# Cross-Linking of Novikoff Ascites Hepatoma Cytokeratin Filaments†

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ABSTRACT: We have investigated the structure of solubilized cytokeratins from Novikoff ascites hepatoma using the cleavable cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) in the presence of 6 M urea to effect partial complex melting. By two-dimensional gel electrophoresis, in which the protein cross-links were broken in the second dimension, we have identified two major complexes as a p39–p56 dimer and a $(p39–p56)_2$ tetramer, p39 and p56 being two of the major cytokeratins in Novikoff ascites hepatoma. Experiments investigating possible relationships between the dimer and tetramer employed immunoblots and two monoclonal antibodies which recognized either p56 or p39 cytokeratins. When very low protein concentrations were cross-linked, the dimer was the predominant product. As protein concentration increased, we noted a decrease in dimers and a corresponding increase in tetramers, suggesting that the dimer may be a precursor to the tetramer. In support of the cross-linking experiments, two-dimensional gel electrophoresis using 4 M urea in the first dimension indicated a predominant association of p56 and p39 in the Novikoff ascites hepatoma cytokeratin complexes.

The cytokeratins are a group of water-insoluble proteins in the molecular weight range of 40 000–70 000 that comprise the 10-nm, cytostructural intermediate filaments of epithelial cells (Lazarides, 1980; Moll et al., 1982). Epithelial cell types can be classified on the basis of the cytokeratin composition (Moll et al., 1982), and recent reports suggest that profiles of cytokeratin expression may be important in the pathodiagnosis of various neoplasms (Debus et al., 1984; Franke et al., 1982). Cytokeratins have been divided into two groups: the larger, more basic type II and the smaller, acidic type I proteins (Fuchs et al., 1981). Every cell type thus far examined contains at least one cytokeratin from each group (Schiller et al., 1982).

Novikoff ascites hepatoma contains three proteins originally described in chromatin (Schmidt et al., 1981) and subsequently identified as cytokeratins (Schmidt et al., 1982), termed p39, p49, and p56 with apparent molecular weights of 39 000, 49 000, and 56 000, respectively. By immunotransfer methodology, p39 was detected in a wide variety of rat carcinomas (Schmidt & Hnilica, 1982), and recent immunohistochemical studies showed the protein to be present in several types of simple and ductular epithelial cell types (Schmidt et al., 1984). On the other hand, p56 was observed in normal rat liver (Schmidt et al., 1981; Venetianer et al., 1983), while in our laboratory, p49 was detected only in Novikoff ascites hepatoma. The latter protein, however, may be related to cytokeratin D characterized in normal liver (Venetianer et al., 1983).

In view of recent data suggesting that cytokeratin filaments from a specific cell type are composed of two kinds of cytokeratins (Schiller et al., 1982; Franke et al., 1983; Geisler & Weber, 1982), it was of interest to investigate the subunit composition of intermediate filaments from Novikoff ascites hepatoma. It has been shown that cytokeratins solubilized in 4 M urea appear as rodlets, 2 nm × 50 nm, by electron microscopy and are thought to be the building blocks of intermediate filaments (Franke et al., 1983). In this study, we have investigated the structure of cytokeratins from Novikoff ascites hepatoma solubilized in urea using a cleavable protein–protein cross-linker, 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP),[1] and two monoclonal antibodies, one highly specific for p39. The results are discussed in light of current models of cytokeratin structure.

## Experimental Procedures

*Materials.* Male Sprague-Dawley rats were purchased from Harlan Industries (Indianapolis, IN). Nucleases and rabbit muscle aldolase (Sigma Chemical Co., St. Louis, MO), 3,3′-dithiobis(sulfosuccinimidyl propionate) and bis(sulfosuccinimidyl suberate) (Pierce Chemical Co., Rockford, IL), ampholines, pH 5–8 (LKB, Gaithersberg, MD), and ultrapure urea (Schwarz/Mann Corp., Cambridge, MA) were obtained from the respective suppliers.

*Preparation of Cytokeratin-Enriched Fraction.* Novikoff ascites hepatoma was maintained by weekly transplantations in male Sprague-Dawley rats. Cells were collected and incubated in 0.17 M $NH_4Cl$ for 10 min on ice to lyse contaminant red blood cells and centrifuged at 800*g* for 7.5 min. Pellets were resuspended in normal saline and centrifuged at 10000*g* for 10 min. Cytokeratin-enriched fraction was prepared as described previously (Schmidt et al., 1984). Briefly, 20 mL of packed hepatoma cells was resuspended in 180 mL of 10 mM Tris, pH 7.4, and 1 mM PMSF, briefly homogenized, and sonicated for three 20-s bursts to disrupt the cells. DNase I and RNase A were added to 50 μg/mL each, $MgCl_2$ was added to 2 mM, and the solution was incubated for 1 h on ice. Next, 180 mL of 4 M NaCl, 0.2% Nonidet P-40, and 10 mM Tris, pH 7.4, and 1 mM PMSF were added to the homogenate, and the solution was homogenized and centri-

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); $BS^3$, bis(sulfosuccinimidyl suberate); SDS, sodium dodecyl sulfate; NAH, Novikoff ascites hepatoma; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton(s).

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fuged at 140000$g$ ($r_{max}$) for 1 h. The pellets were resuspended in 2 M NaCl, 0.1% NP-40, and 10 mM Tris, pH 7.4, and 1 mM PMSF and centrifuged again at 140000$g$ for 1 h. Finally, the pellets were solubilized in 30 mL of 8 M urea and 25 mM HEPES, pH 7.4, stirred for 3 h at room temperature, and centrifuged at 200000$g$ for 1 h. The pellet was discarded and the protein concentration of the supernatant measured by trichloroacetic acid precipitation.

*Cross-Linking Rabbit Muscle Aldolase and Mouse IgG.* Cross-linking experiments were performed as described by Staros (1982) with modifications described in detail in Figure 1. Purified mouse IgG was solubilized to 10 mg/mL with 25 mM HEPES, pH 7.4, and rabbit muscle aldolase was prepared as described by Staros (1982). All reactions were for 30 min, at room temperature with gentle agitation, and stopped by the addition of one-fifth volume of 20 mM *N*-ethylmaleimide, 50 mM ethanolamine, and 50 mM Tris, pH 7.4. After the samples were cross-linked, they were dialyzed into 2 mM Tris, pH 7.4, and prepared for SDS gel electrophoresis as described previously (Schmidt & Hnilica, 1982). Samples treated with 2-mercaptoethanol prior to electrophoresis were incubated with 5% 2-mercaptoethanol at 100 °C for 5 min.

*Cross-Linking Novikoff Cytokeratins.* All cross-linking experiments were performed as described by Staros (1982) with modifications. The cytokeratin-enriched fraction was diluted to the appropriate protein concentration (as indicated in Figures 2–5) in 25 mM HEPES, pH 7.4, and dialyzed to the stated urea concentration (as indicated in Figures 2–6) for 4 h at room temperature. To cross-link, appropriate amounts (see Figures 2–5) of 40 mM DTSSP stock (prepared just before use in 25 mM HEPES, pH 7.4, with the appropriate urea concentration) were added to each protein mixture, incubated for 30 min at room temperature, and quenched with one-fifth volume of 20 mM *N*-ethylmaleimide, 50 mM ethanolamine, and 50 mM Tris, pH 7.4. The solutions were then dialyzed against 2 mM Tris, pH 7.4, and prepared for SDS gel electrophoresis as described previously (Schmidt & Hnilica, 1982).

*Gel Electrophoresis of Cross-Linked Samples.* SDS–polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). One-dimensional gel consisted of a 3% acrylamide stacking gel and a 4–10 acrylamide linear gradient separating gel. To form the gradient, 4% and 10% acrylamide separating gel solutions containing 0.2 mg/mL ammonium persulfate were prepared as described by Laemmli (1970). The two solutions were put into separate chambers of a gradient mixer with the 10% acrylamide solution being in the chamber with the outlet, and 0.1% (v/v) *N,N,N′,N′*-tetramethylethylenediamine was added to each chamber just before pouring of the gel. Two-dimensional gel electrophoresis, in which the protein–protein cross-links were cleaved in the second dimension (Wang & Richards, 1974), was performed by first separating the proteins on a 4–10% acrylamide gradient slab gel and slicing the lanes into individual strips. The strips were then incubated in 0.1 SDS and 62.5 mM Tris, pH 6.8, for 2 h. Finally, each strip was embedded onto a second slab gel containing a 3% stack and 7.5% separating gel, with 1% agarose containing 10% 2-mercaptoethanol. After electrophoresis, the gels were either stained with Coomassie Brilliant Blue or transferred electrophoretically to nitrocellulose and assayed for proteins reactive with each one of the two monoclonal antibodies described below (Schmidt et al., 1984).

*Isoelectric Focusing.* Urea was added to the cytokeratin-enriched fraction described above (10 M urea final concentration) for normal isoelectric focusing, or the sample was dialyzed to 4 M urea for focusing in 4 M urea gels. To both preparations were added 2% Nonidet P-40, 2% (w/v) pH 5–8 ampholines (obtained from LKB), and 5% (v/v) 2-mercaptoethanol. The first-dimension isoelectric focusing gels were poured as described (O'Farrell, 1975) except that some gels contained only 4 M urea (Franke et al., 1983). The samples were then loaded on the appropriate gel, electrophoresed, and separated by SDS–PAGE in the second dimension as described by O'Farrell (1975). The gels were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose and assayed for immunoreactive proteins (Schmidt et al., 1984).

*Monoclonal Antibodies.* Monoclonal antibodies were raised against purified preparations of p39 and p56, as described previously (Schmidt et al., 1984).

## Results

*Cross-Linking with Mouse IgG and Rabbit Muscle Aldolase.* Since all cross-linking studies with Novikoff cytokeratins were performed in 5.5 M urea, it was first necessary to determine the effect of urea on protein–protein cross-linking with DTSSP. This was accomplished with two proteins that had well-established quaternary structures, mouse IgG and rabbit muscle aldolase, and a second cross-linker, $BS^3$. $BS^3$ was identical with DTSSP, except that it lacked the disulfide linker region.

Mouse IgG contained endogenous disulfide cross-links, and solubilization in urea would not dissociate the tetramer complex, made up of two heavy and light chains unless 2-mercaptoethanol was included in the buffer (lanes 1 and 2, Figure 1). However, after being cross-linked with $BS^3$, the noncleavable analogue of DTSSP, in the absence or presence of 8 M urea (lanes 3 and 4, respectively, of Figure 1), the complex remained partially associated even in the presence of 2-mercaptoethanol. These findings indicated that $BS^3$ appeared to be completely reactive in urea concentrations up to 8 M. Since DTSSP and $BS^3$ have identical protein cross-linking moieties, it was concluded that DTSSP was also not inhibited by urea.

Rabbit muscle aldolase existed as a tetramer of four identical subunits in solution and was readily cross-linked by treatment with DTSSP without urea into dimers, trimers, and tetramers (lane 6, Figure 1; Staros, 1982). When aldolase was first solubilized in 8 M urea and then treated with DTSSP, very little cross-linking was observed, because aldolase subunits were not attached by disulfides and high urea concentrations dispersed the tetramer (lane 7, Figure 1). Note that DTSSP cross-links formed without urea were easily cleaved by 2-mercaptoethanol (lane 8, Figure 1). When aldolase was cross-linked with DTSSP in buffer without urea, then solubilized to 8 M urea, and cross-linked a second time with $BS^3$, the cross-linked products were stable in 2-mercaptoethanol, indicating the formation of cross-links by $BS^3$ in 8 M urea (lane 9, Figure 1). We suspect that the slightly lower reactivity of $BS^3$ in this experiment (compare lanes 6 and 9) was due to cross-linking sites on the aldolase partially saturated by DTSSP. Thus, it was possible to demonstrate cessation of cross-linking by dispersal of the target protein complex.

Finally, note that the monomer in lane 9 of Figure 1 was resolved into two bands. This may be due to intramolecular cross-linking, resulting in slightly different mobilities for the same protein (Giedroc et al., 1983).

*Cross-Linking of Cytokeratin Complexes with DTSSP in Various Concentrations of Urea.* To investigate the subunit structure of NAH cytokeratin complexes, we first established urea concentrations which would partially dissociate and

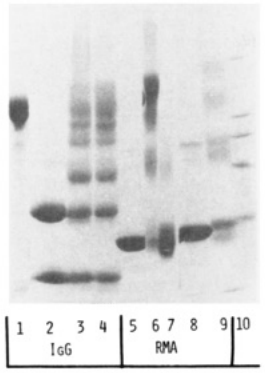


FIGURE 1: Rabbit muscle aldolase and mouse IgG cross-linking with DTSSP and $BS^3$. Mouse IgG and rabbit muscle aldolase were treated with DTSSP or $BS^3$, as described below. Samples were electrophoresed in a 4–10% acrylamide gradient gel, with all samples containing 2-mercaptoethanol on one side of the gel separated from other samples by at least four lanes. This was necessary since we have noted some cleavage of DTSSP resulting from diffusion of 2-mercaptoethanol from neighboring lanes. The gel was then sliced, reorganized for clear presentation, and stained with Coomassie Brilliant Blue. Lane 1, purified mouse IgG electrophoresed under nonreducing conditions; lane 2, IgG incubated with 5% 2-mercaptoethanol prior to electrophoresis; lane 3, IgG reacted with 1 mM $BS^3$ in 25 mM HEPES (1 mg of protein/mL), pH 7.4, and incubated with 5% 2-mercaptoethanol prior to electrophoresis; lane 4, IgG first solubilized in 8 M urea and 25 mM HEPES, pH 7.4, then reacted with 1 mM $BS^3$ (1 mg of protein/mL), and then reduced with 2-mercaptoethanol; lane 5, rabbit muscle aldolase; lane 6, aldolase incubated with 1 mM DTSSP in 50 mM sodium phosphate (0.76 mg/mL), pH 7.4; lane 7, aldolase solubilized in 8 M urea and reacted as in lane 6; lane 8, same as lane 6 except that the sample was incubated with 5% 2-mercaptoethanol prior to electrophoresis; lane 9, aldolase was cross-linked with DTSSP as in lane 6 and dialyzed against 50 mM sodium phosphate, pH 7.4, after the reaction was stopped. The solution was then solubilized to 8 M urea, incubated with 1 mM $BS^3$, and reduced with 5% 2-mercaptoethanol prior to electrophoresis. Lane 10, molecular weight markers (myosin, 200 000; $\beta$-galactosidase, 116 000; phosphorylase *b*, 94 000; bovine serum albumin, 68 000; and ovalbumin, 43 000).

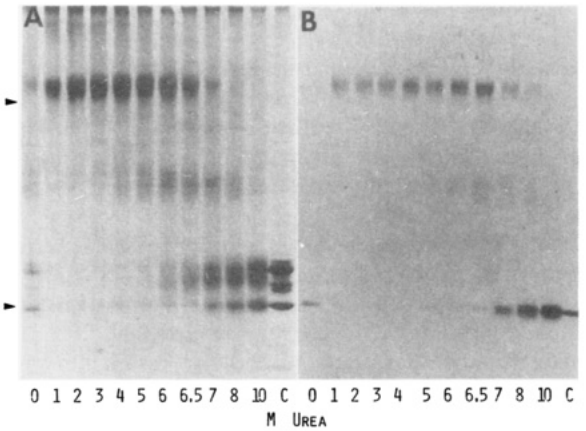


FIGURE 2: Optimal urea concentrations for cross-linking NAH cytokeratins with DTSSP. Novikoff cytokeratin-enriched fractions (1.0 mg of protein/mL) were solubilized in 10 M urea and dialyzed to various concentrations of urea, as indicated in the figure. Solutions were then reacted with 1.0 mM DTSSP (except lane C). Samples were dialyzed, solubilized in SDS gel electrophoresis buffer, and electrophoresed in 4–10% acrylamide gradient gels. The gels were stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose and strained for proteins immunoreactive with a monoclonal antibody specific for p39, anti-p39. (B) Arrowheads denote the molecular weight markers myosin ($M_r$ 200 000) and ovalbumin ($M_r$ 43 000).

solubilize the complexes and allow cross-linking with DTSSP. Figure 2 shows results of experiments in which freshly isolated cytokeratin complexes were solubilized in 10 M urea, dialyzed to 0–10 M urea concentrations, and cross-linked. Control,

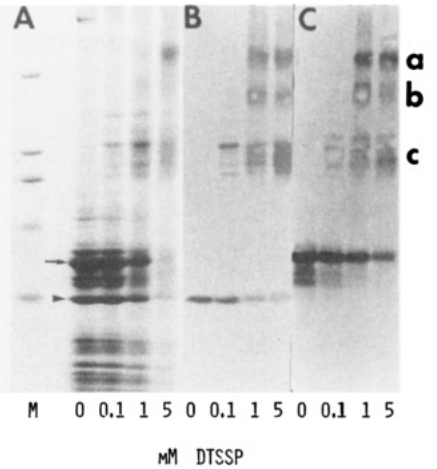


FIGURE 3: Optimal DTSSP concentrations for cross-linking NAH cytokeratins. Novikoff cytokeratin-enriched fractions were solubilized in 5.5 M urea at 0.8 mg of protein/mL and reacted with various concentrations of DTSSP as indicated. Samples were then dialyzed, solubilized in SDS gel electrophoresis buffer, and electrophoresed in 40–10% acrylamide gradient gels. The gels were stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose and stained for proteins immunoreactive to anti-p39 (B) or anti-p56 (C). Arrow, p56; arrowhead, p39.

un-cross-linked preparations showed polypeptide and antigenic profiles which we have described previously (Schmidt et al., 1982, 1984; lane c, Figure 2A). Cross-linking of solubilized cytokeratins with DTSSP resulted in the formation of broad, higher molecular weight bands, putatively cross-linked oligomers (Figure 2A). Cross-linking was progressively enhanced in urea concentrations of 0–4 M, while higher concentrations of urea resulted in a gradual decline in higher molecular weight species. We suspect that the initial increase in cross-linking observed in 0–4 M urea may be due to increased solubilization of the cytokeratins and to the solvent-relaxing tertiary conformations of the complexes, exposing additional cross-linking sites. The decrease of these oligomers in urea concentrations greater than 6 M (lanes 6–10, Figure 2A) indicates the final dissociation and solubilization of the complex (Franke et al., 1983). From these data, we concluded that 6.0 M urea was the optimum concentration of urea for cross-linking, while 8 M urea was sufficient to dissociate higher molecular weight complexes.

Immunotransfer analysis of these experiments confirmed that cross-linked, higher molecular weight complexes contained the p39 cytokeratin (Figure 2B). These immunoblots employed a monoclonal antibody (anti-p39) which we have recently characterized and reported to be highly specific for p39 (Schmidt et al., 1984; Ward et al., 1984). Therefore, a comparison of un-cross-linked polypeptides (lane C, Figure 2B) with cross-linked preparations (lanes 0–10, Figure 2B) indicated p39 as a component of the higher molecular weight complexes.

*Effect of Increasing DTSSP Concentrations on Cytokeratin Cross-Linking.* We next investigated the ability of increasing concentrations of DTSSP (0–5 mM) to effect cross-linking of complexes solubilized in 5.5 M urea. The appearance of broad, high molecular weight cross-linked bands was markedly dependent upon DTSSP concentration (Figure 3) in that the largest species were produced by 5 mM DTSSP. To probe these complexes antigenically, we employed two different monoclonal antibodies, the anti-p39 antibody used above and a new monoclonal antibody recently prepared to purified NAH p56 cytokeratin (panels B and C of Figure 3, respectively). The latter antibody reacted predominantly with p56 but also

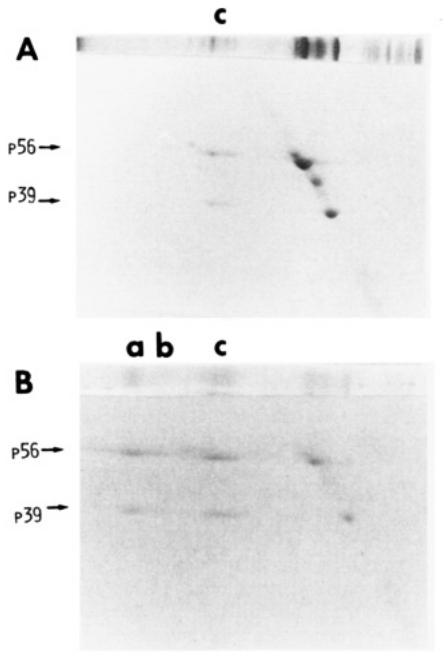


FIGURE 4: Two-dimensional gel electrophoresis of cross-linked NAH cytokeratin. Novikoff cytokeratins (0.8 mg of protein/mL) were reacted with 0.1 mM DTSSP (A) or 1.0 mM DTSSP (1.0 mg of protein/mL) (B). Samples were then electrophoresed in 4–10% acrylamide gradient gels for the first dimension. The gels were cut into strips and polymerized onto 7.5% acrylamide gels with 1% agarose containing 10% 2-mercaptoethanol. After electrophoresis in the second dimension, gels were stained with Coomassie Brilliant Blue. The first-dimension profiles for these gels are shown at the top of each gel.

recognized several other cytokeratins in the molecular weight range of 41 000–56 000; most importantly, however, the antibody did not react with p39 (lane 0, Figure 3C).

Immunoblots stained with either the anti-p39 or the anti-p56 monoclonal antibody showed that the complexes consisted of p39 and most likely p56, although the heterogeneity of the anti-p56 complicated the latter conclusion. These antigenic bands of cross-linked, higher molecular weight species, labeled A, B, and C in Figure 3, reproducibly electrophoresed at 226–204 (A), 175–160 (B), and 110–90 kDa (C).

*Two-Dimensional Electrophoresis of Cross-Linked Samples.* To define the composition of the cross-linked species required two-dimensional gel electrophoresis, in which cross-links were cleaved in the second dimension. These experiments are illustrated in Figure 4. Control, untreated samples electrophoresed on two-dimensional gels showed no cytokeratins migrating off the diagonal (data not shown). Samples cross-linked with 0.1 mM DTSSP (Figure 4A), were only C-size complexes were detected, revealed the oligomer C complex to be composed of p39 and p56 monomers. The fact that the p39 and p56 proteins occupied equivalent vertical positions in the second dimension indicated that the proteins had the same mobility in the first dimension and were likely cross-linked. Oligomers A, B, and C were seen by increasing the cross-linker concentration to 5 mM (Figure 4B). Immunoblots of these experiments with anti-p39 and anti-p56 monoclonal antibodies confirmed that these bands were indeed genuine p56 and p39 cytokeratins (data not shown).

Apparent molecular weights of the three major, cross-linked oligomers were assigned from semilogarithmic plots of molecular weight standards. These were, 226–204 (A), 175–160 (B), and 110–90 kDa (C). These data, together with the identical vertical positions in the two-dimensional gels, permitted calculation of the composition of the cross-linked

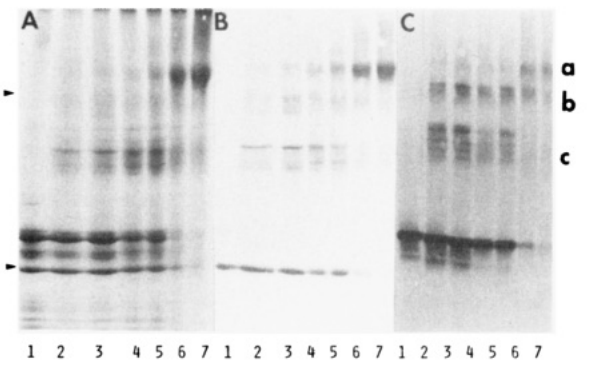


FIGURE 5: Effect of protein concentration on cross-linking of NAH cytokeratins. Freshly prepared cytokeratins were diluted to various protein concentrations, dialyzed to 5.5 M urea, and reacted with 0.1 mM DTSSP (lanes 2 and 3) or 1.0 mM DTSSP (lanes 4–7). Samples were then dialyzed, lyophilized, and solubilized with SDS sample buffer for gel electrophoresis. Samples were electrophoresed in 4–10% acrylamide gradient gels and stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose and stained for proteins immunoreactive to anti-p39 (B) or to anti-p56 (C). Lane 1, control, un-cross-linked sample; lane 2, 0.01 mg of protein/mL; lane 3, 0.2 mg/mL; lane 4, 0.1 mg/mL; lane 5, 0.2 mg/mL; lane 6, 1.0 mg/mL; lane 7, 1.5 mg/mL. Arrowheads denote molecular weight markers myosin ($M_r$ 200 000) and ovalbumin ($M_r$ 43 000).

complexes. Complexes A, B, and C each contained p39 and p56, and in A and C, the ratio of these proteins appeared to be equimolar. Therefore, complex A was probably a $(p39–p56)_2$ tetramer while complex C was a p39–p56 dimer. Although complex B did not resolve as clearly as A and C in the second dimension (even after repeated attempts using a variety of gel lengths and conditions), the composition of this complex was inferred as a $p39(p56)_2$ trimer based on the following observations: (1) the intensity of the Coomassie staining of p56 at the position of complex B in Figure 4B was greater than that of p39 (this was confirmed by densitometer scans), and (2) the apparent molecular weight, 160 000–170 000, was more compatible with a $p39(p56)_2$ trimer than a $(p39)_2p56$ trimer.

*Effect of Protein Concentration on Cytokeratin Cross-Linking.* Having established the optimal conditions for cross-linking and having defined the composition of the various cross-linked complexes, the effect of protein concentration was then investigated. As shown in Figure 5, the amount of $(p39–p56)_2$ tetramer (complex A) increased with increasing protein concentration, with a concomitant decrease in the p39–p56 dimer (complex C) suggesting that the latter is the building block of the tetramer (see lanes 4–7, Figure 5). Furthermore, even at lower cross-linker and protein concentrations (lanes 2 and 3, Figure 5), dimer formation was readily apparent, suggesting that the tetramer formation was dependent upon prior formation of the dimer.

The $p39(p56)_2$ trimer, complex B, decreased antigenically with increasing protein concentration (Figure 5B,C) but generally did not parallel the decrease of the dimer. Although reasons for this behavior are currently unclear, we suspect two possible factors: (1) incomplete cross-linking of the tetramer actually forms the trimer, or (2) it is possible that the trimer may be an obligatory precursor to the tetramer subsequent to the formation of the dimer.

*Isoelectric Focusing of the Cytokeratin Complex.* As a final test of the existence of p39–p56 complexes, a method completely independent of chemical cross-linking was employed (Franke et al., 1983; Eichner et al., 1983). Figure 6A illustrates a typical two-dimensional isoelectric focusing pattern for the Novikoff cytokeratin-enriched fraction. p56 and p39 migrated to their individual isoelectric points: 6.2 and 6.0 for p56 and 5.2 for p39 (Schmidt et al., 1982). When this

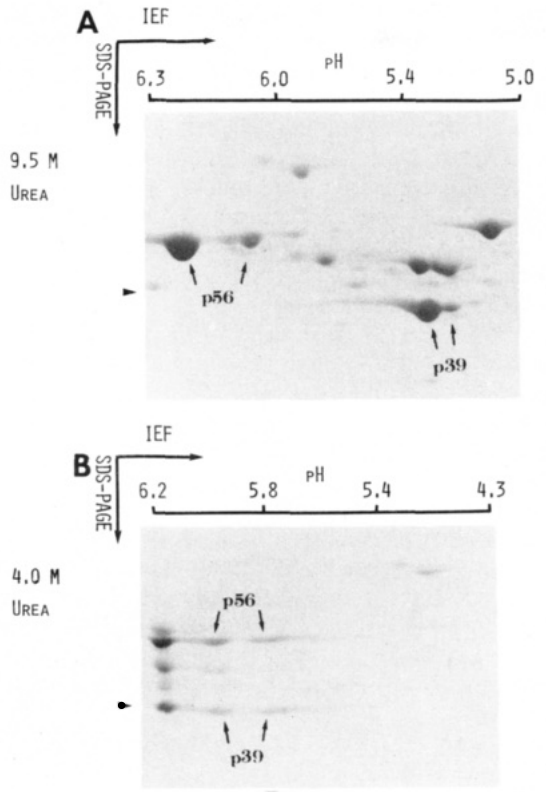


FIGURE 6: Two-dimensional isoelectric focusing in 4 M urea. (A) Novikoff cytokeratin-enriched fraction was solubilized in 9.5 M urea, electrophoresed in isoelectric focusing gels containing 9.5 M urea with pH 5–7 ampholines, and stained with Coomassie Brilliant Blue. (B) Same as in (A) except the cytokeratins were dialyzed to 4 M urea prior to electrophoresis and electrophoresed in isoelectric focusing gels containing only 4 M urea in the first dimension. The band pattern at the left of Figure 6B represents the origin of the first-dimension gel. Arrowheads denote the position of ovalbumin ($M_r$ 43 000) in the second dimension.

preparation was dialyzed to 4 M urea and electrophoresed on an isoelectric focusing gel constructed with 4 M urea, p56 and p39 migrated as an undissociated complex to at least two intermediate isoelectric points, 6.0 and 5.9, as shown in Figure 6B. A small amount of p49 comigrated with the p39–p56 complex, but this was clearly a minor component. The identity of these proteins was confirmed by transferring gels identical with those shown in Figure 6A,B to nitrocellulose and reacting with a combination of anti-p39 and anti-p56 monoclonal antibodies (data not shown).

## Discussion

At least two models describing the association of cytokeratin subunits to form intermediate filaments have been proposed. The model of Steinert (Steinert, 1977; Steven et al., 1982) suggested that three keratin subunits form a coiled-coil $\alpha$-helix and then complex to form the intermediate filament. This was supported by Skerrow and co-workers (Skerrow, 1974; Skerrow et al., 1973). The other model, based on amino acid sequence studies (McLachlan, 1978) and chemical cross-linking studies (Ahmadi & Speakman, 1978), proposed the basic subunit of keratin filaments to consist of four subunit proteins coiled around each other. Aebi et al. (1983) suggested, using electron microscopy, that the 10-nm-diameter cytokeratin filaments were composed of four 4.5-nm-wide protofibrils helically twisted around each other. These protofibrils were shown to be made up of an undetermined number of 2-nm protofilaments, presumably also helically twisted around each other in a coiled-coil fashion (Stewart & McLachlan, 1975; McLachlan, 1978; Parry et al., 1977). According to Steinert's model, this 2-nm protofilament was composed of three protein subunits (Steinert, 1978), while McLachlan's model would describe the protofilament as being made up of a tetramer, composed of two double-stranded helices coiled around each other (McLachlan, 1978).

Recent chemical cross-linking studies with desmin (Geisler & Weber, 1982) and keratin (Ahmadi & Speakman, 1978) indicated that the basic building block of the intermediate filament may have been a tetramer, but neither of these results was confirmed by two-dimensional gel electrophoresis. Under conditions in which the cytokeratin filaments are known to solubilize into 2 nm × 50 nm rodlets (Franke et al., 1983), we have shown that the cytokeratins from Novikoff ascites hepatoma exist primarily as p39–p56 heterodimers and $(p39–p56)_2$ tetramer. Furthermore, by increasing the cytokeratin concentration, which promotes filament formation (Sun & Green, 1978), the disappearance of the p39–p56 dimer was shown to parallel the appearance of the $(p39–p56)_2$ tetramer. These data suggest that the basic building block of the Novikoff cytokeratin filament is a tetramer composed of two p39–p56 heterodimers and support the tetramer model for intermediate protofilaments (McLachlan, 1978).

Previous protein–protein cross-linking studies performed on cytoskeletal proteins have been employed dimethyl suberimidate (Geisler & Weber, 1982; Bretscher & Weber, 1980). This compound is very soluble but has a low half-life in water, which decreases the efficiency of cross-linking (Peters & Richards, 1977). Furthermore, while a method has been developed to cleave protein–protein cross-links resulting from dimethyl suberimidate, this method is harsh on proteins and much more difficult than reduction with 2-mercaptoethanol (Packman & Perham, 1982). Staros (1982) has succeeded in constructing a cross-linker, DTSSP, with the increased efficiency of bis(*N*-hydroxysuccinimide) esters, the high solubility of suberimidates, and a disulfide linkage cleavable by reduction. Finally, since urea is a weak nucleophile, it was possible that such high concentrations of urea would inhibit cross-linking by reacting with DTSSP. Experiments conducted with mouse IgG, however, demonstrated no detectable inhibition of cross-linking with DTSSP by urea. Thus, DTSSP was an ideal cross-linker for the study of in vitro cytokeratin complex formation.

The fact that at 0 M urea less cross-linking was observed than in 1–6.0 M urea was difficult to interpret. It was probable that the insolubility of cytokeratin filaments in aqueous buffer contributed to the decrease in cross-linking at 0 M urea, while partial relaxation of protein tertiary structure exposing additional cross-linking sites increased cross-linking in higher urea concentrations. Nevertheless, since this study concentrated on a well-defined structural subunit of the cytokeratin filament, cross-linking studies were performed at 5.5 M urea. Note that even in 0 M urea, the tetramer and dimer species were seen. At higher concentrations of urea, from 6.5 to 7.0 M, the complex completely dissociated, and cross-linking was markedly reduced (Figure 2). That this phenomenon of urea dissociating a complex was possible was demonstrated with aldolase (lanes 5–9, Figure 1).

Franke et al. (1983) have shown that the intermediate filaments isolated from rat liver and various rat hepatomas are made up of cytokeratins A and D, 55 and 45 kDa, respectively (Venetianer et al., 1983). On the basis of the apparent molecular weight and isoelectric variants, cytokeratin A probably corresponds to the p56 isolated from Novikoff ascites hepatoma (Venetianer et al., 1983; Schmidt et al., 1982). Since our results indicate that in Novikoff ascites hepatoma p56 is

primarily associated with p39, it appears at least one rat hepatoma contains cytokeratin filaments composed of p56 with a protein other than cytokeratin D. A small portion of p56 is associated with p49, which may be related to cytokeratin D, but this is not a major component in Novikoff ascites hepatoma. The significance that p39 apparently replaces cytokeratin D in Novikoff ascites hepatoma intermediate filaments is as yet unclear. It is noteworthy, however, that p56 is a normal component of the hepatocyte but p39 is not[2] (Schmidt & Hnilica, 1982).

Finally, two technical points must be addressed. First, protein–protein interactions not pertinent to a given study often complicate protein cross-linking experiments. Thus, relatively pure samples of proteins are required. In this study, the problem was solved by the immunotransfer method in which only the cross-linked polymers of interest reacted with appropriate antibodies. Second, 2-mercaptoethanol was omitted from the high-salt extraction procedure since it would react with the cross-linker DTSSP (Schmidt et al., 1984; Franke et al., 1978). This did not affect the protein composition of the high-salt extract, however, since two-dimensional isoelectric focusing gels of cytokeratin-enriched fractions prepared in the presence of 25 mM 2-mercaptoethanol were identical with that shown in Figure 6A (data not shown).

Thus, by using a recently developed, more efficient cross-linker, DTSSP, and a rapid procedure for isolating a cytokeratin-enriched fraction from Novikoff ascites hepatoma, we have provided evidence to support the four-stranded model for cytokeratin protofilament structure.

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**Registry** No. DTSSP, 81069-02-5.

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[2] It has been suggested that Novikoff p39 and human cytokeratin p40 are the same proteins (Wu et al., 1982), but monoclonal antibody p39 does not recognize human p40 from colon (Schmidt et al., 1984).