We have previously shown that a DNA topoisomerase I from mouse mammary carcinoma cells is inhibited by heparin. Taking advantage of this enzyme-heparin interaction, we developed a rapid and efficient method of purification of this enzyme to near homogeneity by extraction of chromatin with 0.15 M phosphate buffer followed by two-step column chromatography on heparin-Sepharose and phenyl-Sepharose. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gels revealed that the final preparation is composed of two polypeptides with apparent Mr of 98,000 (p98) and 102,000 (p102). p98 comprising 70% and p102 30%. Extraction and renaturation of the polypeptides from the gel shows that both p98 and p102 seem to possess topoisomerase activity. Partial proteolytic digestion of p98 and p102 with Staphylococcus aureus V8 and chymotrypsin yielded a series of identical peptides, indicating that the two polypeptides are structurally related. The enzyme sedimented through sucrose density gradient with s20, w of 4.0 S, and thus is monomeric in solution.

An enzyme activity from mammalian cells that removes positive and negative superhelicity turns from DNA was first described by Champoux and Dubceco (1972). Similar enzymes were subsequently found in various eukaryotic organisms largely in association with chromatin (Champoux, 1978; Wang and Liu, 1979). The physiological functions of type I topoisomerase, as is termed according to the recommendations of Wang and Liu (1979), have been the subject of speculation (Champoux, 1978; Wang and Liu, 1979; Cozzarelli, 1980; Gellert, 1981). Although they were originally assumed to form a swivel which would facilitate replication and/or transcription, recent discoveries have suggested additional roles: the finding that the bacteriophage λ int protein is a type I topoisomerase (Kikuchi and Nash, 1979) and recent isolation and characterization of Escherichia coli mutants lacking topoisomerase I (Sternaglanz et al., 1981) suggested that one of the possible roles of this enzyme may be in the recombinational processes. Prus et al. (1982) and DiNardo et al. (1982) recently reported results clearly demonstrating that all of the previously isolated topoisomerase I-deficient mutants of Escherichia coli possessed a second additional mutation in either of the two gyrases subunit loci gyr A and gyr B, thus compensating for the lack of topoisomerase I in maintaining the proper degree of superhelicity of chromosomal DNA. Weisbord (1982) and Javaherian and Liu (1983) have shown in Xenopus laevis erythrocytes and in HeLa cells, respectively, that topoisomerase I is bound in active nucleosomes presumably in association with high mobility group proteins. These results strongly suggested that topoisomerase I is an essential enzyme involved in various genetic processes and hence indispensable for survival.

We describe in this paper a rapid and efficient method of purification of the topoisomerase I from cultured mouse mammary carcinoma cells for various studies using purified enzyme. The method involves extraction of purified chromatin with low salt concentration followed by successive chromatography on heparin-Sepharose and phenyl-Sepharose. After the two chromatographic steps, the enzyme was purified to near homogeneity. From 10 g of wet pellet of the mouse mammary carcinoma cells, we typically obtain about 100 μg of topoisomerase within 2 days. The final preparation contained two polypeptides with Mr = 98,000 (p98) and 102,000 (p102), p98 comprising about 70% and p102 ~30%. Both polypeptides seem to possess topoisomerase activity. Furthermore, the two polypeptides were shown to be structurally related by peptide mapping after partial proteolysis by Staphylococcus aureus V8 and chymotrypsin.

EXPERIMENTAL PROCEDURES

Materials—Heparin-Sepharose 6B and phenyl-Sepharose 4B were purchased from Pharmacia; Seikem agarose (ME) was from Marine Colloids, Inc.; acrylamide and methylenebisacrylamide were from Seikagaku Kogyo, Inc.; tetrathymethenediamine was from Wako Pure Chemicals, Inc.; BSA1 was from Bethesda Research Laboratories; S. aureus V8 protease was from Miles; α-chymotrypsin type IA was from Sigma. Col E1 DNA was prepared from E. coli A745met-thy (col E1) by the method of Sakakibara and Tomizawa (1974).

Topoisomerase Assay—The principle of the assay is the decreased mobility in an agarose gel of supercoiled DNA after treatment with topoisomerase (Keller, 1975). Assays contained 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM spermidine, 0.2 mM PMSF, 150 mM sodium chloride, 100 μg/ml of BSA, 10% glycerol, and 1 μg of supercoiled col E1 DNA in a final volume of 50 μl. Reactions were started by addition of 5 μl of the sample to be assayed and were allowed to proceed for 15 min at 37 °C. Reactions were terminated by addition of an equal volume of double strength sample buffer (40 mM Tris-HCl, pH 7.5, 0.4 mM EDTA, 20% sucrose, 2% SDS, and 0.31% bromphenol blue) and incubation at 45 °C for 5 min. The samples were loaded on a 1.2% agarose gel cast in a horizontal slab apparatus. The electrophoresis buffer contained 36 mM Tris-HCl, pH 7.7, 30 mM sodium phosphate, 1 mM EDTA. For discrimination of relaxed closed circular DNA from nicked circular form II DNA, samples were electrophoresed in agarose gels containing chlorogaine as described by Shure et al. (1977).

Purification of Topoisomerase—All steps in the purification were carried out at 0–4 °C. Centrifugations were for 15 min at 7000 g. Chromatin extract containing topoisomerase activity was prepared from cultured mouse mammary carcinoma FM3A cells (Nakano, 1981), taking advantage of this enzyme-heparin interaction.

1 The abbreviations used are: BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PMSF, phenylmethylkane sulfonyle fluoride; NP40, Nonidet P-40.
solution B and centrifuging through the layer of hypotonic buffer A was applied at a flow rate of 50 ml/h. The lysate was purified by suspending in 5 to 10 volumes of NP40 of hypotonic buffer A (0.2 mM sodium phosphate, pH 7.5, 0.5 mM EDTA, 0.2 mM PMSF, pH 7.5). Crude chromatin obtained by centrifugation of the lysate was purified by suspending in 5 to 10 volumes of hypotonic buffer A used was at pH 7.5. The same washing procedure was repeated two more times except that the hypotonic buffer used was at pH 8.5.

Chromatin extract usually containing 150-200 µg of protein/ml was applied at a flow rate of 50 ml/h to a column (0.7 x 1.0 cm) of heparin-Sepharose equilibrated with elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 mM EDTA, 0.2 mM PMSF and 10% glycerol) containing 0.16 M NaCl. Unless otherwise mentioned, solutions used hereafter for heparin- and phenyl-Sepharose chromatography contained the elution buffer. After application, the column was washed with 5 ml of 0.4 M NaCl and eluted with a total of 50 ml of a linear gradient of 0.4-0.9 M NaCl. One-half-ml fractions were collected and 5 µl of appropriately diluted samples were assayed for topoisomerase activity. The fractions containing the bulk of the activity were pooled.

The purified fractions were adjusted to 1 M ammonium sulfate by addition of an equal volume of 2 M ammonium sulfate. This sample, typically 10 ml, was applied to a column (1.0 x 2.0 cm) of phenyl-Sepharose equilibrated with 1 M ammonium sulfate, and the column was washed with 10 ml of 1 M ammonium sulfate. Topoisomerase was eluted from the column with a total of 25 ml of a linear decreasing gradient of ammonium sulfate from 1.0 to 0.2 M. One-half-ml fractions were collected, and 5 µl of appropriately diluted samples were assayed for topoisomerase activity. The fractions containing the bulk of the activity were pooled and stored frozen at -80°C. The activity was maintained practically unaltered for at least 1 month.

Renaturation of Topoisomerase after Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out with the discontinuous buffer system of Laemmli (1970) and with prescription of acrylamide and methylenebisacrylamide according to Blatter et al. (1972). The procedure used for elution of proteins from the gel, removal of SDS, and renaturation of enzymatic activity was as described by Hager and Burgess (1980) and Dynan et al. (1981).

Peptide Mapping Analysis of Topoisomerase—Partial proteolytic digestion with S. aureus V8 and chymotrypsin was done according to Clevland et al. (1977). A sample of about 2 µg of the phenyl-Sepharose fraction was subjected to SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue, and regions corresponding to protein bands were cut out. Each gel slice was placed in a well of another SDS-polyacrylamide slab gel consisting of a stacking gel (3% polyacrylamide) and separating gel (15% polyacrylamide). Various amounts of the protease were added to each well and electrophoresis was carried out. Proteins were partially digested during electrophoresis, and resulting peptides were separated on the gel.

Other Procedures—Protein concentration was determined by the Bio-Rad protein assay kit. Silver staining of proteins on polyacrylamide gels was performed using the Bio-Rad silver stain kit. Sucrose gradient sedimentation of topoisomerase was performed as follows. Two-tenths ml of the phenyl-Sepharose fraction of topoisomerase (50 µg of protein) was overlaid on 5 ml of a 10 to 22% linear sucrose density gradient containing 40 mM Tris-HCl, pH 8.0, 0.25 mM NaCl, and 0.2 mM PMSF, and centrifuged for 27 h at 19,500 x g at 4°C in a Beckman SW 50.1 rotor.

Results
Comments on the Purification—Topoisomerase activity can be detected in whole cell extract, but total activity calculated tends to be lower than that of chromatin extract, suggesting the interference with some materials in the extract. Chromatin extract containing virtually all of the enzymatic activity was prepared according to Germond et al. (1975). In order to recover most of the activity in this fraction, extraction buffer in the preparation and washing of chromatin should contain 0.2 mM PMSF. Briefly, cells were lysed with 0.25% NP40 in hypotonic buffer and resulting chromatin in the lysate was pelleted by centrifugation. The crude chromatin was washed three times by suspending the pellet and centrifuging through the 0.1 M sucrose layer. Since we have previously shown that the topoisomerase is strongly inhibited by heparin (Ishii et al., 1982), we took advantage of this enzyme-heparin interaction and used heparin-Sepharose for the purification of this enzyme. Thus, the chromatin extract was applied to a heparin-Sepharose column.

A typical heparin-Sepharose column profile is shown in Fig. 1. There is no topoisomerase activity in the column flow-through or wash fractions. Since the peak of activity, which elutes between 0.6 and 0.8 M NaCl, contains only about 1.6% of the protein applied to the column, almost 50-fold purification is obtained at this step. As shown in Fig. 1B, a prominent band with apparent Mr = 98,000 (p98) indicated by an arrow seemed to be intimately associated with enzymatic activity.

![Fig. 1. Heparin-Sepharose affinity chromatography of topoisomerase I. A, chromatin extract (240 ml) was prepared from 10 g of mouse mammary carcinoma cells and applied to a heparin-Sepharose column as described under "Experimental Procedures." After washing with 0.4 M NaCl (solid arrow), the enzyme was eluted with a linear gradient of NaCl from 0.4 to 0.9 M (---) as shown by the open arrow. Protein concentration (O) and enzymatic activity (●) were determined. The inset shows the topoisomerase activity of fractions; samples were from left to right starting material, i.e. chromatin extract, flow-through, 0.4 M NaCl wash, and odd-numbered fractions from the gradient elution. FI and FIr denote the positions of supercoiled form I DNA and relaxed form I DNA, respectively. B, SDS-polyacrylamide gel electrophoresis of fractions as indicated. SM and FT denote starting material and flow-through, respectively.](http://www.jbc.org/)
Fig. 2. Identification of topoisomerase activity on SDS-polyacrylamide gels. A, enzymatically active fraction from heparin-Sepharose chromatography was electrophoresed alone (HFr) or as mixture (MIX) with standard marker proteins (ST) on SDS-polyacrylamide gels and stained with Coomassie blue. Marker proteins and their respective molecular weights are as follows: E. coli RNA polymerase holoenzyme (β subunit, 145,000; β' subunit, 155,000; α subunit, 90,000; α subunit, 36,500); rabbit muscle phosphorylase b, 97,000; BSA, 68,000. The sample lane (HFr) was cut into 12 segments as indicated and each segment was extracted and assayed for topoisomerase activity as described under "Experimental Procedures." B, agarose gel pattern of form I col El DNA after incubation with each extract.

Fig. 3. Determination of active polypeptide of topoisomerase on a SDS-polyacrylamide gel. A, enzymatically active fraction from heparin-Sepharose was electrophoresed on a SDS-polyacrylamide gel and stained. Major (b) and minor bands (a) were separately cut out and extracted and assayed for topoisomerase activity as in Fig. 2. B, agarose gel patterns of form I col El DNA after incubation with various amounts of each extract.

To determine which polypeptide on the gel the topoisomerase activity was associated with, we ran a sample of the heparin-Sepharose fraction on an SDS-polyacrylamide gel, cut the region from the top of the gel to the marker dye into 12 sections, and subjected each section to the protein elution and renaturation procedure described under "Experimental Procedures" (Fig. 2). We then correlated activity with the positions of polypeptides. As shown in the lower figure, we found that most of the topoisomerase activity was in region number 5 which contained doublet bands. Fig. 3 shows further that the enzymatic activity seems to be associated with both of the two polypeptides in regions a and b. The possibility of the enzymatic activity associated with the minor band a being a result of the contamination of the major band b, however, cannot be excluded. The molecular weight of the major enzyme was determined to be approximately 98,000 by comparison with marker proteins on SDS-polyacrylamide gels.

The concentration of ammonium sulfate in the pooled heparin-Sepharose fractions was increased to 1 M by addition of 2 M ammonium sulfate solution in the elution buffer, and then applied to a phenyl-Sepharose column. The peak of activity eluted between 0.6 and 0.4 M ammonium sulfate (Fig. 4A). Results shown in the lower figure (Fig. 4B) clearly dem-

Fig. 4. Phenyl-Sepharose hydrophobic chromatography of topoisomerase I. A, pooled heparin-Sepharose fractions were adjusted to 1 M ammonium sulfate and applied to a phenyl-Sepharose column as described under "Experimental Procedures." After washing with starting buffer, the enzyme was eluted with a linear decreasing gradient from 1.0 to 0.2 M ammonium sulfate (- - - - - -). Protein concentration (C) and enzymatic activity (○) were determined. The inset shows the topoisomerase activities of various fractions; samples were from left to right starting materials, i.e. pooled heparin fraction, flow-through, and odd-numbered fractions from the gradient elution. B, SDS-polyacrylamide gel electrophoresis of fractions as indicated. Marker proteins were run in parallel.
Demonstrated that in the final preparation of active fraction the major topoisomerase band was still accompanied by a minor band with apparent $M_t = 102,000$ (p102). The major 98,000-Da polypeptide in the final preparation makes up about 70% of the mass of proteins in the peak fractions shown in Fig. 4A. Table I summarizes the results of this purification. About 180-fold purification with an overall yield of 54% was obtained starting from chromatin extract. The purified topoisomerase was shown to be free of contaminating protease by incubating aliquots of the enzyme either at room temperature for 48 h or at 4 °C for 72 h, and then analyzing by SDS-polyacrylamide gel electrophoresis. Nuclease was not detected in the preparation by incubation of supercoiled col E1 DNA with the enzyme at 20 °C for 4 h and analysis of the product by agarose gel electrophoresis containing chloroquine.

**Table I**

| Purification step           | Total proteins (mg) | Total activity (units/$10^3$) | Specific activity (units/mg) | Yield (%) | Purification (fold) |
|-----------------------------|---------------------|------------------------------|----------------------------|-----------|---------------------|
| Chromatin extract           | 36                  | 240                          | 67                         | 100       | 1                   |
| Heparin-Sepharose           | 0.59                | 175                          | 2,966                      | 73        | 45                  |
| Phenyl-Sepharose            | 0.11                | 130                          | 11,820                     | 54        | 177                 |

**Fig. 6.** Sucrose density gradient sedimentation of purified topoisomerase. A, an aliquot of topoisomerase (topo I) in phenyl-Sepharose fraction was centrifuged alone or as a mixture with marker proteins BSA (4.5 S) and aldolase (7.6 S) for 27 h at 19,500 × g at 4 °C. Fractions were collected from the top and assayed for topoisomerase activity (O) and absorbance at 280 nm (C). The inset shows agarose gel electrophoresis of form I col E1 DNA after incubation of aliquots of fractions indicated below.

**Fig. 5.** Partial proteolytic digestion of p98 and p102 with *S. aureus* V8 and chymotrypsin. Phenyl-Sepharose fractions were electrophoresed on a SDS-polyacrylamide gel, and stained with Coomassie blue. The leading half of the main band polypeptide p98 and all of satellite band p102 were separately cut out and the segments were digested with various amounts of the proteases as described under "Experimental Procedures." Gels were stained with the Bio-Rad silver stain kit. A, digestion by *S. aureus* V8. Track a contains the mixture of p98 and p102, and track j contains 500 ng of the protease alone. Tracks b to e, and f to i show peptides released after digestion of p98 and p102, respectively, with the protease at concentrations of 0 (b, f), 0.1 (c, g), 0.5 (d, h), and 1.0 μg (e, i) per slot. B, digestion by chymotrypsin. Track a contains the mixture of p98 and p102, and track j contains 1.0 μg of chymotrypsin. Tracks b to e and f to i show peptides released after digestion of p98 and p102, respectively, with chymotrypsin at concentrations of 0 (b, f), 0.1 (c, g), 0.5 (d, h), and 1.0 μg (e, i) per slot.

**Peptide Composition of the Two Proteins p98 and p102**—To test the structural similarity of the two proteins p98 and p102, we compared the patterns of their partial proteolytic digests by *S. aureus* V8 and chymotrypsin. As shown in Fig. 5, the electrophoretic patterns of these peptides on SDS-polyacrylamide gels changed with the concentrations of the protease used, but with fixed concentrations the patterns of the products of the two proteins were almost indistinguishable: both p98 and p102 gave rise to several discernable and indistinguishable peptide bands when digested either with V8 protease or chymotrypsin, strongly suggesting that p98 and p102 are structurally similar and related each other.

**Sucrose Density Gradient Centrifugation of Topoisomerase I**—As analyzed by sucrose density gradient centrifugation, phenyl-Sepharose-purified topoisomerase sedimented as a single symmetrical peak on the lighter shoulder of BSA (Fig. 6A). The sedimentation coefficient ($s_{20w}$) of topoisomerase was calculated to be 4.0 S. SDS-polyacrylamide gel electrophoresis of aliquots of fractions demonstrated that topoisomerase is still $M_t = 98,000$ and 102,000 after centrifugation. These results suggest that the topoisomerase assumes rather rod-like conformation and is monomeric in solution.


**DISCUSSION**

In the present work, we described a rapid and convenient method of purification of topoisomerase I from a cultured line of mouse mammary carcinoma FM3A cells. The method consisted of low salt extraction of chromatin followed by two steps of column chromatography on heparin-Sepharose and phenyl-Sepharose. Table I summarizes the result of the purification. We obtained about 180-fold purification with an overall yield of 54% starting from the chromatin extract. Since almost all enzymatic activities are recovered from the chromatin extract, nearly 7000-fold purification was attained starting from whole cell extract. Completion of this purification procedure takes about 48 h in succession. This time can be reduced to one-third with similar yield and purity by introduction of the following modifications. Enzyme is adsorbed to heparin-Sepharose by mixing with chromatin extract. After packing the beads onto a column and washing with 0.53 M NaCl in elution buffer, enzyme is eluted in one step with 1.0 M NaCl. This eluate is adjusted to 1.0 M ammonium sulfate by addition of an equal volume of 2.0 M ammonium sulfate and applied to a column of phenyl-Sepharose. After washing with 0.8 M ammonium sulfate, enzyme is eluted with 0.4 M ammonium sulfate.

The finding that partial proteolytic digestion of the two proteins p98 and p102 in the purified preparation gave rise to a series of identical peptides clearly demonstrated that they are structurally similar and related. One could be a modified form of the other. The modification could be either phosphorylation, glycosylation, or poly(ADP)-ribosylation. This is under investigation. Since the mass ratio of these two peptides does not change with or without the addition of a protease inhibitor PMSF throughout the purification procedure, the possibility of p98 being derived from p102 by partial proteolysis during purification would be negligible. However, if it occurs it would be an in vivo phenomenon. It is relevant to refer to Liu and Miller (1981) who showed that the 67-kDa species of topoisomerase was formed by proteolysis of the 100-kDa species in HeLa cells. We isolated here for the first time the structurally related two polypeptides of topoisomerase I with very close $M_r \sim 100,000$. We could not detect any smaller species with $M_r = 60,000-70,000$ as often reported in various organisms such as human KB cells (Keller, 1975), rat liver (Champoux and McConaughy, 1976), mouse L cells (Tang, 1978), and human HeLa cells (Liu and Miller, 1981).

The establishment of the rapid and efficient method of purification enables us to obtain the enzyme in large quantity for various studies including further structural analyses.

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