to stand for 5 minutes. The resin was then repeatedly rinsed with distilled water until the decanted liquid reached a pH above 3. Following equilibration with 5X the volume to weight of 2X equilibration buffer, the pH was titrated to 8.0. A DEAE-cellulose column (10cm X 100cm) was prepared and equilibrated using 1X equilibration buffer at 4°C. New columns were poured after every 3 to 4 experiments.

Desalted enzyme preparations in equilibration buffer were applied to the DEAE-columns at a flow rate of 1ml/minute, which were then washed with twice the void volume of the column or until no protein was detected in the 2ml fractions as determined by 280/260 spectrophotometric analyses. Bound proteins were eluted in a linear gradient of 0-0.5M KCl in equilibration buffer, with a volume equal to twice the void volume of the column.

Fractions of 2ml were collected and assayed for absorbance at 280nm for protein concentration.

Salt eluted fractions containing enzyme activity were pooled and desalted on a G-50 Sephadex column. Pooled fractions were then concentrated 10- to 20-fold with Centricell ultrafilters at 1,500g in a swinging bucket clinical centrifuge at 4°C and labeled as the DEAE{G-50} fraction for further purification.

Cation Exchange Chromatography

Phosphocellulose resin was prepared, poured, and equilibrated in the same manner as the DEAE-cellulose resin at 4°C. New columns (10cm X 50cm) were poured after every 3 to 4 experiments.

Concentrated enzyme preparations (DEAE{G-50}) in equilibration buffer were applied to the columns at a pump speed of 0.5ml/minute. The columns were then washed with twice their void volume of equilibration buffer or until no protein was detected in 2ml fractions as determined by 280/260 spectrophotometric analyses. Bound proteins were eluted in a linear gradient of 0-1.0M KCl in equilibration buffer equal to twice the void volume of the column. Fractions of 2ml were collected and assayed for absorbance at 280nm for protein concentration.

Fractions obtained in the wash eluate that exhibited enzyme activity were pooled and concentrated to approximately 10ml with a Amicon PM10 filter and labeled as the phospho-wash fraction. Following desalting on a G-50 Sephadex column, fractions which demonstrated enzymatic activity were pooled and concentrated 10- to 20-fold (phospho-salt fraction).

Hydrogen Bonding Chromatography

Hydroxylapatite resin (Whatman) was prepared by dispersing the medium in 10X the volume to weight of equilibration buffer. After allowing the resin to stand 30 minutes, the excess solution and fines were removed by siphoning. The slurry was then poured into a 5cm X 20cm column and the column packed using equilibration buffer. After packing, equilibration was completed by rapidly passing two bed volumes of equilibration buffer containing 2.5M ammonium sulfate. The column was then thoroughly washed with equilibration buffer prior to use.

Concentrated enzyme preparations (DEAE-{G-50}) in equilibration buffer were applied to the columns at a pump speed of 0.5ml/minute. The columns were then washed with twice their void volume and salt eluted as described for cation exchange chromatography.

Wash eluate fractions which exhibited enzymatic activity were treated as outlined for phosphocellulose eluted fractions and appropriately concentrated 10- to 20-fold (hydroxy-salt fraction).

Protein Concentration Methods

Sample pools over 100ml were concentrated to approximately 20ml with an Amicon PM10 filter using an Amicon ultrafiltration cell concentrator unit with a pressure of 30psi and constant stirring at 4°C.

Samples of 20ml were concentrated to approximately 1 to 2 ml using, Centricell centrifugal ultrafilter concentrators (Polysciences Incorporated) in a swinging bucket clinical centrifuge at 1,500g and 4°C.

Samples of 2ml or less were concentrated with an Amicon Centricon microconcentrator to a volume of 50ul or less at 5,000g (7,950 rpm with a JA 20 rotor) and 4°C.

Electrophoretic Analyses

Discontinuous polyacrylamide gels consisting of a lower resolving gel and an upper stacking gel as described by Laemmli (1970) were used to further separate the proteins of the turnip and soybean total protein preparations which were found to contain enzymatic activity. All gel compositions were derived from a mixture of three stock solutions: stock A, stock R, and stock S. Stock solution A contained 29.2% weight to volume acrylamide and 0.8% bis-acrylamide in a total volume of 100ml using distilled water to give a 30% acrylamide/bis-acrylamide stock solution. Stock solution R, resolving buffer, was a 1.5M Tris-HCl solution at a pH of 8.8. Stock solution S, stacking buffer, was a 1.0M Tris-HCl solution at a pH of 6.8. The resolving gel was composed of 9 or 12% polyacrylamide. All gels were cast as 16 X 20cm slab gels 1.0mm thick with either a 20 well comb of 220ul/well capacity or a one well reference preparatory comb with a capacity of 3ml. Nine percent gels were fabricated as follows: 9ml stock A, 13.1ml d.H_2O, 0.15ml of freshly prepared 11% ammonium persulfate (APS), 7.5ml stock R, 0.3ml of 10% sodium dodecyl sulfate (SDS), and 0.015ml N,N,N^1,N^1-tetramethylethylenediamine (TEMED) to give a total gel volume of 30.065ml. Twelve percent gels were prepared as follows: 12ml stock A, 10.1ml d.H_2O, 0.15ml of freshly made 11% APS, 7.5ml stock R, 0.3ml of 10% SDS, and 0.015ml TEMED to yield a total volume of 30ml. Three percent stacking gels consisted of the following: 1.5ml stock A, 7.05ml d.H_2O, 0.1ml of freshly made 11% APS, 1.25ml stock S, 0.1ml of 10% SDS, and 0.005ml TEMED for a total volume of 10ml. In gel preparation procedures APS and TEMED are added last since their addition initiates polymerization of the gel. Prior to the loading of the protein preparations, gels were

pre-electrophoresed at 50mA for 60 minutes in order to deplete any "free" APS, a strong oxidizing agent, from the gels. The electrophoresis buffer was replaced before sample loading. Solvent used to load samples contained 10% glycerol and 0.001% bromphenol blue. Electrophoretic buffer used contained 0.025M Tris-HCl, pH 8.3; 0.192M glycine; and 0.1% SDS in a total volume of 5 liters. Gels were run at 40mA in both denaturing and non-denaturing gel runs. Electrophoresis buffer was changed, when amperage became erratic during a run, in order to prevent acidification of protein bands.

When non-denaturing polyacrylamide gels were used, all SDS was omitted. In gel preparation the SDS was replaced with 42ul of 2-mercaptoethanol/resolving gel and 14ul/stacking gel to give a 20mM concentration. Denaturing (SDS containing) polyacrylamide gels were employed for molecular weight determinations of protein bands from enzyme isolations. Non-denaturing polyacrylamide gels were used when isolating "active" bands of protein.

Gel Staining

In the case of SDS polyacrylamide gels, all of the gel was fixed and stained. Only a small portion (including reference well) of non-denaturing gels were fixed and stained since this procedure would inactivate any enzyme activity associated with the protein bands. Denaturing (SDS) polyacrylamide gels were stained by being placed in a solution consisting of 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid for 30 minutes. The gels were then destained to remove background using a 40% methanol and 10% acetic acid solution for 1 to 3 hours. After

Coomassie Brilliant Blue stained SDS gels were thoroughly examined, they were completely destained in the 40% methanol and 10% acetic acid solution in order to be silver stained.

Gels to be silver stained were fixed in 400ml of 40% methanol/10% acetic acid for 60 minutes, this step was eliminated when gels had been previously stained with Coomassie Brillant Blue and then completely destained, followed by two 30 minute washes in 10% ethanol/5% acetic acid. The gels were then oxidized in 200ml of a nitric acid/potassium dichromate solution (Bio-Rad) for 10 minutes. Following washing with distilled water, the gels were then stained in silver reagent (Bio-Rad) for 30 minutes. Gels were then developed using a sodium carbonate/paraformaldehyde solution (Bio-Rad). The development reaction was terminated with a 5% acetic acid solution. In cases where very little protein was present, it was necessary to silver stain a second time. Silver stained gels were washed for 30 minutes and staining protcol repeated starting at the silver reagent step.

Gel Scans

Gel scans were performed using a Gilford Response Spectrophotometer and a 20cm quartz cuvette. Unstained gel strips were read with a survey scan at 280nm. In the case of Coomassie Brilliant Blue stained gels, the lane of interest was excised and read with a survey scan at 576nm. A survey scan at 400nm was carried out on lanes of interest from silver stained gels. In all cases, a slit width of 4nm was employed.

Electro-Elution of Protein Bands

Concentrated samples of enzyme preparations were applied to 3%/12% non-denaturing polyacrylamide gels with a preparatory comb well and

electrophoresed for approximately 48 hours at 40mA at 4°C. The reference well (molecular weight marker) lane and a small section (1-2cm) of the preparatory well were analyzed spectrophotometrically before and after silver staining. Cross-sections of protein bands were excised and the protein was electro-eluted out of the gel using a Bio-Rad Electro-Eluter apparatus at 4°C. A current strength of 10mA/tube was used for approximately 6-8 hours. The electrophoresis buffer was replaced at the half-way point in order to prevent acidification.

It was important to wear gloves at all times while assembling the Electro-Eluter apparatus and loading excised gel cross-sections into the tubes. Membrane caps were soaked at 60°C in elution buffer (same as electrophoresis buffer) for at least 1 hour prior to use. One pre-wetted membrane cap was placed in the bottom of each silicone adapter needed. The adapters were then filled with buffer, pipetting it up and down to remove any air bubbles around the dialysis membrane. A frit was then inserted into the bottom of each of the glass tubes needed (one tube/fragment), making sure that the frit fitted flush at the bottom of the tube. The silicone adapter with the membrane cup was then secured to the bottom of the glass tube with frit. To insure that all air bubbles had been expelled the silicone adapter was partially pulled on and off a few times. For optimum elution yields, all air bubbles had to be removed from the silicone adapter and frit. Next, the glass tube with attached adapter, frit, and membrane was inserted into a grommet of the electro-eluter module. The top of the tube being made even with the grommet. All empty grommet holes were filled with stoppers.

Each tube was then filled with buffer and gel slices inserted. It was important for gels not to fill the tube higher than 1cm from the frit surface for proper elution. If the gel slice exceeded the 1cm height, it was macerated and packed into the tube with a glass rod to the lowest possible height. Care was taken not to pack with such a force as to dislodge the frit from the bottom of the tube.

Each module was then placed into the buffer chamber containing 600ml of buffer. The lower buffer level had to be above the top of the silicone adapters or air bubbles would form on the surface of the dialysis membrane and disrupt elution. The upper buffer chamber required 100ml of elution buffer. A stirring bar was placed in the lower buffer chamber and the entire unit placed on a magnetic stirrer. Vigorous stirring during the run prevented bubbles from sticking to the bottom of the dialysis membranes.

At the end of the elution run, the silicone adapter with dialysis cup was carefully removed, making sure not to dislodge the frit from the bottom of the tube. It was sometimes necessary to first remove liquid down to the frit level from the tube before removing the adapter. Eluted protein in the adapter and in the dialysis cup was carefully removed. The adapter and cup were then rinsed with their volume of elution buffer. This buffer rinse was then pooled with the protein elution and labeled appropriately.

RESULTS

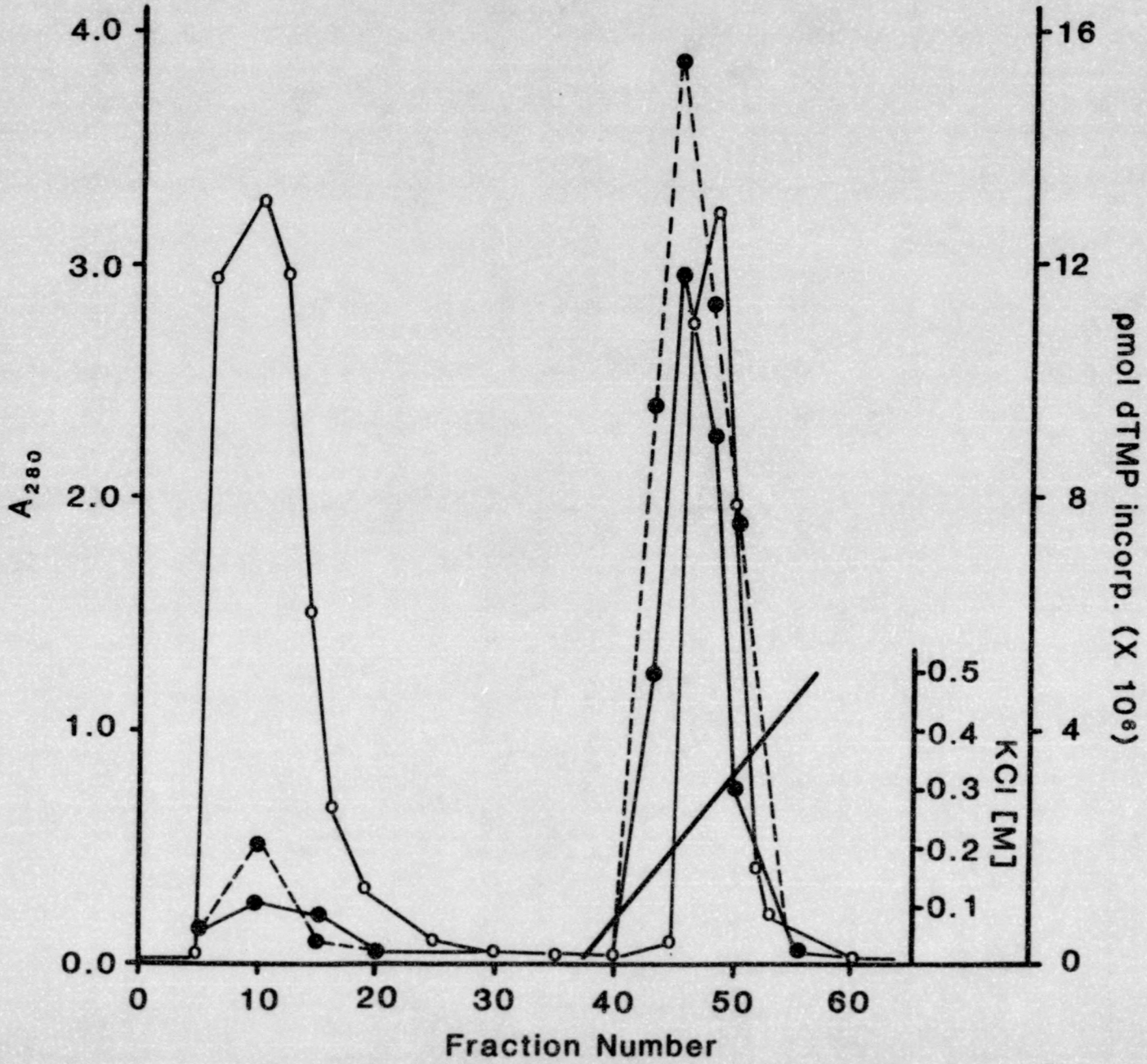
Purification of Soybean DNA Polymerase Alpha and Gamma

Partial purification of DNA polymerase alpha and gamma from DNA polymerase beta was accomplished by centrifugation of a homogenized crude extract in order to separate the nuclei from the cytosol. Further purification of the cytosol fraction, which has been previously determined to contain DNA polymerase alpha (D'Alesandro et al., 1980) and gamma (Dunham and Bryant, 1986), included ammonium sulfate precipitation, G-50 Sephadex, DEAE-Cellulose, Phosphocellulose, and Hydroxylapatite column chromatography. Fractions of enzymatic activity eluted from the cation and anion exchange columns were then further purified by non-denaturing polyacrylamide gel electrophoresis.

Using a 100ml column of DEAE-cellulose, five-hundred to seven-hundred and fifty milligrams of the desalted DNA polymerase preparation was applied to the column. DNA polymerase alpha and gamma activity were eluted in one major peak of enzymatic activity, using a 0 to 0.5M KCl linear gradient, at approximately 0.25M KCl (Figure 3). The fractions comprising the protein peak containing both enzymatic activities were pooled and desalted on a G-50 Sephadex column. Protein fractions were then concentrated and labeled as the DEAE-cellulose post-G-50 fraction (DEAE{G-50}).

The next step of purification was then carried out on either a phosphocellulose or hydroxylapatite column. DNA polymerase alpha and gamma adhered to both columns. However, the salt-eluted protein from the

Figure 3: DEAE-cellulose chromatography of soybean DNA polymerase activity. Five-hundred to seven-hundred and fifty milligrams of a desalted DNA polymerase preparation was applied to a 100ml DEAE-cellulose column. Using a KCl linear gradient from 0-0.5M KCl, one protein peak was eluted which contained both DNA polymerase alpha and gamma activity at 0.25M KCl. Fractions were monitored for protein at 280nm (o——o) and assayed for DNA polymerase alpha (●——●) and gamma activity (●- - - -●).


DEAE-Cellulose (Soybean)
4.0
3.0
2.0
1.0
0.0
A_{280}
0
10
20
30
40
50
60
Fraction Number
16
12
8
4
0
pmol dTMP incorp. (X 10^3)
0.5
0.4
0.3
0.2
0.1
KCl [M]

phosphocellulose column contained twice the enzymatic activity of that eluted from the hydroxylapatite column. Therefore, the phosphocellulose salt-eluted fractions were used for further purification of DNA polymerase alpha and gamma.

Two small aliquots of the DEAE{G-50} fraction were put aside for later use, one to purify on a hydroxylapatite column and the other for characterization of protein content on a non-denaturing polyacrylamide gel. The remaining DEAE{G-50} fraction was applied to a 50ml phosphocellulose column. DNA polymerase alpha and gamma activities both were prevalent in the salt-eluted peak, using a 0 to 1.0M KCl linear gradient, at approximately 0.5M KCl (Figure 4). The wash peak, which contained some enzymatic activity, was pooled, concentrated and labeled as the phosphocellulose wash fraction (P-W). The salt-eluted protein fractions which contained enzymatic activity were pooled, desalted, concentrated and labeled as the phosphocellulose salt-eluted fraction (P-S).

One of the small aliquots of the DEAE{G-50} fraction was then applied to a 20ml hydroxylapatite column. Both enzymatic activities, DNA polymerase alpha and gamma, were associated with the salt-eluted protein peak, using a 0 to 1.0M KCl linear gradient (Figure 5). DNA polymerase alpha was eluted at approximately 0.7M whereas DNA polymerase gamma was eluted at approximately 0.5M KCl. Due to the small amount of protein associated with both enzyme activities, all the fractions of the protein salt-eluted peak with associated enzyme activity were pooled, desalted, concentrated and labeled as the hydroxylapatite salt-eluted fraction (H-S). The wash-eluted peak was concentrated and labeled as the hydroxylapatite wash fraction (H-W).

Figure 4: Phosphocellulose chromatography of soybean DNA polymerase. Thirty-five milligrams of the DEAE{G-50} fraction was applied to a 50ml phosphocellulose column. Using a linear gradient from 0-1.0M KCl, one protein peak was eluted at 0.5M KCl which contained both DNA polymerase alpha (●——●) and DNA polymerase gamma (●- - -●) activities. Fractions were monitored for protein at 280nm (o——o).

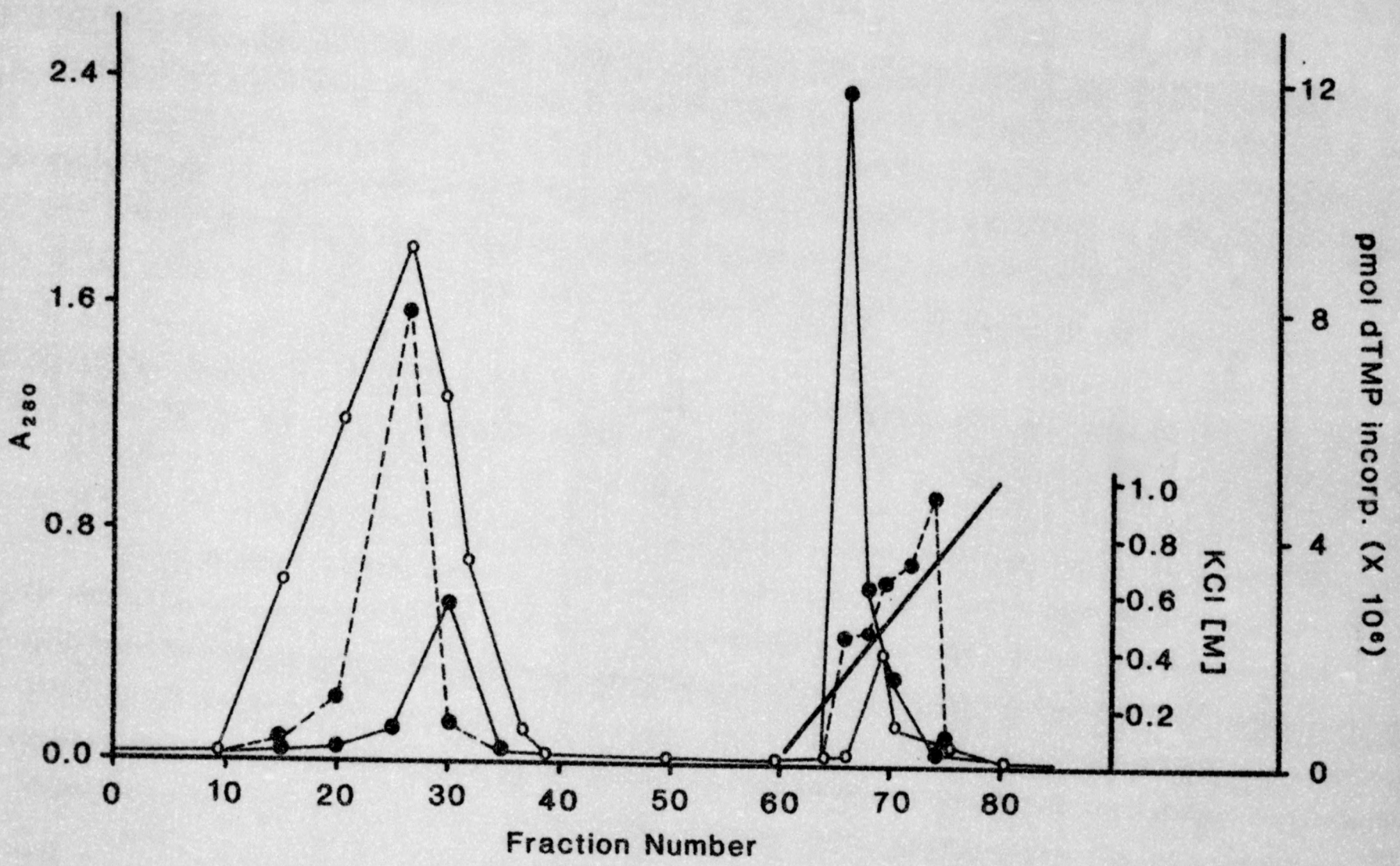
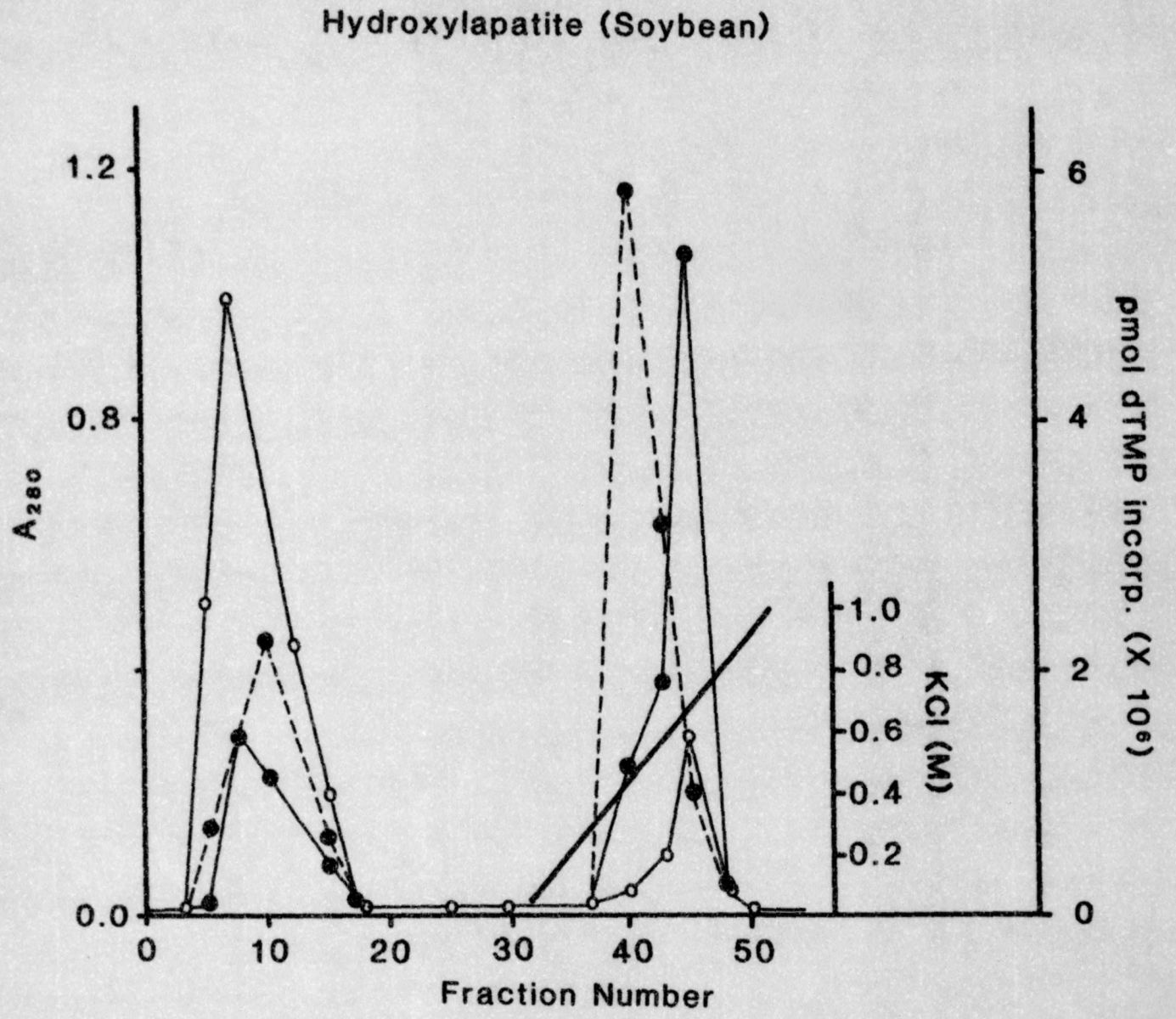

Phosphocellulose (Soybean)
2.4
1.6
0.8
0.0
A_{280}
0
10
20
30
40
50
60
70
80
Fraction Number
1.0
0.8
0.6
0.4
0.2
KCl [M]
12
8
4
0
pmol dTMP incorp. (X 10^3)

Figure 5: Hydroxylapatite chromatography of soybean DNA polymerase. Approximately ten milligrams of the DEAE{G-50} fraction was applied to a 20ml hydroxylapatite column. Using a 0-1.0M KCl linear gradient, one protein peak was eluted at 0.6M KCl which contained both DNA polymerase activites. Fractions were monitored for protein at 280nm (o——o) and assyed for DNA polymerase alpha (●——●) and DNA polymerase gamma (●- - -●).


Hydroxylapatite (Soybean)
1.2
0.8
0.0
A_{280}
0
10
20
30
40
50
Fraction Number
1.0
0.8
0.6
0.4
0.2
KCl (M)
6
4
2
0
pmol dTMP incorp. (X 10^3)

The remaining aliquot of the DEAE{G-50} fraction, as well as the P-W, P-S, H-W, and H-S fractions were then further purified individually on a non-denaturing polyacrylamide gel (Figures 6-8). Three major bands of protein were detected on the non-denaturing gel in the DEAE{G-50}, P-W, H-W and H-S fractions. Only two major bands of protein, which appeared to correlate with two of the three bands mentioned above, were detected in the P-S fraction. The three major protein bands of the phosphocellulose fraction were excised and the proteins electro-eluted. The heaviest of the protein bands was labeled S_1, the "middle" band as S_2, and the lightest weight band as S_3.

In general, both DNA polymerase alpha and gamma enzymatic activities increased with protein purification (Table 2). The post-phosphocellulose fractions showed the greatest increase in activities of any of the column purification steps, 104-fold for alpha and 55-fold for gamma more purified as compared to the crude homogenate. However, the post-polyacrylamide electro-eluted fractions were almost 5-times more purified than the post-phosphocellulose fractions, 490-fold for alpha and 465-fold for gamma. Based on these preliminary assays S_1 and S_3 represents DNA polymerase gamma and alpha respectively. S_2 had no polymerase activity and its identity remains unknown.

Purification of Turnip DNA Polymerase Alpha and Gamma

Turnip DNA polymerase alpha and gamma purification followed the same course as was taken for that of soybean DNA polymerase alpha and gamma. One-hundred to one-hundred and fifty milligrams of the desalted DNA polymerase preparation was applied to a 100ml DEAE-cellulose column. A 0 to 0.5M KCl linear gradient was used to elute the adhered protein from the

Figure 6: Non-denaturing PAGE gel scan of the soybean DEAE{G-50} fraction. Three major protein bands, labeled (S_1, S_2 and S_3), can be seen in the gel photograph. A strip of the gel was scanned unstained at 280nm (A) and then silver stained and scanned at 400nm (B). Note that at beginning and end of gel scans are artifacts of entrance onto and exit off of the gel scan program.

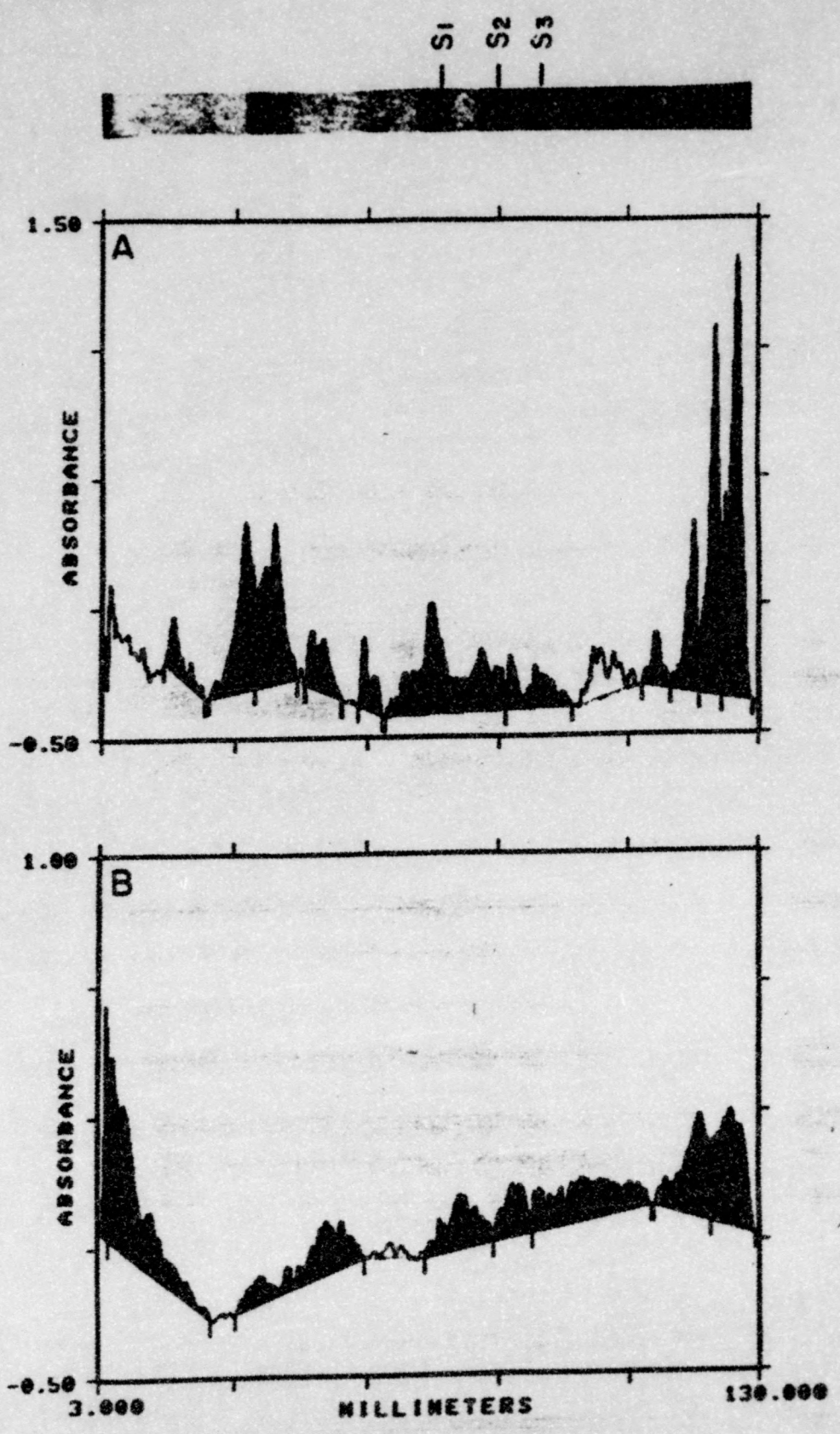

S1
S2
S3
1.50
A
ABSORBANCE
-0.50
1.00
B
ABSORBANCE
-0.50
3.000
MILLIMETERS
130.000

Figure 7: Non-denaturing PAGE gel scan of the soybean P-W fraction. Three major bands of protein can be seen in the gel photograph. A strip of the gel was scanned at 280nm (A) and then silver stained and scanned at 400nm (B). Note that beginning and end of gel contains artifacts previously mentioned.

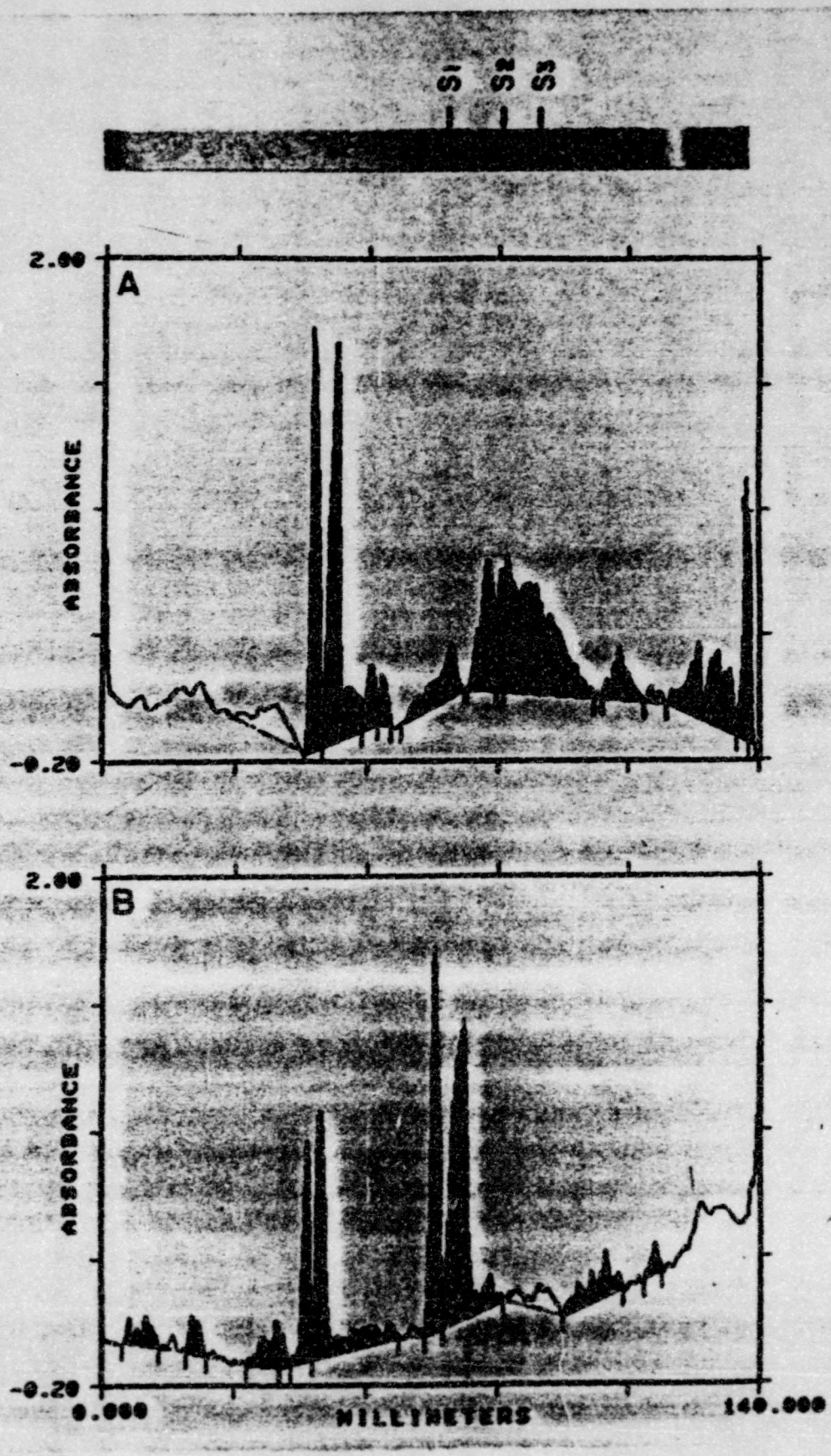

S1
S2
S3
2.00
A
ABSORBANCE
-0.20
2.00
B
ABSORBANCE
-0.20
0.000
MILLIMETERS
140.000

Figure 8: Non-denaturing PAGE gel scan of soybean P-S fraction. Two major bands can be seen in the gel photograph. An unstained gel scan at 280nm (A) was carried out on a strip of the gel which was then silver stained and scanned at 400nm (B).

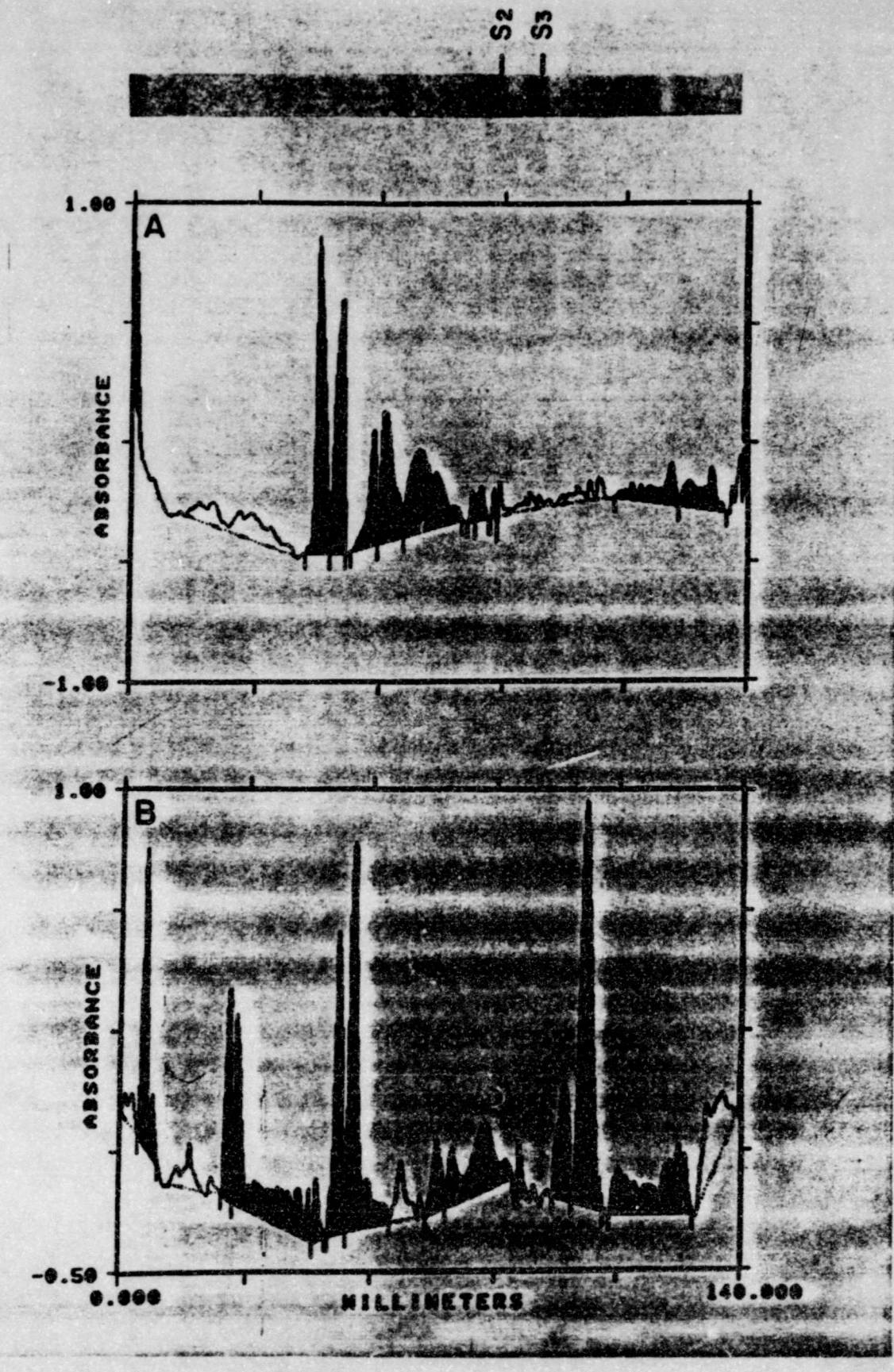

S2
S3
1.00
A
ABSORBANCE
-1.00
1.00
B
ABSORBANCE
-0.50
0.000
MILLIMETERS
140.000

Table 2: Purification of Soybean DNA Polymerase Activities

Polymerase Alpha Activity

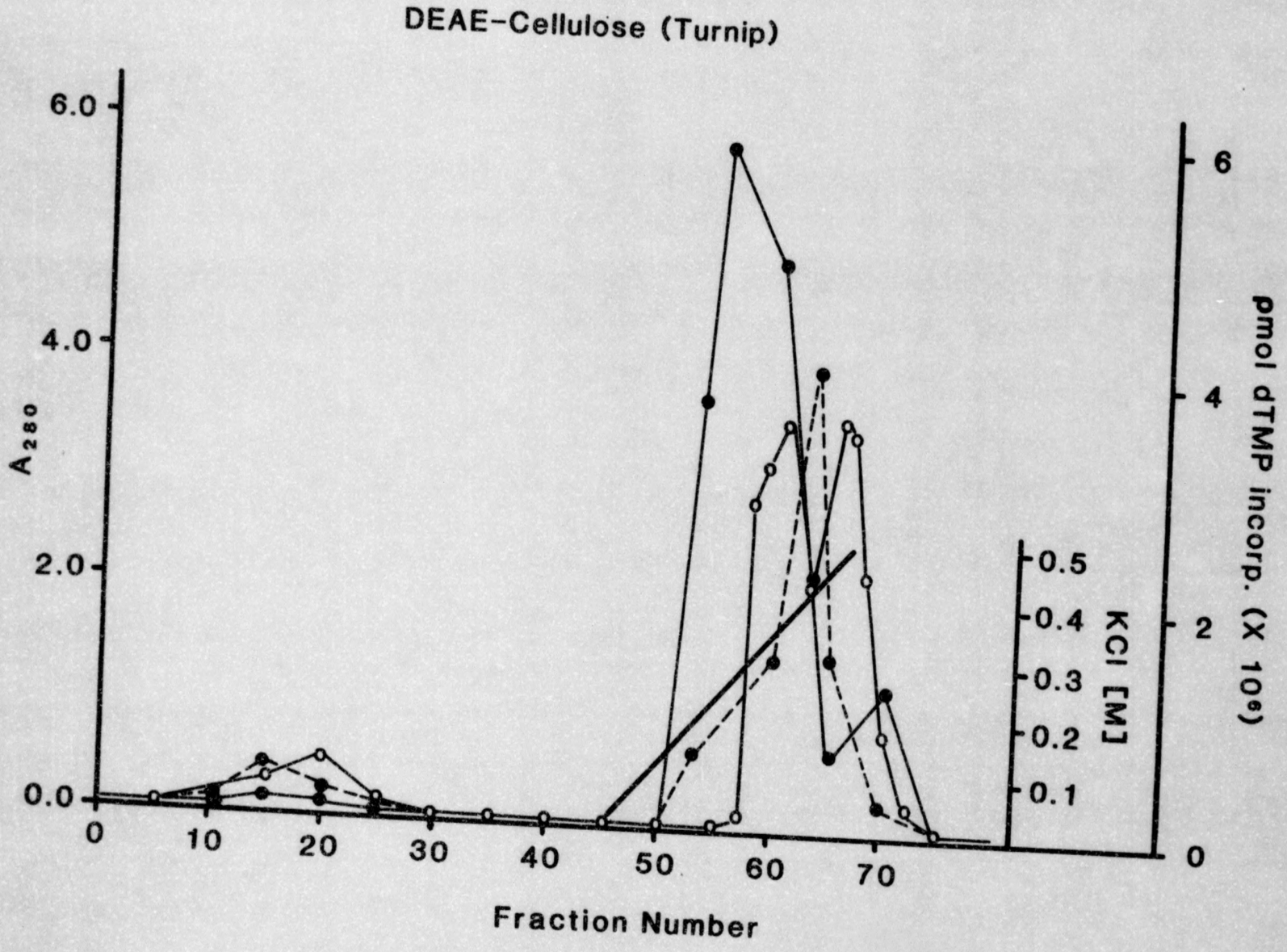
Procedural Step	Total Protein(mg)	pmol dTMP incorp. /20min. (X 10^6)	Specific Activity*	Purification Fold
Crude Homog.	4,273	8	0.3	1
$AmSO_4$ ppt.	679	10	0.8	3
DEAE-Cellulose	42	7	4	15
Phosphocellulose	6	11	30	105
Eluted Band S_3	0.13	9	144	488

Polymerase Gamma Activity

Procedural Step	Total Protein(mg)	pmol dTMP incorp. /20min. (X 10^6)	Specific Activity*	Purification Fold
Crude Homog.	4,273	9	0.3	1
$AmSO_4$ ppt.	679	9	0.6	2
DEAE-Cellulose	42	5	3	9
Phosphocellulose	10	8	19	55
Eluted Band S_1	0.15	10	160	465

*pmol dTMP incorporated/ mg of protein/ minute

Figure 9: DEAE-cellulose chromatography of turnip DNA polymerase activity. One-hundred to one-hundred and fifty milligrams of a desalted DNA polymerase preparation was applied to a 100ml DEAE-cellulose column. Using a KCl linear gradient from 0-0.5M KCl, two protein peaks were eluted which contained both DNA polymerase alpha and gamma activity at 0.35M KCl and 0.45MKCl. Fractions were monitored for protein at 280nm (o——o) and assayed for DNA polymerase alpha (●——●) and gamma activity (●- - -●).


DEAE-Cellulose (Turnip)
6.0
4.0
2.0
0.0
A_{280}
0
10
20
30
40
50
60
70
Fraction Number
0.5
0.4
0.3
0.2
0.1
KCl [M]
6
4
2
0
pmol dTMP incorp. (X 10⁶)

column (Figure 9). DNA polymerase alpha activity was eluted at approximately 0.25M whereas gamma activity was eluted at approximately 0.4M KCl. Once again, due to the low abundance of protein content, all protein salt-eluted fractions with associated enzymatic activity were pooled, desalted, concentrated and labeled as the DEAE-cellulose post-G-50 fraction (DEAE{G-50}).

As in soybean the next step of protein purification was carried out either on a phosphocellulose or hydroxylapatite column. DNA polymerase alpha and gamma both adhered to either column, however, the salt-eluted protein from the turnip hydroxylapatite column contained twice the enzymatic activity of that eluted from the phosphocellulose column. Therefore, the electro-eluted bands for turnip will have originated from the hydroxylapatite column fractions.

A 50ml phosphocellulose column was then used as a small aliquot of the total DEAE{G-50} fraction was applied, an additional aliquot and the remaining DEAE{G-50} fraction were saved for later use. Using a 0 to 1.0M KCl linear gradient, both DNA polymerase alpha and gamma activities were eluted at approximately 0.5M KCl (Figure 10). The salt-eluted protein fractions, which contained enzymatic activity, were pooled, desalted, concentrated and labeled as the phosphocellulose salt-eluted fraction (P-S). The wash-eluted fractions were pooled, concentrated and labeled as the phosphocellulose wash-eluted fraction (P-W).

The remainder of the DEAE{G-50} fraction, minus the small aliquot to be used for protein characterization on the non-denaturing polyacrylamide gel, was applied to a 30ml hydroxylapatite column (Figure 11). The salt-eluted protein peak was determined to contain both enzymatic activities. DNA polymerase alpha was eluted at approximately 0.7M and DNA polymerase

Figure 10: Phosphocellulose chromatography of turnip DNA polymerase. Twenty milligrams of the DEAE{G-50} fraction was applied to a 50ml phosphocellulose column. Using a linear gradient from 0-1.0M KCl, one protein peak was eluted at 0.5M KCl and both DNA polymerase alpha (●——●) and DNA polymerase gamma (●- - -●) were associated with it. Fractions were monitored for protein at 280nm (○——○).

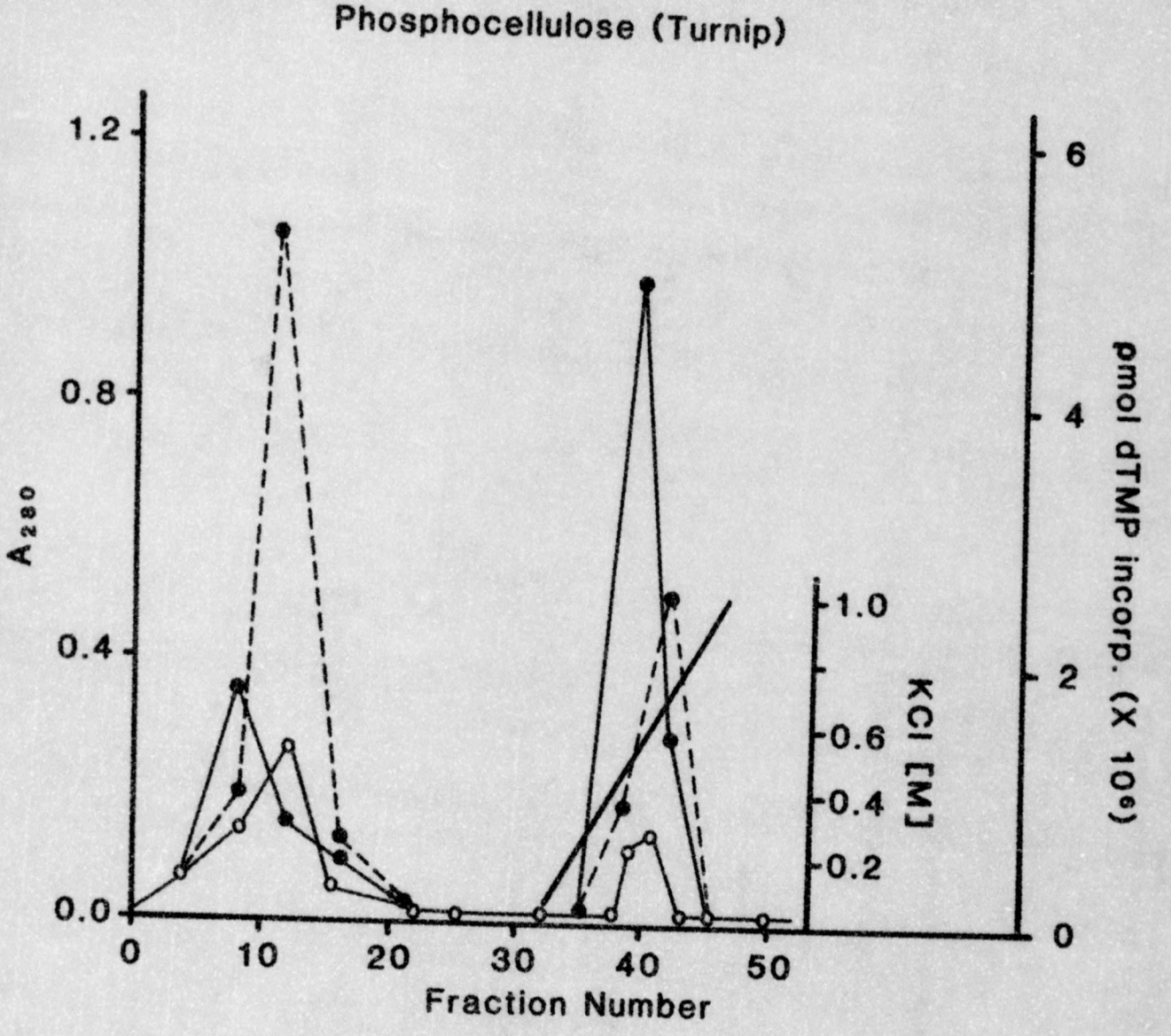
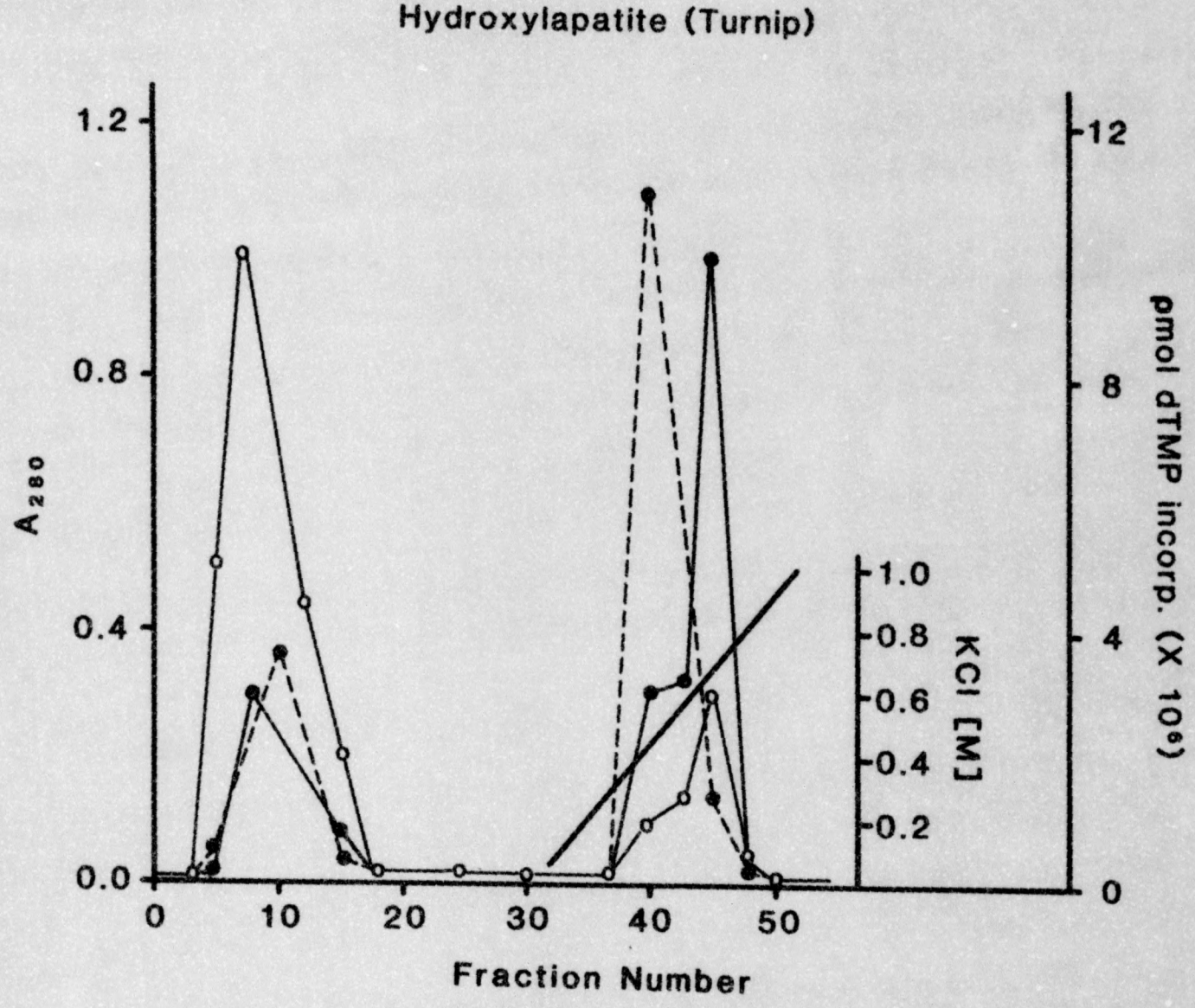

Phosphocellulose (Turnip)
1.2
0.8
0.4
0.0
A_{280}
0
10
20
30
40
50
Fraction Number
1.0
0.6
0.4
0.2
KCl [M]
6
4
2
0
pmol dTMP incorp. (X 10^3)

Figure 11: Hydroxylapatite chromatography of turnip DNA polymerase. Twenty-five milligrams of the DEAE{G-50} fraction was applied to a 25ml hydroxylapatite column. Using a 0-1.0M KCl linear gradient, one protein peak was eluted at 0.6M KCl which contained both DNA polymerase activites. Fractions were monitored for protein at 280nm (○——○) and assyed for DNA polymerase alpha (●——●) and DNA polymerase gamma (●- - -●).


Hydroxylapatite (Turnip)
1.2
0.8
0.4
0.0
A_{280}
0
10
20
30
40
50
Fraction Number
1.0
0.8
0.6
0.4
0.2
KCl [M]
12
8
4
0
pmol dTMP incorp. (X 10^3)

gamma at approximately 0.5M KCl, using a 0 to 1.0M KCl linear gradient. To have enough protein to be detected in the gel purification procedure, the salt-eluted fractions with enzymatic activities were pooled, desalted, concentrated and labeled as the hydroxylapatite salt-eluted fraction (H-S). The wash-eluted protein peak was pooled, concentrated and labeled as the hydroxylapatite wash-eluted fraction (H-W).

Non-denaturing polyacrylamide gel purification was then carried out separately on the remaining aliquot of the DEAE{G-50} fraction and the entire volumes of the P-W, P-S, H-W and H-S fractions (Figure 12-16). Three major bands of protein were detected on the non-denaturing PAGE gel in the DEAE{G-50}, P-W, H-W, and H-S electrophoresed fractions. In the P-S fraction only two major protein bands were visible. Excision of the three major protein bands from the hydroxylapatite fractions was followed by electro-elution of the proteins from the gel. In keeping with the nomenclature used for soybean eluted protein bands, the heaviest weight band was labeled T_1, the "middle" band as T_2, and the lightest weight band as T_3.

In comparison to the activities associated with the crude homogenate, DNA polymerase alpha and gamma activities increased with protein purification (Table 3). The most significant increase in purification was observed in the post-PAGE fractions, 156-fold for alpha and 186-fold for gamma more purified than the crude homogenate. These results indicate that T_1 and T_3 represent DNA polymerase gamma and alpha respectively. T_2 had no polymerase activity and its identity remains unknown.

Figure 12: Non-denaturing PAGE gel scan of the turnip DEAE{G-50} fraction. Three major protein bands, labeled (T_1, T_2 and T_3), can be seen in the gel photographs. A strip of the gel was scanned unstained at 280nm (A) and then silver stained and scanned at 400nm (B). Note that at beginning and end of gel scans are artifacts of entrance onto and exit off of the gel scan program.

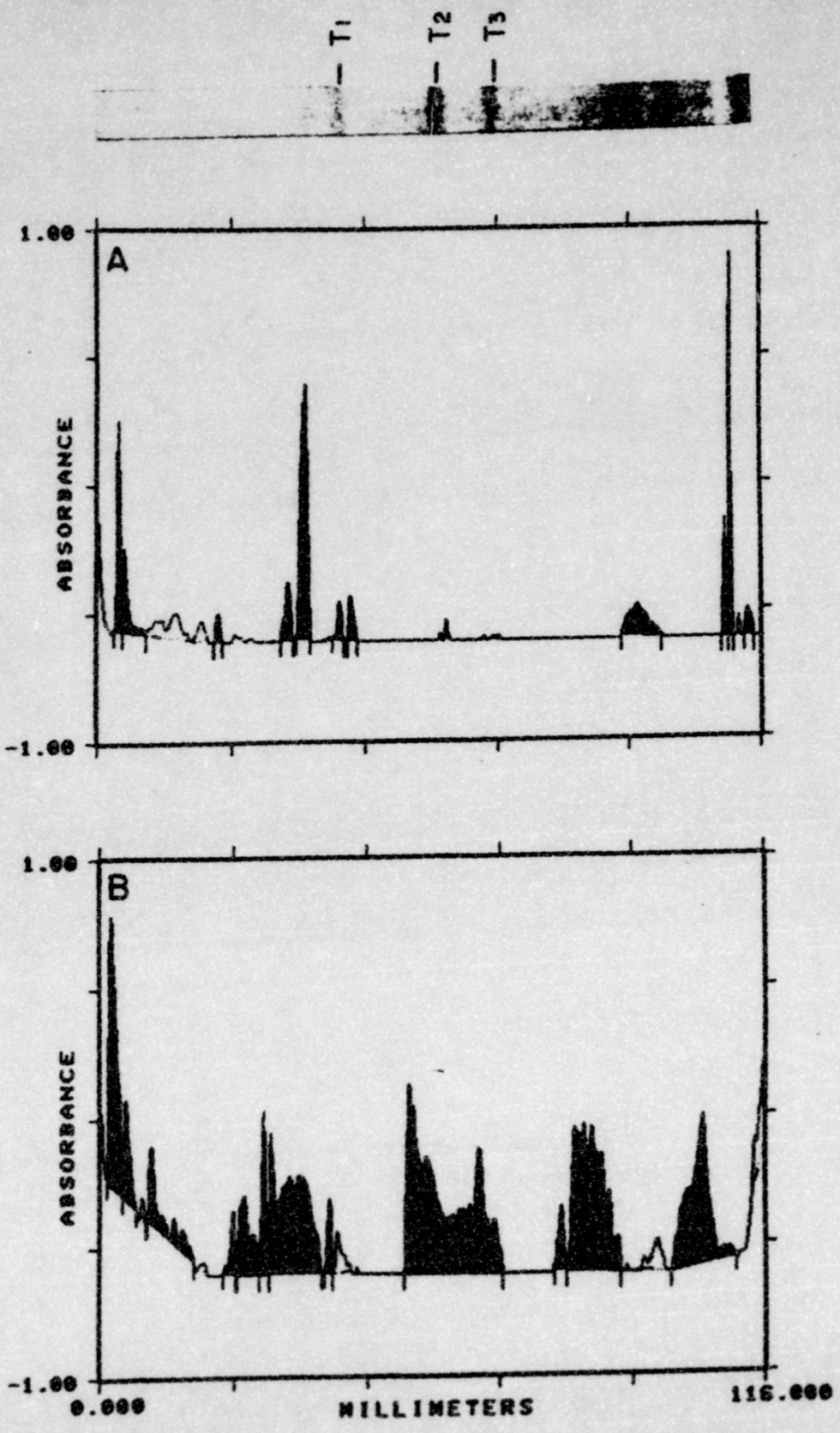

T1
T2
T3
1.00
A
ABSORBANCE
-1.00
1.00
B
ABSORBANCE
-1.00
0.000
MILLIMETERS
116.000

Figure 13: Non-denaturing PAGE gel scan of the turnip P-W fraction. Three major bands of protein can be seen in the gel photograph. A strip of the gel was scanned at 280nm (A) and then silver stained and scanned at 400nm (B). Note that beginning and end of gel contains artifacts previously mentioned.

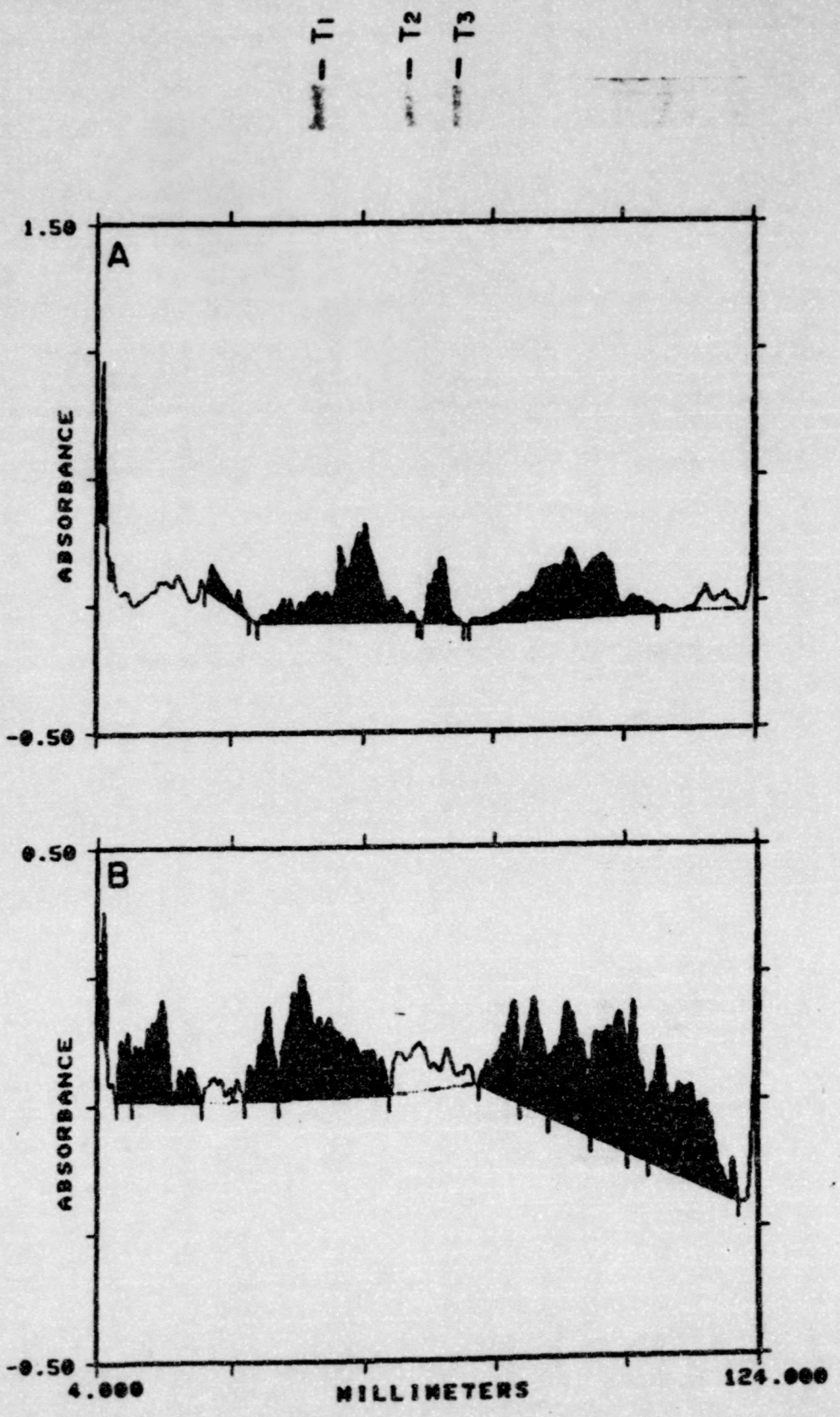

T1
T2
T3
1.50
A
ABSORBANCE
-0.50
0.50
B
ABSORBANCE
-0.50
4.000
MILLIMETERS
124.000

Figure 14: Non-denaturing PAGE gel scan of the turnip P-S fraction. Two major bands can be seen in the gel photograph. An unstained gel scan at 280nm (A) was carried out on a strip of the gel which was then silver stained and scanned at 400nm (B).

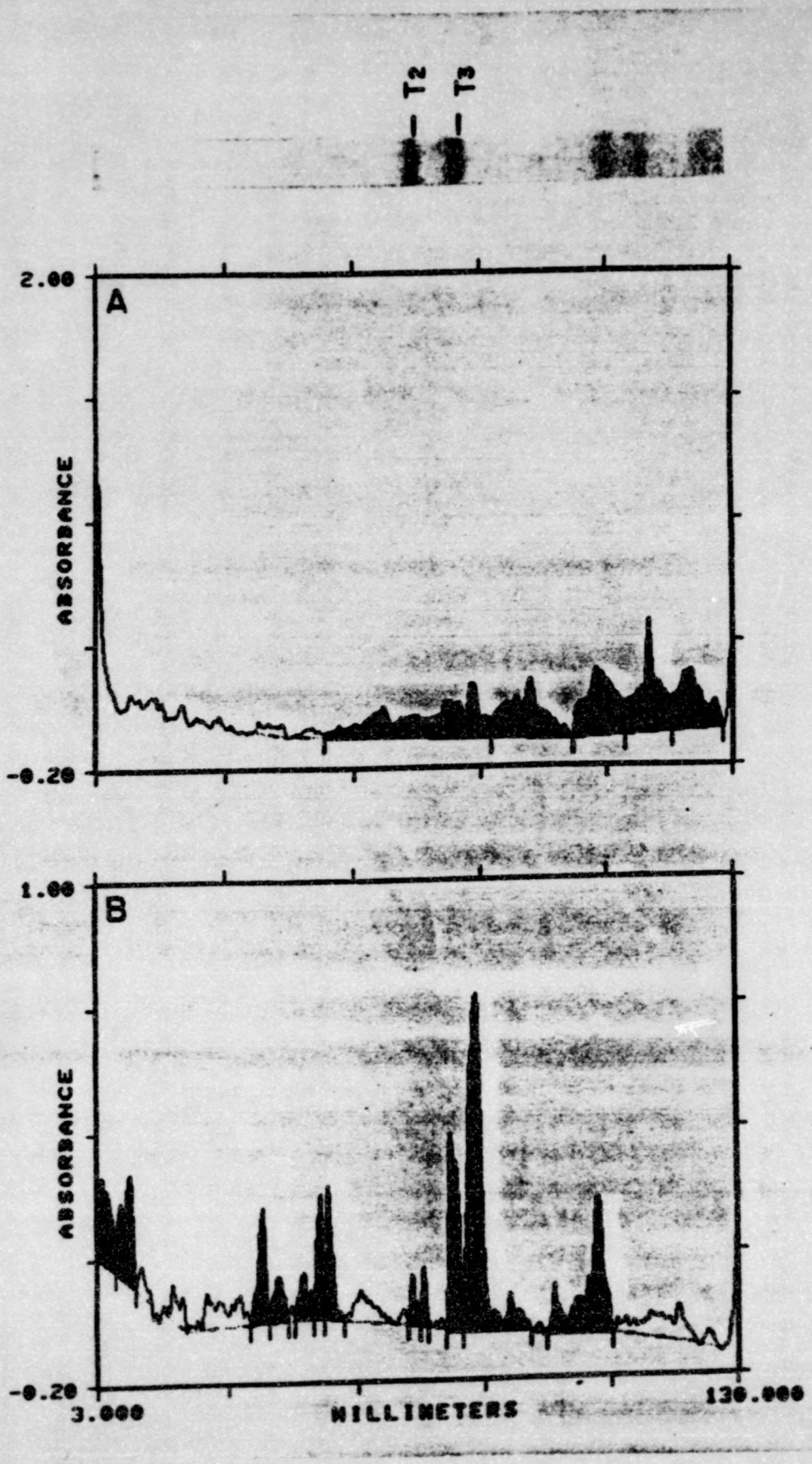

T2
T3
2.00
A
ABSORBANCE
-0.20
1.00
B
ABSORBANCE
-0.20
3.000
MILLIMETERS
130.000

Figure 15: Non-denaturing PAGE gel scan of the turnip H-W fraction. Three major bands of protein can be seen in the gel photograph. A strip of the gel was scanned at 280nm (A) and then silver stained and scanned at 400nm (B). Note that beginning and end of gel contains artifacts previously mentioned.

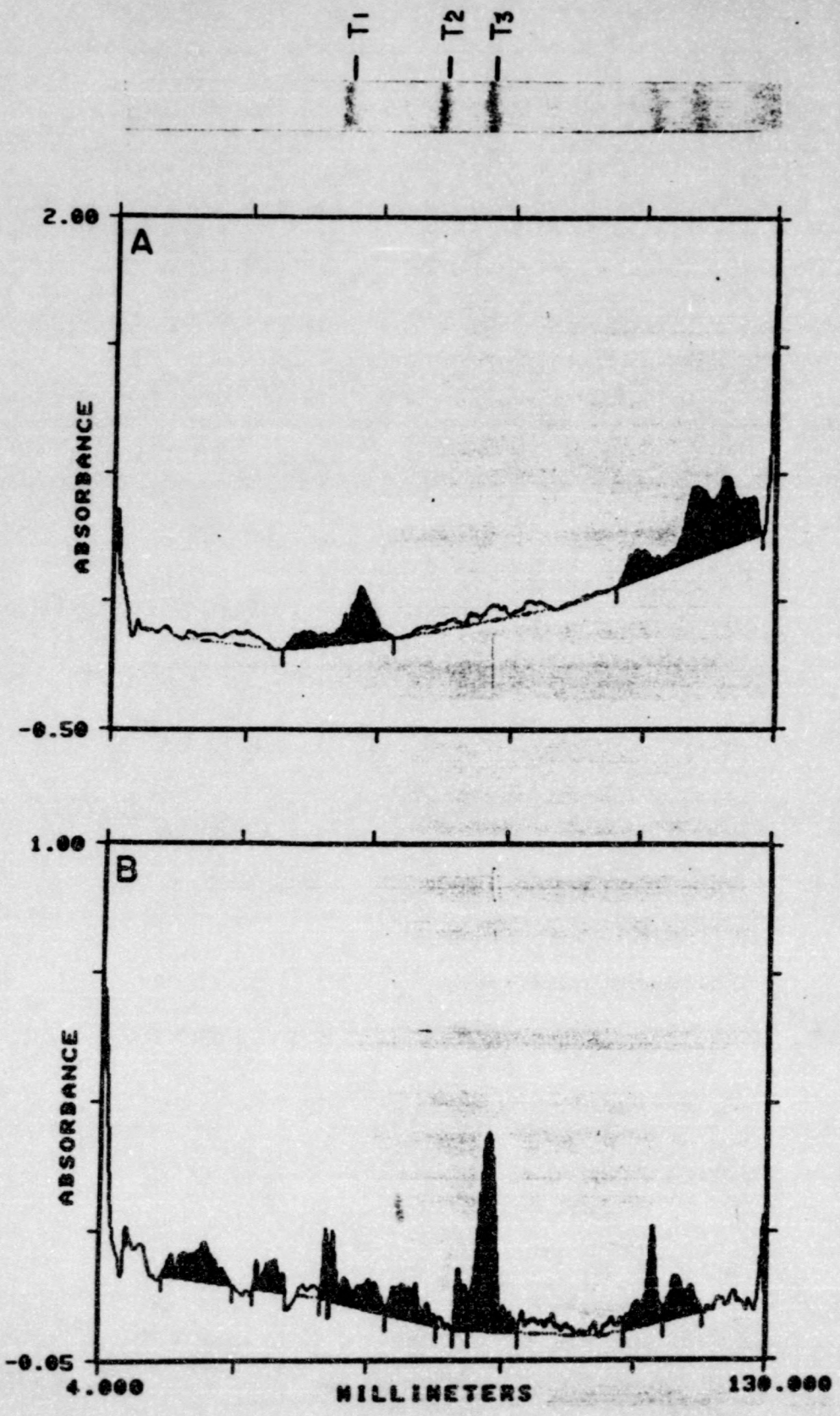

T1
T2
T3
2.00
A
ABSORBANCE
-0.50
1.00
B
ABSORBANCE
-0.05
4.000
MILLIMETERS
130.000

Figure 16: Non-denaturing PAGE gel scan of the turnip H-S fraction. Three major bands can be seen in the gel photograph. An unstained gel scan at 280nm (A) was carried out on a strip of the gel which was then silver stained and scanned at 400nm (B).

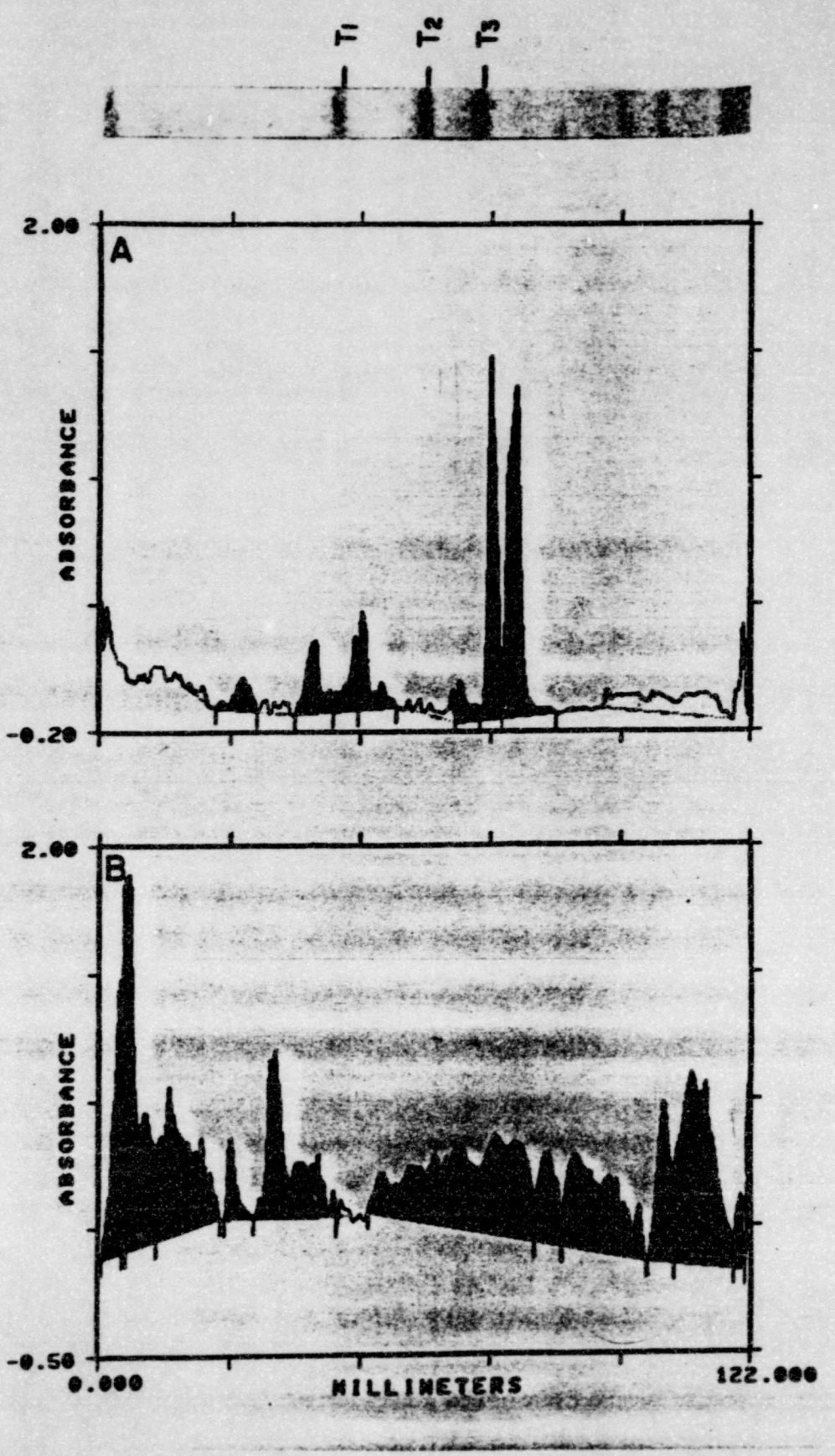

T1
T2
T3
2.00
A
ABSORBANCE
-0.20
2.00
B
ABSORBANCE
-0.50
0.000
MILLIMETERS
122.000

TABLE 3: Purification of Turnip DNA Polymerase Activities

Polymerase Alpha Activity

Procedural Step	Total Protein(mg)	pmol dTMP incorp. /20min. (X 10^6)	Specific Activity*	Purification Fold
Crude Homog.	1,097	7	0.7	1
$AmSO_4$ ppt.	163	10	3	4
DEAE-Cellulose	50	5	6	8
Phosphocellulose	10	6	25	33
Hydroxylapatite	7	7	20	26
Eluted Band T_3	0.056	3.6	119.0	156.1

Polymerase Gamma Activity

Procedural Step	Total Protein(mg)	pmol dTMP incorp. /20min. (X 10^6)	Specific Activity*	Purification Fold
Crude Homog.	1,097	7	0.7	1
$AmSO_4$ ppt.	163	10	3	5
DEAE-Cellulose	50	4	5	6
Phosphocellulose	20	6	14	20
Hydroxylapatite	7	6	17	24
Eluted Band T_1	0.07	4	132	186

*pmol dTMP incorporated/ mg of protein/ minute

Kinetic Assays of Purified Soybean DNA polymerase Alpha and Gamma

In order to further identify the associated enzyme activities of the protein gel eluted bands, time course activity assays for DNA polymerase alpha and gamma were performed on each band. It is known that as long as sufficient template is provided, and there are no nucleases present, DNA polymerases will continue to act in a linear manner with respect to nucleotide incorporation. From Figure 17, it can be seen that in S_1 there is a three-fold linear increase in its DNA polymerase gamma activity (Figure 17A) while its DNA polymerase alpha activity remains essentially the same after 30 minutes. The opposite effect detected for S_1 was observed for S_3, the DNA polymerase alpha activity linearly increased three-fold while its DNA polymerase gamma activity remained relatively unaltered after 30 minutes (Figure 17). Due to the linear action of both S_1 and S_3 it can be stated that nucleases present are not in high concentrations in the preparations and that S_1 represents DNA polymerase gamma and S_3, DNA polymerase alpha. The diminutive and uncharacteristic activity of both the DNA polymerase alpha and gamma activities of S_2 indicate the possible presence of a nuclease as well as an absence of a viable enzyme for DNA polymerase alpha or gamma activity. Its identity is therefore unknown.

Kinetic Assay of Purified Turnip DNA Polymerase Alpha and Gamma

In general, DNA polymerase activity in turnip appears to be less active than its compliment activities in soybean. The time course study for turnip eluted protein bands is represented in Figure 18. A slight increase in DNA polymerase gamma activity was observed for T_1 (Figure 18A) over a

Figure 17: Supplied nucleotide incorporation time course study of soybean electro-eluted bands S_1 (A) and S_3 (B). DNA polymerase alpha activity (●——●) and gamma (●- - -●).

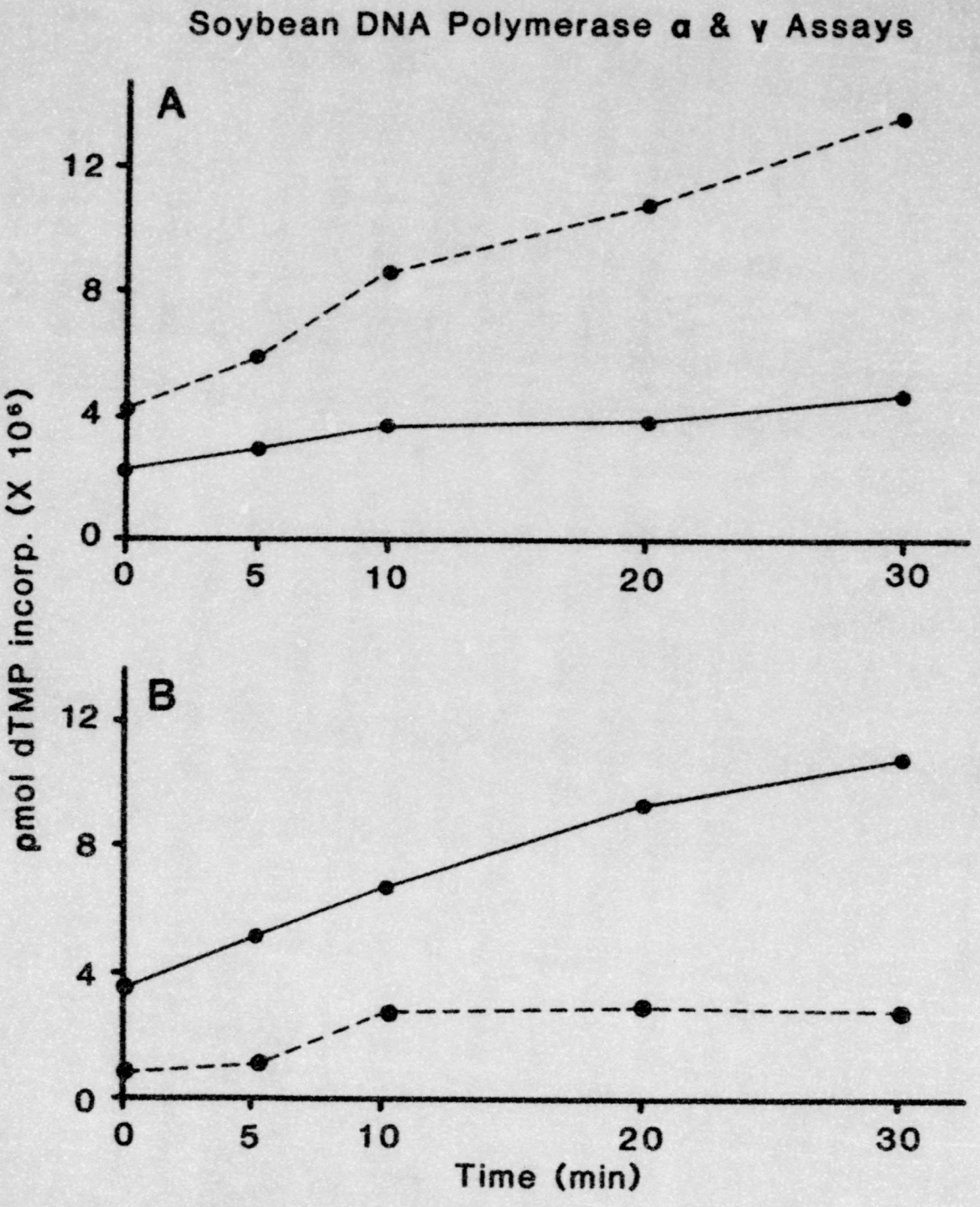
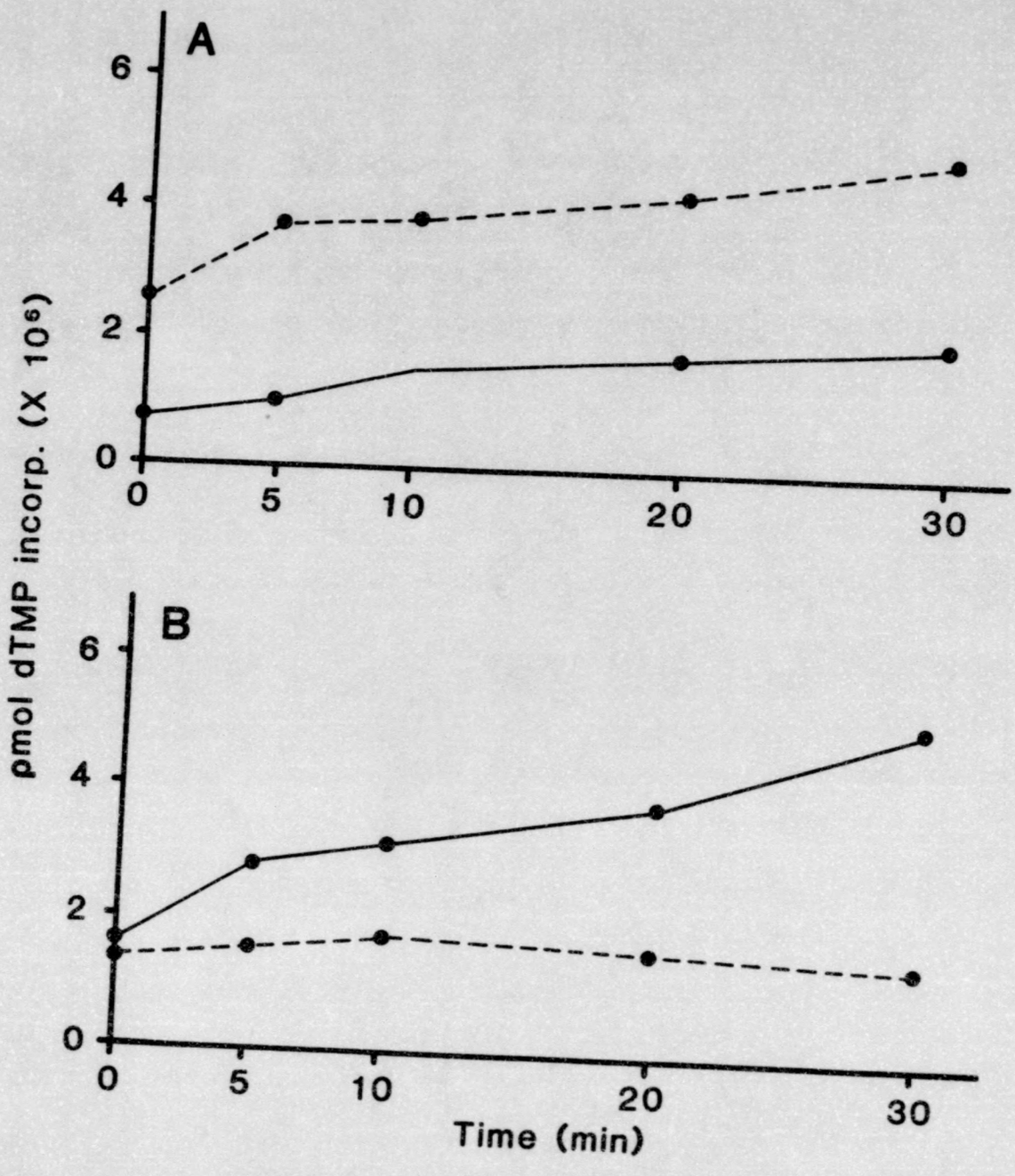

Soybean DNA Polymerase α & γ Assays
A
B
12
8
4
0
0
5
10
20
30
pmol dTMP incorp. (X 10⁶)
Time (min)

Figure 18: Supplied nucleotide incorporation time course study of turnip electro-eluted bands T_1 (A) and T_3 (B). DNA polymerase alpha activity (●——●) and gamma (●- - -●).

Turnip DNA Polymerase α & γ Assays



thirty minute assay time. Eluted protein band T_3 acted linearly with respect to its DNA polymerase alpha activity as its activity increased with time (Figure 18B). The linear activity of T_1 and T_3 with respect to their associated enzyme activities indicates the absence of significant levels of nuclease in the protein preparations. The data for turnip support the statements made for soybean, T_1 represents DNA polymerase gamma and T_3 DNA polymerase alpha, but the identity of T_1 was not as clearly defined as S_1 was in soybean.

Divalent Cation Study of Soybean and Turnip Purified DNA Polymerases

To further characterize the protein electro-eluted bands, alternate divalent cation preference assays were performed (Table 4). Based on previous studies S_1/T_1 and S_3/T_3 are DNA polymerase gamma and alpha respectively. The divalent cation preference studies support these conclusions in that enzymatic activities of fractions S_1/T_1 were enhanced in the presence of Mn^{+2}, whereas the activities of fractions S_3/T_3 were maximum in the presence of Mg^{+2}. Since DNA polymerase alpha is known to require Mg^{+2} and DNA polymerase gamma Mn^{+2}, the above data support the hypothesis that S_1/T_1 are DNA polymerase gamma and S_3/T_3 are DNA polymerase alpha.

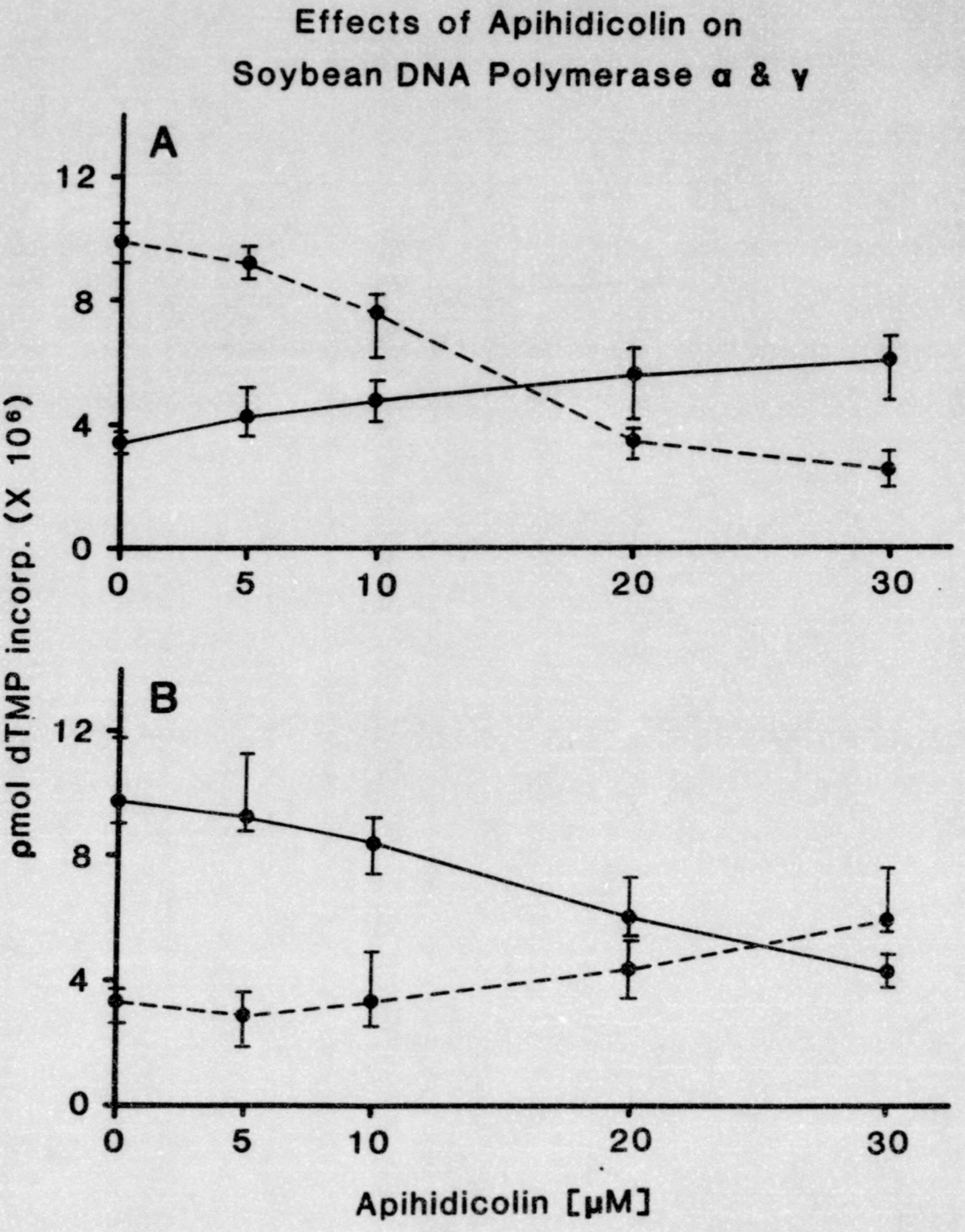
Aphidicolin Inhibition Study on Purified Soybean DNA Polymerases

Aphidicolin is a known specific inhibitor of DNA polymerase alpha in higher plants, as well as in all other systems in which DNA polymerase alpha has been isolated. Based on the aphidicolin data (Figure 19B), S_3 is characteristically inhibited by increasing aphidicolin concentrations. However, soybean DNA polymerase gamma (S_1), based on Figure 19A, also

Table 4: Effects of Divalent Cation on DNA Polymerase Alpha & Gamma Activity

Eluted Protein Band	Alpha Assay pmol dTMP incorp./20min. (X 10^6) w/Mg^{+2}	Alpha Assay pmol dTMP incorp./20min. (X 10^6) w/Mn^{+2}	Gamma Assay pmol dTMP incorp./20min. (X 10^6) w/Mg^{+2}	Gamma Assay pmol dTMP incorp./20min. (X 10^6) w/Mn^{+2}
S_1	3.6	3.1	1.4	9.6
S_3	8.6	6.1	5.1	3.1
T_1	2.0	4.1	2.1	4.9
T_3	5.6	0.6	4.0	2.7

Figure 19: Effects of increasing aphidicolin concentration on the DNA polymerase alpha (●——●) and gamma (●- - -●)activities of soybean electro-eluted protein bands S_1 (A) and S_3 (B). Graphs were determined from the results of assays on six different enzyme preparations.


Effects of Apihidicolin on
Soybean DNA Polymerase α & γ
A
B
12
8
4
0
0
5
10
20
30
pmol dTMP incorp. (X 10⁶)
Apihidicolin [μM]

appears to be sensitive to aphidicolin which may be due to its possible involvement in a complex were DNA polymerase alpha activity is present.

Aphidicolin Inhibition Studies on Purified Turnip DNA Polymerases

The turnip DNA polymerase alpha (T_3) is characteristically inhibited by increaseing concentrations of aphidicolin (Figure 20B). However, the data in Figure 20A do not clearly delineate the identity of the DNA polymerase. It should be noted that this fraction has been isolated from a green tissue and therefore may contain chloroplastic DNA polymerase gamma.

SDS-PAGE Analysis of Soybean & Turnip Purification Steps

Purification of proteins in the chromatographic steps outlined previously resulted in an expected decrease in the number of protein bands in a SDS-polyacrylamide gel (Figure 21A and 22A). It should be noted that the heavier bands present in Figure 21A, 205 kDa/ 110 kDa for soybean and 105 kDa/ 97 kDa for turnip, were not present in preparations over four days old. Even though a protease inhibitor was included in all buffers (phenylmethyl-sulfonyl fluoride) used during purification, the gradual disappearance of these heavier weight bands with age indicates that there was some proteolytic activity present. These heavier weight bands did not independently appear in the non-denaturing gel preparations. Therefore, their possible DNA polymerase activities are unidentified. In the non-denaturing gel system the molecular weights for the S_1/T_1 proteins were estimated to be approximately 75 kDa, S_2/T_2 approximately 55 kDa, and S_3/T_3 appeared as two protein bands too close together to physically cut out as two separate bands (approximately 45 kDa).

Figure 20: Effects of increasing aphidicolin concentration on the DNA polymerase alpha (●——●) and gamma (●- - -●) activities of turnip electro-eluted protein bands T_1 (A) and T_3 (B). Graphs were determined from the results of assays on four different enzyme preparations.

Effects of Apihidicolin on
Turnip DNA Polymerase α & γ

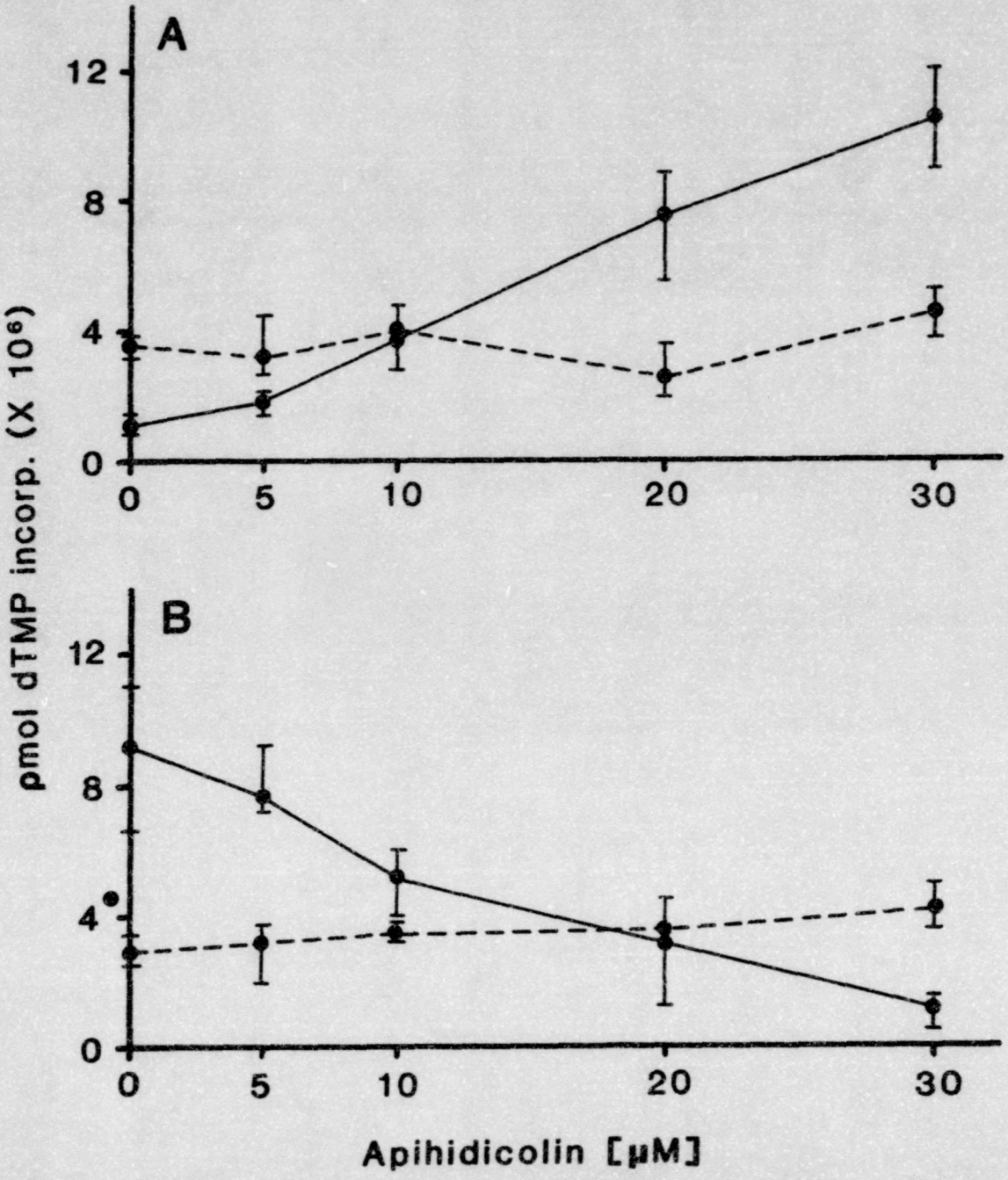


Figure 21: SDS-polyacrylamide gel of soybean purification steps G-50 through H-S (A). Reapplied concentrated electro-eluted protein bands S_1 and S_3 (B). Accompaning molecular weight marker is composed of the following: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), beta-galactosidase (116 kDa), and myosin (205 kDa).

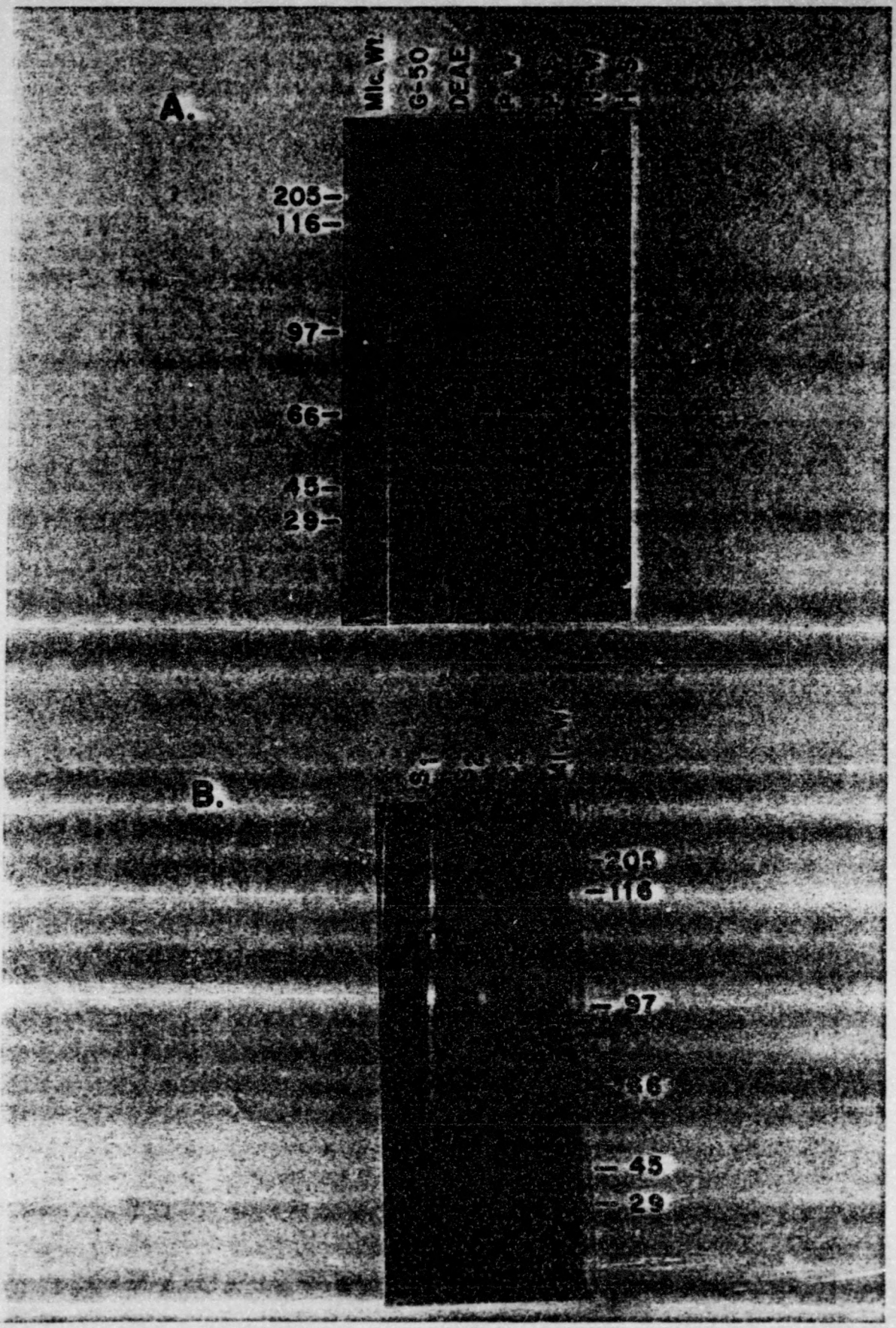
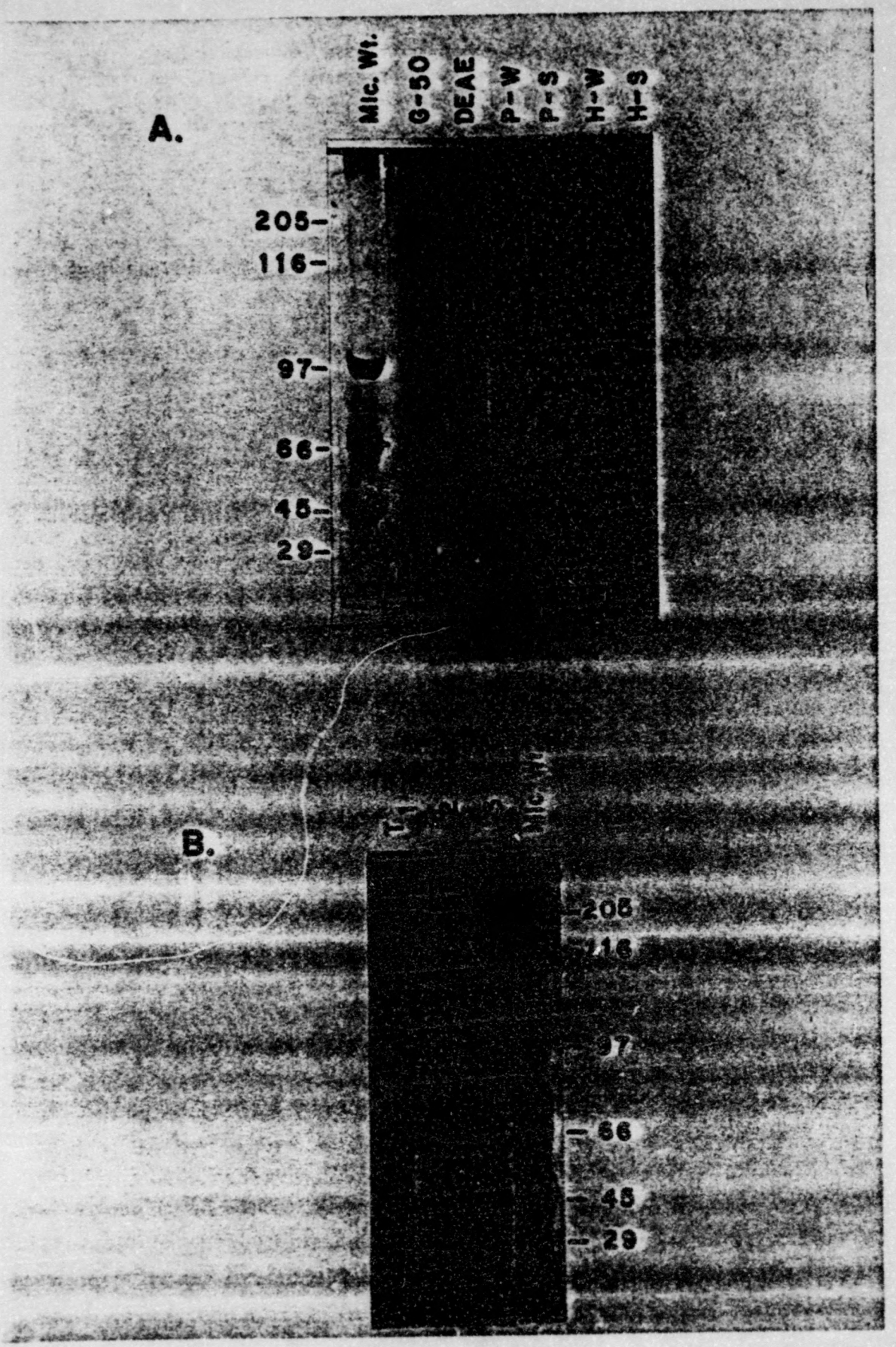

A.
Mic. Wt.
G-50
DEAE
205
116
97
66
45
29
B.
205
116
97
66
45
29

Figure 22: SDS-polyacrylamide gel of turnip purification steps G-50 through H-S (A). Reapplied concentrated electro-eluted protein bands T_1 and T_3 (B). Accompaning molecular weight marker is composed of the following: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), beta-galactosidase (116 kDa), and myosin (205 kDa).


A.
Mic. Wt.
G-50
DEAE
P-W
P-S
H-W
H-S
205-
116-
97-
66-
45-
29-
B.
208
116
97
66
45
29

To further characterize the electro-eluted proteins they were concentrated and applied to a SDS-polyacrylamide gel. All of the protein bands present are listed in Table 5. Several protein bands were shared by both enzymes. By a plot of relative mobility of known molecular weight markers (Figure 23), the molecular weights of the catalytically active electro-eluted proteins, S_1 and S_3 of soybean and T_1 and T_3 of turnip, were determined as shown in Table 6. As stated previously, the catalytic activity of the heavier molecular weight bands is unknown since they did not independently appear in the non-denaturing gels. In both plant systems the T_1 and S_1 electro-eluted proteins were approximately the same molecular weight, 64 to 66 kDa. Electro-eluted proteins representing T_3 and S_3 were also of the same molecular weight (43 to 45 kDa) in both the turnip and soybean plant systems.

Figure 23: Log plot of known molecular weight versus relative mobility from an SDS-Page gel.

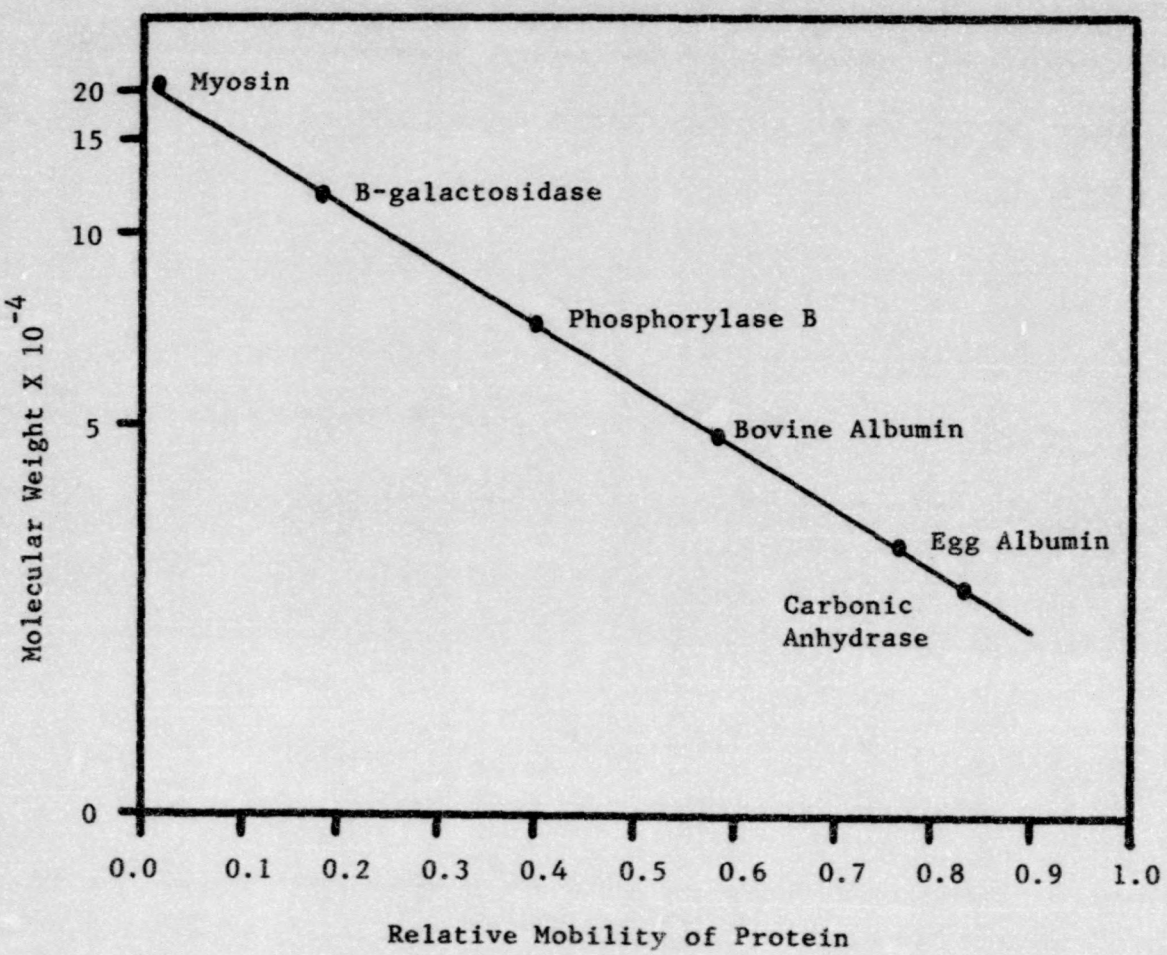

Myosin
B-galactosidase
Phosphorylase B
Bovine Albumin
Egg Albumin
Carbonic
Anhydrase
20
15
10
5
0
Molecular Weight X 10^{-4}
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Relative Mobility of Protein

Table 5: SDS-PAGE Molecular weight Determination of all protein bands associated with fractions S_1/S_3 and T_1/T_3 in kDa.

S_1	S_3	T_1	T_3
	205		
116		116	116
		100	100
97		95	95
66*	66	66*+	66
55		64*+	64
46	46*+	46	46*+
43	43*+		43*+
		29	
		20	20

* one of two catalytically active proteins which appeared in non-denaturing gels

+ bands were too close together to extract and assay individually for catalytic activity in non-denaturing gels

Table 6: SDS-PAGE Molecular Weight Determinations of the Catalytically Active Electro-eluted Protein Fractions.

	S_1	S_3	T_1	T_3
Relative Mobility	0.58	0.75	0.57	0.77
Molecular Weight (KDa)	64	45	66	43

Discussion

To characterize the enzymatic activites of DNA polymerase alpha and gamma in plants, an *in vitro* assay system was employed (Dunham and Bryant, 1986). Enzymes such as DNA polymerase alpha and gamma are capable of synthesizing new strands of DNA when supplied with the required components: nucleotides, appropriate template-primer systems, proper salt concentrations, and divalent cations (Sala et al., 1981). The relative activities of the protein subunits were monitored by the quantitative detection of incorporated thymidine (H^3) using the appropriate template-primer system.

The elution profiles for both DNA polymerase alpha and gamma, for soybean and turnip, from the DEAE-cellulose, phosphocellulose, and hydroxylapatite columns were as expected from data concerning animal systems (Bolden et al., 1977; Filpula et al., 1982; Yamaguchi et al., 1982). Similar DEAE-cellulose elution profiles were observed in plant systems (Chivers and Bryant, 1983; Dunham and Bryant, 1986). The enzymatic activities associated with the eluted protein prior to the salt gradients were due to the overloading of the columns. This was proven by simply applying less protein to the exchange columns which eliminated the enzymatic activities in the column wash protein peak.

Kinetic studies using specific template-primer systems agreed with data previously published (Dunham and Bryant, 1986). The S_1 fraction of the soybean preparation expressed the catalytic activity for DNA polymerase

gamma and S_3, the catalytic activity of DNA polymerase alpha. The catalytic activity of turnips T_1 fraction did not fully support T_1 as being DNA polymerase gamma. However, the incorporation of thymidine was quantitatively higher for the in vitro assay using the synthetic RNA template-deoxy primer than for that which contained the activated DNA template. The T_3 fraction catalytically functioned in thymidine incorporation as DNA polymerase alpha.

Aphidicolin inhibition studies support the presence of DNA polymerase alpha activity by its specific inhibition of the enzyme as originally reported by Spadari and co-workers (1982) and Sala and co-workers (1985). Any effect of aphidicolin on the DNA polymerase gamma activity was probably due to the sensitivity of the initial elongation of the RNA primer to aphidicolin (Sala et al., 1980; 1985) while not effecting the actual DNA polymerase gamma protein. The DNA polymerase alpha assays of the T_3 and S_3 fractions were substantially inhibited by the presence of aphidicolin while DNA polymerase gamma activities remained low and unaffected. This supports T_3 and S_3 as being DNA polymerase alpha proteins.

The responses of the DNA polymerase alpha assay activitites of T_1 and S_1 to aphidicolin were quite different. In both T_1 and S_1, the DNA polymerase gamma activities were unaffected by aphidicolin and initially remained higher in activity than DNA polymerase alpha activities. The DNA polymerase alpha activity of S_1 remained lower than its DNA polymerase gamma activity with increasing aphidicolin concentration. However, the DNA polymerase alpha activity of T_1 gradually increased with increasing aphidicolin concentration and at 10um exceeded its DNA polymerase gamma activity.

Why T_1 exhibited a preference for a template-primer system different than that of S_1 is unclear. DNA polymerase gamma is an organellular enzyme, present in the mitochondria and chloroplast (Litvak and Castroviejo, 1987; Lestienne, 1987). The protein representing T_1 was harvested from light grown plants, whereas S_1 was from etiolated plants. Perhaps the active chloroplasts in T_1 were contributing a different variation of DNA polymerase gamma than that found in the "unactive" chloroplast from the dark-grown soybeans. This variant of DNA polymerase gamma may be able to utilize the activated DNA template and Mg^{+2} in the DNA polymerase alpha assay system more readily than the synthetic RNA template and Mn^{+2} in the DNA polymerase gamma assay system in the presence of aphidicolin. The overall results of the aphidicolin experiments, however, support T_1 and S_1 as being DNA polymerase gamma proteins.

Additional support for T_3 and S_3 being DNA polymerase alpha proteins and T_1 and S_1 DNA polymerase gamma proteins comes from a study of their divalent cation preference (Dunham and Bryant, 1986). The enzymatic activities of T_3 and S_3 exhibit a preference for $Mg+^2$ as their divalent cation which is a characteristic of DNA polymerase alpha. T_1 and S_1 prefer $Mn+^2$ indicating that their enzymatic activities are that of a DNA polymerase gamma.

In animal systems there are a variety of molecular sizes attributed to the catalytic polypeptides of the holoenzyme of DNA polymerase alpha (150 to 1000 KDa) and the core proteins of DNA polymerase alpha, which range from 68 to 180 KDa (Masaki et al., 1985; Vishwanatha et al., 1986; Yagura et al., 1986; Prussak and Tseng, 1987; Zahradka, 1987). These molecular weights were determined from proteins which had been isolated using an antibody-affinity column system. The majority of the reports agree on the

presence of a core polypeptide of approximately 70 kDa and an associated primase of 50 kDa. A 97 kDa catalytic polypeptide isolated from HeLa cells is actually a dimer of a 47 kDa catalytic polypeptide and an associated primase of 70 kDa (Vishwanatha et al., 1986). In spinach, a core DNA polymerase alpha polypeptide has been isolated which has a molecular weight of 160 kDa with dissociated subunits of 70 kDa (Misumi and Weissbach, 1982). Therefore, the most plausible explanation for T_3 and S_3 DNA polymerase alpha activitites is that one of the two bands which appear in the electrophoretic runs of the electro-eluted T_3/S_3 protein band (Figure 21B and 22B) represents the primase and the other band a degradation product of the DNA polymerase alpha core enzyme which is still catalytically active (gels were run two days after electro-elution of proteins). The larger molecular weight bands present in Figures 21A and 22A may represent the holoenzymes of the soybean (205 and 110 kDa) and turnip (105 and 97 kDa) DNA polymerase alpha and or gamma proteins, which have degraded to the smaller core proteins in Figures 21B and 22B. In future studies the association of a primase with the T_3/S_3 proteins can be determined by exposing the possible primase protein to aphidicolin, since it is known that primase associated with DNA polymerase alpha is resistent to aphidicolin (Sala et al., 1985).

DNA polymerase gamma has been reported in the literature to be a catalytic polypeptide of 87KDA $\pm$ 20 kDa in plant systems (Sala et al., 1980; Litvak and Castroviejo, 1987). The molecular weight of the isolated protein which has DNA polymerase gamma activity in both turnip and soybean is approximately 66 kDa, well within the molecular weight range reported in

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