Masters Theses & Specialist Projects   Graduate School

5-1989

Isolation & Characterization of a Type I Topoisomerase from the Hypocotyls of Etiolated Soybeans

Rick Dye
Western Kentucky University

Follow this and additional works at: https://digitalcommons.wku.edu/theses

Part of the Agricultural Science Commons, Agronomy and Crop Sciences Commons, and the Biology Commons

Dye,

Rick B.

1989

ISOLATION AND CHARACTERIZATION OF A

TYPE I TOPOISOMERASE FROM THE HYPOCOTYLS

OF ETIOLATED SOYBEANS

A thesis

Presented to

the Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Rick B. Dye

May, 1989

ISOLATION AND CHARACTERIZATION OF A

TYPE I TOPOISOMERASE FROM THE HYPOCOTYLS

OF ETIOLATED SOYBEANS

Recommended _November 10, 1989_
(date)

Valgene L. Dunham
Director of Thesis

Frank Toman

Martin R. Houston

Approved _November 10, 1989_
(date)

Elmer Gray
Dean of the Graduate College

AUTHORIZATION FOR USE OF THESIS

Permission is hereby

[x] granted to the Western Kentucky University Library to make, or allow to be made photocopies, microfilm or other copies of this thesis for appropriate research or scholarly purposes.

[ ] reserved to the author for the making of any copies of this thesis except for brief sections for research or scholarly purposes.

Signed _Rich B Hye_

Date _11/10/89_

Please place an "X" in the appropriate box.

This form will be filed with the original of the thesis and will control future use of the thesis.

ACKNOWLEDGMENTS

I would like to thank Dr. V. L. Dunham for serving as my thesis advisor. His encouragement stimulated me to return to school to seek my Master's Degree and his enthusiasm for research and the pursuit of knowledge guided my thesis project.

I would also like to thank the other members of my graduate committee, Dr. Frank Toman and Dr. Martin Houston, for their inspiration, instruction, and encouragement.

I would like to acknowledge the help and patience of Dr. Robley Williams of Vanderbilt University for his understanding and assistance and Dr. Neil Osheroff of Vanderbilt for help with photography and densitometry.

I dedicate this thesis to my loving wife and children and to my wonderful and understanding parents and brothers without whose support I could never have completed this endeavor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ISOLATION AND CHARACTERIZATION OF A
TYPE I TOPOISOMERASE FROM THE HYPOCOTYLS
OF ETIOLATED SOYBEANS

Rick B. Dye   May, 1989   80 pages

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

Department of Biology   Western Kentucky University

Topoisomerases are enzymes of critical biological significance. Despite this fact, little data specific to the topoisomerases of higher plants has been published. This research was undertaken to isolate and characterize a higher plant topoisomerase from soybean hypocotyls to further the understanding of the structure and function of these enzymes.

Nuclei were isolated from frozen hypocotyls of 4 day old etiolated soybeans by homogenization and centrifugation, then lysed by gentle stirring in the presence of 1.5 mM ammonium sulfate. The resultant extract was desalted and purified by column chromatography on DEAE sepharose, S-200 sephacryl and CM cellulose. The components of the purified fraction were separated by electrophoresis on non-denaturing polyacrylamide and recovered by electro-elution.

The molecular weight of the native enzyme was determined to be 225 kilodaltons (Kd) by gel filtration and 300 Kd by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Three smaller molecular sub-species, possibly resulting from

dissociation of the native enzyme in the presence of beta mercaptoethanol (BME), were isolated. These smaller molecules had approximate molecular weights of 155 Kd, 29 Kd and 25 Kd. The two smaller polypeptides appeared to re-associate to form a 68 Kd aggregate in the absence of BME. This aggregate was subsequently resistant to denaturation by sodium dodecyl sulfate (SDS).

The purified native enzyme was found to be adenosine triphosphate (ATP) and Mg^{++} (divalent magnesium ion) independent. Although not required, the presence of magnesium (Mg^{++}) stimulated enzyme activity. Manganese (Mn^{++}) stimulated activity to a lesser degree. Enzymatic activity was inhibited by ATP, potassium chloride (KCl) and N-ethylmaleimide (NEM) but not inhibited by novobiocin.

The results indicate that a type I topoisomerase was purified from the nuclei of soybeans (Glycine max). The soybean topoisomerase I has a native molecular weight similar to that of cauliflower (200 Kd) and like cauliflower topoisomerase was inhibited by NEM but not by novobiocin and was stimulated by Mg^{++}. It is similar to vaccinia virus topoisomerase I in being slightly inhibited by ATP. The data suggests that the native enzyme may be composed of enzymatically active domains as small as 27 Kd and thus is similar to vaccinia virus and Ustilago maydis topoisomerase I.

INTRODUCTION

Soon after Watson and Crick discovered the double-
stranded, helical nature of DNA, researchers began to realize
that this structure imposes certain topological restrictions
upon replication. To replicate the molecule, the two strands
of the helix must be unwound. This requires that the molecule
rotate as the unwinding proceeds to relieve the torsion
(supercoiling) that results. With the later discovery that the
genome of Escherichia coli was composed of a single,
covalently-closed, circular molecule, it became evident that
supercoiling must be relieved in front of the replication fork.
The problem can be compared to separating the strands of a
circle of tightly laid rope. This discovery initiated a search
for the "swivel" involved in DNA replication which led to the
discovery of enzymes capable of reducing torsional strain in
covalently-closed DNA molecules. The first such enzyme
discovered was the "Omega" protein of Escherichia coli (Wang,
1971). This and other enzymes like it have been renamed DNA
topoisomerases because they control and modify the topological
states of DNA.

1

Supercoiling is an important molecular characteristic of circular DNA molecules and DNA loops with fastened ends. Any appreciable added or subtracted twist changes the rotation angle of the bases in the helix and leads to some degree of supercoiling. Added twists mean a decrease in the rotation angle of the bases and therefore more bases per turn of the helix while the reverse is true of subtracted twists. If the phosphodiester backbone of one strand of a circle is broken and one twist is inserted or removed before re-ligating the backbone, then one supercoil either negative or positive will have been inserted into the circle. Each of these supercoils can be seen under electron microscopy as a crossover in the circle (Vinograd et al., 1965; White et al., 1987). The numerical value and sign of the twists in a molecule are known as the linking number and it is this linking number which is altered by the action of DNA topoisomerases. A linking number of zero denotes a relaxed molecule (36 degree base angle of rotation) while a linking number of +2 means that positive torsional strain or positive supercoiling has been applied to the relaxed molecule by the introduction of two complete twists (see Figure 1).

All topoisomerases change the topological character of double-stranded DNA through an event that involves transient strand breakage and are divided into two classes based upon their method of cleavage.

Class I topoisomerases catalyze the transient breakage of a single strand of a double-stranded DNA molecule then pass a

FIGURE 1: Linking number changes in plasmid DNA. The forward reaction at the top is catalyzed by gyrase using the energy of ATP. The reverse reaction at the bottom is catalyzed by topoisomerase I.

0   −1   −2   −4
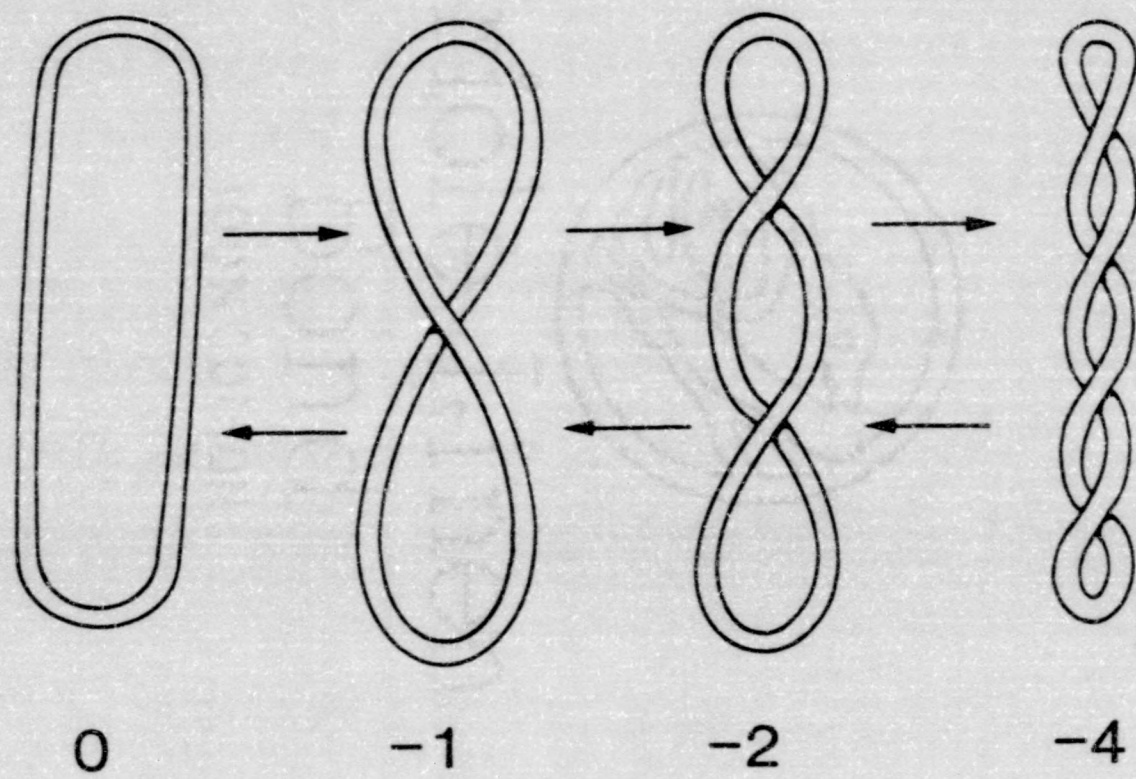
single-stranded section of the same molecule through the gap and ligate the first strand. Since Class I topoisomerases do not require a source of energy, it is postulated that the energy of the cleaved phosphodiester bond must be conserved and used in the ligation reaction. The linking number of the substrate molecule is altered by an increment of one at each strand passage event.

Class II topoisomerases catalyze linking number changes by increments of two. The energy of ATP is required to transiently cleave both strands of DNA, pass another section of the molecule through the gap, and ligate the break.

Reactions of both Class I and Class II enzymes create an intermediate product by the covalent attachment of the enzyme via a phosphotyrosine bond to one end of the cleaved DNA strand (Wang, 1985; Blair and Helinski, 1975; Osheroff and Zechiedrich, 1987).

In addition to the control of supercoiling in DNA, topoisomerases are capable of reactions involving the catenation and de-catenation of circular molecules (Goto and Wang, 1982). Catenation is the linking together of circles into a chain while de-catenation is the reverse reaction, which by necessity, involves the breakage of both strands, passage of a section of another circle through the gap, and ligation. Class II enzymes can catenate native double-stranded DNA but because Class I enzymes cleave only a single strand they require nicked substrates to catalyze the same reaction (Low et al., 1984).

Although the complete roles are at present unclear, topoisomerases are implicated in numerous reactions with critical biological significance. The degree of supercoiling of DNA has biological significance with regard to gene control. Supercoiling effects the tendency of the two strands of native, duplex DNA to dissociate cr "melt" over regions of decreased base pair energy. Because AT base pairs have lower bond energy than GC base pairs, AT-rich regions are the first to dissociate. Positive supercoiling results in a strand which is resistant to melting. Negative supercoiling, on the other hand results in a strand which melts more easily. Furthermore, a single-stranded AT-rich area in a negatively supercoiled circular molecule can relieve the torsional strain and allow the rest of the molecule to assume it's normal 36 degree rotational angle (Figure 2). If an AT-rich area of DNA is also characterized by internal dyad symmetry (a palindrome), then melting can be followed by the formation of stem-and-loop structures. Origins of replication and regulatory genes are known to contain such AT-rich palindromic sequences. Both melting of regulatory sequences and stem-and-loop structures have been implicated in such functions as transcription, replication, and recombination.

Other biologically important topological features of DNA can be effected by the catalytic activity of topoisomerases. The packaging of eukaryotic DNA around nucleosomes to form chromatin involves the formation of coils within the DNA molecule. Because eukaryotic DNA is organized into loops called replicons that are

firmly attached to the nuclear matrix, the introduction of coils changes the linking number of the DNA involved. Nucleosome packaging in eukaryotes therefore involves changes in the linking number of the DNA involved.

Native double-stranded DNA, also known as B-DNA, exists as a right-handed helical molecule. However, it has been known for some time that GC-rich regions of DNA may exist as left-handed helices which possess a unique zig-zag configuration (Z-DNA) (Pohl and Jovin, 1972). These poly-GC sequences will shift from B to Z-DNA under the torsional effect of negative supercoiling (Peck et al., 1982) and can be stabilized in this configuration by DNA binding proteins or by methylation of the G and C bases (Behe and Felsenfeld, 1981). Widespread areas of naturally occurring Z-DNA have been found in most organisms studied to date and indeed there are over 230 such areas in the E. coli genome.

There is specific evidence to implicate topoisomerases in biological functions in eukaryotic organisms, some of which is at present contradictory and confusing. Despite this, the role of topoisomerases in some in vivo functions in eukaryotes is widely accepted. These include: resolution of daughter chromosomes during mitosis by topoisomerase II (DiNardo et al., 1984), involvement of topoisomerase I in initiation of transcription through control of negative supercoiling (Garg et al., 1987; Kaguni and Kornberg, 1984), involvement in initiation of replication through negative supercoiling (Fuller et al., 1981), and relief of supercoiling in front of the replication fork

FIGURE 2: Negative supercoiling in covalently closed circular DNA
can be relieved by local disruption of strand pairing.

ss DNA
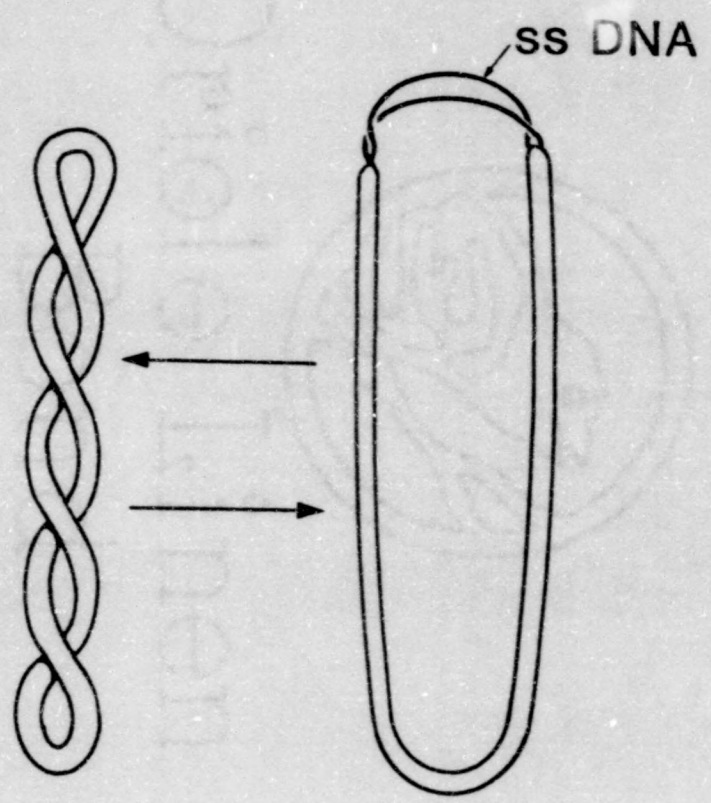
(Jazwinski and Edelman, 1984; Jazwinski, 1987; Nakamura et al., 1986). In addition, there is evidence to support the concept that eukaryotic topoisomerases may be involved in certain less well substantiated functions including: unpackaging and packaging of DNA into nucleosomes (Bryant and Dunham, 1988), catenation and decatenation of naturally occurring interlocked ring structures (in trypanosomes), control of transcriptional elongation through action as a "swivel" (Fleishman et al. 1984), recombination (Halligan et al. 1982), excision of integrated viral elements, relocation of transposable elements, and ultraviolet damage repair. Of these possible roles, the most conclusively proven are the absolute requirement for topoisomerase II for the resolution of daughter chromosomes and the involvement of topoisomerases I in transcription through site-specific control of supercoiling (Wang, 1985; Bryant and Dunham, 1988).

Nuclear topoisomerase activities have been reported from a number of organisms (Gellert, 1981; Wang, 1985; Bryant and Dunham; 1988). Generally speaking eukaryotic class I topoisomerases identified to date are monomers varying from 67 to 100 kilodaltons (Kd) which do not have an absolute requirement for but are never-the-less stimulated by divalent cations. Class II topoisomerases have usually been found to be dimers varying from 300 to 325 Kd with an absolute requirement for a divalent cation (Mg is always optimal).

Tables I and II present the results of characterization studies to date on all forms except those from higher plants which will be dealt with separately.

Topoisomerases have been isolated from mitochondria (Echeverria et al. 1986) and chloroplasts (Siedlecki et al. 1983; Nielson and Tewari, 1988) of higher plants. In keeping with the theory of the evolutionary origin of cell organelles, these enzymes show more similarity to prokaryotic than to eukaryotic topoisomerases and will not be discussed here.

Nuclear topoisomerases from higher plants have been isolated and characterized only from wheat germ (Dyan et al. 1981) and cauliflower inflorescence (Fukata and Fukasawa, 1982 and 1986). Table III below summarizes data concerning these enzymes.

No one will refute the statement that higher plants are organisms of incalculable value and importance to mankind. With that in mind, this research was undertaken to further the understanding of the structure and function of higher plant topoisomerases by isolating to near homogeneity and characterizing a topoisomerase from soybean (Glycine max). As can be seen from the introductory material, higher plant topoisomerases are critically important enzymes about which very little specific information is known. Topoisomerases, because they are essential for DNA replication, are necessary for plant nuclear, mitochondrial, and chloroplastic function. They are also involved in the replication of plant viral DNA and may be

TABLE I

Eukaryotic Class I Topoisomerases

Species or Cell type	Molecular Weight	Subunit Structure	Cation Requirement	Reference
Saccharomyces cerevisiae	90 Kd	Monomeric	None	a
Ustilago maydis			None	b
Plasmodium berghei	70-100 Kd	Monomeric	K or Na	c
Drosophila melanogaster	100 Kd			d
Xenopus laevis	67 Kd	Monomeric	None	e
Human lymphoblastoma	97 Kd			f

a. Goto et al., 1984
b. Rowe et al., 1981
c. Riou et al., 1986
d. Javaherian et al., 1982
e. Attardi et al., 1981
f. Andoh et al., 1987

TABLE II

Eukaryotic Class II Nuclear Topoisomerases

Species or Cell type	Molecular Weight	Subunit Structure	Optimal Cation	Reference
Saccharomyces cerevisiae	328 Kd	Homodimer	Mg++	a, b
Plasmodium berghei	320 Kd	Homodimer	Mg++	c
Drosophila melanogaster			Mg++	d, e
Calf thymus	300-325 Kd	Heterodimer		f
Human leukemia cells	180 Kd 170 kd	Two forms reported		g

a. Goto et al., 1984
b. Giaever et al., 1986
c. Riou et al., 1986
d. Osheroff et al., 1983
e. Hsieh and Brutlag, 1980
f. Schomburg and Grosse, 1986
g. Drake et al., 1987

TABLE III
Nuclear Topoisomerases of Higher Plants

CLASS I TOPOISOMERASE

Species or Cell type	Molecular Weight	Subunit Structure	Cation Requirement	Inhibited by	Reference
Cauliflower	200 Kd	Unknown	None	EDTA 3 mM NEM (N, ethylmaleamide)	a
Wheat germ					c

CLASS II TOPOISOMERASE

Species or Cell type	Molecular Weight	Subunit Structure	Cation Requirement	Inhibited by	Reference
Cauliflower	223 Kd	Unknown	10 mM Mg++	EDTA 2 mM NEM spermidine novobiocin	b

a. Fukata and Fukasawa, 1982
b. Fukata and Fukasawa, 1986
c. Dyan et al., 1981

involved in many other crucial plant cellular functions including genetic recombination and repair. It is hoped that this research will add to the body of specific information regarding plant topoisomerases and make it somewhat less necessary to draw inferences regarding higher plant topoisomerase characteristics and functions from research on prokaryotic and animal topoisomerases.

MATERIALS AND METHODS

MATERIALS

Unless otherwise specified, all chemical and chromatography materials were purchased from Sigma Chemical Company, St. Louis, Missouri. Seeds of Glycine max, variety SB 4000, were purchased from Stewart Seed Company, Greensburg, Indiana. Vermiculite used in seed germination was Terra-Lite, coarse grade.

PLANT TISSUE

Soybean (Glycine max) seeds were germinated and grown for 4 to 6 days in the dark using the following protocol. A beaker was used to measure approximately 400 ml of seed which was placed on top of a bed of 2000 ml of vermiculite in a 12" x 24" non-drain plastic flat. The seeds were covered with an additional 2500 ml of vermiculite and sprinkled with 2000 ml of tap water. Flats were placed either in an incubator at 31^0 C. under high humidity or were covered with another flat to exclude light, wrapped in black plastic and placed in a greenhouse. Sections approximately 1-2 cm long of the actively growing region (hypocotyl and attached epicotyl, if elongated) were harvested, immediately chilled to 4^0 C, weighed, wrapped in parafilm, and frozen.

14

BUFFERS

Buffers referred to in the text were of the following

compositions:

Homogenization Buffer
     50 mM Tris - HCL, pH 8.0
   600 mM sucrose
    10 mM 2-mercaptoethanol (BME)
     5 mM phenylmethyl-sulfonyl fluoride (PMSF)

Solubilization Media for Nuclei
    40 mM Tris - HCl, pH 8.0
   1.5 mM ammonium sulfate
  0.02 mM BME
  0.02 mM magnesium chloride ($MgCl^2$)

Equilibration Buffer for Column Chromatography
   100 mM Tris - HCl, pH 8.0
     5 mM BME
   0.2 mM PMSF
    25% glycerol

Topoisomerase Assay Buffer
    50 mM Tris - HCl, pH 7.5
    10 mM $MgCl_2$

Agarose Gel Electrophoresis Buffers
    40 mM Tris - HCl, pH 7.8
    20 mM sodium acetate
     2 mM ethylenediamine tetra-acetate (EDTA)
           or
    89 mM Tris - HCl, pH 7.8
    89 mM borate
    10 mM EDTA

Polyacrylamide Gel Electrophoresis Buffer
    25 mM Tris - HCL, pH 8.3
   192 mM glycine
  0.1% lauryl sulfate (SDS) (denaturing gels only)

TOPOISOMERASE ISOLATION

Extraction of Soluble Proteins

Isolation of soybean nuclei was by the method of Dunham and Bryant (1986). All steps of the protocol were carried out at 4° C. Frozen soybean hypocotyls (40-50 g) were placed in cold homogenization buffer (3 ml/g of tissue) with 2-3 drops of anti-foam agent and homogenized in a Sorval Omni-mixer on setting six for 30 s.

The resulting homogenate was filtered through two layers of miracloth (Calbiochem) arranged with fibers at right angles and pre-wet with cold homogenization buffer. Each preparation was allowed to drain 15 min then very gently squeezed to extract remaining liquid. The filtrate was collected and centrifuged at 2500 x g for 30 min in a refrigerated centrifuge at 4° C.

The pellet containing nuclei was re-suspended in 1-2 ml solubilization media. Re-suspension was accomplished by stirring with a glass rod, gently drawing up three times into a pasteur pipette and followed by 6 strokes in a Wheaton 7 ml Dounce homogenizer using pestle A. Tubes were rinsed with an additional 1 ml of solubilization media.

The re-suspension was transferred to a 10 ml pyrex beaker and gently stirred for 2 h to lyse nuclei and suspend soluble components. The lysate was centrifuged at 35,000 x g for 30 min to remove insoluble debris. The supernatant containing extracted nuclear proteins was drawn off with a pasteur pipette and saved for further purification by column chromatography.

Column Chromatography

G-50 Sephadex

Small molecules were removed from the crude nuclear extract by passing samples through Sephadex G-50 columns (prepared by pre-soaking dry G-50 in equilibration buffer for 48 h) then packed in a 3 cm diameter plastic column to a height of 7 cm. Samples containing the highest protein content as determined by absorbance at 280 nM were pooled (Figure 3).

DEAE Sepharose

The anion exchange column was prepared using pre-swollen DEAE Sepharose. To ensure thorough packing, beads were suspended in approximately 500 ml of equilibration buffer and gently stirred while being pumped with a peristaltic pump through a 2 cm column at a flow rate of 2-3 ml/min. In order to obtain uniform suspension of the slurry, it was necessary to maintain constant stirring during packing. The prepared column measured 2 x 10 cm and was washed with at least 10 volumes of equilibration buffer before use.

Desalted enzyme preparations were applied to the column at a flow rate of less than 1 ml/min followed by washing of the column with at least twice the measured bed volume of the column or until absorbance readings at 280 nm reached a minimum.

Bound proteins were eluted either with a 0.0 to 1.0 M KCl linear gradient or a 0.7 M KCl step elution. A volume of equilibration buffer (containing KCl) equal to at least twice the void volume of the column was used for the elutions. Equal

FIGURE 3: A typical G-50 column profile. The column was used to desalt and remove small molecules from crude nuclear extracts. The indicated fractions were pooled and subjected to further purification.
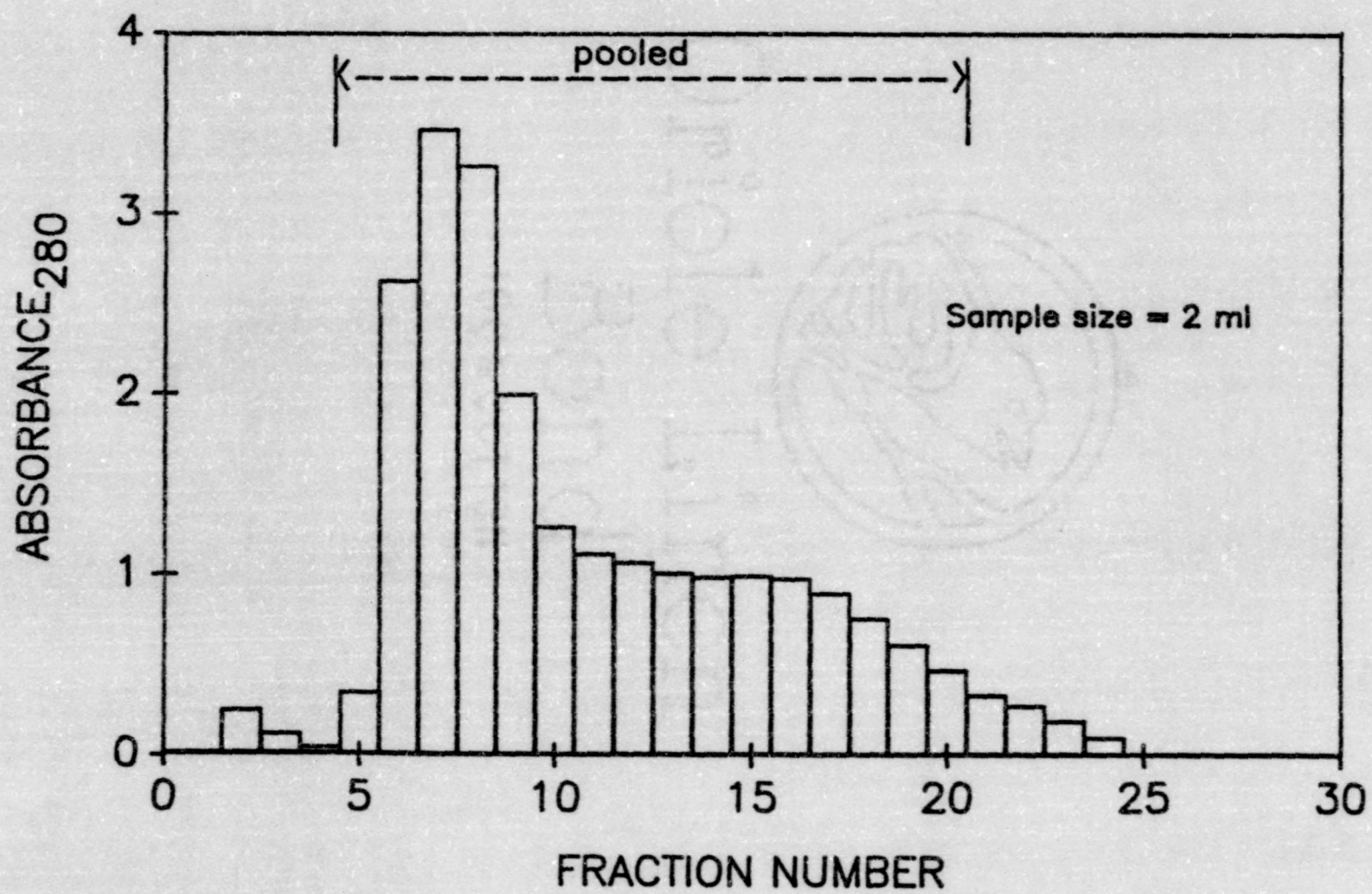
fractions of 2-4 ml were collected, read for absorbance at 280 nm and assayed for topoisomerase activity.

CM Cellulose

A cation exchange column was prepared by stirring dry CM cellulose in equilibration buffer for 48 h. The column was then packed at a flow rate of 1 ml/min by pumping the slurry through a 2 cm diameter column to a final column height of 10 cm. In order to maintain uniform suspension of the slurry, it was necessary to maintain constant, slow stirring during packing. The column was washed with at least 10 volumes of equilibration buffer before use. Application of enzyme to the column and elution of the bound fraction was identical to the procedure already described for DEAE Sepharose. Uniform samples of 2-4 ml were collected, read for absorbance at 280 nm and assayed for topoisomerase activity.

Sephacryl S-200

Proteins were separated by molecular weight using a column prepared from pre-swollen Sephacryl S-200. Proper column packing was ensured by mixing the pre-swollen beads with 500 ml of equilibration buffer and pumping the gently stirred slurry through a 2 cm column at a flow rate of 1 ml/min. The column was washed with at least 10 volumes of equilibration buffer before use. Concentrated samples of 1-3 ml were applied to the column and followed by equilibration buffer at a flow rate of 1 ml per

minute. Uniform samples of 2-4 ml were collected, read for

absorbance at 280 nm, and assayed for topoisomerase activity.

Electrophoretic Separation of Proteins

Following separation by column chromatography, proteins

were further separated on non-denaturing, polyacrylamide gels of

the type described by Laemmli (1970). These gels consisted of a

lower resolving gel and an upper stacking gel. All such gels

were mixed using three stock solutions, the compositions of which

were:

Stock A (Acrylamide)

29.2 g acrylamide
0.8 g bis acrylamide
De-ionized (DI) water to 100 ml

Stock R (Resolving Buffer)

1.5 M Tris - HCl, pH 8.8

Stock S (Stacking Buffer)

1.0 M Tris - HCl, pH 8.8

The stacking and resolving gels were mixed from these

stock solutions as follows:

3% Stacking Gel - 1.5   ml stock A
                   7.05   ml DI water
                   0.1   ml 11% ammonium persulfate[1]
                   1.25   ml stock S
                   0.005 ml TEMED[2]
                   0.1   ml DI water
                   10%   lauryl sulfate (SDS)[3]
                   _____
                   10.005 ml total volume

```
9% Resolving Gel -   9.0    ml stock A
                    13.1    ml DI water
                     0.15   ml 11% ammonium persulfate[1]
                     7.5    ml stock R
                     0.015  ml TEMED[2]
                     0.3    ml DI water
                    10%     SDS[3]
                    _____
                    30.065 ml total volume
```

Notes:
1. (N,N,N1,N1-tetramethylethylenediamine)
2. Used only for denaturing gels.
3. To be made no more than 1 h in advance.

All gels were 16 x 20 cm vertical stab gels, 1.0 mm thick.
When mixing gels, ammonium persulfate and TEMED were added last
because they cause polymerization to begin. Gels were
pre-electrophoresed at 50 mA for 2 h to remove free ammonium
persulfate, and the buffer was changed before loading. Samples
were prepared for loading by the addition of a solution of 40%
glycerol and 0.25% bromphenol blue in electrophoresis buffer to
bring final concentrations to 8% glycerol and 0.05% bromphenol
blue. Non-denaturing gels for protein separation were run at 40
mA or lower. Electrophoresis buffer was changed in order to
prevent acidification of the solution when erratic amperage
indicated buffer exhaustion. This required buffer changes at
intervals of 4-5 h.

Recovery of Proteins from Polyacrylamide Gels

Protein bands were recovered from polyacrylamide gels by a
process that involved silver staining of the reference lane and a
1 cm wide band on either side of the preparatory well, placing
the stained sections alongside the unstained gel center, and

carefully cutting out the bands from the unstained section of the gel. These bands from the unstained section were then electrophoresed at 4° C using a Bio-Rad Electro-Eluter apparatus. The apparatus was prepared and loaded according to instructions contained in the operation manual. In addition, it was important to wear gloves at all times during loading and unloading of the apparatus to prevent contamination of the gels or membranes. The electrophoresis buffer was the same as that listed for polyacrylamide gel electrophoresis (see page 16), and the electro-elution was carried out at 10 mA per tube for 6-8 h. The Bio-Rad operation manual indicated that, if the gel level in the tubes exceeded 1 cm, additional time might be required to elute all the protein. Because some tubes were filled to a height of 5-6 cm, the elution was repeated. Protein was carefully removed according to the Bic-Rad instructions after each 6-8 h period.

PROTEIN DETERMINATION METHODS

Relative protein content of fractions in column profiles was determined by reading absorbance of the sample at 280 nm. Protein content of samples for the purpose of specific activity determinations was calculated by the method of Waddell (1956) and checked using a Bio-Rad colorimetric protein determination kit. All spectrophotometry for both methods was done on a Gilford Response spectrophotometer. It was necessary to

calculate and use cuvette offsets when using the multiple cuvette holder to obtain accurate results.

PROTEIN CONCENTRATION METHODS

When necessary, sample pools were concentrated by one of two methods, depending upon volume. Samples of over 10 ml were concentrated using Centricell centrifugal ultrafilter concentrators (Polysciences Incorporated) with a retention limit of 10,000 daltons in a swinging bucket clinical centrifuge at 1,500 x g at 4° C. Samples of 10 ml and under were concentrated using Amicon Centricon microconcentrators with a retention limit of 10,000 daltons in a Beckman refrigerated centrifuge (JA 20 rotor) at 5,000 x g at 4° C.

ASSAY FOR TOPOISOMERASE ACTIVITY

Enzyme activity was assayed using the procedure of Fukata and Fukasawa (1986), based on the relaxation of negatively supercoiled pBR322 DNA. The DNA was electrophoresed in agarose and stained with ethidium bromide. The composition of the standard reaction mixture and stock solutions is shown in Table IV. The standard reaction volume was 25 ul. However, a minigel apparatus was at times employed using gels poured on 2.6 x 6 cm glass microscope slides. The wells in these gels hold only 10 ul so the reaction volume was reduced to 12.5 ul when the minigel was used. All volumes shown in Table IV were halved for the 12.5 ul assay. Use of the minigel apparatus saved both time and

TABLE IV

Assay Component	Stock Concentration	ul of Stock Added	Final Concentration
Tris-HCl buffer, pH 7.5	250 mM Tris 50 mM $MgCl_2$ 0.1 ug/ul pBR322	5	50 mM Tris 10 mM $MgCl_2$ 0.5 ug/assay
Bovine Serum Albumin (BSA)	800 ug/ml	5	160 ug/ml
Dithiothreitol (DTT)	5 mM	5	1 mM
Adenosine Triphosphate (ATP)	2.5 mM	5	0.5 mM
Enzyme Sample		5	

plasmid DNA. The assay was prepared in 1.5 ml polyethylene
microcentrifuge tubes by adding stock solutions as shown in Table
IV. The mixture was kept at 4° C. during preparation and the
enzyme and pBR322 were added last to control length of
incubation. The polyethylene tubes were spun briefly in an
Eppendorf desk top centrifuge to mix the reactants and initiate
the reaction. The tubes were then incubated in a water bath for
1 h at 35° C. The reaction was stopped by adding 2 ul of a
solution containing 13.5% SDS and 10% glycerol (to increase
density) plus 0.5 mg/ml bromphenol blue (tracking dye) to
facilitate loading into the wells of the agarose gel. Wells
were loaded while the gel was submerged in electrophoresis
buffer.   Separation of the reaction products was carried out by
agarose gel electrophoresis on either 2.5 x 6 cm (minigel) or 7 x
10 cm (standard) slab gels approximately 0.5 cm thick poured from
0.9% agarose. Agarose was mixed using the same buffer that was
to be used as electrophoresis buffer during the run. Tris-
borate buffer (more resistant to exhaustion) was used when gels
were to be run at higher than 40 v. The agarose was brought to a
vigorous boil to eliminate small air bubbles then allowed to
cool and maintained at 60° C. during pouring.

Electrophoresis was performed in 250 ml electrophoresis
tanks for the large gels and in a 50 ml tank for the smaller
gels. The smaller gels were run at 80-100 v for 1.0 h with a
change of buffer after 0.5 h to prevent buffer exhaustion, which

resulted in acidic conditions, heat buildup, and gel melting. The bromphenol blue tracking dye was useful for detecting the onset of acidification in the gel. The larger gels were initially electrophoresed at 40 v for 4-6 h. It was later found that these gels, if poured and electrophoresed using 89 mM Tris-borate buffer, could also be run for 1.0-1.5 h at 80-100 v with buffer changes each 0.5 h. Gels could be run overnight or for other extended periods by lowering the voltage to 5-10 v. This helped to alleviate the problem of constant tending.

Gels were stained with a solution of 0.5 ug/ml ethidium bromide for 1 h and, if necessary to reduce background florescence (which was worse on thicker gels), destained in 1 mM magnesium sulfate for 1 h. The gels were photographed with a polaroid camera using number 660 film and an orange filter to increase contrast.

MOLECULAR WEIGHT DETERMINATION

Molecular weights of fractions isolated by column chromatography were determined by fractionation through the S-200 sephacryl column. The column was first calibrated using a mixture of carbonic anhydrase, ovalbumin, BSA, phosphorylase b, beta-galactosidase and myosin (Sigma MW-SDS-200 kit) (Figure 4). The molecular weight of the protein was graphed against eluant in ml and a linear regression analysis determined on the data . Using this as a standard curve, the fraction in which the

FIGURE 4:   Profile of S-200 sephacryl column loaded with Sigma MW-
SDS-200 kit marker proteins.

        A. Myosin, 205 Kd.

        B. Beta-Galactosidase, 116 Kd.

        C. Phosphorylase b, 97.4 Kd.

        D. Bovine Serum Albumin, 66 Kd.

        E. Ovalbumin, 45 Kd.

        F. Carbonic Anhydrase, 29 Kd.

Sample size = 1.5 ml
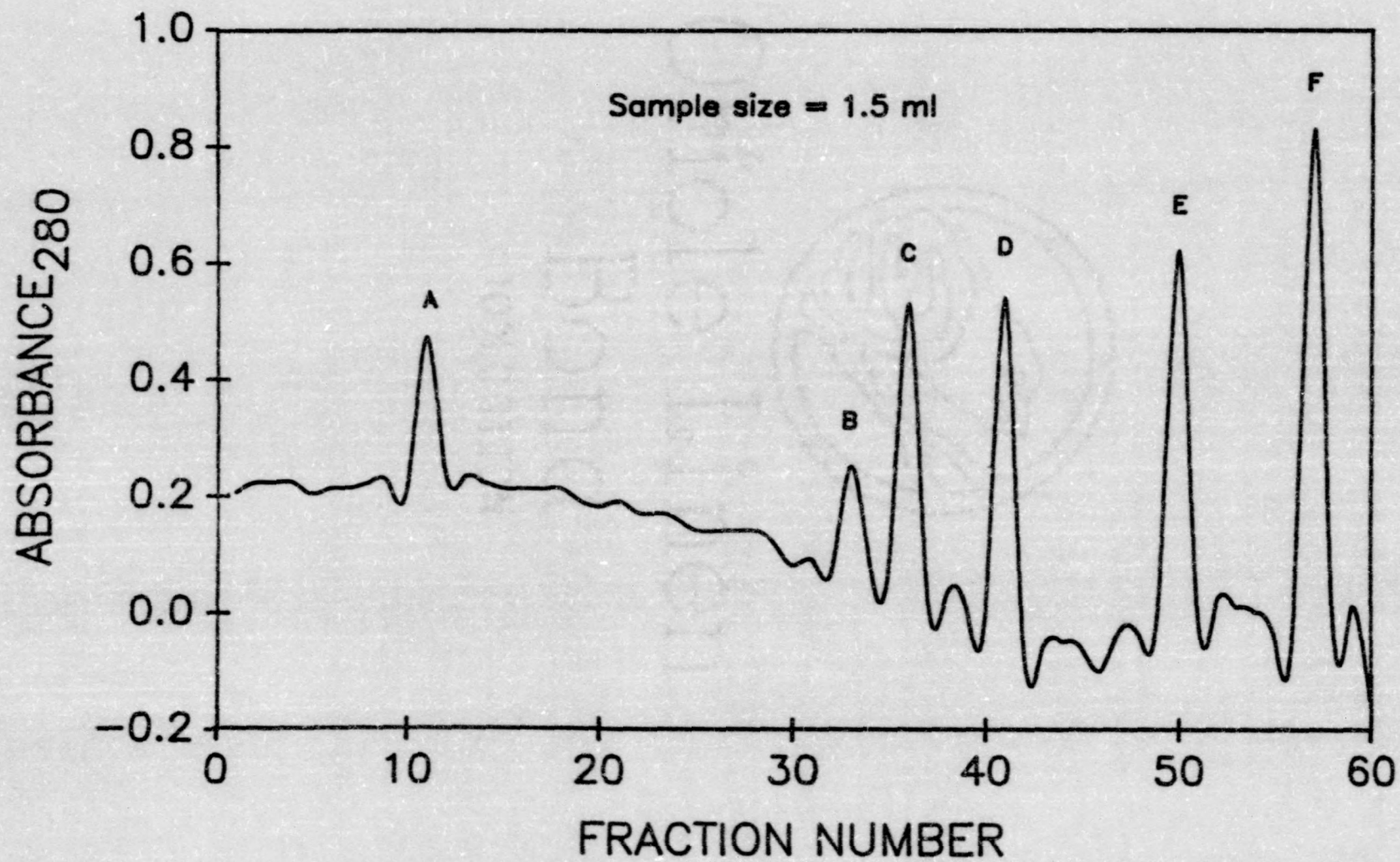
topoisomerase activity emerged was plotted to obtain a rough calculation of the molecular weight of the partially refined native enzyme (Figure 5).

Samples of the enzyme were further purified by separation on non-denaturing polyacrylamide gels and were recovered by electro-elution. These samples were then analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight markers (Sigma MW-SDS-200 kit) were used to construct a standard curve by graphing Kav against the log of the molecular weight (log MWt). Molecular weights were then interpolated from the line graph obtained.

STAINING OF ACRYLAMIDE GELS

All acrylamide gels were stained using Bio-Rad silver stain. A simplified chart of the procedure showing volumes for a 16 cm x 20 cm x 1 mm gel is shown in Table V.

Gels measuring 16 x 20 cm stained best in a standard 21 x 21 x 5 cm pyrex baking dish. Gels were photographed with a polaroid camera and 660 film with no filter. Because some proteins do not stain on the first cycle, all gels were stained a second time (recycled) and re-photographed.

FIGURE 5: Molecular weights of MW-SDS-200 proteins shown in
Figure 4 were graphed against eluant in ml as determined by their
peak absorbance. The line represents a linear regression analysis
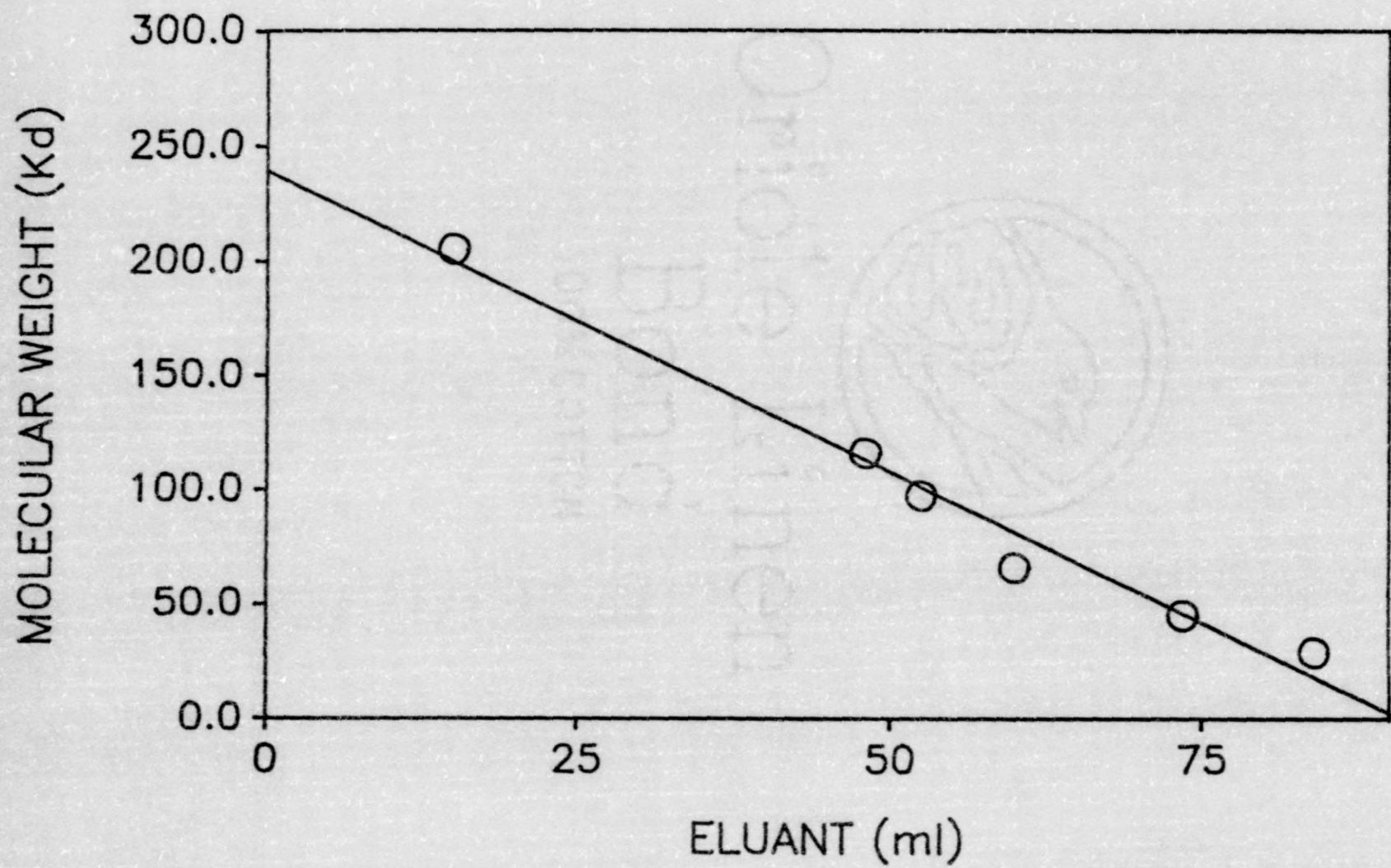of the data.

TABLE V

	Reagent	Volume in ml	Duration in min.
1.	First fixative [1]	400	30
2.	Second fixative [2]	400	15
3.	Second fixative	400	15
4.	Bio-Rad oxidizer [3]	200	5
5.	DI water	400	5
6.	DI water	400	5
7.	Bio-Rad silver reagent	200	20
8.	DI water	400	1
9.	Bio-Rad developer [4]	200	0.5
10.	Bio-Rad developer	200	5
11.	Bio-Rad developer	200	5

1. 40% methanol, 10% acetic acid (v/v).
2. 10% ethanol, 5% acetic acid (v/v).
3. Caution: contains potassium dichromate and nitric acid.
4. Caution: contains sodium carbonate and paraformaldehyde.

DENSITOMETRY OF GEL PHOTOGRAPHS

Polaroid positive images of agarose gel assays for
topoisomerase activity were re-photographed to obtain negatives
for analysis by densitometry. The negatives were then scanned on
a scanning densitometer in the laboratory of Dr. Neil Osheroff,
Vanderbilt University. Areas under the curves representing DNA
peaks were carefully cut out and weighed to the nearest 0.0001 g
on a Sartorius analytical balance. Weights of the curves were
used as an approximation of the relative amount of DNA contained
in the band. The accuracy of this method was evaluated by
comparing relative amounts of relaxed and supercoiled DNA in
control lanes on different gels where pBR322 DNA was from the
same stock (agreement was within +\- 1.0 percent). Relaxed DNA
bands were compared to relaxed DNA bands only and quantity was
expressed as a percentage of the relaxed DNA present in the
control lane. The same inter-comparison was made between
supercoiled DNA bands. No attempt was made to determine the
absolute amounts of supercoiled versus relaxed DNA present in a
single lane because of the inherent problem of differential
intercalation of ethidium bromide into supercoiled versus relaxed
DNA (Dr. Neil Osheroff, personal communication).

DETERMINATION OF SPECIFIC ACTIVITY

Samples of topoisomerase for specific activity were diluted 1:10 and 1:100 with appropriate buffer. The resulting 1.0X, 0.1X and 0.01X stocks of each sample were then reacted under standard assay conditions as described above. Gels were photographed to obtain a positive image, later re-photographed to obtain a negative, and scanned by densitometry as described above. One unit of topoisomerase activity is defined as the amount of enzyme required to fully relax 0.5 ug of supercoiled pBR322 DNA in 1 h at 35° C. The relative amount of pBR322 DNA existing in the plasmid stock was determined by spectrophotometric analysis of the control lane rather than densitometry of the photographs for the reason already cited. The gel scan was performed on a Gilford Response spectrophotometer at 260 nm. The area under the curve provided by the Gilford programming was considered representative of the relative amount of DNA existing in each of the two forms. The stock contained 64% supercoiled and 36% relaxed plasmid and each lane contained 0.5 ug of stock. Therefore each control lane contained 0.32 ug of supercoiled DNA. The amount of supercoiled DNA per reaction lane was determined as a percentage of the supercoiled DNA in the control lane by calculation of the relative amounts of ethidium bromide staining as described above. From this data, the ug weight of supercoiled pBR322 relaxed by the enzyme was determined. The weight of DNA relaxed divided by 0.5 yielded the units of topoisomerase

activity per lane. This number divided by the mg amount of enzyme present in a lane yielded the specific activity. The formulas for specific activity are shown below:

TA = DA / 0.5

Where;

TA = topoisomerase activity in units

DA = ug of supercoiled DNA relaxed

0.5 = ug DNA relaxed per unit

SA = TA / WP

Where;

WP = ug of protein per assay

SA = specific activity

RESULTS

PLANT TISSUE

Electron photomicrograph studies indicated that hypocotyls
of 4 day old seedlings consist of rapidly dividing,
undifferentiated meristematic cells that contain proplastids
approximately 1.5 to 2.0 um in diameter. No fully developed
chloroplasts were found.

Topoisomerase was extracted from both fresh and frozen
hypocotyl tissue. No readily observable difference was found
between topoisomerase isolated from fresh hypocotyls and that
isolated from hypocotyls frozen as described in materials and
methods.

NUCLEAR ORIGIN OF THE ENZYME

Light microscopy of methylene blue stained smears of the
nuclear pellet revealed mostly intact nuclei along with some
debris. No intact nuclei were detected in the supernatant
above the nuclear pellet. Light microscopy of methylene blue
stained smears of the nuclear lysate showed no intact nuclei.
Due to the absence of chloroplasts in the parent tissue and
light microscopic examination of stages of the extraction
procedure it was concluded that the extract was of nuclear
origin and free of significant contamination by chloroplasts.

34

RESULTS OF COLUMN CHROMATOGRAPHY

S-200 Sephacryl Gel Filtration Chromatography

When S-200 filtration immediately followed filtration through G-50 sephadex, topoisomerase activity was detected at peaks from 20 ml to 40 ml (Figure 6). These samples retained activity for up to 90 days at 4° C. in the elution buffer. Comparison to the column calibration curve (Figure 5) indicated that all topoisomerase activity eluted between 205 Kd and 116 Kd. Because of the excellent resolution with which the sephacryl column separated marker proteins (Figure 4) it was concluded that this range of activity must be due to an enzyme pool with poly-disperse apparent molecular weights with regard to gel filtration. Among the possible causes for such a pool are; degradation of a multi-subunit enzyme into lighter fractions, aggregation of a holoenzyme or it's products into larger units or the existence of forms of the enzyme with differing molecular weights.

In later experiments, samples were subjected to affinity column chromatography before filtration on S-200 sephacryl. When this was done, shelf life was considerably shorter (7 to 14 days). The cause of this shorter shelf life was not determined, although removal of essential components of the buffer by the affinity column was considered. Samples were not subjected to buffer exchange following affinity chromatography to determine if this improved enzyme life.

FIGURE 6: Profile of a typical S-200 sephacryl column loaded with post G-50 nuclear lysate. Peaks were assayed for topoisomerase activity. (+) indicates a positive assay. (-) represents no topoisomerase activity. Samples between A and B were pooled and subjected to further purification. Low molecular weight samples (below point B) were not tested for activity.
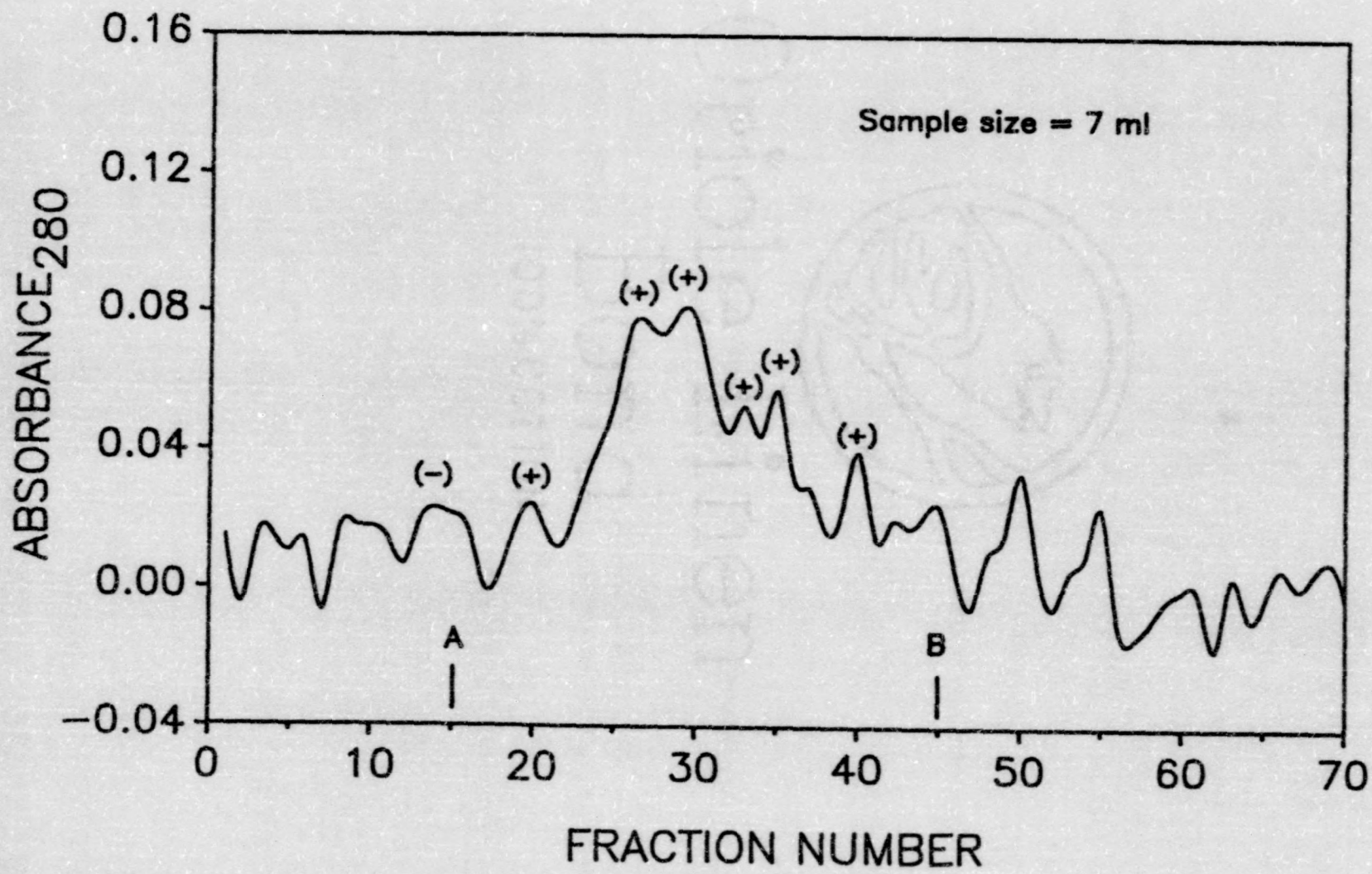
Purification on G-50 sephadex and DEAE sepharose prior to S-200 sephacryl improved resolution of the topoisomerase activity (Figure 7). Maximum activity was observed in the second sample which corresponded to a molecular weight of approximately 220 Kd. A second and distinct topoisomerase activity was located in a 4 ml sample emerging between ml 32 and ml 36, corresponding to a molecular weight of approximately 155 Kd. This sample was separated from the earlier eluting sample by two 4 ml samples which tested negative for topoisomerases. This pattern of activity suggests that two distinct molecular forms of the enzyme existed at this stage of the purification procedure. A sample subjected to G-50 sephadex, DEAE sepharose, and CM cellulose prior to filtration through S-200 sephacryl also showed a major protein peak corresponding with the strongest enzyme activity. This peak corresponded to a molecular weight of approximately 220 Kd (Figure 8). Samples in the 116 Kd range of this column profile were not concentrated and assayed to ascertain activity.

DEAE Sepharose Ion Exchange Chromatography

All topoisomerase activity was found to bind to DEAE sepharose at pH 8.0 and to elute in a range from 250-350 mM KCl (Figure 9). No topoisomerase activity was detected in the wash fraction of the profile either with or without 0.5 M ATP, indicating that all topoisomerase activity was bound to the column. Samples from this gel were assayed for topoisomerase activity (Figure 10). Activity in the absence of ATP was detected from approximately 0.1 M to 0.45 M KCl (samples 5-23).

FIGURE 7: Profile of an S-200 sephacryl column loaded with
nuclear lysate purified through G-50 sephadex, and DEAE sepharose.
Sample size is 4 ml. Samples 1 through 10 were assayed for
topoisomerase activity. A + indicates samples with detectable
topoisomerase activity. Relative size of the + indicates strength
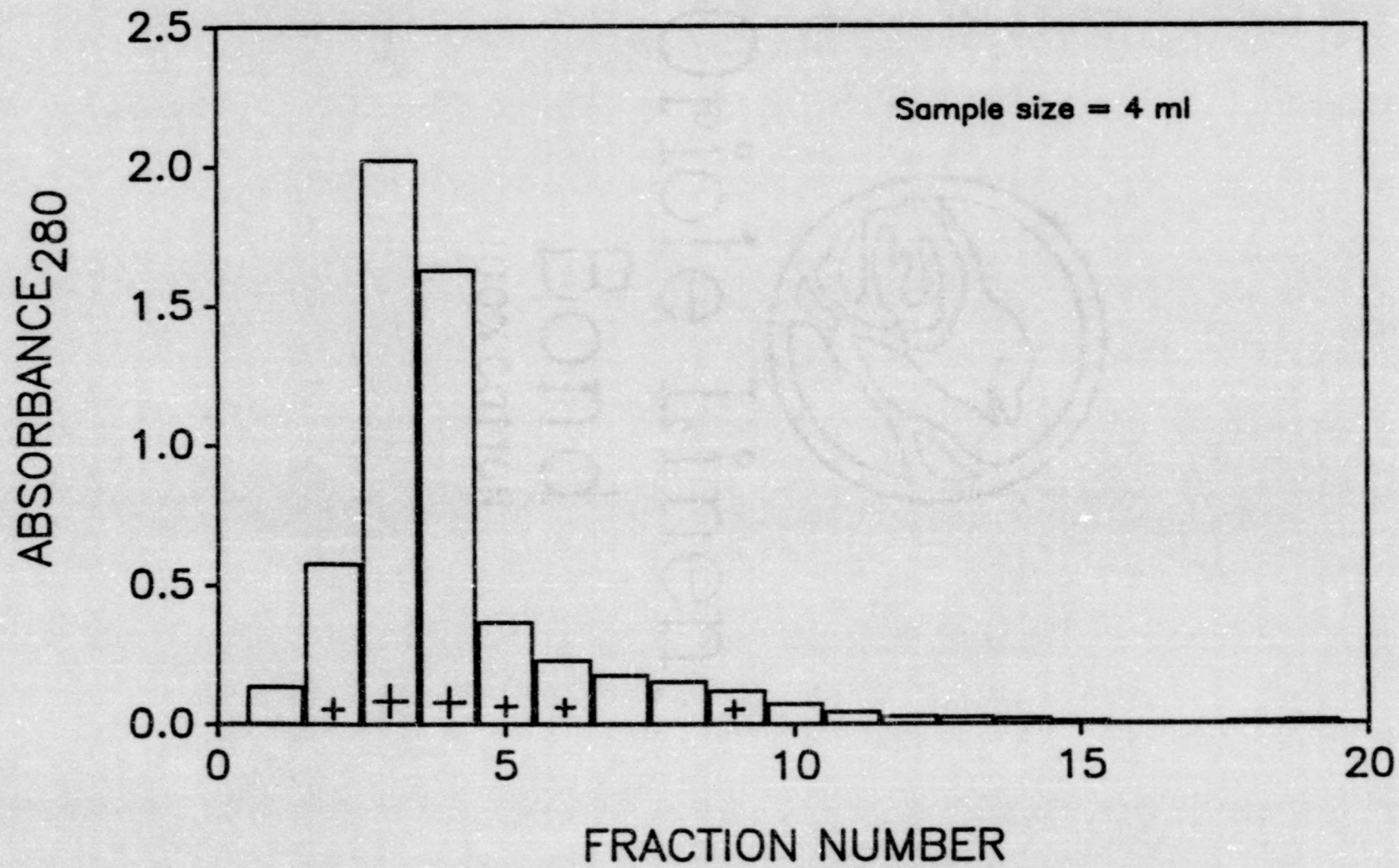of the reaction as determined by visual inspection of the gels.

FIGURE 8: Profile of a S-200 sephacryl column loaded with nuclear lysate purified through G-50 sephadex, DEAE sepharose and CM cellulose. High molecular weight peaks were assayed for enzyme activity. A + indicates activity and the size of the + indicates the relative strength of the reaction as determined by visual inspection of the gels.
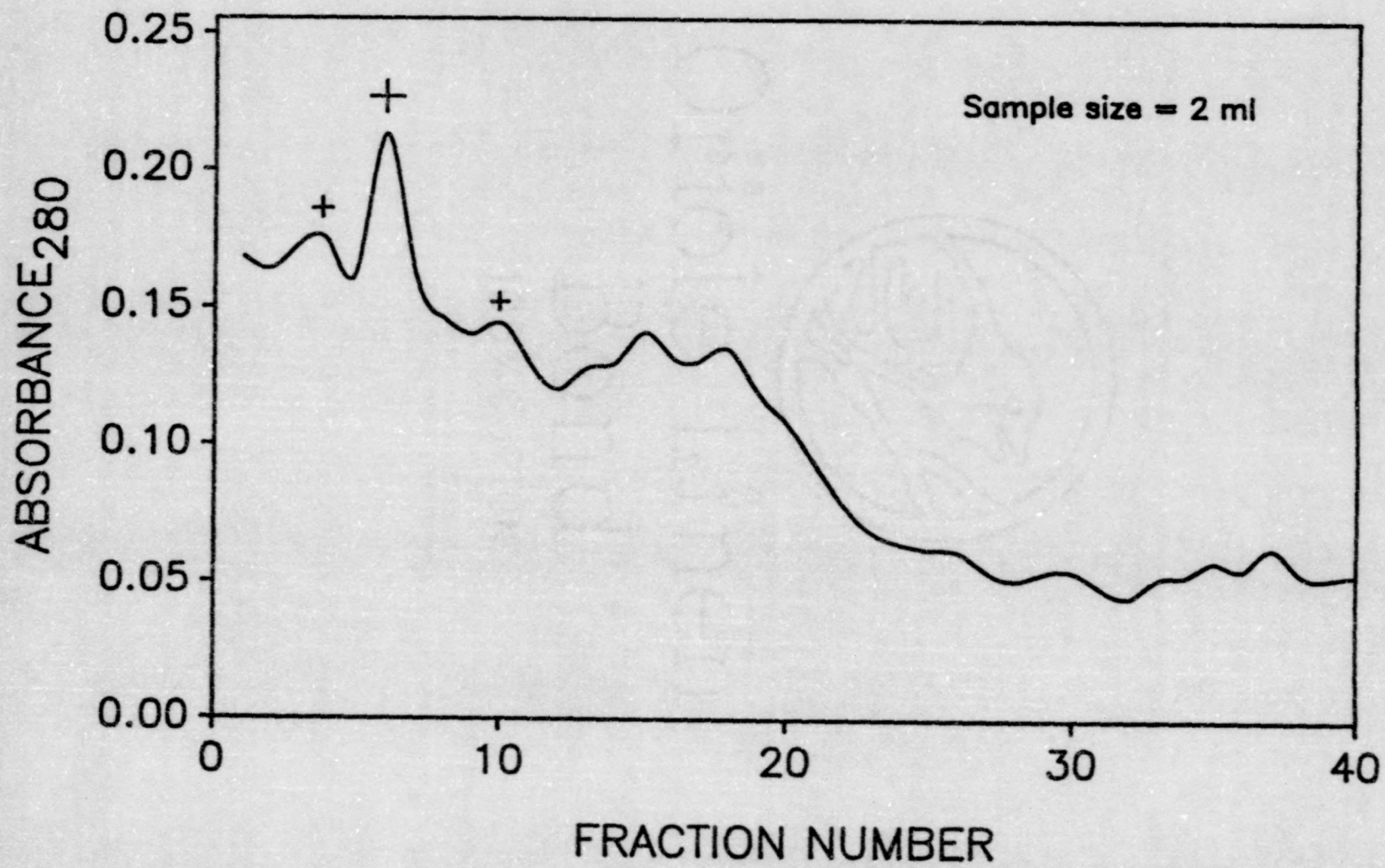
FIGURE 9: Profile of a DEAE sepharose column loaded with post
G-50 nuclear lysate, pooled and concentrated to 1 ml. Wash
fraction was pooled and tested for topoisomerase activity. The
(-) indicates insufficient activity to detect. The bound fraction
was eluted with a 0 to 1.0 M linear gradient of KCl. Every third
2 ml sample was assayed for enzyme activity. Activity was
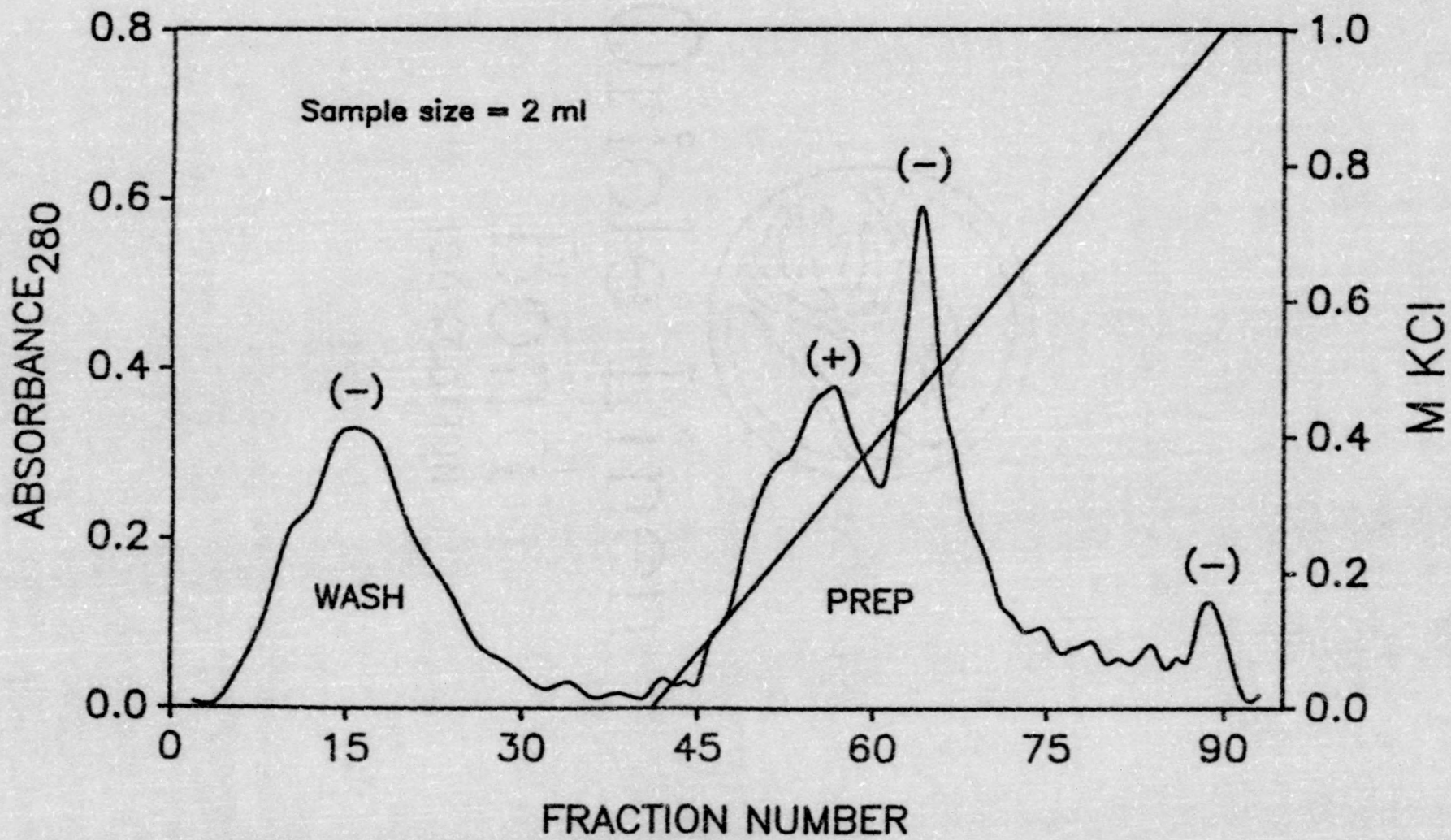detected in the first peak which eluted between 0 and 0.45 M KCl.

FIGURE 10: Topoisomerase assays on samples eluted from DEAE
sepharose with a 0 to 1.0 M linear gradient of KCL. See Figure 9
for column profile. Samples were 2 ml each. Lanes are numbered 1
through 15 from right to left. Top gel represents assay without
ATP. Lanes 1 - 15 are: Lane 1, Control; Lanes 2 - 15, Samples 5,
8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41 and 51 respectively.
Bottom gel represents the assay in the presence of 0.5 mM ATP.
Lanes are the same as in the top gel with the exception of lanes 1
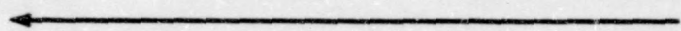and 2. Lane 1 is Sample 5. Lane 2 is the control.

DARK

DOCUMENTS

"May Not Film
"
Well.
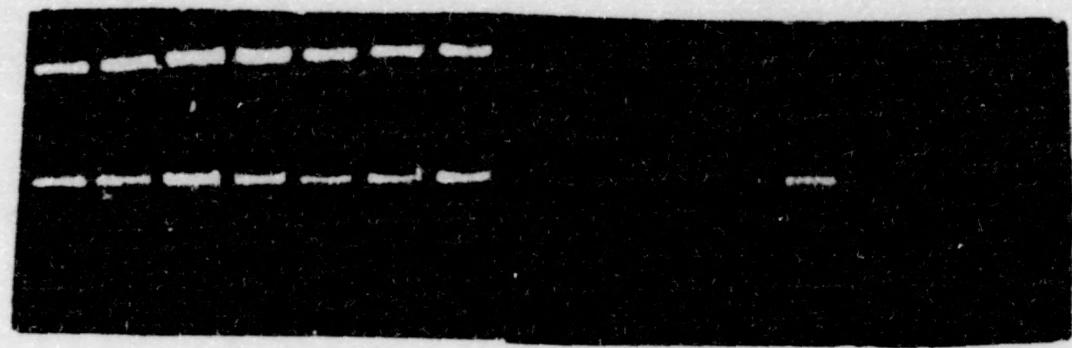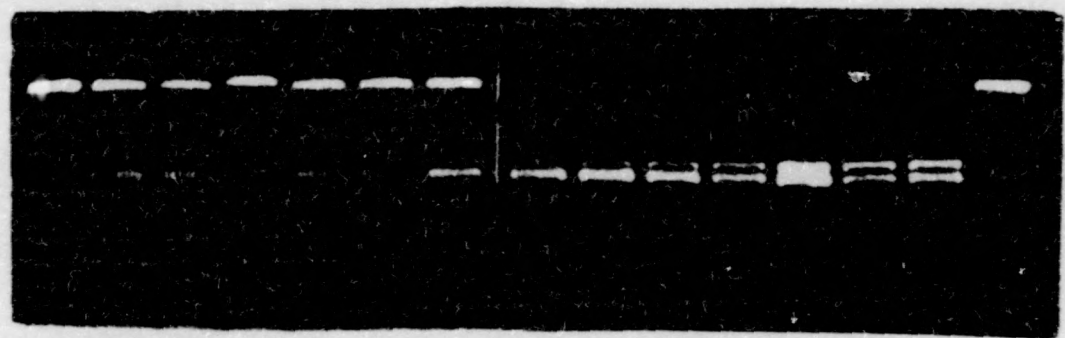
15 ⟵————————————— 1

Slightly less activity was detected when ATP was present (samples 5-17, 0.1 to 0.35 M KCl). On the basis of this profile, further preparations were eluted with a 0.50 M KCl step elution. Most of the topoisomerase activity in these preparations was restricted to the salt eluted protein peak (Figure 11). However, a very small amount of topoisomerase activity was detected in the wash (non-binding) fraction when concentrated 64 fold.

CM Cellulose Ion Exchange Column Chromatography

Topoisomerase did not bind to CM cellulose at pH 8.0 under the conditions of this study (Figure 12). This indicated that all detectable forms of the enzyme present from nuclear extracts under the conditions described are anionic.

Non-Denaturing Polyacrylamide Gel Electrophoresis

Non-denaturing polyacrylamide gels of frac ions purified through G-50, DEAE, S-200, CM, and DEAE in that order showed two closely-spaced high molecular weight bands appearing after one staining with Bio-Rad silver stain. Recycling in silver stain revealed two additional closely spaced bands in the low molecular weight range (Figure 13). The two high molecular weight bands migrated on either side of myosin (205 Kd), while the two low molecular weight bands migrated slightly slower than carbonic anhydrase (29 Kd).

Molecular weight determination of these bands was attempted from plots of Kav versus log MWt (Figure 14). Rough approximations of the molecular weights obtained in this way

FIGURE 11: Profile of a DEAE sepharose column eluted with 0.5 M
KCl. Topoisomerase activity was contained within the major
protein peak of the preparation. The wash fraction showed only a
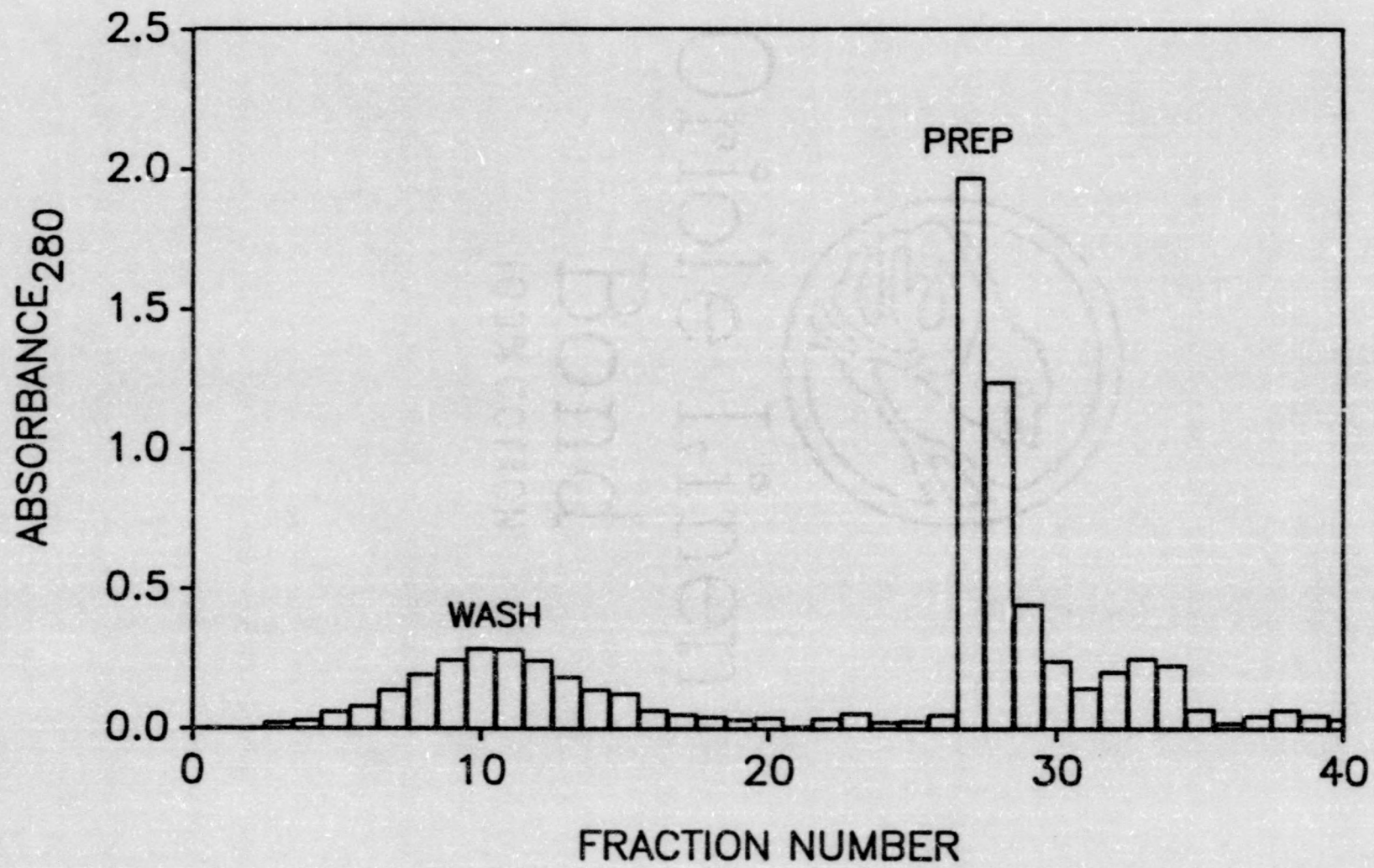trace of topoisomerase activity after 64X concentration.

FIGURE 12: Profile of a CM cellulose column loaded with nuclear lysate purified through G-50 sephadex and DEAE sepharose. The wash fraction was pooled and assayed positive for topoisomerase. The bound fraction was eluted with a 0 to 1.0 M KCl linear gradient. No activity was detected in the eluted fraction.
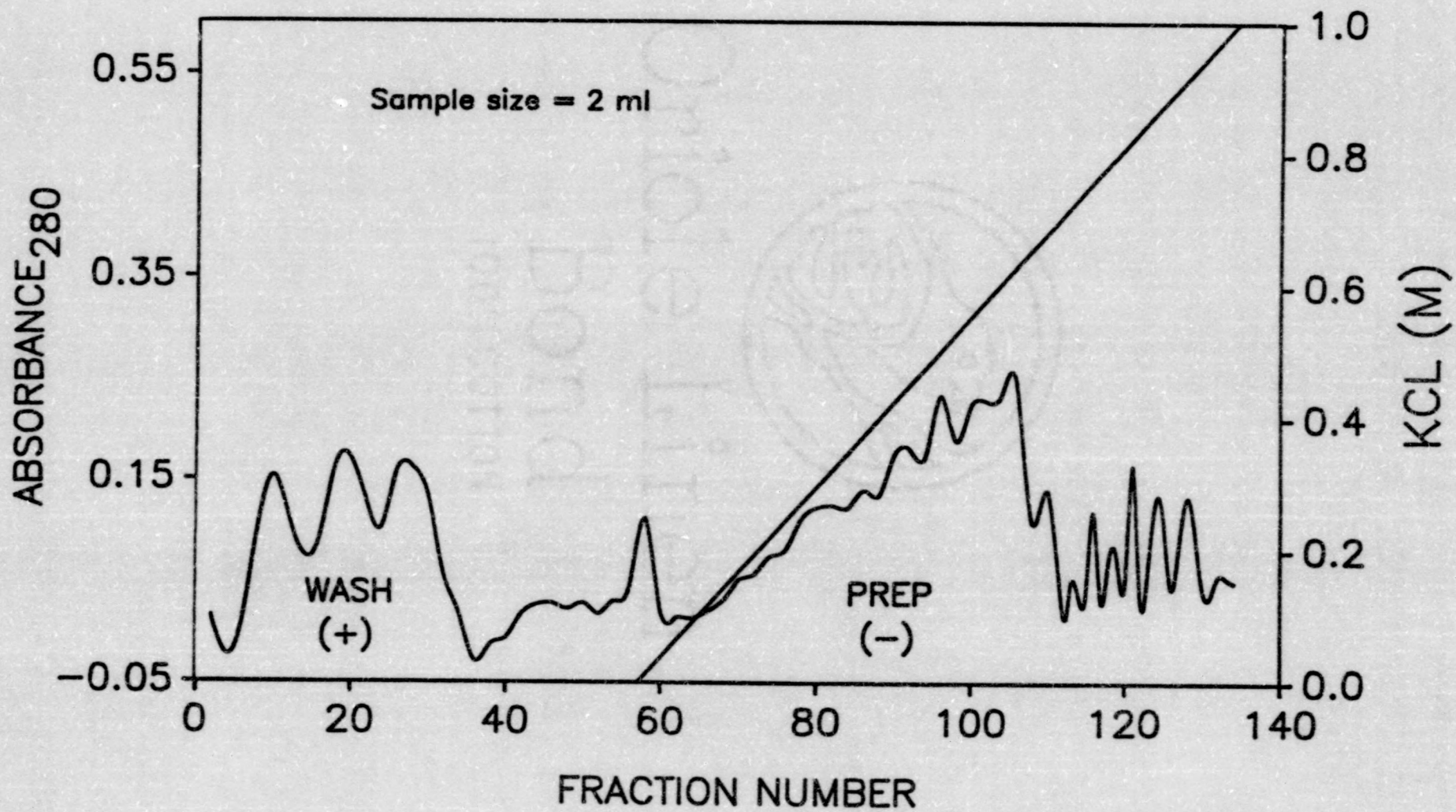
FIGURE 13:Gel # 6221.   Non-denaturing polyacrylamide gel loaded
with samples at stages of purification.   Lanes 1 - 10 represent:

1. MWt markers.

2. Crude nuclear lysate (1X).

3. G-50 sephadex (4X).

4. First DEAE sepharose (30X).

5. S-200 sephacryl (225 Kd enzyme activity, 4X concentration).

6. S-200 sephacryl (155 Kd enzyme activity, 10X concentration).

7. S-200 sephacryl (samples from lanes 5 and 6 combined).

8. CM cellulose wash (2X).

9. Second DEAE sepharose (major activity fraction, 4X).

10.   MWt markers.

Marker lanes contain myosin, beta-galactosidase,
phosphorylase b, bovine serum albumin, ovalbumin and carbonic
anhydrase.

DARK
DOCUMENTS

"MAY NOT FILM
WELL."
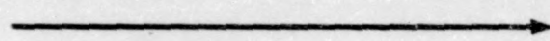
1 ⟶ 10

FIGURE 15: Topoisomerase assay of bands B1,B2,B3 and B4 eluted from non-denaturing polyacrylamide. Lanes 1 - 6 are:

1. Control.

2. B1.

3. B2.

4. B3.

5. B4.

6. Control.

After elution from polyacrylamide, the samples were concentrated 20, 12, 32 and 35 fold respectively. Specific activities were virtually the same for all bands (see Table 4). Different apparent activities shown are the result of concentration differences.

were: Band 1 = 265 Kd, Band 2 = 178 Kd, Band 3 = 27 Kd,
Band 4 = 24 Kd. The proteins present in the closely spaced, low
molecular weight bands may be distinct polypeptides or they may
represent a cellular pool consisting of one polypeptide which
exists in two states which migrate slightly differently on
acrylamide, a possibility that is much less likely for the
proteins represented by the closely spaced, high molecular weight
bands. These four bands were purified by electroelution from
non-denaturing acrylamide for further characterization.

Electroelution of Gel Bands

Proteins were removed from the excised preparatory gel
bands as described in Materials and Methods. These bands were
labeled B1, B2, B3, and B4 in order of descending apparent
molecular weight and were concentrated 20, 12, 32 and 35 fold
respectively by centrifugal ultrafiltration (exclusion limit 10
Kd). All four bands tested positive for topoisomerase activity
(Figure 15) indicating at least three distinct enzyme activities.
On the basis of these results, a new sample was prepared and
purified through G-50 sephadex, bound to DEAE sepharose, eluted
with 0.50 molar KCl, and electrophoresed on two identical non-
denaturing polyacrylamide preparatory gels. Samples of the
proteins from bands 1 through 4 were concentrated 25 fold by
centrifugal ultrafiltration and assayed for topoisomerase
activity in the presence and absence of 0.5 mM ATP. Activity was
greatest in the absence of ATP, indicating that the enzyme is
probably a class I enzyme and that it is slightly inhibited by
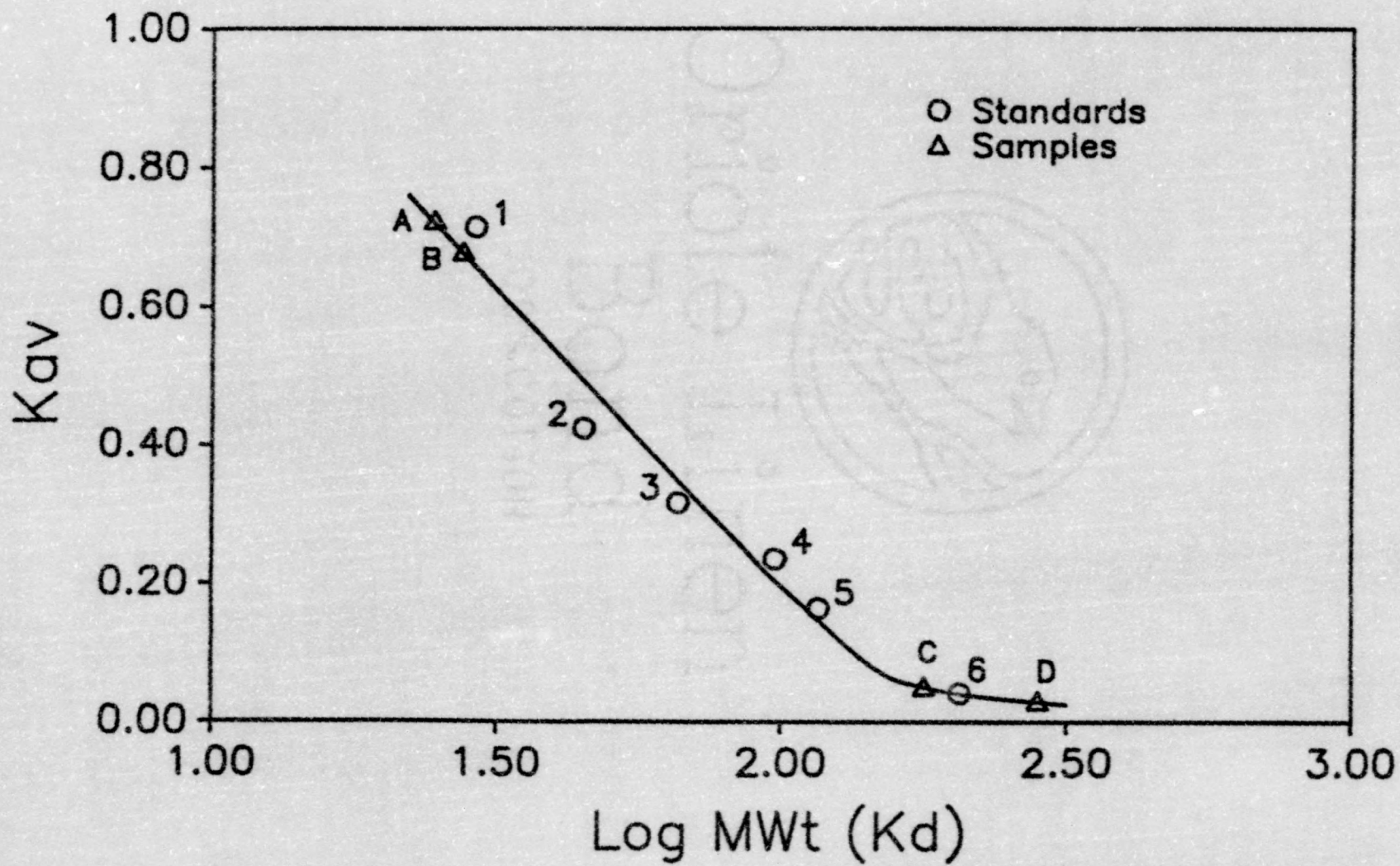
FIGURE 14:   Calibration curve of Gel 6221. (a non-denaturing polyacrylamide gel).   Samples 1 through 6 represent molecular weight markers as follows:

1. Myosin, 205 Kd.   2. Beta-galactosidase, 116 Kd.

3. Phosphorylase b, 97.4 Kd.   4. Bovine Serum Albumin, 66 Kd.

5. Ovalbumin, 45 Kd.   6. Carbonic Anhydrase, 29 Kd.

    Samples A through D represent bands B1 through B4.

Approximate molecular weights are:

A. B1, 251-282 Kd.   B. B2, 178-179 Kd.

C. B3, 26-28 Kd.   D. 23-25 Kd.

DARK

DOCUMENTS

"May Not Film Well."

1 2 3 4 5 6

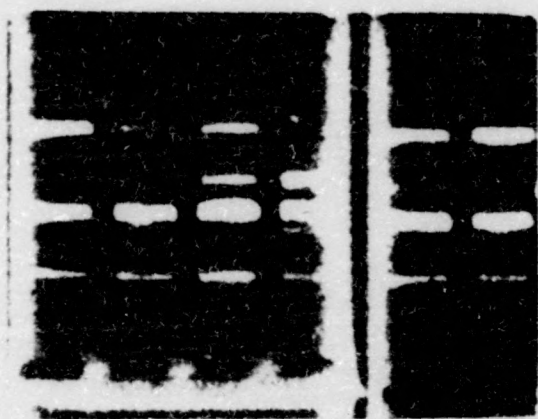ATP.   Concentrations, amounts of protein extracted, and specific activities for each band are shown in Table VI.   The low specific activity of the samples probably results from denaturation of the enzyme during electrophoresis and electroelution.

SDS Polyacrylamide Gel Electrophoresis of Eluted Bands

Samples from bands 1 through 4 were concentrated 30 fold by centrifugal ultrafiltration and subjected to analysis on SDS-PAGE.   Each well was loaded with 35 ul of sample and 5 ul of tracking dye.   Total amounts of protein were as follows: B1 = 65 ug, B2 = 85 ug, B3 = 37 ug, B4 = 50 ug.   The center of the gel exhibited poor staining, rendering B2 (lane 4) unreadable. Sample B1 (lane 3) resolved into four distinct bands: a single band migrating slower than myosin (205 Kd), a single band approximately co-migrating with bovine serum albumin (66 Kd) and two bands migrating in the region of carbonic anhydrase (29 Kd). Sample B3 (lane 5) was identical to sample B1 but lacked the high molecular weight band.   Sample B4 (lane 6) contained the same three lower bands as B1 and B3 but displayed a fourth band in the mid-range slightly above the single mid-range band found previously (Figure 16).

The two lower bands present in lane three are assumed to have arisen as dissociation products of the high molecular weight form.   The closely spaced, low molecular weight bands in lane five and six indicate that the two low molecular weight species may indeed be one molecule that equilibrates between two forms migrating differently on acrylamide.   This modification however

Table VI

Band #	B1	B2	B3	B4
Volume [1]	2.3	2.3	1.3	1.2
Concentration [2]	76.1	90.8	36.3	52.6
Mass of protein [3]	172.9	206.8	46.3	62.6
Topoisomerase assay	+	+	+	+
Specific activity [4]	48.5	48.4	41.1	51.3

Units:

1. ml
2. ug/ml
3. ug of protein
4. units of topoisomerase activity per mg total protein

FIGURE 16:   Gel # 7281.   SDS-Polyacrylamide gel electrophoresis of samples B1, B2, B3 and B4 from Figure 14.   Samples had been stored for 9 days at 4 degrees C. in a solution with no BME prior to SDS-PAGE analysis.   Lanes 1 through 6 are:

1.   Marker lane containing BSA and Beta-galactosidase.
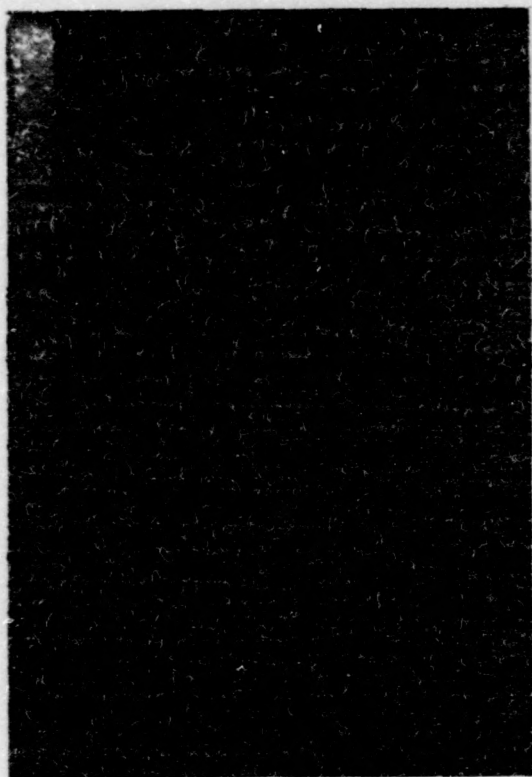
2.   Marker lane containing Myosin, Beta-galactosidase, Phosphorylase b, BSA, Ovalbumin and Carbonic Anhydrase.

3. B1.

4. B2.

5. B3.

6. B4.

Bands marked with arrows from top to bottom represent points A, B, C, D and E in Figure 16.   Over-stained marker lanes are the result of recycling in silver stain.

DARK
DOCUMENTS

"May Not Film
Well."

$1 \longrightarrow 6$

must have been non-enzymatic to have occurred in samples purified by excision and electroelution from a non-denaturing gel. The mid-range bands in lanes five and six could only have occurred by aggregation or assembly of the lighter molecules following electroelution. Nine days elapsed between electroelution and analysis on SDS-PAGE. During this time the samples were stored in electroelution buffer and did not have SDS or BME present. Furthermore, the samples were not heat denatured in the presence of SDS prior to SDS-PAGE analysis. These conditions could have been conducive to reformation of disulfide bridges.

CHARACTERIZATION

Molecular Weight Determination

Molecular weight was determined by a combination of SDS-PAGE and gel filtration. Molecular weights of the purified enzyme fractions were determined from the final SDS gel above. When Kav for these proteins was graphed against log MWt in Kd (Figure 17), the mid-range proteins showed a linear relationship, as expected, with a sigmoidal foot in the high molecular weight range (myosin). Beta galactosidase was a distinct flyer in the relationship and was excluded in drawing the curve. Kav values for the resolved fractions indicated a total of five distinct molecular species in the three sample fractions. The highest apparent molecular weight species (A) occurred only in B1 and the second highest weight species (B) occurred only in sample B4. The other three species (C,D & E)

FIGURE 17:   Calibration curve of SDS-PAGE Gel # 7281.   Standards

1-6: Carbonic anhydrase, ovalbumin, bovine serum albumin,

phosphorylase b, beta-galactosidase and myosin respectively.

Bands

A-E represent bands on gel # 7281 (Figure 15) from top to bottom

respectively.   Molecular weights are found in Table VII.

Beta-galactosidase did not migrate in a linear relationship with

the other markers and was not incorporated in the linear

regression analysis represented by the solid line.
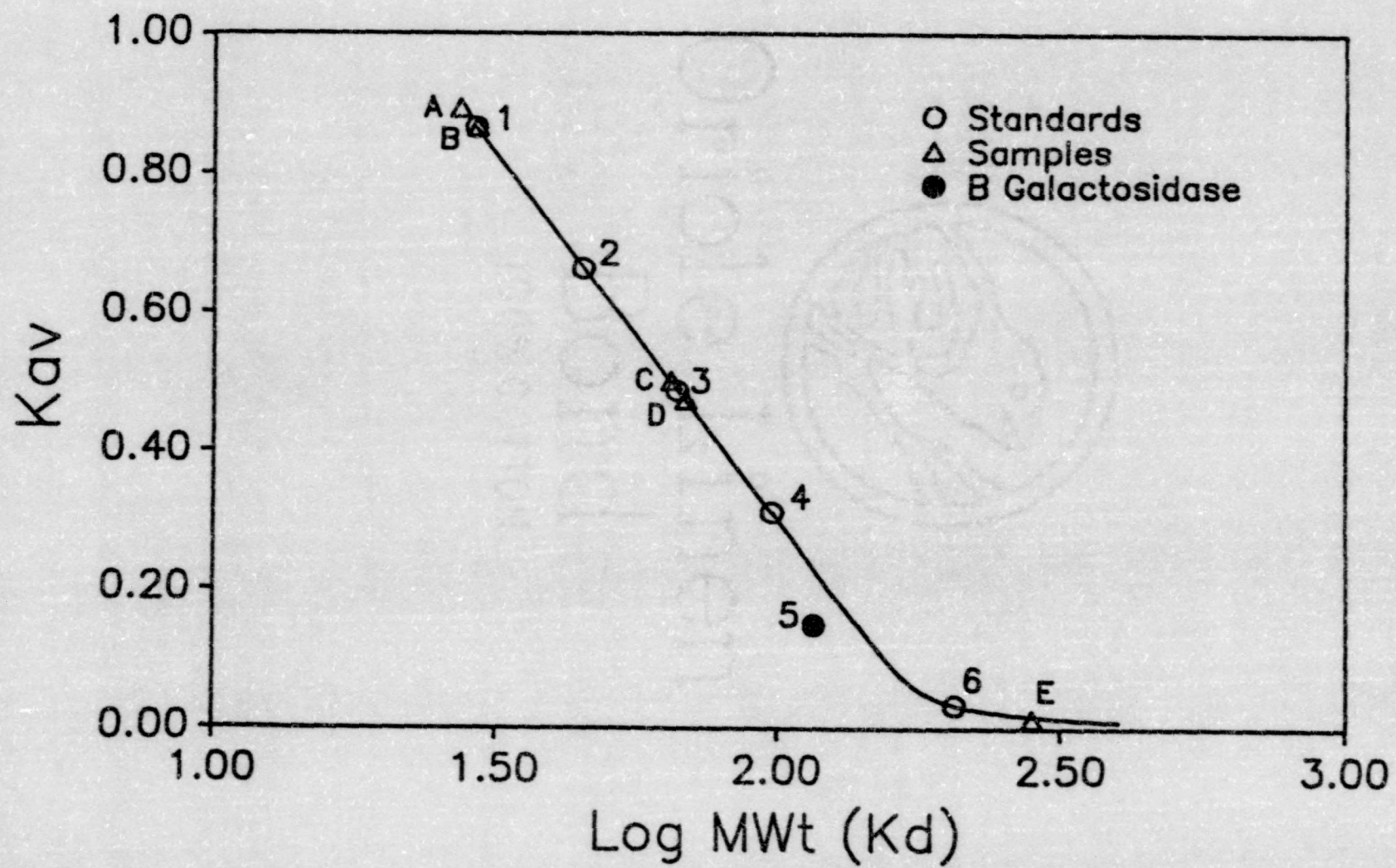
were common to all three samples, as shown previously in Figure 16. Results of molecular weight determination of these five bands based on the calibration curve are shown in Table VII. Note that bands B and C have roughly twice the weights of bands D and E supporting the idea that they represent dimeric species (68 Kd) reassembled from lighter subunits or domains (29 Kd). Theoretically four such units could form the native enzyme (300 Kd).

The molecular weight of the native enzyme was determined by gel filtration on a S-200 sephacryl column calibrated with proteins of known molecular weight. When cellular extracts that had not been subjected to gel electrophoresis were purified through S-200, topoisomerase activity always emerged in the range of 210-225 Kd. Because this molecular weight fell within the sigmoidal area of the calibration curve, the error involved in the estimation of this molecular weight is large. A second, much smaller peak of activity was detected at 150-160 Kd. This second peak was separated from the main activity by two fractions testing negative for topoisomerase (Figures 7 and 18).

Effect of 0.5 mM ATP on Electroeluted Enzyme Activities

Proteins eluted from bands 1 through 4 were assayed for topoisomerase activity in the presence and absence of 0.5 mM ATP (Figure 19). Each sample exhibited slightly reduced activity in the presence of 0.5 mM ATP. This same inhibition had been noted earlier in the experimentation when the profile of enzyme eluted

TABLE VII

BAND	Kav	MWt
A	0.014	300 Kd
B	0.473	68 Kd
C	0.500	65 Kd
D	0.865	29 Kd
E	0.435	27 Kd

FIGURE 18: Topoisomerase assay of S-200 sephacryl column shown in Figure 7. Lanes 1 through 12 represent:

1. Control.	2. Sample 1.	3. Sample 2.
4. Sample 3.	5. Sample 4.	6. Sample 5.
7. Sample 6.	8. Sample 7.	9. Control.
10. Sample 8.	11. Sample 9.	12. Sample 10.

Samples 4 and 5 represent the peak of an enzyme activity at 225 Kd. Sample 9 represents a separate activity eluting at 155 Kd.

DARK
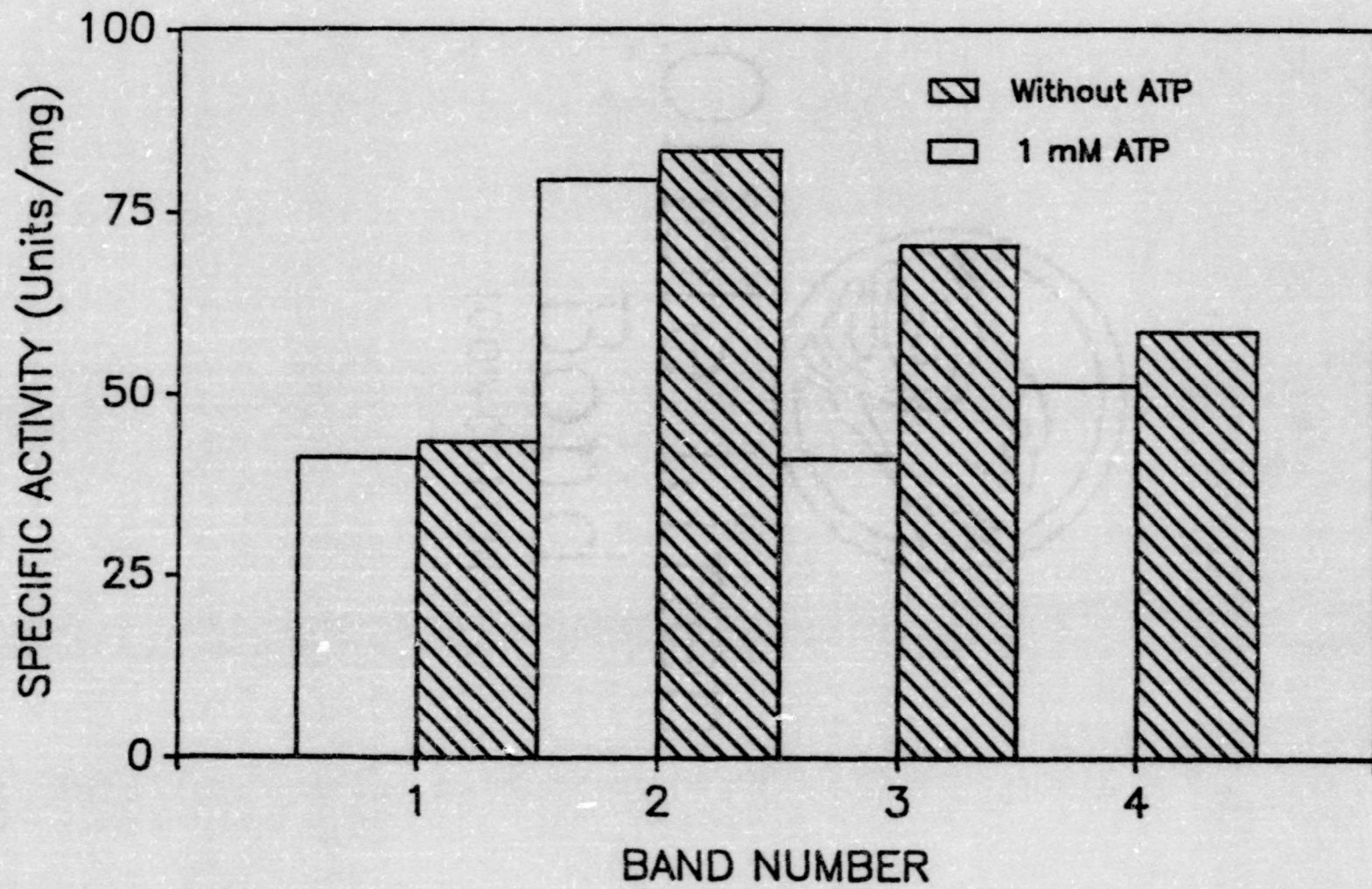# DOCUMENTS

"May Not Film Well."

1 2 3 4   5 6 7 8   9 10 11 12

FIGURE 19: Inhibition of topoisomerase by 0.5 mM ATP. Bands B1, B2, B3 and B4 eluted from non-denaturing polyacrylamide were immediately concentrated and assayed for topoisomerase activity in the presence and absence of 0.5 mM ATP. Figure 14 is a photo of the gel without ATP. Enzyme molecular weights are shown in Table VII.

from DEAE sepharose was tested in the presence and absence of 0.50 mM ATP (see Figure 10).

Effects of Magnesium and Manganese Divalent Cations

Following assays of bands 1 through 4, only band 1 (sample B1) was subjected to further characterization. The specific activity of the B1 enzyme was determined under the following conditions: in the presence of varying concentrations of Mg^{++} and Mn++ from 1 mM to 14 mM, in the absence of a divalent cation, and with 5.0 mM EDTA to chelate divalent cations (Figure 20). The topoisomerase isolated here exhibited a preference for magnesium over manganese at concentrations less than 14 mM. The exception was at a concentration of 6 mM where manganese stimulates activity more than magnesium. This concentration was simultaneously the maximum for manganese and the minimum for magnesium. At or above 14 mM equal activity was exhibited with either cation. The two sets of points in Figure 20 represent two separate assays conducted on two separate dates, thereby greatly reducing the possibility that the points at 6 mM were an artifact. The enzyme exhibited full activity in the presence of 10 mM $MgCl_2$ but substantially reduced activity in the absence of Mg^{++} or in the presence of 5 mM EDTA. Strict Mg^{++} dependence was not observed, that is to say that activity was not zero in the absence of magnesium; therefore, it was concluded that the enzyme is stimulated by the presence of magnesium.

FIGURE 20: Effects of concentrations of Mg++ and Mn++ on specific

activity of the native enzyme (B1 in Gel # 6221). Magnesium and

manganese assays were run on separate gels and on separate days.
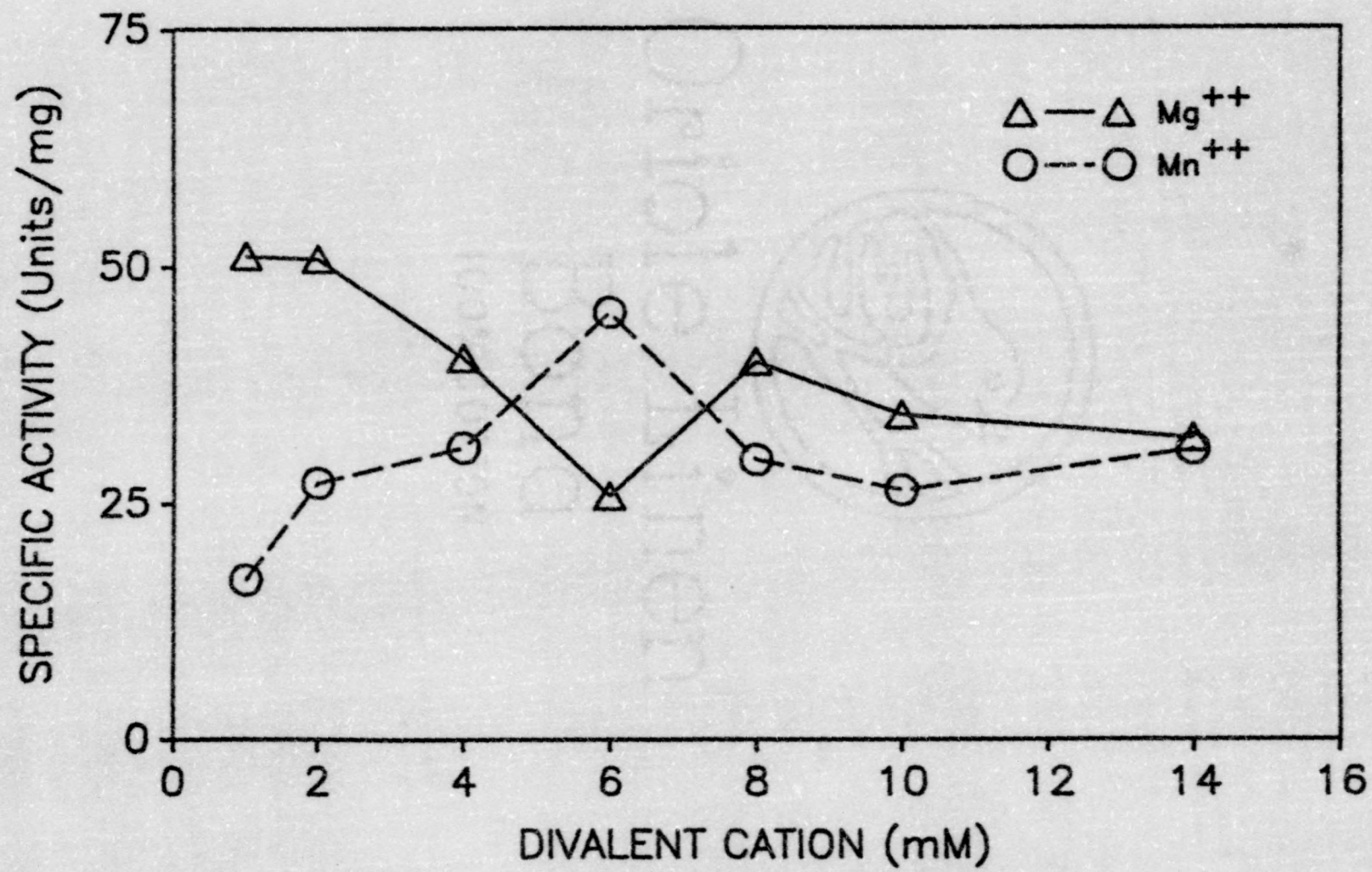
Effects of KCl Concentration

Increased concentrations of KCl from 0.04 to 0.50 M
resulted in decreased topoisomerase activity (see Figure 21).
Compared to a control lane, no activity difference was noted
between 0.04 M KCl and 0.00 M KCl reactions. At 0.10 M KCl,
inhibition was first detected and at 0.50 M KCl inhibition was
greater than 85 percent. Inhibition by KCl was not discovered
until after the work done with elution of enzymes from affinity
columns. Because this topoisomerase was almost completely
inhibited at 0.5 M KCl, it is possible that enzymatic activity
was present in the column profiles of DEAE sepharose and/or CM
cellulose above 0.5 M KCl (Figures 9 and 12). This may account
for the failure to detect a class II topoisomerase in post
affinity preparations, although some indication of an ATP
dependent activity had been detected in early experiments
involving only G-50 sephadex and S-200 sephacryl (data not
shown). However, KCl was probably not the cause of inhibition in
the ATP containing assays of samples eluted from DEAE sepharose
discussed above, especially considering later evidence that ATP
inhibited the enzyme in the absence of KCl.

FIGURE 21:   Effects of the concentration of KCl on specific
activity of the native enzyme.   The curve shown represents a
polynomial regression drawn on the data points.
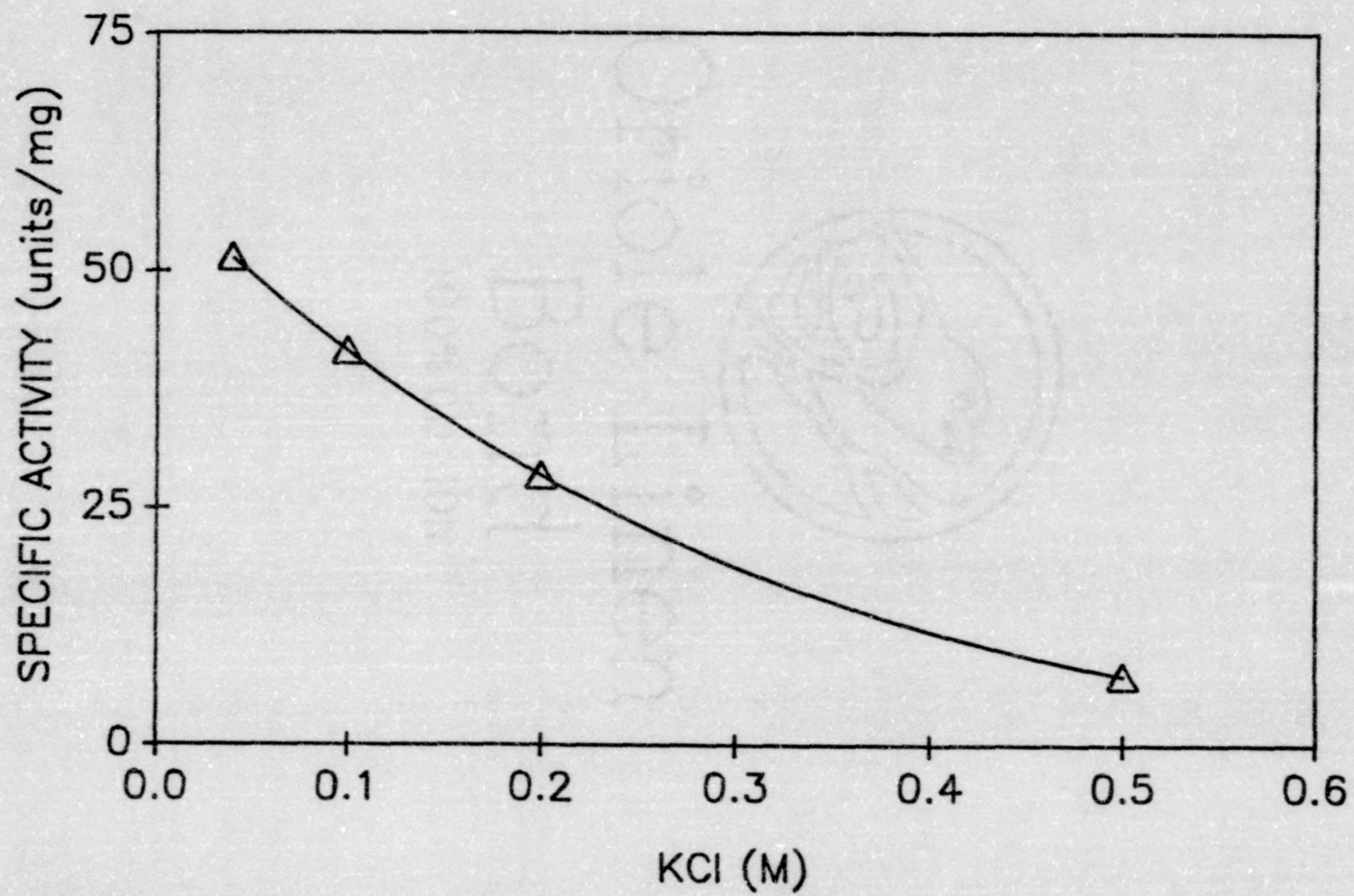
Effects of NEM and Novobiocin

The enzyme was inhibited by mM concentrations of NEM (N, ethyl maleamide). Reactions were carried out in 0, 1, 5 and 40 mM concentrations of NEM. Inhibition was maximal at 1 mM (Figure 22).

The enzyme was not inhibited by 100 uM novobiocin to an extent measurable by the assay used (Figure 23). Both control and 100 uM novobiocin containing enzyme samples fully relaxed the plasmid DNA substrate.

FIGURE 22: Effects of NEM on the specific activity of the native soybean topoisomerase. Data points represent 0, 1, 5 and 40 mM NEM. Maximum inhibition was detected at 1 mM NEM. Also see Figure 19.
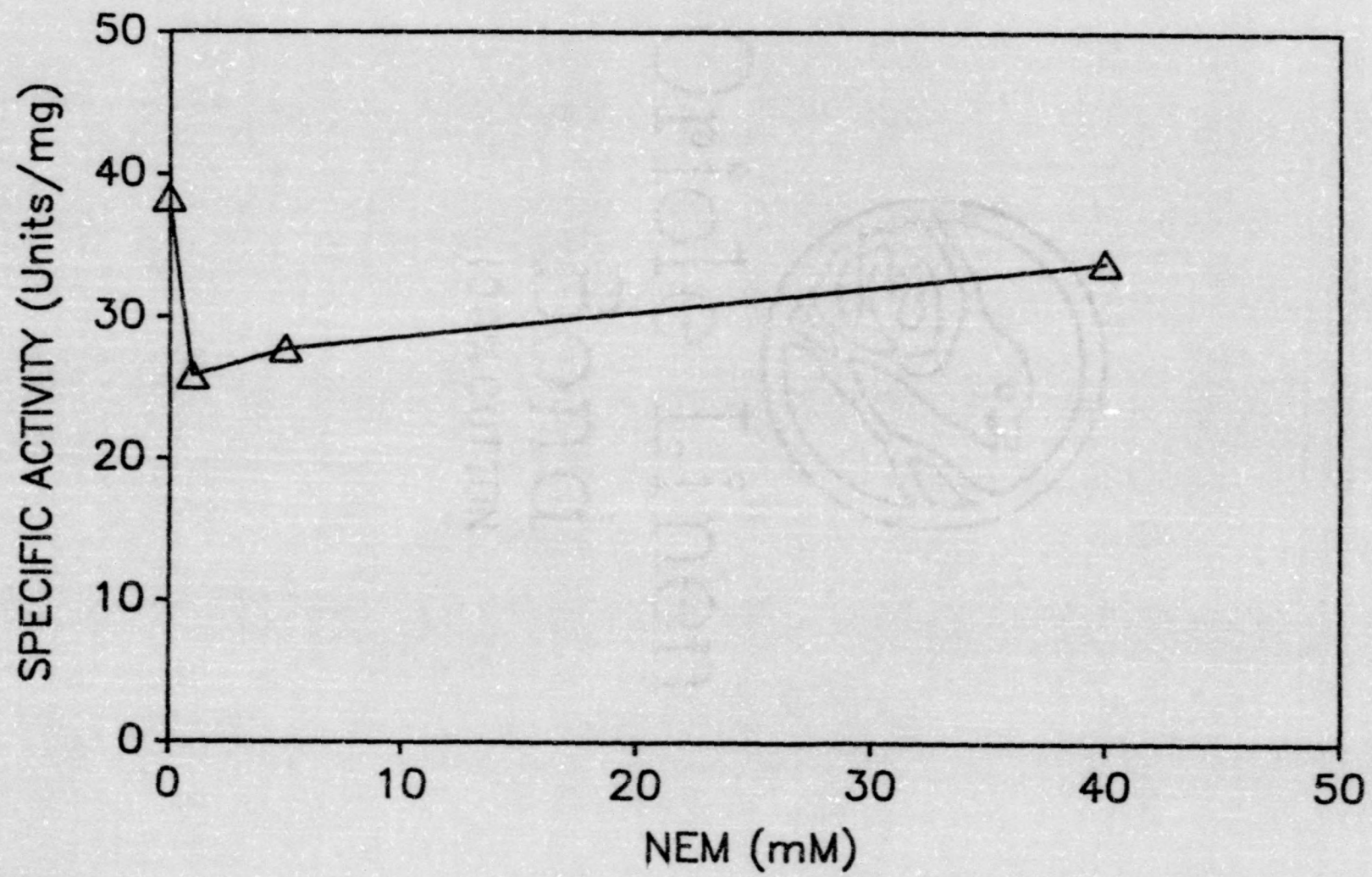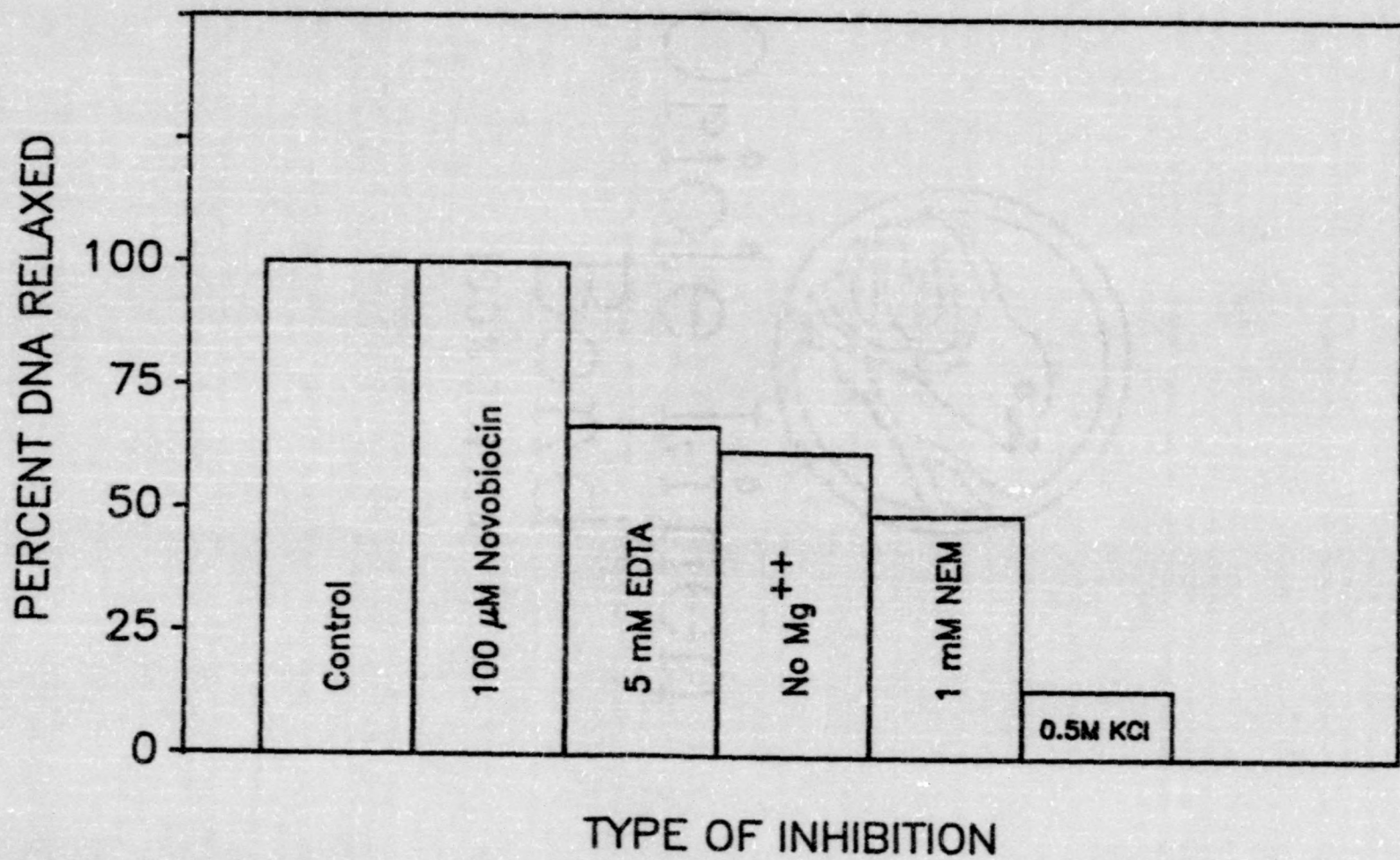
FIGURE 23: Effects of inhibitors on activity of the native enzyme expressed as percent of supercoiled DNA relaxed.

DISCUSSION

ENZYME CLASSIFICATION

Class I eukaryotic topoisomerases from the cell nucleus are capable of relaxing supercoils in covalently closed double-stranded DNA without the energy of ATP. They have largely been found not to be dependent upon Mg++ or the presence of other divalent cations but have been shown to be stimulated by them. These enzymes are not sensitive to inhibition by 100 uM novobiocin, which does inhibit class II enzymes. These three characteristics and the inability to catenate covalently closed circles in the presence of a condensing agent have become accepted as methods for the characterization and identification of class I topoisomerases (Wang, 1985).

The nuclear enzymes isolated to date from higher eukaryotic organisms have native molecular weights in the range of 220 Kd to 325 Kd and are most often characterized as homodimers based upon an apparent molecular weight on SDS-PAGE of 175 Kd to 150 Kd (Tables I and III).

The results of this experimentation indicate that a class I topoisomerase has been isolated from soybean nuclei. This enzyme readily relaxes negatively supercoiled pBR322 DNA in vitro and in the absence of ATP. The enzyme is not measurably inhibited by 100 uM novobiocin and is not magnesium dependent.

SOYBEAN TOPOISOMERASE I INHIBITION

Soybean topoisomerase I is inhibited by NEM. Of the concentrations tested (0, 1, 5 and 40 mM), 1 mM causes maximum inhibition (Figure 22). The threshold for sensitivity is between 0 and 1 mM NEM and maximum inhibition can only be narrowed to the range of 0-5 mM from the data available. Cauliflower nuclear topoisomerase I has been shown to be sensitive to inhibition by NEM at 3 mM (Fukata and Fukasawa, 1986). All aspects of the assay used in these experiments were identical to those of Fukata and Fukasawa except the source of reagents. Yeast topoisomerase I is reported to be completely inhibited by 0.5 mM NEM (Goto et al. 1984).

Assays of the enzyme in the presence and absence of 0.5 mM ATP indicate that soybean topoisomerase I is slightly inhibited by ATP (Figure 19). Similar inhibition of a completely purified enzyme has been found for the vaccinia virus 32 Kd topoisomerase I-like enzyme (Shaffer and Traktman, 1987). It is notable that the smallest activity found here (29 Kd) is likewise inhibited by ATP (data not shown).

Soybean topoisomerase I activity declines markedly as KCl concentration increases (Figure 21). Inhibition is greater than 85% in the presence of 0.5 M KCl (Figure 23). Vaccinia virus topoisomerase has been found to be remarkably similar to eukaryotic type I topoisomerase and is inhibited by elevated levels of NaCl (Shaffer and Traktman, 1987). The vaccinia topoisomerase (Shaffer and Traktman, 1987) and the topoisomerase

of the protozoan parasite <u>Plasmodium</u> <u>berghei</u> (Riou <u>et</u> <u>al</u>., 1986) have optimal salt concentrations of 100 to 150 mM. Soybean topoisomerase does not have an absolute requirement for salt and if there is an optimum concentration for KCl it must lie between 0 and 40 mM (see Figure 21). At 40 mM KCl, relaxation of plasmid DNA was 98 percent of the control lane containing 0 KCl. A data point is not shown in Figure 21 for the control lane because specific activity was incalculable for lanes where 100 percent of the plasmid DNA was relaxed.

EFFECTS OF MG^{++} AND MN^{++} ON SOYBEAN TOPOISOMERASE I

The vast majority of eukaryotic type I topoisomerases isolated to homogeneity to date do not have an absolute requirement for a divalent cation but are stimulated as much as 10-20 fold by the presence of magnesium (Gellert, 1981; Rowe <u>et</u> <u>al</u>., 1981; Riou <u>et</u> <u>al</u>., 1986). Type II topoisomerases by contrast absolutely require a divalent cation. In both cases, Mg^{++} has proven to be the most effective cation with Mn^{++} slightly less effective (Wang, 1985; Riou <u>et</u> <u>al</u>., 1986; Shaffer and Traktman, 1987).

The topoisomerase isolated from soybeans in these experiments is stimulated to a greater degree by Mg^{++} than by Mn^{++} at concentrations lower than 14 mM at which point stimulation was equal for either cation. The interesting aspect of enzyme activity in the presence of divalent cations is the existence of a single concentration (6 mM) which represents both

the peak concentration for enzyme activity in the presence of Mn^{++} and the concentration of minimum activity for Mg^{++}. In the range of approximately 5 to 7 mM Mn^{++} and only in that range, $Mn+^{+}$ is more efficient than Mg^{++} in stimulating the activity of soybean topoisomerase I. No similar observation was found in the literature regarding other topoisomerases. Further research in this area needs to include a more detailed study of this phenomena especially in the range around 6 mM.

APPARENT NATIVE MOLECULAR WEIGHT

The apparent molecular weight of this enzyme is approximately 225 Kd as measured by S-200 sephacryl gel filtration and approximately 300 Kd as measured by SDS-PAGE. This compares with a native molecular weight of 200 Kd for the soybean topoisomerase (Fukata and Fukasawa, 1982).

INTRACELLULAR LOCATION

Eukaryotic topoisomerase I is a known component of the nuclear scaffold and indeed had been identified as a major nuclear scaffolding protein before Wang's discovery of the Omega protein (Champoux, 1978; Wang, 1985). The enzyme described here is assumed to be a nuclear protein. Chloroplasts and mitochondria were effectively excluded by etiolation and centrifugation. A small amount of activity was present in the cytoplasmic fraction of the preparation after precipitation by ammonium sulfate and filtration through S-200 sephacryl (to

separate the activity from nucleases which interfere with the assay). This activity level could be expected to occur due to nuclear leakage during sample homogenization and nuclear isolation. No conclusion can be drawn concerning the in vivo existence of an active cytoplasmic enzyme.

ENZYME PURIFICATION

The soybean enzyme binds to DEAE sepharose but not to CM cellulose at pH 8.0 indicating that the entire enzymatic pool detectable under these assay conditions is cationic at this pH. This creates a rapid and very inexpensive method for the purification of the enzyme. DEAE and CM affinity materials are widely available and easy to work with. If CM cellulose is employed first, the wash fraction can be applied directly to a DEAE sepharose column. The bound fraction can be eluted using a step or linear KCl gradient. Because KCl in excess of 0.5 M severely inhibits soybean topoisomerase I activity, it cannot be concluded from this study whether some topoisomerase activity, perhaps topoisomerase II, elutes between 0.5 and 1.0 M KCl from affinity columns. Cauliflower topoisomerase I and II bind to CM sephadex and elute differentially with topoisomerase II eluting in the high salt portion of the elution gradient (Fukata and Fukasawa, 1982). Further work in this area should include desalting and assay of fractions eluted from DEAE sepharose by a 0.0 to 1.0 M KCl gradient both in the presence and absence of 0.5 mM ATP.

It was noted during experimentation that enzyme activity remained high in samples purified by gel filtration only but rapidly declined in fractions that had passed through DEAE sepharose affinity columns. Further study should be done concerning possible effects of these columns. Removal of Mg^{++} and changes in buffer composition are two possible results of affinity chromatography which could result in more rapid denaturation of the purified enzyme. Phosphocellulose columns are known to remove Mg^{++} and this has been a problem with protein stability in the isolation of tubulin (Dr. Robley Williams, personal communication). Both the homogenization media and the solubilization buffer in these experiments contained Mg^{++} but the equilibration and elution buffers did not.

Although specific activity data is not available, enzyme activity in progressively purified samples remained high despite constantly decreasing total protein content (as judged by sample absorbance at 280 nm). This level of purification by column chromatography revealed only the four bands previously noted when analyzed on non-denaturing polyacrylamide gels and was therefore concluded to be essentially homogeneous. The enzyme fractions declined drastically in activity after separation on acrylamide and electroelution from the gel, presumably because of protein denaturation. Total yield was approximately 0.5 mg of total protein from 168 grams of fresh tissue. This was contained in four activity bands with the majority of the protein (approximately 0.4 mg) contained in the two high molecular

weight bands. Specific activity was approximately the same for all samples but was consistently low at about 50 units/mg. Gel filtration was a gentler procedure and resulted in less pure but more stable preparations. If enzyme activity can be stabilized following affinity chromatography, then gel filtration on Sephacryl S-200 would be the favored technique for separation of purified enzyme. This study relied on the use of a simple yes or no test for topoisomerase activity in major protein peaks on the S-200 profiles in the range of reported molecular weights. Further work should also involve a complete activity profile of affinity purified preparations applied to a S-200 sephacryl column in light of the existence of low molecular weight species with topoisomerase activity. Such profiles, if conducted with appropriately diluted samples, could be used to construct a quantitative enzyme activity curve of final S-200 purified samples. This method of purification in conjunction with density gradient sedimentation (which unfortunately was not available during these experiments) would also give a more accurate estimate of the molecular weights of the four enzymatically active molecules isolated in these experiments.

OTHER ACTIVE SUBUNITS OR DOMAINS

The native topoisomerase I of soybean nuclei has a molecular weight of 225 or 300 Kd depending upon the method of estimation employed. This conclusion agrees well with a molecular weight estimated to be greater than 200 Kd (Fukata and

Fukasawa, 1982) for topoisomerase I isolated from cauliflower
inflorescences. That study was based entirely on data from
column chromatography and reported no data concerning subunit
structure.

There is evidence in the literature that polypeptides of
considerably less mass than 200 Kd are capable of removing
superhelical twists from DNA. A 67.7 Kd carboxyl-terminal
fragment from the human topoisomerase I gene has been shown to
exhibit topoisomerase I activity when expressed as a fusion
protein linked to a 32 Kd fragment of the bacterial TrpE protein
(D'Arpa et al., 1988). Vaccinia virus encapsidates an even
smaller polypeptide with the properties of a eukaryotic type I
topoisomerase (Shaffer and Traktman, 1987). This 32 Kd
polypeptide has a region homologous to a region of the 80 Kd type
I topoisomerase from the yeast Saccharomyces cerevisiae
suggesting that active domains in eukaryotic organisms may have
been evolutionarily conserved (Shuman and Moss, 1987). More
specifically, a dissociation product of 30 Kd exhibiting
nicking-closing activity has been isolated from an ATP
independent nuclear topoisomerase from the lower eukaryote
Ustilago maydis (Rowe et al., 1981).

Data collected in these experiments suggest that the native
soybean topoisomerase I may be composed of subunits or active
domains as small as 27 Kd. The native enzyme appears to
dissociate into three smaller molecular subspecies which retain
enzymatic activity as measured by relaxation of negatively

supercoiled pBR322 DNA. These three molecular subspecies together with the native enzyme were present in non-denaturing polyacrylamide analysis of fractions from the crude nuclear extract through the most pure samples obtained by column chromatography (Figure 13). The molecular weights of these subunits as estimated by electrophoresis on non-denaturing polyacrylamide were 180, 30 and 25 Kd. The fact that they were present in fractions at all stages of purification (including very pure samples resolving in the range of 225 Kd on S-200 sephacryl) suggest they resulted from the dissociation of the native enzyme. A separate molecular weight estimate of 155 Kd for the heaviest of these three subspecies was obtained from S-200 sephacryl gel filtration data (see Figure 7). More accurate molecular weights of 29 and 27 Kd for the two lightest subspecies were obtained by SDS-PAGE analysis of the most purified preparations after electroelution from non-denaturing polyacrylamide gels. Unfortunately no such data is available for the heavier subspecies. It is further assumed that these molecular subspecies are the result of spontaneous dissociation, not enzymatic proteolysis, because even those samples that had been separated by gel electrophoresis and recovered by electroelution partially dissociated when analyzed on SDS-PAGE.

Some of the data presented in the Results section is confusing, but what is certainly clear from these experiments is that the native enzyme has a molecular weight of between 225 and

300 Kd but dissociates under the conditions of these experiments into enzymatically active molecular subspecies of approximately 155, 29 and 27 Kd. Furthermore it may be hypothesized that the dissociation is the result of the cleavage of disulfide bonds. It is likely that the two lightest subspecies are a single polypeptide that exists in two forms with slightly different Kav values on acrylamide gels. This light form is able to reassemble in the absence of BME and SDS to form a dimeric unit.

It might be postulated that the topoisomerase I enzyme from soybean consists of a 68 Kd basic unit composed of two 29 Kd domains linked by disulfide bonds. The native enzyme may then be a homotetramer composed of these units or a homodimer that in vitro is associated on the nuclear matrix with other topoisomerase dimers and thus extracts as aggregates of two such enzymes. There is no evidence in the literature concerning the subunit structure of higher plant class I topoisomerases to either refute or support such a hypothesis. The evidence collected here is tentative and other models are possible; however, nothing in this research would rule out such a hypothesis.

SUMMARY

In summary, it can be concluded that we have purified to apparent homogeneity a class I topoisomerase from the nuclei of soybean hypocotyls. The native enzyme is similar to the type one topoisomerase of cauliflower in having a molecular weight falling somewhere in the range of 225 to 300 Kd. This enzyme, like other eukaryotic enzymes of its genre, does not require the energy of ATP for the relaxation of negatively supercoiled DNA, does not require but is stimulated by the presence of Mg^{++}, is not inhibited by 100 uM concentrations of the class II topoisomerase inhibitor novobiocin, is inhibited by mM amounts of NEM, and is slightly inhibited by the presence of ATP at 0.5 mM concentrations.

Furthermore, a body of data has been presented concerning the relative efficiency of stimulation by Mn^{++} as compared to Mg^{++}. This enzyme is stimulated more by Mn^{++} in a narrow concentration range on either side of 6 mM.

Evidence from these experiments indicates that an active domain of approximately 29 Kd exists in the native soybean enzyme which is capable of nicking-closing activity. In the presence of BME, the native enzyme is capable of dissociating to yield this 29 Kd polypeptide. The existence of an intermediate dissociation product of approximately 155 Kd coupled with evidence that the 29 Kd polypeptide can re-aggregate to form a 68 Kd unit makes it seem unlikely that the enzyme is a simple monomer.

LITERATURE CITED

Attardi, D.G., De Paolis, A. and G.P. Tocchini-Valentini. 1981. Purification and characterization of Xenopus laevis type I topoisomerase. J. Biol. Chem., 256: 3654-3661.

Andoh, T., Ishii, K., Suzuki, Y., Ikegami, Y., Kusunoki, Y., Takemoto, Y., and K. Okada. 1987. Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc. Natl. Acad. Sci. USA, 84: 5565-5569.

Behe, M. and G. Felsenfeld. 1981. Effects of methylation on a synthetic polynucleotide: The B-Z transition in poly (dG-m5d)*poly(dG-m5dC)." Proc. Natl. Acad. Sci. USA, 78: 1619-1623.

Blair, D.G. and D.R. Helinski. 1975. Relaxation complexes of plasmid DNA and protein: Strand-specific association of protein and DNA in the relaxed complexes of plasmids ColE1 and ColE2. J. Biol. Chem., 250: 8785-8789.

Bryant, J.A. and V.L. Dunham. 1988. The Biochemistry of DNA Replication. In: DNA Replication in Plants. Bryant, J.A. and V.L. Dunham editors., CRC Press Inc., Boca Raton, Florida.

Champoux, J.J. 1978. Proteins that effect DNA conformation. Ann. Rev. Biochem., 47: 449-479.

D'Arpa, P., Machlin, P. S., Ratrie, H., III, Rothfield, N. F., Cleveland, D. W. and W. C. Earnshaw. 1988. cDNA cloning of human DNA topoisomerase I: Catalytic activity of a 67.7 Kd carboxyl-terminal fragment. Proc. Natl. Acad. Sci. USA, 85: 2543-2547.

DiNardo, S., Voelkel, K. and R. Sternglanz. 1984. DNA Topoisomerase II mutant of Saccharomyces cerevisiae: Topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. Proc. Natl. Acad. Sci. USA, 81: 2616-2620.

Drake, F.H., Zimmerman, J.P., McCabe, F.L., Bartus, H.F., Per, S.R., Sullivan, D.M., Ross, W.E., Mattern, M.R., Johnson, R.K., Crooke, S.T. and C.K. Mirabelli. 1987. Purification of Topoisomerase II from amsacrine-resistant P388 leukemia cells. J. Biol. Chem., 262: 16739-16747.

Dunham, V.L. and J.A. Bryant. 1986. DNA polymerase
activities in healthy and cauliflower mosaic
virus-infected turnip (Brassica rapa) plants. Ann.
Bot., 57: 81.

Dyan, W.S., Jendrisak, J.J., Hager, D.A. and R.R. Burgess.
1981. Purification and characterization of wheat germ
DNA topoisomerase I. J. Biol. Chem., 256: 5860-5875.

Echeverria, M., Therese, M., Ricard, B. and S. Litvak.
1986. A DNA topoisomerase type I from wheat embryo
mitochondria. Plant Molecular Biol., 6: 417-427.

Fleishman, G., Pflugfelder, G., Steiner, E.K., Javaherian,
K., Howard, G.C., Wang, J.C. and S.C.R. Elgin. 1984.
Drosophila topoisomerase I is associated with
transcriptionally active regions of the genome. Proc.
Natl. Acad. Sci. USA, 81: 6958-6962.

Fukata, H. nd H. Fukasawa. 1982. Isolation and partial
characterization of two distinct DNA topoisomerases
from cauliflower inflorescence. J. Biochem., 91:
1337-1342.

Fukata, H. and H. Fukasawa. 1986. Isolation and
characterization of DNA topoisomerase II from
cauliflower inflorescences. Plant Molecular Biol., 6:
137-144.

Fuller, R.S., Kaguni, J.M. and A. Kornberg. 1981.
Enzymatic replication of the origin of E. coli
chromosome." Proc. Natl. Acad. Sci. USA, 78:
7370-7374.

Garg, L.C., DiAngelo, S. and S.T. Jacob. 1987. Role of
DNA topoisomerase I in the transcription of supercoiled
rRNA gene. Proc. Natl. Acad. Sci. USA, 84: 3185-3188.

Gellert, M. 1981. DNA Topoisomerases. Ann. Rev. Biochem.,
50: 879-910.

Giaever, G., Lynn, R., Goto, T. and J.C. Wang. 1986. The
complete nucleotide sequence of the structural gene TOP2
of Yeast DNA topoisomerase II. J. Biol. Chem., 261:
12488-12454.

Goto, T. and J.C. Wang. 1982. An ATP-dependent type II
topoisomerase that catalyzes the catenation,
decatenation, knotting, and relaxation of double-
stranded DNA rings. J. Biol. Chem., 257: 5866-5872.

Goto, T., Laipis, P. and J.C. Wang. 1984. The purification
and characterization of DNA topoisomerases I and II of
the yeast Saccharomyces cerevisiae. J. Biol. Chem.,
259: 10422-10429.

Halligan, B.D., Davis, J.L., Edwards, K.A. and L.F. Liu.
1982. Intra- and intermolecular strand transfer by
HeLa DNA Topoisomerase I. J. Biol. Chem., 257:
3995-4000.

Hsieh, T. and D. Brutlag. 1980. ATP dependent DNA
topoisomerase from D. melanogaster reversibly catenates
duplex DNA rings. Cell, 21: 115-125.

Javaherian, K., Tse, Y.C. and Jose Vega. 1982. Drosophila
topoisomerase I: isolation, purification and
characterization. Nucleic Acids Res., 10: 6945-6955.

Jazwinski, S.M., and G.M. Edelman. 1984. Evidence for
participation of a multi-protein complex in yeast DNA
replication in vitro. J. Biol. Chem., 259: 6852-6857.

Jazwinski, S.M. 1987. Participation of ATP in the binding
of a yeast replicative enzyme complex to DNA.
Biochem. J., 246: 213-219.

Kaguni, J.M. and A. Kornberg. 1984. Topoisomerase I
confers specificity in enzymatic replication of the E.
coli chromosomal origin. J. Biol. Chem., 259:
8578-8583.

Laemmli, U.K. 1970. Cleavage of structural proteins during
the assembly of the head of bacteriophage T4. Nature,
227: 680-685.

Low, R.L., Kaguni, J.M. and A. Kornberg. 1984. Potent
catenation of supercoiled and gapped DNA circles by
Topoisomerase I in the presence of a hydrophilic
polymer. J. Biol. Chem., 259: 4576-4581.

Nakamura, H., Morita, T. and C. Sato. 1986. Structural
organizations of replicon domains during DNA synthetic
phase in the mammalian nucleus. Exp. Cell Res., 165:
291-297.

Nielsen, B.L., and K.K. Tewari. 1988. Pea chloroplast
topoisomerase I: Purification, characterization, and
role in replication. Plant Mol. Biol., 11: 3-14.

Osheroff, N., Shelton, E.R. and D.L. Brulag. 1983. DNA
topoisomerase II from Drosophila melanogaster.
J. Biol. Chem., 258: 9536-9543.

Osheroff, N. and L. Zechiedrich. 1987. Calcium-promoted
    DNA cleavage by eukaryotic topoisomerase II: Trapping
    the covalent enzyme-DNA complex in an active form."
    Biochemistry, 26: 4303-4309.

Osheroff, Neil pHD Vanderbilt University, Department of
    Biochemistry, Nashville, TN. Personal Communication.

Peck, L.J., Nordheim, A., Rich, A. and J.C. Wang. 1982.
    Flipping of cloned d(pCpG)n*d(pCpG)n DNA sequences
    from a right- to a left-handed helical structure by
    salt, Co(III), or negative supercoiling. Proc. Natl.
    Acad. Sci. USA, 79:
    4560-4564.

Pohl, F.M. and T.M. Jovin. 1972. Salt-induced
    co-operative conformation change of a synthetic DNA:
    Equilibrium and kinetic studies with poly (dG-dC). J.
    Mol. Biol., 67:
    375-396.

Riou, J.-F., Gabillot, M., Phillippe, M., Schrevel, J. and
    G. Riou. 1986. Purification and characterization of
    Plasmodium berghei DNA topoisomerases I and II: Drug
    action, inhibition of decatenation and relaxation, and
    stimulation of DNA cleavage. Biochemistry, 25:
    1471-1479.

Rowe, T.C., Rusche, J.R., Brougham, M.J. and W.K. Holloman.
    1981. Purification and properties of a topoisomerase
    from Ustilago maydis. J. Biol. Chem., 256:
    10354-10361.

Schomburg, U., and F. Grosse. 1986. Purification and
    characterization of DNA topoisomerase II from calf
    thymus associated with polypeptides of 175 and 150 KDa.
    Eur. J. Biochem., 160: 451-457.

Siedlecki, J., Zimmerman, W. and A. Weissbach. 1983.
    Characterization of a prokaryotic topoisomerase I
    activity in chloroplast extracts from spinach.
    Nuc. Acids Res., 11: 1523-1536.

Shaffer, R. and P. Traktman. 1987. Vaccinia virus
    encapsidates a novel topoisomerase with the properties
    of a eukaryotic type I enzyme. J. Biol. Chem., 262:
    9309-9315.

Shuman, S. and B. Moss. 1987. Identification of a vaccinia
    virus gene encoding a type I DNA topoisomerase. Proc.
    Natl. Acad. Sci. USA, 84: 7478-7482.

Vinograd, J., Lebowitz, R., Radloff, R., Watson, R. and P. Laipis. 1965. The twisted circular form of polyoma DNA. Proc. Natl. Acad. Sci. USA, 53: 1104- 1111.

Waddell, W.J. 1956. A simple ultraviolet spectrophotometric method for the determination of protein. J. Lab. Clin. Med., 48: 311-314.

Wang, J.C. 1971. Interaction between DNA and an Escherichia coli Protein W (omega). J. Mol. Biol., 55: 523-533.

Wang, J.C. 1985. DNA Topoisomerases. Ann. Rev. Biochem., 54: 665-697.

White, J.H., Millet, K.C. and N.R. Cozzarelli. 1987. Description of the Topological entanglement of DNA catenanes and knots by a powerful method involving strand passage and recombination. J. Mol. Biol., 197: 585-603.

Williams, Robley pHD. Vanderbilt University, Department of Molecular Biology, Nashville, TN. Personal Communication.