

## Two-dimensional gel electrophoretic analysis of human lens proteins

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### ABSTRACT

Human lens proteins from clear lenses were separated and identified using two-dimensional polyacrylamide electrophoresis. Isoelectric focusing, both equilibrium and non-equilibrium, was performed in the first dimension and SDS electrophoresis in the second dimension. Proteins were identified by Western blotting and sequencing techniques and by comparison with patterns obtained with purified crystallin fractions. Analyses were performed on total urea soluble proteins of lenses varying in age from fetal to 73 yr. Several hundred protein spots representing crystallins, cytoskeletal proteins and enzymes were resolved in the fetal lens. In the older lenses there was a dramatic increase in the number of protein species in the molecular weight range of the crystallins and a reduced number of discrete protein species visible at molecular weights greater than 50,000. Conversely, a number of proteins below approximately 15 kDa were visible even in the fetal lens. The number and amount of polypeptides in this molecular weight range were increased in the older lenses. Many of these low molecular weight species could be assigned to either the  $\alpha$ -,  $\beta$ - or  $\gamma$ -crystallin fractions. An age dependent increase in the number of acidic species of both crystallins and other proteins, such as, glyceraldehyde 3-phosphate dehydrogenase was observed as well as the loss or mobility change of  $\gamma$ -crystallin. Two-dimensional gel electrophoresis provides a sensitive and practical technique for characterizing all of the proteins of the human lens.

### INTRODUCTION

The recent change from the intracapsular to the extracapsular cataract extraction technique has resulted in a nearly total lack of intact human cataractous lenses for use in research in the United States (1). To date few attempts have been made to analyze the lens fragments and aspirated material which can be obtained following extracapsular extraction of human cataracts. In the hope that the

ability to perform such analyses would provide a new avenue of research that quite possibly could yield valuable insight into the processes involved in the formation of human cataracts, we have sought a highly sensitive technique capable of resolving not only the abundant lens crystallins, but also the enzymes and the cytoskeletal proteins of the lens.

The technique which we have chosen is two-dimensional gel electrophoresis (2-DGE). In various tissues this method has been used to separate and quantitate hundreds of proteins from very small samples. 2-DGE has been applied to lens proteins by various laboratories, but these studies have dealt primarily with non-human material and generally only with the major proteins of the lens, the crystallins (2-6). Two factors make lens a difficult tissue for analysis by 2-DGE. First, the presence of a large group of extremely abundant proteins (crystallins) makes it difficult to resolve simultaneously both these proteins and the myriad low abundance enzymes. Secondly, the extremely low rate of turnover of proteins in the lens and the resultant accumulation of post synthetically modified forms greatly complicates the pattern, making resolution and quantitation much more difficult than with most other tissues. Furthermore, there is relatively little known about the human lens proteins with regard to structure or the changes that occur with aging.

The present study is an attempt to optimize the 2-DGE system for lens tissue and characterize the proteins of normal human lens. Since it has long been

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recognized that the proteins of the lens undergo extensive age-related changes, we have compared samples from lenses of widely varying age in order to develop baseline normal data with which to compare samples from cataract. We also have identified some of the major components in the gels by immunoblotting and protein sequencing techniques.

#### MATERIALS AND METHODS

Urea, ampholytes, sodium dodecyl sulfate, acrylamide, bisacrylamide, glycine, Tris buffer, TEMED, and sodium persulfate were all purchased from BDH Chemicals LTD (Poole, England). The antisera to vimentin and actin were purchased from Sigma (St. Louis, MO). The antibodies to human fetal  $\alpha$ -crystallin, bovine  $\beta$ B2-crystallin, and the peptide corresponding to residues 33-52 of bovine  $\beta$ B1-crystallin were gifts of J. Horwitz, (Jules Stein Eye Institute, UCLA School of Medicine). Antisera to  $\gamma$ -crystallin was prepared using bovine low molecular weight fraction. In addition to those listed several preparations of antibodies made against  $\alpha$ -,  $\beta$ B2- and  $\gamma$ -crystallins have now been used. Each of these antisera was highly specific for its respective targeted crystallin (data not shown). Antiserum to glyceraldehyde 3-phosphate dehydrogenase (GAPD) was made against the human red cell enzyme.

Twenty one pairs of human eyes were obtained in moist chambers from National Disease Research Interchange within forty-eight hours of death. The ages of the eyes were fetal, newborn, 17 months and 6, 9(2), 11, 19, 20, 24, 35, 38(2), 45, 56, 59(2), 64, 67, 71 and 73 years. The clear lenses were removed intracapsularly, weighed and stored at  $-70^{\circ}$  prior to use. Optical clarity was determined by stereoscopic photography (7). The whole lenses were then homogenized in eight volumes of 9M urea, two percent NP-40 (8:1V:W). Samples were homogenized by hand in Eppendorf tubes using the

pestles fitted to these tubes. Every effort was made to minimize air oxidation of the sample. Dithiothreitol or  $\beta$ -mercaptoethanol was included during homogenization of some samples to check the technique. The homogenates were centrifuged at 15,000 x g for fifteen minutes and the supernatants were taken for analysis. For optically clear lenses this method of solubilization yielded extremely small pellets. Furthermore, there was not a notable increase in the size of the pellet with older lenses. The absence of dithiothreitol or  $\beta$ -mercaptoethanol during homogenization did not alter the pellet size. For the purpose of determining the appropriate amount of sample to apply to the gel, protein content was estimated by reading the absorbance at 278 nm against the urea, NP-40 solution. 300-350 milliabsorbance units of protein were routinely applied to the first dimension gel.

Fractionation of human lens water soluble extracts was performed on Sephadex G-200 as previously described (8). Whole lenses were homogenized in 7 volumes of 50 mM Tris buffer, 100 mM KCl, 1 mM EDTA and 10 mM  $\beta$ -mercaptoethanol at pH 7.2. The Sephadex G-200 column (2.6 x 80 cm) was eluted by gravity and 5.0 ml fractions were collected with continuous monitoring of the absorbance at 280 nm. To obtain highly purified samples only the peak fraction from the  $\alpha$ - and  $\gamma$ -crystallin peaks were taken and each were rechromatographed on a Superose 12 gel exclusion column fixed to an LKB-HPLC system. For  $\beta$ -crystallin the peak fractions from each  $\beta$ -crystallin HPLC peak were combined to give a single sample representative of the entire  $\beta$ -crystallin protein fraction. The purity of peaks obtained from the Superose 12 column was established by SDS electrophoresis.

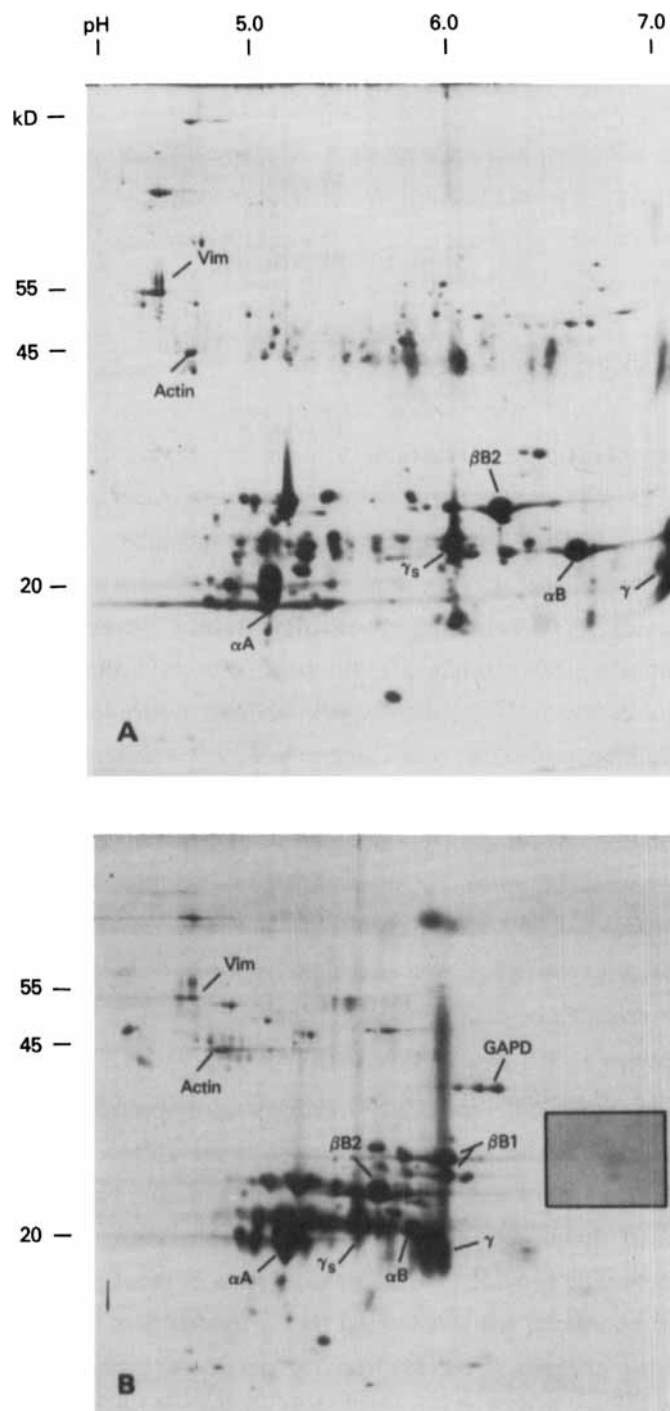
Electrophoresis was performed with the ISO-DALT system (Hoeffer Scientific Instruments). The 2-

dimensional electrophoresis procedure, which is based on the original O'Farrell technique, was done as described for the ISO-DALT system (9-11). Unless otherwise indicated the ampholytes used were pH 4-8.

Isoelectricfocusing (equilibrium method) was done by loading the sample to the basic end of the gel and running for 14,000 volt-hours. Alternatively, the proteins were separated in the first dimension using non-equilibrium pH gradient electrophoresis conditions (NEPHGE) (12). The sample was loaded at the acidic end of the gel and was run for 4,000 volt-hours. The second dimension was run on a 17 x 17 cm, 12-16.6% acrylamide gradient SDS slab gel. This gradient was selected to separate the proteins in the molecular weight range of the crystallins but still visualize proteins with molecular weights up to about 200,000. Gels were stained with a colloidal Coomassie blue G(13).

Proteins were transferred to Immobilon P or nitrocellulose membrane for immunoreaction and sequencing. The blots were stained with Coomassie blue or Ponceau S and were photographed to document the protein pattern. For immunolabeling experiments the membranes were blocked with 0.2% milk protein in 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl at 37° for 1 hour prior to incubation with antibody. The blots were incubated with 1:200 dilutions of antibody in 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl and 0.1% milk protein for 12 hours at 5°. The blots were then incubated with peroxidase labeled secondary antibodies for three hours at 37°. Substrate for the peroxidase reaction was diaminobenzidene. The iodine-125 probed blots were exposed for 12 hours at -70° with X-omat R film.

Protein sequencing was done by Harvard Microchemistry Facility, Cambridge, MA. The proteins, after transfer to Immobilon P, were treated with cyanogen bromide to generate free amino terminal residues before sequencing.



**Figure 1** Two-dimensional electrophoresis gels of fetal human lens using (A) equilibrium and (B) non-equilibrium techniques. Inset in panel B shows the immunoreactivity on a Western blot after probing with an antibody against  $\beta$ B1-crystallin. The numbers indicate the position of the proteins sequenced.

Table 1. Comparison of proteins with reported lens crystallin sequences

Protein 1	E I I D D D V P S F H A H G Y Q E K V S	
	104	
$\beta$ B2-Bovine	E V I D D D V P S F H A H G Y Q E K V S	
	104	
$\beta$ B3-Bovine	E V I D D D V P S L W A H G F Q D R V A	
Protein 2	D I A I H H P W I	R L E K D R F S V N
	2	69
$\alpha$ B-Human	D I A I H H P W I	R L E K D R F S V N
Protein 3	D V T I Q H P - F	L T F * G P K I Q
	2	139
$\alpha$ A-Human	D V T I Q H P W F	L T F C G P K I Q

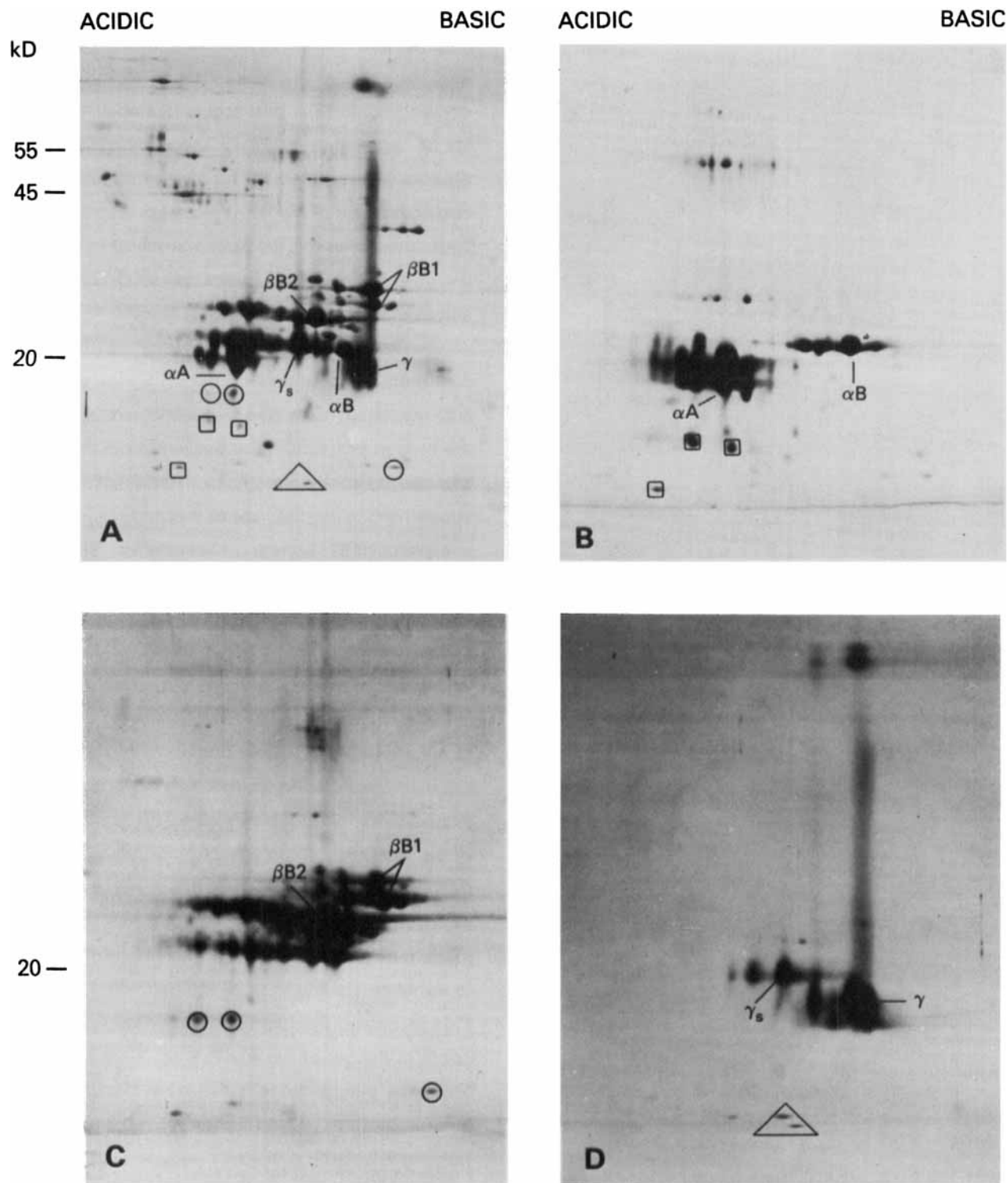
The proteins sequenced are indicated by numbers in Figure 1. The sequences obtained were aligned with previously reported crystallins.  $\beta$ -crystallin residues were numbered to align motifs (15).  $\alpha$ -crystallins were compared with and numbered according to published sequences (16-18). \*Glycine was also found at these positions but at a low level.

## RESULTS

Two different 2-DGE methods were applied to the lens tissue; representative results are shown in Figure 1. In panel A fetal human lens proteins have been analyzed by the equilibrium method. This technique provided excellent resolution of most protein components, but was limited by the tendency of highly basic proteins to be lost from the gel due to instability of the basic end of the ampholyte generated gradient. In the non-equilibrium pH gradient electrophoresis (NEPHGE) method shown in panel B the problem of loss of the basic species was overcome as is seen by the presence of GAPD and  $\beta$ B1-crystallin, proteins which were not present in the gel run under equilibrium

conditions. Likewise, the  $\gamma$ -crystallins were always on the gel whereas in the equilibrium system they were frequently lost completely or partially as in Figure 1A. The protein spots which are labeled were all identified by immunoblotting. As an example, the immunoreaction to  $\beta$ B1-crystallin is shown in the inset. The two reactive spots probably represent the polypeptides corresponding to the  $\beta$ B1a- and  $\beta$ B1b-crystallins which have been characterized from bovine lens (14).

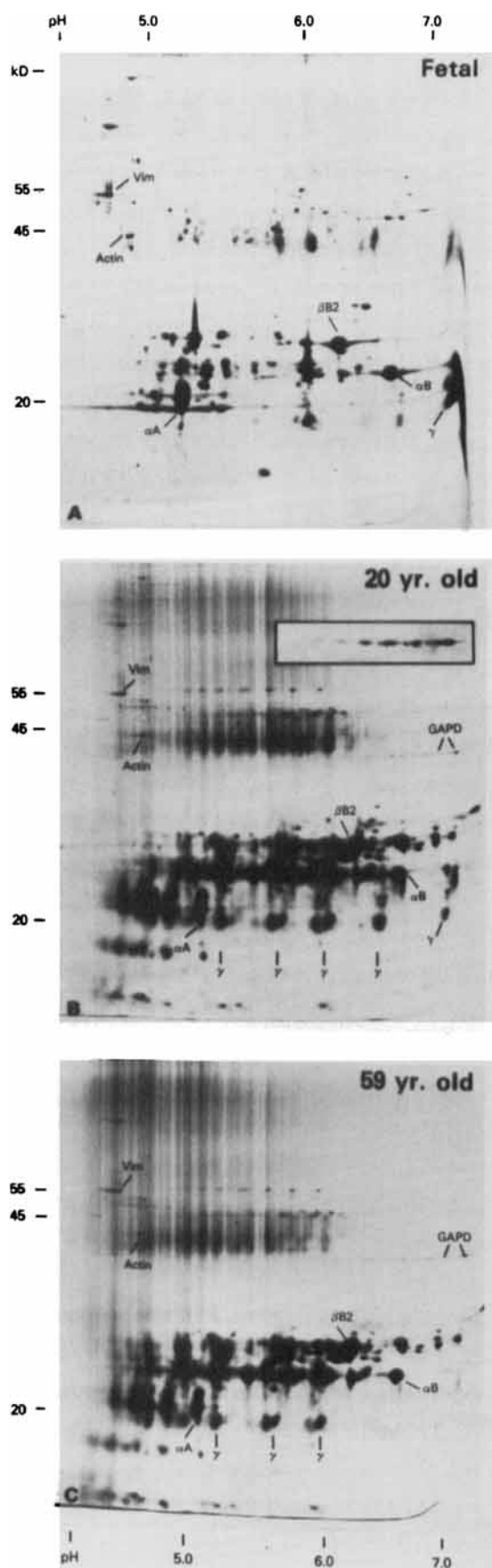
The identification of  $\alpha$ A-,  $\alpha$ B- and  $\beta$ B2-crystallins was also confirmed by sequencing the proteins. Because these proteins have blocked amino terminal residues the protein spots were treated with cyanogen bromide prior to sequencing to generate peptides with



**Figure 2** Non-equilibrium gels demonstrating (A) whole lens, (B)  $\alpha$ -crystallin fraction, (C)  $\beta$ -crystallin fraction and (D)  $\gamma$ -crystallin fraction. Symbols indicate those

polypeptides less than 20 kD that eluted with (□)  $\alpha$ -crystallin, (○)  $\beta$ -crystallin and (△)  $\gamma$ -crystallin.





free amino terminal residues. In Table I, the sequences obtained are compared with sequences reported for basic bovine  $\beta$ -crystallins (15) and the human  $\alpha$ -crystallins (16-18). Two sequences each were obtained for  $\alpha$ A- and  $\alpha$ B-crystallins. These were aligned to optimize comparison with the reported sequences. In each case one of the sequences corresponds to the sequence following the amino terminal methionine and the other corresponds to the sequence following the single internal methionine.

The  $\beta$ -crystallin was identified as  $\beta$ B2-crystallin based on comparison with the sequence of bovine  $\beta$ B2-crystallin. Only one sequence was obtained with the human protein in contrast to the bovine protein that has two internal methionines. The absence of a second sequence may mean that only one methionine is present in the human  $\beta$ B2-crystallin. Between the bovine and human  $\beta$ B2-crystallins there was only one amino acid difference in the twenty amino acid sequence obtained. The human protein had an isoleucine in place of a valine at residue 105 (15).

In Figure 2, we have attempted to delineate in general terms the protein components present in the  $\alpha$ -,  $\beta$ - and  $\gamma$ -crystallin fractions as separated by gel exclusion chromatography. Panel A is a NEPHGE gel of the total urea soluble fraction of newborn lens. Panels B, C and D are the purified  $\alpha$ -,  $\beta$ - and  $\gamma$ -crystallin fractions from a 17 month old lens. A heavier loading of protein was used with the purified crystallins to enhance visualization of the individual polypeptides. This facilitated the identification of the corresponding spots in the gel of the whole lens. Labeled on each gel are major polypeptides which were identified by immunoblotting. Also in Figure 2, we indicate the polypeptides with molecular weights less than 20,000 which co-chromatographed with either  $\alpha$ -,  $\beta$ -, or  $\gamma$ -

Figure 3 Equilibrium gels of samples from (A) fetal, (B) 20 year old and (C) 59 year old human lenses. Inset within panel B shows the immunoreactivity on a Western blot after probing with an antibody against GAPD.

crystallin fractions. Stained spots present in the whole lens pattern (panel A) but not in any of the purified fractions (panels B, C and D) probably represent urea soluble polypeptides which were not water soluble and thus were absent from the Sephadex G-200 fractionated samples.

Proteins from about 20 human lenses ranging in age from fetal to 90 years have now been analyzed by 2-DGE. Figure 3 displays the equilibrium patterns for the total urea soluble proteins from fetal, 20 year old and fifty nine year old lenses. Minor differences were observed between lenses of the same age but the data shown here are representative of the results obtained with the entire group of lenses and demonstrate the age related changes observed. Upon comparison of these protein patterns it was immediately apparent that the quality of resolution of the protein spots decreases as a function of aging. The spots become more diffuse, background staining increases especially on the upper half of the gel and there was an increase in the number of spots visible on the lower half of the gel. These changes which were already so striking by 20 years of age (Figure 3B) begin to occur very early in life. Samples from a 17 month old lens already showed clear evidence of these changes (data not shown).

## DISCUSSION

The increased number of polypeptide species in the molecular weight range of the crystallins (below 45 kDa) in the adult lenses relative to the fetal sample was striking. Some of the new species, particularly those basic proteins with molecular weights between 20,000 and 35,000 may represent synthesis of proteins not expressed in the fetal lens. Ontogenetic patterns in crystallin expression have been reported (19-21). However, most of the new species appear to be modifications of polypeptides present in the fetal lens. Supporting this are recent observations which demonstrate that in older lenses only a few species are synthesized and these correspond to the unmodified forms of the crystallins (6). Multiple more acidic forms

of  $\alpha$ A-crystallin, along with its 17 kDa cleavage product,  $\beta$ -crystallin polypeptides and the  $\gamma$ -crystallins were observed with increasing age. These results are consistent with the earlier observation of the progressive acidification of human lens proteins with age (9,21). The inclusion of thiols during sample preparation did not significantly change the protein patterns of these samples. Glyceraldehyde 3-phosphate dehydrogenase (GAPD) is a particularly good example of such multiple acidic modifications. In a newborn lens sample (Figure 1B) GAPD appeared as a chain of 3 or 4 spots at the extreme basic end of the gel. By 20 years of age it was seen as at least 10 spots with a more acidic range of isoelectric points all of which were reactive with antibody to GAPD (Figure 3B and inset). In older lens samples further acidification of GAPD was apparent. The acidification process was also clearly shown in the  $\gamma$ -crystallins. The loss of the  $\gamma$ -crystallins from their original location in the gels of fetal lens (Figure 3A) was evident when one compared that pattern with the 20 year and 59 year patterns. In this 59 year old lens virtually no  $\gamma$ -crystallin remained on the gel in that original position. However, in both the 20 and 59 year patterns a repeating series of protein spots corresponding to the  $\gamma$ -crystallin pattern seen in the young lens occurred at regular intervals in more acidic positions. That these are  $\gamma$ -crystallin species that have become modified has been confirmed by immunoblotting. These results are consistent with earlier observations using single dimension analysis (22).

In the fetal lens a large number of discrete proteins were visible in the molecular weight range above 40,000. With increasing age these became less apparent. This may be a result of the increase in diffuse staining and streaking in this region of the gel. The streaking and background staining was apparently the result in large part of the formation of crosslinks between crystallin polypeptides. This was indicated by the fact that diffuse immunoreactivity was present throughout this region when gels from adult samples

were probed with antibodies to crystallins (data not shown). Another factor contributing to this phenomenon is a tendency of  $\gamma$ -crystallin to aggregate. This is not a problem in one dimensional SDS gels because samples are routinely heated to 100° in the presence of SDS and reducing agents and these non-covalent aggregates are dissociated. In the 2-DGE system it was not possible to do this and as a result dimers and higher oligomers of  $\gamma$ -crystallin were evident. This was particularly obvious on the NEPHGE gels (Figure 2B, D) where these forms appeared as a streak extending to the top of the gel. Microwave heating of the first dimension gels before application to the second dimension gel was tried, but did not alleviate the problem.

Interestingly, numerous low molecular weight components (<20,000) were present in all lens samples. The same pattern of spots consistently appeared on the gels. Several of these species eluted with the oligomeric  $\alpha$ - and  $\beta$ -crystallin fractions on gel filtration, suggesting strong associations with proteins from within these respective fractions. The nature of such associations remains to be determined. Although we suspect that most of the species represent crystallin degradation products, in contrast to previous reports, they have proven to be largely non-reactive with antibodies to crystallins (23-24). Definitive proof of their identities will require partial sequence analysis. The acidic shifting noted with increasing age in the crystallins and most other lens proteins was not apparent in these low molecular weight proteins. This process, seen so well with GAPD, may be absent in these species because their half-life in the soluble fraction is relatively short. That is, because they are inherently less stable than the intact crystallin polypeptides, they may be proteolytically degraded or become aggregated as part of the lens insoluble protein before undergoing significant acidic modification. Since most of these species are located on the

acidic side of the gel, it is also conceivable that they are formed from crystallin polypeptides largely after the process of acidic modification is complete.

2-DGE, in combination with immuno-techniques and protein sequencing, provides the resolving power and sensitivity to identify and characterize the proteins from human lens including enzymes as well as the crystallins. Not included in these analyses are the intrinsic membrane proteins which will likely not be solubilized. This study described the results obtained on optically clear lenses of various ages. These results will serve as a basis for comparison for future studies on aging related protein modifications and on cataracts. Only one ampholyte range and acrylamide gradient was used for the results described in this study. These were chosen so that both crystallins and other cellular proteins could be visualized. By altering the ampholyte range we consistently resolved the more basic crystallins using the equilibrium method. The range used was dependent on the source of the ampholytes and other reagents. By changing the acrylamide gradient we have been able to optimize separation of proteins in any molecular weight range.

We are now attempting to optimize this technique for lens tissue removed during extracapsular cataract extraction. Specifically the analysis will be applied to material obtained from a group of patients for which extensive data on the progression of their cataracts has already been obtained at the NEI. Included are those individuals with cataracts of various etiologies including steroid induced, congenital and cataracts associated with retinal degenerative diseases as well as aging-related cataracts of idiopathic origin. Our aim is to determine if specific changes in the protein patterns will correlate with particular cataract types. Identification of any such characteristically modified proteins using the techniques described above should provide insight into the mechanisms involved in the cataractogenic process.



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