NMR Analyses of the Cold Cataract

III. $^{13}$C Acrylamide Studies

Sidney Lerman, Judith M. Megaw, and Michael N. Moran

$^{13}$C-enriched acrylamide was employed to further delineate the action of this compound in preventing the cold cataract phenomenon when it is incorporated (in vitro) into young human and rabbit lenses. The extent of acrylamide incorporation, in the dark and with concurrent UV exposure, was monitored by $^{13}$C NMR spectroscopy. These studies provide further evidence that UV exposure causes permanent acrylamide photobinding within the lens. In such lenses, the gamma crystallin fraction of the soluble lens proteins is affected to the greatest extent. It appears to become aggregated and/or combined with the alpha and beta fractions resulting in an apparent loss of most of the gamma monomers. There is also an age-related effect with respect to the amount of acrylamide that can be incorporated into the lens. The decrease in acrylamide incorporation with age directly parallels the age-related decline in gamma crystallin levels. Invest Ophthalmol Vis Sci 26:1349–1353, 1985

We have recently completed a series of NMR analyses on protein/water interactions in the intact lens and in extracted lens protein solutions from various species and ages; human (1st–9th decade), weanling and 1-year-old rat, and 2-week-old rabbit lenses.1–4 Young lenses incubated in media containing acrylamide will rapidly incorporate this compound and such lenses will no longer undergo the cold cataract phenomenon.1–3 Our proton pulse magnetization studies demonstrated that incubating the normal young human, rat, or rabbit lens with acrylamide affects both the ambient temperature transverse relaxation time ($T_2$) and its temperature response.1,3 Our studies on the total soluble protein fraction (TSP) and its separated components (alpha, beta, and gamma crystallins) suggest that the age-related development of the cold cataract and its location may be associated with the relative concentration of one or more of the soluble gamma crystallins within the lens and their localization to specific regions.1–4 Only the young gamma crystallin fraction and the young lens TSP solutions undergo cold precipitation while the young alpha, beta, and the old TSP or individual crystallins are not similarly affected.2,3,6 The unique response of young gamma crystallins in these experiments suggests a significant role for this protein fraction in the phenomenon associated with cold cataractogenesis. This is consistent with previous observations that the relative concentration of the gamma crystallins decreases significantly with age and the hypothesis that this decline is mainly due to an age-related loss of one or more of the individual gamma crystallins.6–9 It is possible that acrylamide exerts its role by crosslinking to one or more of the gamma crystallins, thereby altering the normal protein/water relationship within the lens and preventing the cold cataract phenomenon. In order to further elucidate the acrylamide effect and the role of UV radiation exposure on acrylamide binding within the lens, we utilized $^{13}$C-enriched acrylamide as a convenient marker for our NMR experiments.

Materials and Methods

The normal human lenses were obtained from donor eyes (Georgia Lions Eye Bank) within 8 hr postmortem. These specimens ranged in age from 6 days to 80 yr and were all incubated as soon as they were obtained. Rabbit lenses were derived from albino rabbits, aged 2–3 wk. Treatment of the animals adhered to the ARVO Resolution on the Use of Animals in Research.

Paired lenses from one donor were used in all the experiments to compare the UV exposed versus dark incubations. Thus one lens was incubated in 1 ml Earle’s medium, containing 1% $^{13}$C-acrylamide and 4% unlabeled acrylamide in the dark for 1 hr, while the contralateral lens was similarly incubated and also exposed to ca 0.5 mW/cm$^2$ broadband UV radiation (300–400 nm) for one hour.10 The $^{13}$C NMR experiments were performed in a Bruker 200 MHz spectrometer (Bürcker Instruments; Manning Park, Biller-
Fig. 1. A, $^{13}$C NMR spectra of a young rabbit lens incubated in the dark showing the three resonances (C.A. 131, 132, and 172 ppm) characteristic of free acrylamide; B, $^{13}$C NMR spectra of the contralateral rabbit lens exposed to UV radiation during the incubation period, showing an additional resonance at ca 180 ppm which represents the photopolymerized acrylamide.

A total of 26 human (13 pair) and 12 rabbit lenses were employed in these studies. Eighteen human and six rabbit lenses were removed from the incubation media, and were frozen upon completion of the NMR studies. Six human and six rabbit lenses were retained and dialyzed against Earle's media overnight and $^{13}$C NMR spectra were repeated on these lenses to monitor for retention of free and/or photobound acrylamide. All the lenses were frozen and retained for protein extraction and separation by sephacryl chromatography as reported previously.

Anti (human) gamma crystallin antibodies were produced in New Zealand white rabbits. After bleeding to obtain preimmune serum, the rabbits were injected subcutaneously with 0.5 ml of a 1:1 v/v emulsion of Freund's complete adjuvant and 1.2 mg/ml human gamma crystallin in sterile water. Further injections were performed 10 days and 30 days after the first administration. Forty days after the first injection, the rabbits were bled. Thirty days later the animals were again injected and injections occurred at 30-day intervals thereafter, with bleeding being performed 10 days after each injection. The sera were tested for reactivity with the immunizing antigen by immunodiffusion assays employing the Ouchterlony technique. After 6 mo, distinct reactivity was detectable, and these sera were then employed for all the immunodiffusion assays.

The separated alpha and beta crystallins from the UV exposed lenses were tested for cross reactivity with the rabbit antiggamma crystallins serum by the Ouchterlony immunodiffusion assays. The alpha, beta, and gamma fractions from the contralateral lenses maintained in the dark were also tested in this manner. As will be evident in the following section, little if any, gamma crystallin was obtained by sephacryl chromatography on the total soluble protein fractions derived from the UV exposed lenses.

Results

$^{13}$C NMR spectra performed on individual rabbit lenses kept in the dark showed the three residues characteristic of acrylamide (Fig. 1), while the UV-irradiated lenses showed an additional peak at C.A. 180 ppm which is due to acrylamide photopolymerization (Fig. 1B). In our studies, we have always noted that the signal to noise ratio in the human lens is usually worse compared with the spectra we (and other laboratories) obtain on freshly excised rabbit lenses. Aside from the size differential, the degree of freshness of human tissue can not be similar to lenses obtained from experimental animals. All human lenses must be obtained from donor material, which at best is at least 6 hr postmortem when the experiments commence.

Representative spectra obtained on human lenses (ranging in age from 6 days to 80 yr) are shown in Figures 2 and 3. There is an apparent age-related correlation with the amount of acrylamide incorporated into the lens. In the 6-day-old lens kept in the dark, the 171 ppm resonance peak was the highest and it diminished with age (Figs. 2A-D). A similar situation prevailed with the 180 ppm resonance (representing the photobound acrylamide) as shown in Figures 3A-D. In the lenses maintained in the dark and subjected to overnight dialysis, no acrylamide could be demonstrated by repeat $^{13}$C NMR spectroscopy, irrespective of age or species. However none of the photobound material was lost from the UV-exposed lenses following dialysis as evidenced by complete retention of the 180 ppm peak heights.

There was a marked change in the elution profiles of the total soluble protein fractions derived from the
lens incubated with acrylamide and concurrently exposed to UV irradiation (Fig. 4B). In contrast with a representative profile derived from contralateral lenses (incubated with acrylamide and maintained in the dark) in which the characteristic alpha, beta, and gamma peaks can be seen (Fig. 4A), the gamma crystallins appear to have virtually disappeared when such lenses are concurrently exposed to UV radiation. However, the Ouchterlony assays demonstrated that the apparent loss of the gamma crystallins in such lenses was an artifact, since the beta fractions (to a large extent) and the alpha crystallins could be shown to cross react with the anti (human) gamma antiserum (Fig. 5) while the alpha and beta fractions from the contralateral unirradiated lenses showed no such cross reactivity.

13C NMR spectra on the alpha and beta crystallins derived from UV-exposed lenses contain the 180 ppm (photo-bound) acrylamide resonance. On the other hand, while 13C NMR spectra obtained on homogenates of unirradiated lenses prior to fractionation clearly contain the 171 ppm resonance, none of the acrylamide peaks (180 or 171 ppm) could be demonstrated in the alpha, beta, or gamma crystallins ex-
Fig. 4. A, Elution profile of a second decade total soluble protein fraction extracted from a lens incubated in the dark. B, Elution profile of the total soluble protein fraction extraction from the contralateral lens (Fig. 4A) which was exposed to UV irradiation during the incubation period. Note the apparent marked loss of the gamma crystallins with a concomitant change in the beta profiles, and to a lesser extent, in the alpha fraction.

tracted from these lenses. This is to be expected, since in the dark the acrylamide is believed to exert its action by noncovalent binding to lens proteins and would, thus, be removed during the extraction and separation procedure.

Discussion

The foregoing experiments provide further evidence linking the gamma crystallins with the cold cataract phenomenon. As previously reported, this phenome-
non requires a threshold concentration of this protein fraction to become manifest in the whole lens or the total soluble protein extract.6 In the intact lens, this condition is only met in the core of the very young lens in which the gamma crystallins are the major soluble proteins. The relative concentration of gamma crystallins rapidly declines (compared with the other soluble crystallins) as the lens ages.7-9,13,14 This decline is paralleled by a decrease in the amount of acrylamide which becomes incorporated in the lens during the in vitro incubation experiments. The 13C NMR spectra demonstrate an age-related decline in acrylamide incorporation both in the dark and with UV exposure. With UV radiation exposure, the acrylamide becomes permanently photobound within the lens and can no longer be removed by dialysis. The apparent loss of gamma crystallin evidenced by the elution profiles of the total soluble proteins derived from the UV-exposed lenses appears to be due to changes occurring in the gamma crystallins during these experiments. The Ouchterlony immunodiffusion studies clearly demonstrate cross reactivity of antigamma antiserum with the alpha and beta crystallin fractions derived from UV-exposed lenses, and with gamma crystallin from unexposed lenses. This indicates that some form of aggregation (polymerization) of the gamma monomers has occurred in the UV-exposed lenses due to the incorporated photobound acrylamide.

Blundell et al have demonstrated that at least one of the gamma fractions contains a significant number of hydrophobic surface residues.15,16 Such a polypeptide would become less soluble when the temperature is lowered, resulting in the phase separation phenomenon. Thus, it is not unreasonable to suggest that UV-induced photopolymerization of acrylamide within the intact lenses also causes aggregation of one or more of the gamma monomers, resulting in a decrease in hydrophobicity and an altered temperature response. A recent report indicates that the gamma 2 fraction, and to some extent the gamma 1 fraction, are the ones that are cryoprecipitable, and decline in concentration with age. Furthermore, there is also some evidence that the human gamma 2 fraction contains significantly more hydrophobic residues than the other crystallins.19

These and other studies have clearly demonstrated a correlation between lens age, acrylamide incorporation, and the relative concentration of gamma crystallin present in the lens.14,15 The marked decrease of gamma crystallin with age has been attributed to "loss" by aggregation (or to proteolysis) of at least one of the four gamma fractions. The decline in the ability to incorporate acrylamide with increasing lens age may thus be due to the preferential binding of acrylamide to this fraction.

**Key words:** cold cataract, acrylamide, gamma crystallin, 13C NMR spectroscopy, UV irradiation

**References**