N-Terminal Sequences of α -Crystallin

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The amino acid sequences at the *N*-terminal ends of the chains of the lens protein, α -crystallin, were studied. Both the main kinds of chain in bovine α -crystallin (A chains and B chains) have an *N*-terminal methionine residue, and the amino group is acetylated. Selective purification of the peptides in a tryptic digest of bovine α -crystallin gave a preparation consisting largely of the *N*-terminal peptide from the A chains, and the sequence of this peptide was elucidated. Subsequently, the *N*-terminal peptides were prepared from separated A and B chains. The proposed sequences are: A chain, acetyl-Met-Asp-Ile-Ala-Ile-Gln-His-Pro-Trp-Phe-Lys; B chain, acetyl-Met-Asp-Ile-Ala-Ile-His-(Pro,Trp)-Ile-Arg. The similarity between the sequences supports the hypothesis that the A and B chains are derived evolutionarily from a common precursor.

α -Crystallin is one of the characteristic 'bulk' proteins of the vertebrate lens (Waley, 1969*a*). Bovine α -crystallin has a molecular weight of about 1 000 000, and the α -crystallin formed from the outer layer (cortex) of calf lens is built up from chains of two main kinds (Schoenmakers & Bloemendal, 1968*a,b*), called the A and B chains (Bloemendal, 1969; Waley, 1969*b*). The situation in the lens from older animals is more complicated, and is discussed below. Little is known of the relationship between the A and B chains. Their amino acid compositions (Schoenmakers, Matze, van Poppel & Bloemendal, 1969) are sufficiently similar to suggest that they may be structurally related. There is one *N*-terminal acetyl group per chain (Mok & Waley, 1968*a*; Hoenders, Schoenmakers, Gerding, Tesser & Bloemendal, 1968), and the *N*-terminal dipeptide sequence is acetyl-Met-Asp- in both A and B chains (H. J. Hoenders & H. Bloemendal, personal communication). The tetrapeptide sequence acetyl-Met-Asp-Ile-Ala had previously been obtained from 'whole' α -crystallin (i.e. mixtures containing both kinds of chain) (Mok & Waley, 1968*b*; Hoenders, van Tol & Bloemendal, 1968). The work described below extends our knowledge of the *N*-terminal part of the polypeptide chains and permits a limited comparison of the sequences of the A and B chains.

The existence of the *N*-terminal methionine enables one to exploit the reactivity of the methionine towards alkylating agents as a help in purifying the *N*-terminal peptides. Two approaches suggest themselves: to use radioactive iodoacetamide or to use the selective purification (diagonal) method suggested by Mikes & Holeysovsky (1958) and developed by Tang & Hartley (1967). Both ap-

proaches have been explored with 'whole' α -crystallin and in addition the *N*-terminal peptides have been isolated by conventional methods from the separated A and B chains. The fact that both chains have only two residues of methionine (Schoenmakers *et al.* 1969) and that cleavage with cyanogen bromide has established the sequences Met-Asp and Met-Leu (Schoenmakers, Hoenders & Bloemendal, 1968) facilitates identification of the *N*-terminal peptide.

A preliminary account of part of this work has been given (Waley, 1969*c*).

MATERIALS AND METHODS

α -Crystallin. This was prepared from ox lenses as previously described (Mok & Waley, 1967). When the protein was to be for the preparation of A and B chains, calf lenses were used, and the precipitation by $(\text{NH}_4)_2\text{SO}_4$ was omitted, and instead the protein was purified by chromatography on Sephadex G-200 (Schoenmakers *et al.* 1968).

Isolation of N-terminal peptide from (unfractionated) α -crystallin. (a) Diagonal method. Tris (6g.) was added to a solution of α -crystallin (20ml., 41mg./ml.) and the pH adjusted to 8.6 with acetic acid. Mercaptoethanol (30 μ l.), EDTA (5mg.) and then recrystallized urea (18g.) were added, and the solution was treated with three portions of ethyleneimine (0.12ml. each) at intervals of about $\frac{1}{4}$ hr. After 4 hr. the nitroprusside test was negative; the protein was precipitated with 300ml. of ethanol and washed with ethanol and then acetone. Amino acid analysis confirmed that the thiol group had reacted (Table 1).

The protein was suspended in 20ml. of water containing 0.5g. of NH_4HCO_3 and 1ml. of trypsin (10mg./ml. in 1mM-HCl) added. After 3 hr. at 37°, the pH was lowered to 4.4, the precipitate was separated and washed and the combined supernatants were evaporated. The residue, in 2.5ml.

Table 1. *Amino acid analysis of α -crystallin after reaction with ethyleneimine*

The values for α -crystallin are taken from Wisse, Zweers, Jongkind, Bont & Bloemendal (1966).

	Amino acid composition (moles/100 moles)																	
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	
α -Crystallin treated with ethyleneimine	8.8	3.1	9.8	10.2	7.6	6.1	4.3	0.75*	5.7	1.3	4.3	7.8	3.5	7.1	5.2	4.7	7.8	
α -Crystallin	9.3	3.2	10.6	11.1	7.6	6.0	4.0	0.7†	5.8	1.1	5.2	8.4	2.9	7.9	4.5	4.4	7.8	

* Determined as *S*-(2-aminomethyl)cysteine (there was no cystine present).

† Determined as cysteic acid.

of acetic acid–water–pyridine (3:12:10, by vol.) containing a little dye (Light Green), was spread over the starting lines of four sheets of Whatman no. 3 paper and the sheets were run in BAWP* overnight.

The Ehrlich-positive band (the *N*-terminal peptide contains tryptophan) that travelled just ahead of the dye was cut out from the dried paper and dipped through iodoacetamide (280 mg.) in acetone (30 ml.) and pH 3.5 buffer (10 ml.), and the strip kept moist in the dark overnight. The strips were then dried, rinsed with acetone, sewed into fresh sheets of paper and run again in BAWP. The main band, which was Ehrlich-positive, and also gave a slow reaction with the ninhydrin reagent, was eluted with aq. 15 mM-NH₃ solution. A portion was hydrolysed, and the amounts of aspartic acid and glutamic acid were determined on paper after electrophoresis at pH 4 (Cliffe & Waley, 1958): Asp, 4.86 μ moles; Glu, 5.04 μ moles.

(b) 'Labelling' method. *S*-Aminoethyl- α -crystallin (116 mg.) in 0.6 ml. of 8 M-urea in pH 3.5 buffer was treated with recrystallized iodo[1-¹⁴C]acetamide (8 mg., 0.8 μ C/ μ mole) at 40° for 43 hr. The protein was precipitated with 10 ml. of ethanol, and washed with ethanol and acetone. The radioactivity of the solid amounted to about 10 μ moles of ¹⁴C/116 mg.; there are two methionine residues/chain, and the molecular weight of the chains is about 20000 (Schoenmakers *et al.* 1969), and so this result is consistent with both methionine residues having reacted. Methionine is the only residue expected to react with iodoacetamide at pH 3.5 (Tang & Hartley, 1967).

The protein was digested with 0.4 mg. of trypsin in 4 ml. of 1% (w/v) NH₄HCO₃ for 2.5 hr.; 90% of the radioactivity was in the pH 4.5 supernatant, which was concentrated before chromatography on a column (120 cm. long $\times$ 1 cm. diam.) of Sephadex G-25 equilibrated with 0.5% formic acid. The peptide in the second main radioactive peak was purified as described in the Results section.

Separation of A and B chains. After alkylation of the thiol groups (Waley, 1965) the chains were separated by chromatography on sulphoethyl-Sephadex (Schoenmakers & Bloemendal, 1968a). The first peak comprises the A chains and the second peak the B chains. The pooled fractions were desalted by chromatography on a column (19 cm. long $\times$ 7.5 cm. diam.) of Sephadex G-25 equilibrated with 0.5% formic acid; up to 200 ml. of solution could be desalted like this, the urea being detected with the Ehrlich reagent. The desalted solution was freeze-dried: 230 mg. of

A chain and 140 mg. of B chain were obtained from 650 mg of alkylated α -crystallin. The relative amounts (and the total recovery) are similar to those obtained by Schoenmakers *et al.* (1969).

Digestion of A chains with trypsin. Two-thirds of a solution of trypsin (5 mg.) in mM-HCl–mM-CaCl₂ (0.5 ml.) was added to A chain (230 mg.) in water (5 ml.) at 37° and at pH 7.6; the pH was kept at 7.6 with M-NaOH. After 45 min. the consumption of alkali had greatly decreased and the rest of the trypsin was added. After 1.5 hr. the pH was lowered to 4 with acetic acid, and the precipitate removed at the centrifuge.

Digestion of B chains with trypsin. The protein was first treated with citraconic anhydride to block the amino groups of lysine residues (Dixon & Perham, 1968); this gave a water-soluble digest. Citraconic anhydride (0.4 ml.) was added to B chains (140 mg.) in 2% (w/v) Na₂B₄O₇·10H₂O (4 ml.) at 4°, and the pH kept at about 9.5 by adding 5 M-NaOH. After 10 min. the solution was left at room temperature for 4 hr., and then dialysed overnight at 4° against NH₄HCO₃ (8 mg./ml.). The protein was then digested with trypsin (1 mg. for $\frac{1}{2}$ hr., then 0.4 mg. for 2 hr.) at 37°.

Paper electrophoresis. This was carried out at 10 v/cm. at pH 4 (Grassmann, Hannig & Plöckl, 1955) or pH 7 (Newton & Abraham, 1954) or at 50–100 v/cm. in a cooled-plate apparatus at pH 1.9, pH 3.5, pH 6.5 or pH 8.9, with the buffers described by Ambler (1963).

Paper chromatography. This was carried out in BAWP, in butan-1-ol–acetic acid–water (40:9:20, by vol.), or in pyridine–3-methylbutan-1-ol–aq. 0.1 M-NH₃ (6:3:5, by vol.) (Harris & Hindley, 1965).

Peptides were detected by dipping the paper in ninhydrin–cadmium reagent (Heilmann, Barollier & Watzke, 1957); the Ehrlich reaction for tryptophan was as described by Smith (1960), and the Pauly reagent for histidine utilized diazotized sulphanilic acid (prepared from equimolar amounts of sulphanilic acid and NaNO₂).

Amino acid analysis. This was done qualitatively by paper chromatography in butanol–acetic acid; the isatin test was used for proline (Atfield & Morris, 1961). The quantitative amino acid analyses were done on a Technicon amino acid analyser or an EEL amino acid analyser.

Enzymes. Trypsin (twice-crystallized, salt-free) and carboxypeptidase A treated with di-isopropyl phosphorofluoridate were both from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Subtilisin was from Novo Terapeutisk Laboratorium, Copenhagen, Denmark. Thermolysin was from Chugai Boyaki Co. Ltd. (P.O. Box Higashi, no. 106, Osaka, Japan).

* Abbreviation: BAWP, butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953).

Carboxypeptidase. Digestions with carboxypeptidase were carried out as described by Smillie & Hartley (1966).

RESULTS

N-Terminal peptide from mixed chains

The diagonal procedure of Tang & Hartley (1967) was used to isolate the *N*-terminal peptide from a tryptic digest of α -crystallin, after the protein's thiol groups had been blocked by reaction with ethyleneimine. The basis of this method for the selective purification of peptides containing methionine is that reaction with iodoacetamide converts the thio ether into a sulphonium salt; this conversion changes the electrophoretic properties of the peptide containing methionine. Here, paper chromatography instead of paper electrophoresis was used, since larger amounts of the digest could be fractionated by paper chromatography in BAWP.

Fig. 1. Paper chromatography of tryptic digest of amino-ethyl derivative of bovine α -crystallin. The strip (carrying about 4 mg. of peptides) was run in the horizontal direction first, treated with iodoacetamide and then sewn into a fresh sheet and run in the vertical direction. The solvent was BAWP. The 'off-diagonal' peptide 1 gave a slow reaction with ninhydrin. The origin is at the top left-hand corner.

Reaction of free methionine with iodoacetamide decreased the R_F in BAWP from 0.5 to 0.2, the decrease being due to the conversion of a non-polar thio ether into a polar sulphonium salt.

There were three off-diagonal peptides (Fig. 1) detected by ninhydrin (and no extra ones were detected by the Sakaguchi test); the peptides corresponding to the spots marked 2 and 3 contained free *N*-terminal amino groups, but the peptide corresponding to spot 1 did not. This suggested that the last was the *N*-terminal peptide (peptide 1), and this was confirmed by the fact that this peptide contained aspartic acid, but did not contain leucine, and, as already mentioned, the peptide sought should have a Met-Asp rather than a Met-Leu bond.

Although the isolation so far was easy, and the yield of 3–8 μ moles/g. of α -crystallin not unreasonably low (the molecular weight of the chains is approx. 20 000), further purification proved difficult. The recovery after paper electrophoresis at pH 3.5, pH 6.5 or pH 8.9 was poor; *S*-carboxyamidomethylmethionine is known to be rather unstable (Colman, 1968). Moreover, amino acid analysis on portions purified by these methods (Table 2) indicated that the samples still contained several peptides, perhaps closely related.

Several points were not clear at this stage, namely (1) why do the peptides whose analysis is given in Table 2 differ in charge, but not in composition?; (2) why is there a fractional amount of arginine?; (3) why is the value for phenylalanine low?; (4) is the methionine wholly present as the sulphonium salt? The analytical values for methionine are not informative; *S*-carboxyamidomethylmethionine decomposes on hydrolysis and gives appreciable amounts of methionine (Stark & Stein, 1964).

Answers to these questions emerged from further structural work, which is now described. The peptide contains tryptophan, and there is probably one tryptophan residue (the evidence for this is discussed below), and we provisionally assumed that the peptide contained 11 amino acid residues. The

Table 2. *N-Terminal peptide from α -crystallin*

The peptide was isolated by diagonal electrophoresis, and further fractionated by electrophoresis at pH 8.9 [sample 1(9)], followed by electrophoresis at pH 6.5 [sample 1(9,6)]. Sample 1(9) had low anionic mobility at pH 8.9, and was essentially uncharged at pH 6.5; sample 1(9,6) had little or no charge at both pH values. The average values from all six samples analysed are also given. All the samples contained tryptophan.

Peptide	Amino acid composition (moles/mole of peptide)									
	Asp	Glu	Pro	Ala	Met	Ile	Phe	Lys	His	Arg
1(9)	0.99	0.96	1.13	1.01	0.41	2.18	0.65	0.79	0.91	0.14
1(9,6)	0.97	1.02	1.02	1.04	0.23	1.93	0.61	1.12	0.92	0.12
1 (average)	0.99	0.83	1.27	1.03	0.28	2.01	0.58	0.82	0.98	0.27

Table 3. *Peptides obtained by subtilisin or thermolysin digestion of N-terminal peptide*

The peptides were separated as described in the text.

Peptide	Ionic state		Amino acid composition											<i>N</i> -Terminus*	<i>C</i> -Terminus
	pH 4	pH 7	Met	Asp	Ile	Ala	Glu	His	Pro	Trp	Phe	Lys			
S1	Acidic		+	++	++	++									
S2	Slightly basic	Neutral			+		+							Ile	
S3	Basic	Slightly basic						+	+	+				His	Trp
S4	Basic	Basic			+		+	+	+	+				Ile	Trp
S5	Basic	Basic									+	+		Phe	
Th1	Acidic		+	+											
Th2	Slightly basic				+	+								Ile	
Th3	Basic				+		+	+	+	+				Ile	
Th4	Basic										+	+		Phe	

* The 'dansyl' method (Gray, 1967) was used for the subtilisin peptides, and the subtractive method with acrylonitrile (Fletcher, 1966) was used for the thermolysin peptides. The 'dansyl'-amino acids were identified by the method of Woods & Wang (1967) or Waley (1965).

Fig. 2. *N*-Terminal sequence of bovine α -crystallin. The enzymes used were subtilisin B (S), and thermolysin (Th); $\leftarrow$ denotes amino acids released by carboxypeptidase A. The state of the methionine residue is discussed in the text.

structural work was done on samples of peptide 1 obtained by the diagonal procedure given above.

Peptide 1 was digested with subtilisin, and the ninhydrin-positive peptides were separated by paper electrophoresis at pH 4, paper chromatography in butanol-acetic acid, and then paper electrophoresis at pH 7; for the ninhydrin-negative peptide, only the first step was carried out. The composition of the peptides (Table 3) was determined qualitatively by paper chromatography on butanol-acetic acid, which was adequate since the quantitative amino acid analysis (Table 1) had shown that there was only one residue of each amino acid (apart from the two residues of isoleucine, one of which is in a sequence already known).

Digestion of peptide 1 with carboxypeptidase gave tryptophan, phenylalanine and lysine; since peptide S5 is Phe-Lys, this gives the sequence -Trp-Phe-Lys, and since peptide S3 is His-Pro-Trp, the sequence -His-Pro-Trp-Phe-Lys is obtained. From its mobility, peptide S2 must contain gluta-

mine rather than glutamic acid, and so its structure is Ile-Gln. Peptide S4 provides the overlap required for ordering peptides S2 and S3, and enables the sequence to be written as -Ile-Gln-His-Pro-Trp-Phe-Lys. The *N*-terminal tetrapeptide sequence in bovine α -crystallin is acetyl-Met-Asp-Ile-Ala (Mok & Waley, 1968b; Hoenders *et al.* 1968), and hence the sequence in Fig. 2 is obtained. Digestion of peptide 1 with thermolysin, and purification of the peptides by electrophoresis at pH 4 (followed by electrophoresis at pH 3.5 for peptide Th4) gave the confirmatory results shown in Table 3 and Fig. 2. The dipeptide Phe-Lys was accompanied by a smaller amount of Ile-Arg, a finding discussed below.

B. A. Lang & B. Meloun (cited in Bloemendal, 1969) have studied peptides obtained by digestion of α -crystallin that had been treated with 4-nitrophenylsulphenyl chloride, a reagent that gives coloured derivatives with cysteine and tryptophan residues. Their peptides correspond in composition to residues 5-11 and residues 7-11 in the sequence given in Fig. 2, and confirm the presence of one tryptophan residue in the peptide.

Form of the methionine residue. Although the diagonal procedure for isolating peptide 1 was meant to convert the methionine into its sulphonium salt, it became clear that this was not the main product. Thus the cleavage by heating at 100° in aqueous solution (Tang & Hartley, 1967) released *N*-terminal aspartic acid in only very low yield. The peptide Th1 (Fig. 2) had an electrophoretic mobility at pH 4 of -0.75, greater than that of glutamic acid (-0.54); a lower mobility would be expected if the methionine were present as the sulphonium

salt. (These are anionic mobilities, measured from the neutrals, relative to lysine.) These arguments are, however, only suggestive, so an attempt was made to settle the issue by isolating the radioactive sulphonium salt form of peptide 1 from a tryptic digest of *S*-aminoethyl- α -crystallin treated with iodo[1- 14 C]acetamide in 8M-urea at pH 3.5.

The digest was fractionated by chromatography on Sephadex G-25 in 0.5% formic acid, and the material from the second radioactive peak was further purified by paper chromatography in BAWP and then electrophoresis at pH 6.5. A radioactive peptide was obtained, although in low yield, and this was compared with peptide 1 by electrophoresis at pH 4 (Fig. 3). The radioactive spot gave positive reactions with ninhydrin, and Pauly and Ehrlich reagents, as expected for the sulphonium salt corresponding to peptide 1, and it coincided with a minor spot in the preparation of the peptide obtained by the diagonal procedure. The main spot in the preparation of this peptide had a lower mobility, again suggesting that methionine was not present as the sulphonium salt. This conclusion was later confirmed when the version of peptide 1 with unmodified methionine (see below) was found to have the lower mobility (Fig. 3).

The main product in the preparations of peptide 1 obtained by the diagonal method may well contain the methionine residue as the sulfoxide. Oxidation of methionine in peptides has been noted before (Baglioni, 1960). Oxidation may take place during the drying of the paper chromatogram, and

during treatment with iodoacetamide. The diagonal procedure would be expected to separate methionine peptides from other peptides because the sulfoxide would have a lower R_F than the thio ether; apparently the sulfoxide and sulphonium salt forms have similar R_F values. Methionine sulfoxide is largely converted into methionine during acid hydrolysis (Ray & Koshland, 1960) and so amino acid analysis does not detect the sulfoxide. Both the sulphonium salt form and the sulfoxide form could have approximately zero net charge at pH 6.5 if the pK of the imidazole in the peptide is relatively low (say 5.8) in the sulphonium salt form and fairly high (say 7.5) in the sulfoxide form, but there is also the possibility that other factors are operative. It is possible that carboxyamidomethylmethionine deamidates in the diagonal procedure. It is also possible that there is appreciable adsorption on to paper, and so the mobilities are misleading. Milstein & Milstein (1968) have obtained variant forms of peptides, and have called attention to other instances of this phenomenon, which seems to have been noted especially often with peptides containing aspartic acid.

N-Terminal peptide from A chains of α -crystallin

The soluble peptides from the tryptic digestion of the A chains (see the Materials and Methods section) were fractionated on a column (150 cm. long $\times$ 2 cm. diam.) of Sephadex G-50 equilibrated with 0.05M-ammonia. Three main peaks were detected by u.v. absorption; the contents of the

Fig. 3. Electrophoresis (pH 4, 10 v/cm., 4 hr.) of variants of *N*-terminal peptide. Number 1 refers to the preparation obtained by the diagonal method, [14 C]-1 refers to the preparation obtained by the use of iodo[14 C]acetamide, and 1A refers to the preparation from A chains.

Fig. 4. Chromatography on Sephadex G-25 of *N*-terminal peptide from A chains. The bar shows the Ehrlich-positive fractions taken.

tubes comprising the second peak were concentrated, and refractionated on a column (130 cm. long $\times$ 0.8 cm. diam.) of Sephadex G-25 (the same solvent was used) (Fig. 4). The fractions comprising the main peak gave a positive Ehrlich reaction and these fractions were further purified by paper chromatography in BAWP followed by paper electrophoresis at pH 4; the peptide containing tryptophan had R_F 0.68, and electrophoretic mobility 0.13 (see Fig. 3). The analysis of this peptide is given in Table 4, where the peptide is referred to as 1A since it is derived from the A chains. Peptide 1A (in contradistinction to peptide 1 from the mixed chains) contains no arginine, and contains an integral number of phenylalanine residues. Hence the sequence in Fig. 2 represents the *N*-terminal sequence of the A chains. This at once suggests that the fractional amounts of arginine and phenylalanine in peptide 1 from mixed chains might be due to a variant version from the B chains.

N-Terminal peptide from B chains

The preparation of B chains was treated with citraconic anhydride (Dixon & Perham, 1968)

before digestion with trypsin; the peptides were now all soluble. After chromatography on Sephadex G-50 the fractions were tested by paper chromatography in BAWP, and the fractions that gave an Ehrlich-positive spot of high R_F were further purified by paper chromatography in pyridine-3-methylbutan-1-ol-aq. 0.1M-ammonia (6:3:5, by vol.) (Harris & Hindley, 1965). The peptide was treated with pH 3.5 buffer at room temperature overnight (to remove any citraconyl groups), and then further purified by paper electrophoresis at pH 3.5. The peptide, which was ninhydrin-negative, was obtained in very low yield, and consequently the analysis (Table 4) is only approximate, but the results are consistent with a variant of peptide 1 (called 1B).

The remainder of the peptide (0.08 μ mole) was digested with thermolysin, and the peptides were separated by electrophoresis at pH 4 and chromatography in BAWP (Table 5). The results in Table 4, taken together with the *N*-terminal sequence acetyl-Met-Asp, which is common to A and B chains, and the specificity of trypsin, which entails the arginine residue being *C*-terminal, suggest the sequence shown (Fig. 5).

Table 4. *Peptides obtained from A chains (peptide 1A) and B chains (peptide 1B)*

The isolation of the peptides is described in the text. Both peptides contained tryptophan. Peptide 1B contained proline, but the amount of peptide was too low for proline to be estimated in the hydrolysate.

Peptide	Amino acid composition (moles/mole of peptide)										
	Asp	Glu	Pro	Ala	Met	Ile	Phe	His	Lys	Arg	Trp
1A	1.00	1.02	0.96	1.12	0.76	1.78	0.99	1.01	1.29		+
1B	1.0		+	0.8	0.6	2.3		1.4		1.6	+

Table 5. *Peptides obtained by thermolysin digestion of peptide 1B*

The digest was fractionated by paper electrophoresis at pH 4, followed by paper chromatography in BAWP, of the neutral and positively charged peptides. The negatively charged peptides were eluted after electrophoresis. The neutral and positively charged peptides were detected by treating the paper with dilute ninhydrin (0.02% in acetone). The mobilities were measured relative to lysine at pH 4, and a negative sign indicates the charge. The mobilities at pH 2 were measured relative to serine.

Peptide	R_F	Mobility		Amino acid composition								<i>N</i> -Terminus	Sequence
		pH 4	pH 2	Met	Asp	Ile	Ala	His	Pro	Trp	Arg		
1		-0.5		+	+								
2	0.7	+0.2				+	+					Ile	Ile-Ala
3	0.6	+0.7	1.3*			+		+	+	+		Ile	Ile-His-(Pro-Trp)†
4	0.5	+0.64	1.7*			+					+	Ile	Ile-Arg

* The mobilities of peptides 3 and 4 at pH 2 when compared with the plot of Offord (1966) show that these peptides had only one residue of histidine and of arginine respectively.

† Measured by the 'dansyl'-Edman method as described by Gray (1967).

	1	2	3	4	5	6	7	8	9	10	11
Peptide 1B	Acetyl-Met	Asp	Ile	Ala	Ile	-	His	(Pro,Trp)	-	Ile	Arg
Peptide 1A	Acetyl-Met	Asp	Ile	Ala	Ile	Gln	His	-	Pro	Trp	Phe-Lys

Fig. 5. Suggested sequence for peptide 1B. A gap is shown in the sixth position, to emphasize homology with peptide 1A.

DISCUSSION

Earlier work had established the presence of different chains in α -crystallin (Waley, 1969a), but their separation has proved difficult. Indeed, reasonably satisfactory methods (Schoenmakers & Bloemendal, 1968a) only became available after the work described here had been started. Moreover, these methods are not satisfactory for α -crystallin from the lens of adult animals, and even with calf lens α -crystallin the recovery is not good. Thus most of the work was done with digests of mixed chains, which contain a complex mixture of peptides, and so the selective (diagonal) method of purification was a great help. However, when different forms of the *N*-terminal peptide were obtained it was not at first clear whether this 'polymorphism' was due to the presence of mixed chains, or due to different forms of a peptide derived from one kind of chain. In fact, both complications were encountered. The methionine residue was present partly as the sulphonium salt and partly as the sulfoxide, and the fractional amounts of certain amino acid residues (Table 1) were due to the presence of both kinds of chain. Nevertheless, it proved possible to deduce a sequence (Fig. 2) and, after the *N*-terminal peptide had been isolated from the A chains, its composition showed that the sequence could be assigned to the A chains. Since the ratio of the weight of the A chains to B chains is about 2:1, the sequence was that of the main component. This sequence is consistent with the compositions of the pentapeptide and the heptapeptide isolated by B. A. Lang & B. Meloun (cited by Bloemendal, 1969).

Work on the peptide from the mixed chains suggested the presence of a variant form in which residues 10 and 11 were isoleucine and arginine instead of phenylalanine and lysine, and this was confirmed when the peptide from the B chains was studied (Fig. 5). The B chain form is probably also one residue shorter, and comparison of the sequences suggests a gap in the sixth position. This feature, and the conservative nature of the amino acid replacements, are common occurrences on comparing homologous polypeptide chains, such as the α and β chains in haemoglobin. Hence it seems likely that the A and B chains in bovine α -crystallin are derived evolutionarily from a common precursor.

The evolution of lens proteins has been very thoroughly studied by use of antigenic relationships between species (Manski, Halbert, Auerbach-Pascal & Javier, 1967; Manski, Halbert & Javier, 1967; Manski, Halbert, Javier & Auerbach-Pascal, 1967). Indeed, analysis of the results on lens antigens led to a scheme demonstrating the phylogenetic relationships between vertebrate classes. Several antigenic determinants have been preserved throughout the course of vertebrate evolution, and at least one of these determinants is attributed to an α -crystallin. Zwaan & Ikeda (1965) have put forward a model for the phylogeny of lens proteins that could account for the antigenic relationships, and the work described above is a beginning towards providing information about the structural basis of these organ-specific antigens.

The present results are consistent with the suggestion that the four chains in calf α -crystallin may be assigned to two classes. The two members of the A chain class (called A₁ and A₂; Waley, 1969b) are very similar, their amino acid analyses being indistinguishable (Schoenmakers & Bloemendal, 1969). It is not known whether the B₁ and B₂ chains are also so similar, nor is anything known about the structure of the X chain (Waley, 1969b), which is present only in lenses from adult animals (Palmer & Papaconstantinou, 1968). Calf lenses also contain a minor α -crystallin fraction that is separated from the bulk of the α -crystallin by chromatography on DEAE-cellulose and characterized by its high specific radioactivity after incubation of the lens with radioactive amino acids (Spector, Wandel & Li, 1968). Spector *et al.* (1968) suggested that the biosynthesis of α -crystallin takes place in two stages: the chains are assembled in the first stage, and then acetylation at the *N*-terminus takes place in the second stage. Knowledge of the sequence at the *N*-terminus of the chains may help in testing this hypothesis.

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REFERENCES

- Ambler, R. P. (1963). *Biochem. J.* **89**, 349.
- Atfield, C. N. & Morris, C. J. O. R. (1961). *Biochem. J.* **81**, 606.
- Baglioni, C. (1960). *Biochim. biophys. Acta*, **48**, 392.
- Bloemendal, H. (1969). *Exp. Eye Res.* **8**, 227.
- Cliffe, E. E. & Waley, S. G. (1958). *Biochem. J.* **69**, 649.
- Colman, R. F. (1968). *J. biol. Chem.* **243**, 2454.

- Dixon, H. B. F. & Perham, R. N. (1968). *Biochem. J.* **109**, 312.
- Fletcher, J. C. (1966). *Biochem. J.* **98**, 34c.
- Grassmann, W., Hannig, K. & Plöckl, M. (1955). *Hoppe-Seyl. Z.* **299**, 258.
- Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 469. Ed. by Hirs, C. H. W. New York: Academic Press Inc.
- Harris, J. I. & Hindley, J. (1965). *J. molec. Biol.* **13**, 894.
- Heilman, J., Barollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
- Hoenders, H. J., Schoenmakers, J. G. G., Gerding, J. T. T., Tesser, G. I. & Bloemendal, H. (1968). *Exp. Eye Res.* **7**, 291.
- Hoenders, H. J., van Tol, J. & Bloemendal, H. (1968). *Biochim. biophys. Acta*, **160**, 283.
- Manski, W., Halbert, S. P., Auerbach-Pascal, T. & Javier, P. (1967). *Int. Arch. Allergy*, N.Y., **31**, 38.
- Manski, W., Halbert, S. P. & Javier, P. (1967). *Int. Arch. Allergy*, N.Y., **31**, 475.
- Manski, W., Halbert, S. P., Javier, P. & Auerbach-Pascal, T. (1967). *Int. Arch. Allergy*, N.Y., **31**, 529.
- Mikes, O. & Holeysovsky (1958). *Coll. Czech. chem. Commun.* **23**, 524.
- Milstein, C. P. & Milstein, C. (1968). *Biochem. J.* **109**, 93.
- Mok, C.-C. & Waley, S. G. (1967). *Biochem. J.* **104**, 128.
- Mok, C.-C. & Waley, S. G. (1968a). *Exp. Eye Res.* **7**, 148.
- Mok, C.-C. & Waley, S. G. (1968b). *Symp. Biochemistry of the Eye, Tutzing Castle, 1966*, p. 268. Basle: S. Karger.
- Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **53**, 103.
- Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
- Palmer, W. G. & Papaconstantinou, J. (1968). *Nature, Lond.* **219**, 171.
- Ray, W. J. & Koshland, D. E. (1960). *Brookhaven Symp. Biol.* **13**, 135.
- Schoenmakers, J. G. G. & Bloemendal, H. (1968a). *Biochem. biophys. Res. Commun.* **31**, 257.
- Schoenmakers, J. G. G. & Bloemendal, H. (1968b). *Nature, Lond.*, **220**, 790.
- Schoenmakers, J. G. G. & Bloemendal, H. (1969). *Proc. 6th Meet. Fed. Europ. biochem. Soc., Madrid*. London: Academic Press (Inc.) Ltd. (in the Press).
- Schoenmakers, J. G. G., Hoenders, H. J. & Bloemendal, H. (1968). *Exp. Eye Res.* **7**, 172.
- Schoenmakers, J. G. G., Matze, R., van Poppel, M. & Bloemendal, H. (1969). *Int. J. Protein Res.* **1**, 19.
- Smillie, L. B. & Hartley, B. S. (1966). *Biochem. J.* **101**, 232.
- Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 96. London: William Heinemann Ltd.
- Spector, A., Wandel, T. & Li, L.-K. (1968). *Invest. Ophthalm.* **7**, 179.
- Stark, G. R. & Stein, W. H. (1964). *J. biol. Chem.* **239**, 3755.
- Tang, J. & Hartley, B. S. (1967). *Biochem. J.* **102**, 593.
- Waley, S. G. (1965). *Biochem. J.* **96**, 722.
- Waley, S. G. (1969a). In *The Eye*, vol. 1, 2nd ed., p. 299. Ed. by Davson, H. New York: Academic Press Inc.
- Waley, S. G. (1969b). *Exp. Eye Res.* **8**, (in the Press).
- Waley, S. G. (1969c). *Proc. 6th Meet. Fed. Europ. biochem. Soc., Madrid*. London: Academic Press (Inc.) Ltd. (in the Press).
- Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.
- Wisse, J., Zweers, A., Jongkind, J., Bont, W. S. & Bloemendal, H. (1966). *Biochem. J.* **99**, 179.
- Woods, K. R. & Wang, K.-T. (1967). *Biochim. biophys. Acta*, **133**, 369.
- Zwann, J. & Ikeda, A. (1965). In *The Structure of the Eye, 2nd Symp.*, p. 419. Ed. by Rohen, J. W. Stuttgart: Schattauer-Verlag.