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# Elastin Biosynthesis and Cross-Link Formation in Rabbit Aortic Smooth Muscle Cell Cultures†

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ABSTRACT: Rabbit aortic smooth muscle cells in culture produce insoluble elastin which can be purified by treatment with hot alkali. These cells, when maintained in the same flask for long periods of time, continue to accumulate elastin. Both desmosine and isodesmosine, cross-links unique to insoluble elastin, have also been found to increase with time in culture. The results from pulse–chase studies with radiolabeled proline and lysine confirm these observations. All the data indicate that the appearance of the desmosines in the elastin in these cell cultures is a relatively slow process, while the lysine-derived aldehydes appear quite rapidly.

The formation of insoluble elastin in in vitro cell culture systems has been the focus of several laboratories in recent years. In vivo, the disruption and/or lack of synthesis of the elastin fiber component leads to devastating diseases, such as pulmonary emphysema and, possibly, atherosclerosis. Thus, studies on the biosynthesis of the insoluble elastin fiber in cell cultures can provide valuable information to our understanding of the formation and turnover of this important connective tissue component.

Since elastic fibers are extracellular connective tissue components, the biosynthesis of the insoluble protein component has to have its origin in the intracellular protein synthetic machinery, followed by secretion from the cell and, finally, incorporation into the fiber. By examination of the end product, the insoluble elastin, one is able to learn something of a cell's ability to (1) synthesize protein, (2) hydroxylate prolyl residues, (3) transport macromolecules to the extracellular milieu, (4) assemble larger protein aggregates, and (5) form connective tissue cross-links. Studies of this type will give some insight into the mechanisms of elastin turnover.

The study of elastin synthesis in cell cultures has been limited since very few culture systems are capable of producing insoluble elastin. Thus far, the only cells which have been reported capable of producing insoluble elastin in culture are vascular smooth muscle cells (Ross, 1971; Daoud et al., 1974; Faris et al., 1976; Rucker & Tinker, 1977; Burke & Ross, 1979), human endothelial cells from umbilical cord veins (Jaffe et al., 1978), chondroblasts (Quintarelli et al., 1979), and fibroblasts from bovine ligamentum nuchae (Mecham, 1981).

As noted by several laboratories, aortic smooth muscle cells in culture are capable of synthesizing both insoluble collagen and elastin. The soluble precursors to these connective tissue proteins have also been identified in these same cell cultures (Burke et al., 1977; Scott et al., 1977; Rosenbloom & Cywinski, 1976; Uitto et al., 1976; Foster et al., 1978). Collagen has been shown to accumulate over long periods of time (Salcedo & Franzblau, 1981), and recently these cultures have been shown to accumulate glycosaminoglycans in exactly the same proportion as found in the donor rabbit aorta (Namiki et al., 1980). Few, if any, of the more recent studies have focused on the formation of the insoluble elastin in these cultures.

The studies reported here describe relatively long-term pulse–chase experiments involving the incorporation of lysine and proline into the insoluble fractions of elastin derived from the aortic smooth muscle cell layer. The increase in the total elastin content associated with the cell layer at various times in culture is also examined. The data suggest that desmosine formation is quite slow although cross-link formation (lysine-derived aldehydes) begins rapidly. Hopefully, these data will serve as the base line for future experimental protocols related to elastin fibrogenesis in cell culture systems.

## Materials and Methods

*Smooth Muscle Cell Cultures.* Rabbit smooth muscle cells were isolated and grown from the aortic arch of weanling rabbits as described previously (Faris et al., 1976). The

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cultured cells were maintained in 75-cm$^2$ tissue culture flasks with 20 mL of Dulbecco's modified Eagle's medium (Morton, 1970) which contained 3.7 g/L sodium bicarbonate, 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). The medium was changed twice weekly. Approximately 1.5 × 10$^6$ cells in second passage were placed in 75-cm$^2$ tissue culture flasks. Under these conditions, confluency is reached in 4–5 days. After 18 days in culture, the cells were used for the experiments described below. At this time, the flasks usually contained approximately 5 × 10$^6$ cells.

*Pulse Procedure.* The cells were divided into two groups, some to be pulsed with [$^{14}$C]proline and others to be pulsed with [$^{14}$C]lysine. At the time of pulsing, the spent medium was aspirated off, and the cell layers were washed twice with calcium- and magnesium-free Puck's Saline G solution. The cells were then incubated for 1 h at 37 °C in medium free of either proline or lysine and fetal bovine serum but containing penicillin, streptomycin, and sodium bicarbonate as in the maintenance period, as well as 50 μg/mL sodium ascorbate. The medium in each flask was then replaced with 10 mL of medium which contained either 1 μCi/mL [$^{14}$C]proline (sp act. 250–260 Ci/mol; Schwarz/Mann) or 1 μCi/mL [$^{14}$C]-lysine (sp act. 312 Ci/mol; Schwarz/Mann), but was otherwise identical with that used in the 1-h period described above. The pulse period in all cases was 24 h.

*Chase Procedure.* After the pulse period, the spent radioactive medium was aspirated off and replaced with the routine maintenance medium containing fetal bovine serum as described above. Each flask of cells was changed twice weekly with 20 mL of medium for the specified chase period. At the time of harvesting, the medium was removed from the cells, and each cell layer was washed with 2 mL of 0.9% NaCl. Pooled cell layers from two flasks of [$^{14}$C]proline-pulsed cells were harvested with the aid of a rubber policeman, dialyzed vs. $H_2O$, and lyophilized. In the case of the [$^{14}$C]lysine-pulsed cells, groups of five flasks were pooled, dialyzed, and lyophilized. The lyophilized [$^{14}$C]lysine-pulsed cells were then homogenized in 5.0 mL of $H_2O$ in a motorized glass homogenizer and separated into two aliquots corresponding to two and three flasks of cells, respectively. That portion of the homogenate which represented two flasks of cells was lyophilized directly, while the aliquot of homogenate corresponding to three flasks of cells was reduced with $NaBH_4$ in the following manner before lyophilization.

*$NaBH_4$ Reduction.* To each 3.0-mL aliquot of cell homogenate to be reduced was added 0.5 mL of a solution containing 0.5 mg of $NaBH_4$ in 0.001 N NaOH, and the reaction was allowed to proceed at 4 °C with occasional shaking for 90 min. The reaction mixture was maintained at pH 7.0–9.0 for the 90-min duration, then acidified with 50% acetic acid, and lyophilized. It is rather important that the reduction of the cross-links with $NaBH_4$ or $NaB^3H_4$ be carried out before any base treatment of the tissue is undertaken. If this is not done, serious losses of the cross-linking amino acids do occur (unpublished experiments).

*Insoluble Elastin Preparation.* All lyophilized samples, reduced or not reduced, were suspended in 0.1 N NaOH at 98 °C for 45 min with occasional shaking according to the method of Lansing et al. (1952). Suspensions were centrifuged, and the insoluble residues were washed with $H_2O$ and then hydrolyzed.

*Quantitation of Radioactivity.* The presence of radioactive hydroxyproline, desmosine, and lysine in the cell fractions was determined by hydrolyzing the samples in 6 N HCl at 110 °C for 20 h. The hydrolysates were placed on a Jeolco 6AH automatic amino acid analyzer with a split-stream arrangement. Those cell layers which were reduced with $NaBH_4$ were hydrolyzed with 2 N NaOH at 110 °C for 20 h and analyzed on a Technicon amino acid analyzer equipped with a split-stream arrangement. A gradient system described previously for the lysine-derived aldehydes in elastin (Lent et al., 1969) was employed. Radioactivity was monitored in a Packard Tri-Carb liquid scintillation spectrometer.

Table I: Radioactive Proline and Hydroxyproline Distribution in [$^{14}$C]Proline-Pulsed Aortic Smooth Muscle Cells in Culture

| | total Hyp/flask (cpm × 10$^{-3}$) | total Hyp in elastin fraction (cpm × 10$^{-3}$) | Hyp in elastin fraction (cpm/mg of total protein) |
|---|---|---|---|
| 24-h pulse | 181 | 4.5 | 410 |
| 1-week chase | 206 | 18.9 | 1150 |
| 2-week chase | 195 | 39.3 | 2030 |
| 3-week chase | 308 | 55.7 | 2230 |

Table II: Lysine-Derived Cross-Links in the NaOH-Insoluble Elastin Fraction from Cell Cultures

| | cpm × 10$^{-2}$ | | | ALD (Lys equiv) |
|---|---|---|---|---|
| | DES/flask[a] | HNL/flask[b] | ALD/flask[c] | HNL (Lys equiv) |
| [$^{14}$C]Lys expt | | | | |
| 24-h pulse | trace | 7.3 | 6.6 | 0.5 |
| 1-week chase | 5.2 | 8.6 | 41.4 | 2.4 |
| 3-week chase | 18.9 | 12.5 | 89.6 | 3.6 |
| 5-week chase | 19.4 | 12.4 | 98.0 | 4.0 |
| $NaB^3H_4$ reduced | | | | |
| 18-day culture | | 3500 | 15400 | 4.4 |

[a] DES, isodesmosine + desmosine. [b] HNL, ε-hydroxynorleucine. [c] ALD, reduced aldol condensate. [d] ALD (Lys equiv) = ALD/2, since there are two lysines per aldol residue. HNL (Lys equiv) = HNL/1.

*Elastin Content in Cell Layer Fractions.* In a separate experiment, flasks of rabbit aortic smooth muscle cells at various times in second passage were analyzed for insoluble elastin content. The cell layers (not pulsed) were harvested and then extracted with chloroform–methanol (2:1, v/v). This was followed by washes with ethanol and ether, after which the pellets were allowed to air-dry. The residues were extracted 2 times with 0.1 N NaOH at 4 °C for 1 h with shaking followed by treatment with 0.1 N NaOH at 98 °C for 45 min. The resulting residue was washed with $H_2O$, hydrolyzed in 6 N HCl, in vacuo, at 110 °C for 20 h, and analyzed for amino acid composition. The total content of amino acids was used to determine the total content of elastin.

## Results

*Proline-Pulsed Cells.* As seen in Table I, the total [$^{14}$C]-hydroxyproline associated with the cell layer remains constant throughout the initial phases of the chase period and only increases somewhat in the final week. However, the radioactive hydroxyproline which remains insoluble after the hot NaOH procedure (elastin fraction) increases continually during the chase period whether expressed on a per flask or total protein basis. When measured by ninhydrin, the hydroxyproline and proline contents of these fractions are 7 and 110 residues/1000 residues, respectively. These values are compatible with those of insoluble elastin.

*Lysine-Pulsed Cells. (a) Not Reduced.* Trace amounts of radioactive desmosine and isodesmosine are present in the

Table III: Formation of Lysine-Derived Cross-Links in the NaOH-Insoluble Elastin Fraction from [$^{14}$C]Lysine-Pulsed Aortic Smooth Muscle Cells in Culture[a]

| | cpm × $10^{-3}$ | | | |
|---|---|---|---|---|
| | total cpm/ flask[b] | HNL/ nmol of Leu | ALD/ nmol of ALD | nmol of ALD/ flask |
| 24-h pulse | 4.5 | 17.3 | 101.0 | 6.5 |
| 1-week chase | 6.9 | 10.3 | 286.0 | 14.5 |
| 3-week chase | 15.7 | 6.5 | 398.0 | 22.5 |
| 5-week chase | 15.7 | 5.7 | 287.0 | 34.1 |

[a] HNL, ε-hydroxynorleucine; ALD, reduced aldol condensate. [b] Total cpm = cpm Lys + cpm HNL + cpm ALD.

insoluble elastin fraction of the cell layer after the 24-h pulse. As shown in Table II, the radioactivity in these cross-linking amino acids increases significantly over the first 3 weeks of the chase period. Between 3 and 5 weeks, the radioactivity in the desmosine appears to have leveled off.

(*b*) *Reduced.* The ε-hydroxynorleucine (HNL) and reduced aldol condensate (ALD) fractions, when monitored by radioactivity, appear to be of the same order of magnitude after the 24-h pulse (Table II). One should of course be aware of the fact that two lysyl residues are required for each aldol cross-link formed whereas only one lysine is incorporated into a hydroxynorleucine residue. Thus, the aldol residue contains twice the number of lysine equivalents per molecule when compared to a molecule of HNL. During the entire chase period, the total radioactivity in HNL increases less than 2-fold while the increase in the radioactivity associated with the ALD is 15-fold. As in the case of the desmosines, there is a leveling off of the radioactivity in the ALD and HNL between 3 and 5 weeks after the pulse. On a molar basis, with the assumption of two radioactive lysyl residues per aldol, there is approximately 3.5–4.0 times as much radioactive ALD as HNL in the elastin obtained from 3–5 week chase. In a separate series of experiments, the cell layer was not pulsed with lysine but reduced with $NaB^3H_4$ (New England Nuclear) after 18 days in second passage. In this case (also shown in the table), there is 4.4 times as much radioactivity associated with the ALD when compared to the HNL. This latter value represents the reduced elastin that has accumulated during the entire culture, not just during the pulse period. Since only one tritium atom is incorporated into either the HNL or the aldol cross-link upon reduction, these data are consistent with the lysine pulse studies described above.

The total radioactivity in the elastin fraction from these cell cultures (lysine + HNL + ALD) attains a maximum level between 3 and 5 weeks after pulsing. The values obtained for the specific activity of the lysine-derived cross-links suggest a continual increase in the elastin accumulation (Table III). This observation is corroborated by the continual increase noted in the total ALD concentration present as measured by ninhydrin. Because of the difficulties in accurately calculating the concentration of HNL with the chromatographic conditions employed in these experiments, the radioactivity of HNL is calculated on a per nanomole leucine basis. The decrease in the specific activity of the HNL with chase time would suggest rapid conversion to the more complex cross-links such as the aldol and possibly lysinonorleucine. Since there is a continual increase in elastin accumulation in the cultures and an apparent increase in the total aldol concentration, the specific activity of the ALD would be expected to increase with time, and then begin to decrease as the desmosines are being formed. Thus, the data are compatible with the slow formation of the desmosines.

Table IV: Distribution of Radioactivity (%) between Lysine and Lysine-Derived Cross-Links in the NaOH-Insoluble Elastin Fraction from Lysine-Pulsed Aortic Smooth Muscle Cells in Culture[a]

| | Lys | DES | HNL | ALD |
|---|---|---|---|---|
| 24-h pulse | 75 | 0.1 | 13 | 12 |
| 1-week chase | 22 | 7.0 | 12 | 58 |
| 3-week chase | 28 | 11.3 | 7.5 | 54 |
| 5-week chase | 24 | 11.1 | 7.3 | 58 |

[a] DES, isodesmosine + desmosine; HNL, ε-hydroxynorleucine; ALD, reduced aldol condensate.

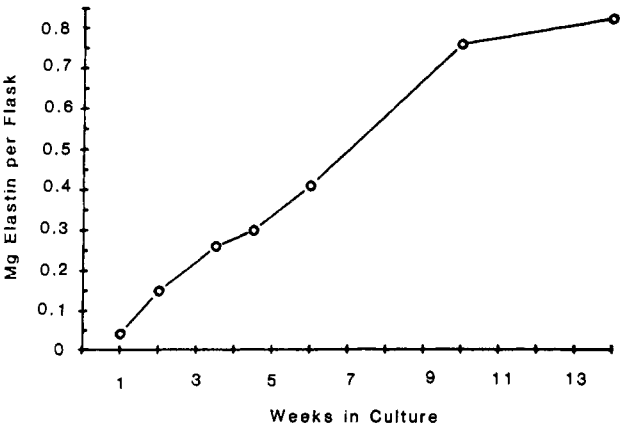


FIGURE 1: Accumulation of insoluble elastin from aortic smooth muscle cells in culture at different periods of time after the second subcultivation.

The distribution of the radioactivity between lysine and the various lysine-derived cross-links with time after the pulse is given in Table IV. The percent distribution of the total radioactivity in lysine sharply decreases while that of the ALD increases within 1 week after the pulse. The percent distribution of the radioactivity in HNL is shown to decrease gradually, while that in the desmosines, which is barely measurable after the pulse, reaches a maximum of approximately 11%. Again, the data indicate that the appearance of desmosine in the elastin in these cell cultures with time is a relatively slow process, while the lysine-derived intermediate cross-links (aldehydes) appear quite rapidly.

*Elastin Content in Cell Layer Fractions.* The total elastin content in the cell layers increases with time (Figure 1). However, the desmosine content in the elastin from the cell cultures, while significant, is less than that found in aortic or ligament elastin. The cell elastin examined here contains 10–15% as much desmosine as that found in the elastin from the other tissues, while the ALD content in the cell elastin is considerably higher (Faris et al., 1976). The amino acid composition of the insoluble elastin is quite consistent throughout the age of the culture, except at the very early and late time periods in second passage. Early in the passage the amino acid composition of the insoluble elastin preparation from the cultures contains more acidic amino acids than normal. This probably reflects a higher proportion of the microfibrillar component in the early developing elastic fiber (Hinek & Thyberg, 1977). Older cultures also contain higher concentrations of polar amino acids, suggesting accumulation or association with other glycoprotein components.

## Discussion

The accumulation of insoluble elastin in rabbit aortic smooth muscle cells has been studied in a series of pulse–chase experiments employing radiolabeled proline or lysine. The fact that one observes no decrease in the total [$^{14}$C]hydroxyproline

content during the chase periods studied suggests that the turnover of collagen and elastin is quite slow in these cultures. The data also suggest that the elastin synthesized during the pulse period becomes insoluble during the chase period as noted by the continual increase in [$^{14}$C]hydroxyproline associated with the insoluble elastin fraction.

Data from the lysine-labeled experiments again indicate the accumulation of insoluble elastin in rabbit aortic smooth muscle cell cultures with time. As noted above, the lysine-derived aldehyde intermediates in cross-link formation appear rapidly while desmosine formation is observed much later during the chase period.

The relative amounts of HNL and ALD present some rather interesting data. One notes that there is approximately 7.5 times as much radioactivity in the ALD compared to the HNL when examining the cell layers 3–5 weeks after the pulse with [$^{14}$C]lysine. If one reduces a similar but not pulsed cell layer with $NaB^3H_4$, thus radiolabeling all of the accumulated elastin cross-links, the ALD contains 4.4 times as much radioactivity as the HNL. This is compatible with the lysine pulse since there are two lysine moieties and one tritium atom incorporated into an ALD residue, but only one lysine and one tritium atom incorporated into each HNL.

The two sets of data actually represent two distinct pools of insoluble elastin. The data from the lysine-pulsed cells represent the fate of those elastin molecules synthesized only during the pulse period, while data from the $NaB^3H_4$-reduced cells (not pulsed) represent the accumulated elastin cross-links formed during the entire growth period. If one examines the lysine label after the 1-week chase, the ALD/HNL ratio is 2.4, while the 24-h pulse yields a ratio of approximately 0.5. After the 3–5-week chase period, the data suggest that the lysine-derived cross-link profile in this elastin fraction has reached a steady state. This ratio (ALD/HNL), 3.6–4.0, is comparable to that found in the elastin fraction obtained from $NaB^3H_4$-reduced cells which were in culture for 18 days. Thus, in culture, a measure of the ratio of these two entities at various times yields information concerning active elastin synthesis.

Radioactive desmosines are not readily apparent in the cell layer after the 24-h pulse of [$^{14}$C]lysine under the conditions we employed. They do appear in the cell layer in reasonable quantities within 1–3 weeks after the pulse period.

Another interesting point to be made relates indicates the definition of insoluble elastin. Examination of the data in Table III indicates that less total radioactivity in lysine or its derivatives is present after the 24-h pulse and the 1-week chase than after the 3- or 5-week chase. This suggests that the hot alkali treatment, which is used to isolate insoluble elastin, is probably too harsh for the less extensively cross-linked newly synthesized insoluble elastin molecules. Thus, a significant amount of this material is solubilized during the alkaline treatment, even after the reduction with $NaBH_4$. One has to be extremely cautious in defining insoluble elastin in tissue cultures. We believe that with these limitations in mind the hot alkali treatment can continue to serve as one definition of insoluble elastin.

The data suggest that in smooth muscle cell cultures the insoluble elastin contains significantly more ALD, but less desmosine and isodesmosine, than that found in aortic elastin. If the formation of the desmosines occurs by the condensation of an ALD moiety and a dehydrolysinonorleucine moiety, one could suggest that the presence of the latter may be rate limiting in the formation of the desmosines since the cells display little or no dehydrolysinonorleucine. Regardless of the mechanism, an explanation for the low content of the desmosines in the cultures cannot be offered at this time. If the desmosines are the true cross-linking units in the insoluble elastin and not some reduced form of the same as suggested by Paz et al. (1974), then an additional oxidation, possibly catalyzed by an enzyme system different from the lysyl oxidase, could be required. It may well be that this system is not functional in the cell cultures. Whether one examines desmosine formation in vivo or in vitro, it is an extremely slow process. The question always arises as to whether or not this final step in the formation of the desmosines is catalyzed by an enzyme.

Vascular smooth muscle cells from many species do not produce elastin in culture under the same conditions. Schwartz et al. (1980) have shown that calf smooth muscle cells do not produce insoluble elastin. Addition of different agents at various times during the life of a culture has pronounced effects on insoluble elastin formation in the culture. Of interest is a recent report (DeClerk & Jones, 1980) which suggests that ascorbate feeding of rat smooth muscle cells stimulates the formation of insoluble collagen fibers, and at the same time inhibits the accumulation of insoluble elastin. The mechanisms involved in insoluble elastin formation and accumulation are quite important and may well relate to the collagen and proteoglycan components of the connective tissue matrix. Having a cell culture which can predictably produce insoluble elastin allows one to evaluate in a systematic manner elastin biosynthesis and turnover.

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# Five Structurally Related Proteins from Affinity-Purified *Maclura pomifera* Lectin†

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ABSTRACT: Affinity-purified *Maclura pomifera* lectin (MPL) elutes from a gel filtration column as a single symmetrical peak with characteristics expected for a single protein of approximately 40 000 daltons. This material can be dissociated into two dissimilar polypeptide chains of approximately 10 000 daltons. Ion-exchange chromatography on DEAE-cellulose resolves affinity-purified MPL into five components. These proteins are structurally related and contain varying proportions of the two polypeptide chains. Two of these tetrameric lectins, each composed solely of one of these chains, display differences in mobility during discontinuous polyacrylamide gel electrophoresis and ion-exchange chromatography, but display no detectable differences in hemagglutination of human erythrocytes and interactions with carbohydrates.

Aqueous extracts of *Maclura pomifera* seeds contain a potent hemagglutinating activity which has been purified by specific adsorption onto insolubilized A+H active hog gastric mucin (Bausch & Poretz, 1977). The affinity-purified hemagglutinin is a lectin of approximately 40 000 g/m which displays a preferential reactivity with α-glycosides of *N*-acetyl-D-galactosamine and D-galactose. Preliminary characterization of the lectin (Bausch & Poretz, 1977) indicates that it is a tetramer composed of two each of two dissimilar polypeptide chains associated by noncovalent bonds. The preparation elutes from an analytical gel filtration column as a single symmetrical peak and focuses in a pH gradient as a narrow band centering at pH 4.75.

We now report that the affinity-purified lectin preparation may be resolved into five structurally related protein components of similar hemagglutinating and carbohydrate binding properties.

## Materials and Methods

Affinity purification of the *M. pomifera* lectin (MPL) was accomplished by a procedure described previously (Bausch & Poretz, 1977).

Ion-exchange chromatography of the affinity-purified MPL was performed by using a glass column (0.9 × 140 cm) packed with DEAE-cellulose powder equilibrated with 0.01 M phosphate buffer, pH 7.8. After application of the protein solution, fractionation was accomplished with a linear gradient of NaCl (0–0.4 M) in 0.01 M phosphate buffer, pH 7.8. The optical density of every 5-mL fraction and the electrical conductivity of every third fraction were determined.

Polyacrylamide gel electrophoresis was accomplished by using stacking and separation gel components as described by Davis (1964) except that the gels were cast as slabs rather than cylinders. Electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was performed in 10% polyacrylamide gels with 0.1% $NaDodSO_4$ as reported by Laemmli & Favre (1973).

Hemagglutination and saccharide inhibition of agglutination were accomplished as described earlier (Bausch & Poretz, 1977). Amino acid analysis was performed on 22-h HCl hydrolysates of proteins as described previously (Bausch & Poretz, 1977).

## Results

Polyacrylamide gel electrophoresis of affinity-purified MPL yields five closely spaced protein staining bands (Figure 1, lane M). These related proteins may be separated by DEAE-cellulose chromatography with a linear salt gradient. Figure 2 shows a relatively symmetrical elution profile of protein from the ion-exchange column. However, gel electrophoresis of select fractions (numbers 63, 73, 81, 92, and 100) demonstrates resolution of the five protein components (Figure 1, lanes A–E). Separation of the proteins is difficult, and only small quantities of the individual components have been obtained. Most fractions of the eluate consist of two or more of the components. However, judicious selection of individual fractions, which may be pooled, results in a yield of purified components equivalent to up to 30% of the applied protein. Ion-exchange chromatography with DEAE-cellulose columns as short as 40 cm has produced resolution of the proteins

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