Further Studies on the Sub-Units of α -Crystallin

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1. A new procedure is described for the purification of α -crystallin, including: preparative zone electrophoresis, density-gradient centrifugation and gel filtration. The total amino acid composition of highly purified samples prepared according to this procedure has been determined. 2. Evidence is presented for the presence of intermediates in the urea-induced splitting of α -crystallin into sub-units. A possible mechanism for this splitting is proposed. 3. The recombination of sub-units has been studied by polyacrylamide-gel electrophoresis and ultracentrifugal analysis. As judged from these criteria, only a partial recovery of starting material was obtained. 4. The origin of the minor bands in the electrophoretic pattern of α -crystallin on 7M-urea-polyacrylamide gel has been investigated. No evidence was found that their presence is due to carbamoylation or sulphide-disulphide interchange. They probably arise from isomerization. 5. The mean molecular weight of the sub-units was calculated to be 24 000 (Archibald's method). Determination of the sedimentation-diffusion equilibrium revealed a value of 21 000 at the meniscus. Assuming that all sub-units contain one cysteine residue/molecule, 23 000 can be derived for the mean molecular weight.

In previous papers we have shown that α -crystallin, a main fraction of the water-soluble lens proteins, when treated with urea, can easily be split into sub-units (Bloemendal, Bont, Jongkind & Wisse, 1962*a,b*; Bont, Jongkind, Wisse & Bloemendal, 1962). On removal of urea by dialysis these sub-units recombined to a compound resembling α -crystallin as far as electrophoretic (starch gel) and ultracentrifugal characteristics were concerned (Bont *et al.* 1962; Bloemendal *et al.* 1962*b*).

When urea-treated α -crystallin was subjected to either starch-gel or polyacrylamide-gel electrophoresis in 7M-urea, a number of bands of widely different intensities were observed. On the other hand, α -crystallin dissolved in 7M-urea solution showed only one, though slightly polydisperse, peak in the analytical ultracentrifuge. By using Archibald's method the mean molecular weight was calculated to be 26 000 (Bont *et al.* 1962). As this value may easily be subject to errors owing to, among other factors, uncertainties about the value of the partial specific volume, the total number of sub-units constituting the α -crystallin molecule could not be determined with accuracy.

To establish whether the multiplicity of bands

observed in 7M-urea-gel electrophoresis could be derived from a single protein, the purity of the starting material was of major importance, and we have therefore reinvestigated the purification of α -crystallin as well as the effect of urea on highly purified samples.

In this paper we present a detailed account of our new purification procedure, the various steps of which were followed by electrophoretic analysis on polyacrylamide gel. The effect of variation of the urea concentration on the sub-unit pattern was also studied. Recombination experiments of urea-treated α -crystallin are discussed and compared with previous findings. A tentative reaction scheme for the urea-induced splitting of α -crystallin is proposed.

MATERIALS AND METHODS

Starting material. Bovine eyes were obtained fresh from the slaughterhouse and the lenses were removed immediately. To 12 lenses 150 ml. of 0.01M-phosphate buffer, pH 7.5, was added. The water-soluble proteins were extracted by gentle stirring until the cortex was dissolved. After decanting from lens nuclei, the turbid solution was centrifuged for 15 min. at 9000*g* (10 000 rev./min. in rotor S30 of a Beckman-Spinco L50 ultracentrifuge). The resulting clear solution was stored at -70° until required for the subsequent isolation procedure.

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Isolation of α -crystallin. Preparative vertical-block electrophoresis was carried out as described by Bloemendal & ten Cate (1959), except for the use of Pevikon C870 (Superfosfat Bolaget, Stockholm, Sweden) instead of starch. Approx. 600 mg. of protein was applied on a block and two blocks were run simultaneously.

α -Crystallin was eluted from the appropriate Pevikon segment by washing twice with a volume of 0.01 M-phosphate buffer equal to that of the segment. Traces of Pevikon were removed from the combined washings by centrifugation (for 15 min. at 3000g). The resulting solution was freeze-dried.

Purification of α -crystallin. To remove the bulk of the phosphate present in the freeze-dried product described above, 300 mg. of this substance (containing approx. 100 mg. of α -crystallin) was dissolved in 5 ml. of distilled water and dialysed in Visking 8/32 in. tubing against 0.01 M-phosphate buffer, pH 7.5. After dialysis the volume increased to 9 ml. A 3 ml. sample was layered on a sucrose gradient [8–22% (w/v) sucrose in phosphate buffer, pH 7.5] in each of the three Lusteroid centrifuge tubes fitting the SW 25 rotor of the Spinco L50 preparative ultracentrifuge. After centrifugation at maximum speed for 16 hr., the tubes were punctured and 1 ml. fractions were collected. Extinctions were read at 280 m μ .

By plotting extinction against fraction number, curves usually showing two minima were obtained. Only fractions in between these minima were pooled, except for two fractions nearest to each minimum, which were discarded (compare Fig. 2a, hatched area). The pooled fractions were subjected to column chromatography on Sephadex G-50, coarse grade (50 cm. standard column with cooling jacket; Pharmacia, Uppsala, Sweden). After swelling in 0.01 M-phosphate buffer, the gel particles were poured into the column up to a height of 30 cm. The sample (20–25 ml.) was layered on top of the column, underneath a layer of phosphate buffer initially covering the gel. The same buffer was used for elution. Fractions (2 ml.) were collected and the extinction was read at 280 m μ .

The fractions constituting the first peak in the elution diagram were combined, those fractions with extinction values lower than 1.0 being omitted. A fixed protein/phosphate ratio in the final preparation was obtained by the following procedure: after the fractions had been freeze-dried water was added so that a 1% solution of protein was obtained. To remove excess of phosphate, this solution was dialysed against 0.01 M-phosphate and freeze-dried again. The final product contained 0.055 mg. of phosphate/mg. of protein. Corrected for the presence of phosphate, $E_{1\text{cm}}^{1\%}$ at 280 m μ was 8.85 for freshly prepared samples of α -crystallin. On storage for months, even at temperatures as low as -15° , this value decreased steadily.

All manipulations involved in the isolation and purification steps were carried out at $0-4^\circ$.

Polyacrylamide-gel electrophoresis. Polyacrylamide gels were prepared by the procedure of Barka (1961), except for changes in the concentration of *NN*-methylenebisacrylamide. Gels containing 0.2% of this compound are denoted as 'small-pore' gels, those with 0.05% as 'large-pore' gels (Jonkink, Wisse & Bloemendal, 1962).

The following chemicals were purchased and generally used without further purification: urea from Brocades-Stheeman and Pharmacia (Meppel, The Netherlands); ammonium persulphate (A.R.) from British Drug Houses

Ltd. (Poole, Dorset); acrylamide, *NN*-methylenebisacrylamide and dimethylaminopropionitrile from Fluka A.-G. (Buchs, Switzerland); potassium cyanate (p.a.) from Union Chimique Belge (Ruisbroek, Belgium). Dithioerythritol was a generous gift from Dr W. W. Cleland of the University of Wisconsin, U.S.A. (this compound is now commercially available from Calbiochem, Los Angeles, Calif., U.S.A.).

In a single experiment urea was freed from cyanate by passing a 7 M-urea solution through a column of Dowex MB-1 resin. Cyanate was determined by the method of Marrier & Rose (1964). Electrophoretic runs were performed in small tubes (inner diam. 5 mm.) at a constant current of 5 ma, supplied by either a CVC-A (Pleuger, Wijnegem, Belgium) or a Duostat (Beckman, Fullerton, Calif., U.S.A.) power pack.

Urea-containing gels were prepared as mentioned above, except for substituting the buffer solution by the appropriate urea-containing buffer solutions. The upper buffer vessel contained the same urea-buffer solution, whereas the lower was filled with the same buffer solution only. Aronsson buffer (Aronsson & Grönwall, 1957) (tris-EDTA, pH 8.9; LKB Produkter, Stockholm, Sweden) was used throughout.

Gels were either stained for 1 hr. in water-methanol-acetic acid (5:5:1, by vol.) containing Amido Black (0.7%), and de-stained by electrophoresis, or subjected to the following procedure.

Re-electrophoresis on polyacrylamide gel. Unstained gels were sliced in a cutting device similar to that described by Lewis & Clark (1963). However, to facilitate the positioning of the gel in the apparatus and to avoid distortion during cutting, it was made of two Lucite (polymethyl methacrylate) blocks with half-cylindrical slots which, when screwed together, fitted the gel perfectly, except for a small open area at the upper side. Perpendicular to the slots, grooves (width 2 mm.) were sawn; the distance between grooves was approx. 1 mm. A wetted razor blade was used for cutting. The gel slices were placed on top of a new gel, prepared either with or without urea, and again subjected to electrophoresis.

Recombination experiments. α -Crystallin was dissolved in 0.1 M-phosphate buffer solution containing the appropriate amount of urea (range of protein concentrations 0.1–2%). After standing for 3 hr. at 20° , urea was removed from the solution by dialysis against 0.1 M-phosphate buffer at 4° for up to 96 hr. During the first 24 hr. the buffer was changed three times and once during every additional 24 hr. period. The same procedure was followed by dialysis against Aronsson buffer.

Ultracentrifugal analysis. All runs were made in a Spinco model E analytical ultracentrifuge. Ultraviolet extinction curves were registered. An ultraviolet-transmitting filter (maximum transmission at 277 m μ) (van Es & Wisse, 1963) was used in combination with an automatic scanning system over the length of the cell (W. L. van Es & W. S. Bont, unpublished work).

All sedimentation coefficients were converted into $S_{20,w}$ values by applying corrections for differences in temperature and urea concentration.

The sedimentation-diffusion equilibrium experiments for the determination of the weight-average molecular weight were carried out as described by Bloemendal, Bont, Benedetti & Wisse (1965).

Partial specific volume in 7 M-urea. The term $(1 - \bar{v}_p)$ was

obtained through the use of the density-gradient column of Linderström-Lang & Lanz (1938). The slope of the plot of the urea densities versus protein concentrations was determined.

Amino acid analysis. After deionization by dialysis, protein samples were hydrolysed in 6N-HCl at 110° for 24 hr. (3 mg./2 ml.) in evacuated Pyrex-glass tubes. For subsequent analysis a Beckman amino acid analyser was used. Cysteine content was measured by amperometric titration (Benesch, Lardy & Benesch, 1955) and spectrophotometrically (Jocelyn, 1962). Both determinations were performed in 7M-urea solutions. Tryptophan and tyrosine were determined by the method of Goodwin & Morton (1945).

Spectrophotometric analysis. A Cary model 15 recording spectrophotometer was used throughout this investigation.

RESULTS

Preparation of α -crystallin. As mentioned in the Materials and Methods section, the following purification steps were applied: (a) preparative electrophoresis; (b) density-gradient centrifugation; (c) gel filtration.

The electrophoretic pattern on polyacrylamide gels of α -crystallin, isolated from total lens protein by preparative electrophoresis on Pevikon C870, was similar to that found previously when starch was used as a stabilizing medium (Bloemendal & ten Cate, 1959). In both small-pore and large-pore gels multiple bands were observed (Figs. 1a and 1b). However, after subjecting this preparation to

density-gradient centrifugation a practically homogeneous product was obtained (Figs. 1c and 1d). The band with the highest mobility in the small-pore gel vanished completely; the band near the origin of the large-pore gel also disappeared. Whereas the former may consist of material of low molecular weight, the latter is probably due to a compound (or compounds) of high molecular weight. This view is supported by the curve shown in Fig. 2(a). Density-gradient centrifugation also removed nearly all nucleotide material (compare Figs. 2c and 2b). Finally, to remove the sucrose introduced during density-gradient centrifugation and the small amount of nucleotide material still left, the preparation was subjected to gel filtration on Sephadex G-50. As the gel-electrophoretic patterns were not clearly affected by this treatment, only those of the final preparation are shown (Fig. 1).

Though it is possible to obtain a pure preparation of α -crystallin by using the density-gradient centrifugation step only (Bloemendal, Bont, Jongkind & Wisse, 1964), the purification sequence mentioned above was applied as much larger quantities of total lens protein could be handled.

Amino acid analysis. Amino acid analyses of three samples of α -crystallin, prepared from different batches of lens extract, are shown in Table 1. As the differences between the samples could be due to analytical error, mean values are also included.

Except for the differences in glycine and phenylalanine, our data are similar to those obtained by Björk (1963) (Table 1, last column). Close agreement exists between our data and those of Waley (1965). The values for half-cystine, determined by automatic analysis, are also shown, although the accuracy of this method is known to be poor. The cysteine value obtained by amperometric titration was considerably higher than that reported by Firfarova & Levdikova (1961) and was in closer agreement with the values determined by Thomann (1958) and by Waley (1965).

Spectrophotometric determination of the molar ratio of tyrosine to tryptophan in sample C yielded a value of 3.8, in close agreement with the results of François, Rabaey & Recoules (1963) but higher than reported by Waley (1965).

Aging of α -crystallin. When α -crystallin was stored for several months at -15° , an appreciable decrease in the extinction at $280\text{m}\mu$ was observed. Whereas we found a value of 8.85 ($E_{1\text{cm}}^{1\%}$) for freshly prepared samples, after 7 months only 8.00 was measured. Changes in electrophoretic behaviour could be observed on both small-pore and large-pore polyacrylamide gels. On small-pore gel an additional diffuse band with higher mobility became visible; on large-pore gel one elongated diffuse zone, quite different in appearance from that in

Fig. 1. Polyacrylamide-gel electrophoresis (5 mA/tube in Aronson buffer, pH 8.9) of α -crystallin on (a) and (c) small-pore gels and (b) and (d) large-pore gels. (a) and (b), Samples obtained after preparative electrophoresis on Pevikon; (c) and (d) samples obtained after preparative electrophoresis followed by density-gradient centrifugation and gel filtration.

Fig. 2. Purification steps of α -crystallin after preparative electrophoresis. (a) Sucrose-density-gradient centrifugation (hatched area: fractions collected for gel filtration); (b) gel filtration of α -crystallin purified on a sucrose density gradient; (c) gel filtration of α -crystallin (purification on density gradient omitted); inset: spectrum of fraction 74, $\lambda_{\max}$ at 260 m μ .

Table 1. *Amino acid analyses of α -crystallin*

Method	Amino acid	Amino acid composition (moles/100 moles)				
		Sample A	Sample B	Sample C	Mean of samples	Values obtained by Björk*
Amino acid analyser; acid hydrolysis	Asp	9.3	9.3	9.2	9.3	9.7
	Thr	3.2	3.2	3.2	3.2	3.2
	Ser	10.5	10.6	10.6	10.6	9.8
	Glu	11.6	11.1	10.5	11.1	11.6
	Pro	7.8	7.8	7.2	7.6	7.3
	Gly	6.1	5.9	6.1	6.0	4.5
	Ala	4.0	4.0	4.1	4.0	4.1
	CyS	(—)	(0.1)	(0.6)	(0.2)	
	Val	5.9	5.7	5.9	5.8	6.4
	Met	0.9	1.1	1.2	1.1	1.2
	Ile	5.2	5.2	5.2	5.2	5.4
	Leu	8.4	8.4	8.4	8.4	8.8
	Tyr	2.9	2.9	3.0	2.9	3.0
	Phe	7.9	7.9	7.8	7.9	8.9
	Lys	4.4	4.6	4.6	4.5	4.4
Sulphur analysis	His	4.3	4.5	4.4	4.4	4.2
	Arg	7.7	7.7	7.9	7.8	7.7
	CyS+Met			2.0		1.9
Amperometric	CySH			0.64		
Spectrophotometric	CySH			0.60		
Spectrophotometric	Tyr/Trp			3.8†		
	Trp			0.9		

* Calculated for α' -crystallin (Björk, 1963)

† Molar ratio.

Fig. 1(d), was detected. However, no differences were found between 7M-urea-gel patterns of aged and freshly prepared samples.

Effect of urea concentration on the electrophoretic pattern. In Fig. 3 the effect of increasing concentrations of urea in the protein sample and in the

polyacrylamide gels is shown. (To the major bands in the 7M-urea-gel roman numerals have been assigned; TL indicates a zone strongly present in total lens protein.) At the lower urea concentrations the bands were always diffuse. As soon as the bands became sharper at the higher urea concentrations,

Fig. 3. Polyacrylamide-gel electrophoresis of α -crystallin in various urea concentrations, as indicated. To facilitate a comparison between protein patterns, all drawings are normalized, taking origin and dye front as references.

less drastic changes were noted with increasing urea concentration, indicating that the action of urea was near completion. Similar observations have been reported by Björk (1964).

The effect of urea appeared to be time-dependent (see the Discussion section).

On incubation for 5 days at 25° the minor bands between I and II and between II and III became more prominent, whereas the intensity of band I decreased. Moreover, the region of bands III, IV and V was blurred.

When α -crystallin was incubated without urea for 12 days, and then treated with 7M-urea, a pattern similar to that shown in Fig. 3 was found, except for the disappearance of band I. To avoid

Fig. 4. Re-electrophoresis of α -crystallin:

Expt.	Urea concn. in gel used for sectioning	Urea concn. in gel used for analysis of sections
(a)	No urea	7M
(b)	No urea*	7M*
(c)	3M	7M
(d)	4M	7M

* Total lens protein used for comparison. The major bands are indicated by roman numerals.

bacterial contamination, thymol had been added to the solution before incubation.

On removal of traces of cyanate from urea by ion-exchange treatment, no changes occurred in the pattern of α -crystallin on a 7M-urea-gel. Cyanate ions did not affect the pattern even at much higher concentrations (0.1M) than those present in a freshly prepared 7M-urea solution (0.003M).

On treatment of α -crystallin with a 7M-urea solution containing dithioerythritol (100-fold excess of thiol groups), no change in the 7M-urea-gel pattern was observed.

Re-electrophoresis of isolated bands. In Fig. 4 the patterns of gels containing different urea concentrations are compared with those of the sections after re-electrophoresis in 7M-urea-gels.

On re-electrophoresis of sections from a 7M-urea-gel, the resulting patterns showed only the original zones.

Re-electrophoresis on 7M-urea-gels has also been performed with sections from gels of total water-soluble lens protein. Two different patterns were obtained: one from the α -crystallin region (section 2) identical with that shown in Fig. 3 and another from the β -crystallin region (section 13). Up to section 6 the resulting gels showed the first pattern; from sections 12 to 16 the second (β -crystallin pattern) was obtained. Intermediate patterns were

observed for sections 7 to 12; in addition, there was a slow-migrating band present in sections 7, 8 and 9. The band marked TL (because it is the major

Fig. 5. Polyacrylamide-gel electrophoresis of recombinant α -crystallin on small-pore gels. (a) Protein dissolved in 7M-urea-phosphate buffer, pH 7.5, dialysed against phosphate buffer, pH 7.5; (b) protein dissolved in 7M-urea-tris buffer, pH 8.9, dialysed against tris buffer, pH 8.9; (c) protein dissolved in 1M-urea-phosphate buffer, pH 7.5, dialysed against phosphate buffer, pH 7.5.

Table 2. $S_{20,w}$ values of α -crystallin in buffer and buffered urea solutions and on recombination from urea solutions by dialysis

A. Experiments in 0.08M-tris buffer, pH 8.9

Expt. no.	Concn. of protein (%)	Concn. of urea (M)	Time of dialysis* (hr.)	$S_{20,w}^\dagger$ (s)	Remarks
1	0.1	—	No dialysis	17.8	
2	0.1	1	No dialysis	6.0, 11.0	
3	0.2	1	No dialysis	7.4, 13.7	
4	0.2	1	No dialysis	5.5, 11.2	
5	0.2	1	No dialysis	5.6	After standing for 48 hr. at 4°
6	0.1	1	48	7.5	
7	0.1	7	64	7.3	

B. Experiments in 0.1M-phosphate buffer, pH 7.5

8	2.0	—	No dialysis	18.5	
9	0.2	1	No dialysis	14.7	
10	0.2	1	No dialysis	11.9	After standing for 48 hr. at 4°
11	0.1	1	48	17.3	
12	0.2	7	20	14.5	
13	0.1	7	24	14.0	
14	2.0	7	48	16.5	
15	2.0	7	96	14.0	
16	2.0	7	96	17.5	Thymol added before dialysis; dialysis at 20°

* Against the same buffer; temperature 4° except in Expt. 16.

† Concentration in the cell of the analytical ultracentrifuge: 0.1%, except in Expts. 3, 4, 5 and 10, where it was 0.2%.

band in the 7M-urea-gel total-lens pattern) is only faintly present in the 7M-urea-gel patterns of α -crystallin whereas it dominates the β -crystallin pattern (Fig. 4b).

Recombination of sub-units. Our data on the recombination of sub-units, obtained with the analytical ultracentrifuge, are shown in Table 2. Some of these experiments were also followed by electrophoresis on polyacrylamide gel (Fig. 5). Only the pattern obtained from α -crystallin subjected to the action of 1M-urea-phosphate buffer, and dialysed against the same buffer, was approximately identical with that of the starting material. In all other cases the strongest band of the recombined sub-units was located at a much greater distance from the origin.

No differences were noted between gel patterns of recombined sub-units from short or from prolonged incubated α -crystallin in 7M-urea solutions.

α -Crystallin, dissolved in 1M-urea-tris buffer, pH 8.9, showed two peaks in the analytical ultracentrifuge when measured directly after dissolution. A striking phenomenon was the disappearance of the peak with the higher sedimentation coefficient on dialysis against the same buffer (Expts. 3 and 6 in Table 2) or when the solution was stored for 48 hr. at 4° (Expt. 5 in Table 2). This strongly contrasts with the behaviour of α -crystallin in a 1M-urea-phosphate buffer, pH 7.5. Dialysis against the latter buffer resulted in a peak of 17.3s (Expt. 11 in Table 2). A slow-sedimenting peak was also found after removal of urea from the 7M-urea-tris buffer, pH 8.9, by dialysis but not in a dialysed 7M-urea-phosphate solution of α -crystallin at pH 7.5 (Expts. 7 and 13 in Table 2).

Molecular weight of sub-units. The mean molecular weight of the sub-units present in 7M-urea has been redetermined. By using the experimentally determined value: $1 - \bar{v}\rho = 0.180$, a molecular weight of about 24 000 has been calculated. Further, it may be concluded that the partial specific volume, being 0.74, has not changed in 7M-urea. It has, however, to be kept in mind that corrections for non-ideality still may affect the value of the molecular weight.

Sedimentation-diffusion equilibrium experiments were performed in 7M-urea solution, as described for untreated purified α -crystallin (Bloemendal *et al.* 1965). The value calculated at the meniscus according to this method was 21 000.

DISCUSSION

Although at present there is ample evidence that α -crystallin is split into sub-units by treatment with urea, the relation between these sub-units and the bands observed in 7M-urea-gels after electrophoresis is not clear.

The question arises whether or not all of these bands, which differ in intensity, represent different sub-units. Among the possible explanations for the complexity of the gel patterns the following seem most feasible: (a) α -crystallin is contaminated with small amounts of other proteins (e.g. proteins of the β -crystallin complex); (b) the action of 7M-urea causes α -crystallin to dissociate into a small number of sub-units, each of which isomerizes rather slowly to one or more final products; (c) side reactions other than isomerization occur, including carbamoylation and sulphide-disulphide interchange; (d) α -crystallin is a mixture of proteins of equal molecular weight, each consisting of different sub-units; the number of different sub-units in each protein is very small; (e) the splitting of α -crystallin in 7M-urea is incomplete.

By the present purification procedure, which includes density-gradient centrifugation and gel filtration, the possibility (a) has been largely eliminated. No heterogeneity of any importance was found when a sample of α -crystallin obtained by this method was subjected to electrophoresis on a large-pore polyacrylamide gel, in contrast with previous findings. However, after electrophoresis on 7M-urea-gel the pattern did not differ substantially from that obtained from samples purified by preparative electrophoresis only. This finding indicates that the components present in samples prepared by preparative electrophoresis only are structurally closely related. From the re-electrophoresis experiment shown in Figs. 4(a) and 4(b) it is clear that our new purification procedure eliminates contamination with proteins of the β -crystallin complex. Only when relatively high amounts of α -crystallin are subjected to electrophoresis in 7M-urea-gels does a faint band (marked TL) appear which might be identical with the main band in the 7M-urea-gel patterns of total lens proteins and the β -crystallin complex.

Both amino acid analyses and urea-gel electrophoresis patterns show that our preparation of α -crystallin and that prepared by Björk (1963) are comparable, although they were obtained by widely different methods. If α -crystallin should be contaminated as supposed under (a) or could be defined as described under (d), the similarity of amino acid content and electrophoretic behaviour of samples prepared by various methods as described above could hardly be expected. However, in immunoelectrophoresis one single precipitation line was observed (Bloemendal *et al.* 1965), in contrast with the findings by Björk (1964) and Manski, Halbert & Auerbach (1961). It is well known that cyanate ions are present in appreciable amount in a 7M-urea solution, and may react with proteins and peptides to form carbamoylated derivatives. However, our electrophoresis experiments provide

no evidence for carbamoylation. As the analytical data (Table 1) and the electrophoresis experiments also indicate that sulphide-disulphide interchange cannot play an important role in the urea-induced splitting of α -crystallin, neither of the possibilities mentioned under (c) seems to contribute to the complexity of the 7M-urea-gel pattern.

The presence of incompletely dissociated α -crystallin in 7M-urea as found by Spector & Katz (1965) cannot explain the complexity of the gel patterns either. These authors reported a mean molecular weight of 40 000 whereas we obtained a value of 21 000 in 7M-urea. This discrepancy may be due to the temperature and time-dependence of the dissociation.

The changes observed in 7M-urea-gel patterns of α -crystallin subjected to prolonged incubation in 7M-urea suggest that after a rapid disaggregation reaction the sub-units initially released are slowly transformed into molecular species of different mobility. We have no evidence that this transformation is associated with changes in molecular weight, and therefore only changes in secondary and tertiary structure seem to occur. Hence the reaction products formed are designated isomers. In view of the low molecular weight of the sub-units differences in mobility will result from differences in charge, rather than from differences in size. Although some of the bands in the gel pattern may correspond to sub-units of different amino acid composition (Björk, 1964; Spector, 1965) other bands represent sub-units that differ in shape only.

From our prolonged incubation experiments we also infer that some sub-units are more stable and isomerize more slowly than others, probably reflecting differences in primary structure. Especially the sub-unit corresponding to band I seems to have a structure different from the others. A similar observation was made by Björk (1964) on the isolated sub-unit I, which corresponds to our band I.

Our present results are in favour of the assumption made under (b) and provide the most plausible explanation for the complexity of the 7M-urea-gel pattern.

With the ultimate aim of obtaining some insight in the mechanisms of dissociation of α -crystallin in 7M-urea, several re-electrophoresis experiments were performed. In 1M-urea two bands were observed; on re-electrophoresis in 7M-urea each of the two bands gave essentially the same pattern. This observation provides evidence that both bands represent components consisting of the same sub-units. Ultracentrifugal analysis in 1M-urea-tris buffer, pH 8.9, showed that these components corresponded to peaks with lower sedimentation coefficients than that of α -crystallin. As no constant values were found in different experiments (Expts. 2, 3 and 4 in Table 2) we assume that at least the

component with the higher sedimentation coefficient has a labile structure and may represent an extended form of α -crystallin that is easily converted into another, more stable, intermediate. The former is designated the 10–14s intermediate, and the latter the 7s intermediate. The pH-dependent instability of the 10–14s intermediate is clearly demonstrated in Expts. 5 and 10 in Table 2.

The intermediates discussed above seem also to be formed, but at a very low rate, during storage of samples of α -crystallin, an assumption that is supported by the changes found in the electrophoretic behaviour of aged samples. In this context the heterogeneity of isoelectric preparations observed by Bon (1961) can be explained. This investigator found two peaks of, respectively, 15s and 7s, the former being the more labile of the two.

Our re-electrophoresis experiments of sections from 3M- and 4M-urea-gels indicate that, although at these concentrations the 7s intermediate is still present, dissociation into sub-units proceeded markedly. From these experiments we have no substantial evidence that intermediates of still lower sedimentation coefficients are involved in the splitting of α -crystallin by urea. However, a component of about 3.5s has been observed in short-time incubations in 7M-urea and in 0.1% solutions of sodium dodecyl sulphate (Bloemendal *et al.* 1965). Its presence was also noted in recombination experiments (Bloemendal *et al.* 1962a).

Although our previous experiments based on starch-gel electrophoresis and ultracentrifugal analysis suggested that the action of urea on α -crystallin was reversible, the present results, obtained with the aid of polyacrylamide-gel electrophoresis, show that the action of urea might be completely reversible only under a carefully selected set of conditions, including pH, ionic strength, temperature and concentration. The influence of ionic strength on the recombination has been studied by Björk (1964), who did not succeed in arriving at the original *S* value for his recombination product. Our ultracentrifugal studies show a similar result, although we investigated some other variables: time of dialysis and pH. Varying the time of dialysis between 20 and 96 hr. (Expts. 12, 13, 14 and 15 in Table 2) did not give products with *S* values exceeding 16.5s. Components with sedimentation coefficients in the range 14–17s are too large to migrate. However, the gel patterns of recombination products yield only a faint zone near the origin and a strong band with higher mobility.

In view of our experiments at pH 8.9 we now assume that electrophoresis at this pH leads to dissociation of 14–16s recombination products into

Our estimate of the mean molecular weight of the sub-units obtained by the Archibald method is 24 000. Determination from sedimentation-diffusion equilibrium gave a value of 21 000 at the meniscus. Assuming that all sub-units contain one cysteine residue/molecule we calculate $23\,000 \pm 1000$ for the mean molecular weight. These values are in close agreement with the findings of Waley (1965), who found one mole of thiol groups/20 000 g. of protein.

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REFERENCES

- Aronsson, T. & Grönwall, A. (1957). *Scand. J. clin. Lab. Invest.* **9**, 338.
- Barka, T. (1961). *J. Histochem. Cytochem.* **9**, 542.
- Benesch, R. E., Lardy, A. H. & Benesch, R. (1955). *J. biol. Chem.* **216**, 663.
- Björk, I. (1963). *Exp. Eye Res.* **2**, 339.
- Björk, I. (1964). *Exp. Eye Res.* **3**, 1.
- Bloemendal, H., Bont, W. S., Benedetti, E. L. & Wisse, J. H. (1965). *Exp. Eye Res.* **4**, 319.
- Bloemendal, H., Bont, W. S., Jongkind, J. F. & Wisse, J. H. (1962a). *Nature, Lond.*, **193**, 437.
- Bloemendal, H., Bont, W. S., Jongkind, J. F. & Wisse, J. H. (1962b). *Exp. Eye Res.* **1**, 300.
- Bloemendal, H., Bont, W. S., Jongkind, J. F. & Wisse, J. H. (1964). *Biochim. biophys. Acta*, **82**, 191.
- Bloemendal, H. & ten Cate, G. (1959). *Arch. Biochem. Biophys.* **84**, 512.
- Bon, W. F. (1961). *J. biol. Chem.* **236**, 81.
- Bont, W. S., Jongkind, J. F., Wisse, J. H. & Bloemendal, H. (1962). *Biochim. biophys. Acta*, **59**, 512.
- Firfarova, K. F. & Levdikova, G. A. (1961). *Biokhimiya*, **26**, 626.
- François, J., Rabaey, M. & Recoules, N. (1963). *Amer. J. Ophthalm.* **56**, 265.
- Goodwin, T. W. & Morton, R. A. (1945). *Biochem. J.* **42**, 239.
- Hurlbert, R. E. & Jakoby, W. B. (1964). *Biochim. biophys. Acta*, **92**, 202.
- Jocelyn, P. C. (1962). *Biochem. J.* **85**, 480.
- Jongkind, J. F., Wisse, J. H. & Bloemendal, H. (1962). *Protides of the Biological Fluids*, 10th Colloq., Brugge, p. 77. Amsterdam: Elsevier Publishing Co.
- Lewis, U. J. & Clark, M. O. (1963). *Analyt. Biochem.* **6**, 303.
- Linderstrøm-Lang, K. & Lanz, H. (1938). *C.R. Trav. Lab. Carlsberg, Ser. chim.* **21**, 315.
- Manski, W. J., Halbert, S. P. & Auerbach, T. P. (1961). *Arch. Biochem. Biophys.* **92**, 512.
- Marrier, J. R. & Rose, D. (1964). *Analyt. Biochem.* **7**, 304.
- Spector, A. (1965). *Invest. Ophthalm.* **4**, 579.
- Spector, A. & Katz, E. (1965). *J. biol. Chem.* **240**, 1979.
- Stellwagen, E. & Schachman, H. K. (1962). *Biochemistry*, **1**, 1056.
- Thomann, H. (1958). *v. Graefes Arch. Ophthalm.* **160**, 219.
- van Es, W. L. & Wisse, J. H. (1963). *Analyt. Biochem.* **6**, 135.
- Waley, S. G. (1965). *Biochem. J.* **96**, 722.