Exp. Eye Res. (1962) **1,** 436-442

Some New Compounds in the Lens

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The discovery in calf lens and characterization of three new tissue constituents is reported. These are *S*-(α,β-dicarboxyethyl)-cysteine, *S*-(α,β-dicarboxyethyl)-glutathione and arginyllysylglycine. Ergothioneine has been identified as a constituent of the eye for the first time. A method is described for the analysis of whole extracts of lens on one paper by electrophoresis and chromatography. This could be applied to other tissues.

This paper is concerned with the identification of a number of peptides, amino acids and related compounds present as minor constituents of calf lens. Studies on the distribution of free amino acids, mostly those which normally occur in proteins, in other parts of bovine eye such as retina (Kubíček and Dolének, 1958), vitreous body (Wootton, Young and Williams, 1954) and aqueous humour (François, Rabaey and Recoulès, 1960) have been published. A series of papers (Malatesta, 1952) describe the identification of amino acids in lens, aqueous humour and vitreous body by paper chromatography.

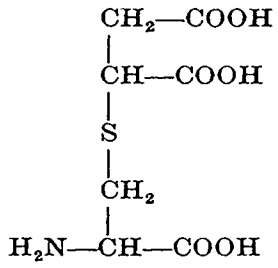
Acidic peptides

A number of peptides closely related to glutathione has been isolated from calf lens (Waley, 1956, 1957, 1958, 1959). These have the general structure γ-glutamyl-X-glycine, where X is cysteine in glutathione, α-amino-n-butyric acid and its lower homologue, alanine, in ophthalmic acid and norophthalmic acid respectively, and *S*-sulphocysteine in *S*-sulphoglutathione. There is evidence that the enzyme system by which these peptides are synthesized is capable of incorporating other amino acids into the X position (Cliffe and Waley, 1958), but the corresponding peptides have not been detected in lens.

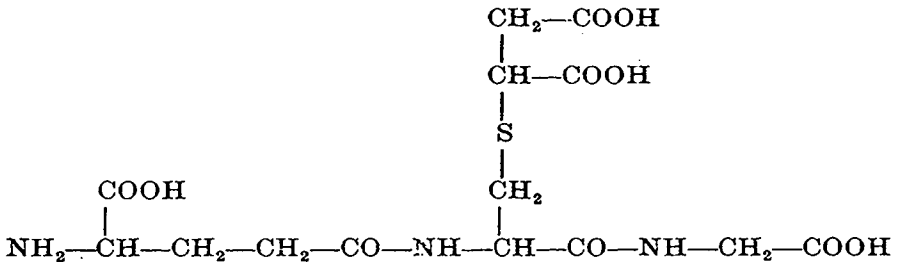
At least one other acidic tripeptide was known to be present in calf lens. In order to characterize it, lenses were deproteinized by treatment with trichloracetic acid and the extract fractionated by two different methods. In the first, the extract was run in a continuous electrophoresis apparatus at pH 4, and fractions pooled to give the unknown with little contamination. The more highly charged species were lost since they migrated off the paper; these included ethanolamine and many phosphorus-containing compounds, such as nucleotides.

The second fractionation was performed by ion exchange chromatography on a Dowex 1 (acetate) column. Basic and neutral species were not adsorbed and were collected as the extract flowed through the column. Acidic compounds were eluted with volatile pyridine–formate buffers and fractions pooled according to their content as shown by paper electrophoresis. The apparently homogeneous material obtained by electrophoresis was eluted in two peaks from the column. These peaks were given by different compounds thus showing the importance of using two isolation procedures. The two substances have been identified as the free amino acid *S*-(α,β-dicarboxyethyl)-cysteine, and the corresponding peptide *S*-(α,β-dicarboxyethyl)-glutathione. Both compounds can be synthesized by addition of fumarate to the free

thiol groups of cysteine and glutathione. The reaction is faster with cysteine than with glutathione, but in both cases is slower than the reaction of maleate with the thiols which was already known (Morgan and Friedmann, 1938) and gives the same product. The synthetic compounds have identical electrophoretic mobility at two different pH values, but are separated by paper chromatography. Since fumarate is



S—(α,β-Dicarboxyethyl)-cysteine



γ-Glutamyl-*S*-(α,β-dicarboxyethyl)-cysteinylglycine
[*S*-(α,β-dicarboxyethyl)-glutathione)]

present in lens, addition to the thiols could occur during preparation of lens extracts. *N*-Ethylmaleimide reacts very rapidly with free thiol groups and a lens extract has been prepared in the presence of this substance to remove all free cysteine and glutathione. *S*-(α,β-dicarboxyethyl)-cysteine and the corresponding glutathione compound are still present in this extract so they are probably normal constituents of lens. Their total concentration is about 0.5 mM. The concentration of glutathione is about 7–10 mM in calf lens.

The extract contains the *N*-ethylmaleimide adduct of glutathione, also traces of oxidized glutathione. The amount of the latter is about 3–5% of the total glutathione content of the lens, in agreement with Kinoshita and Masurat (1957) but contrary to an earlier report that all free glutathione is in the reduced form (Herrmann and Moses, 1945). These authors also found that added oxidized glutathione (GSSG) is rapidly reduced by a number of eye tissues, including the lens. The amount of GSSG found probably represents the proportion normally present.

Cysteine

It is normally assumed that lens contains no free cysteine, but the lens extract prepared in the presence of *N*-ethylmaleimide appears to contain traces of *S*-(*N*-ethylsuccinimido)-cysteine, and probably cystine. The amount of free cysteine indicated by this method is about the same as that of oxidized glutathione, about 3–5%of GSH, which would not be sufficiently significant to alter the amount of GSH determined by other methods. Cysteine is usually found only in trace amounts in tissues.

Basic peptides

One other new peptide has been detected in lens. This probably has the structure arginyllysylglycine and is present at a concentration of about 5×10^{-5} M (less than 1 mg per 100 g lens). It is weakly basic at pH 4 and was first encountered because of its characteristic position on paper electrophoresis between β-alanine (less basic) and γ-aminobutyric acid. It appears to be the first naturally occurring tripeptide containing lysine, but the peptide leucylarginylleucine has been isolated from pig pituitary gland (Gros, de Garilhe, Costopanagiotis and Schwyzer, 1961). The function and origin of the lens peptide is completely unknown.

Basic peptides are rather uncommon tissue constituents, the exceptions being carnosine (β-alanylhistidine) and anserine (the 3-methylhistidine analogue) which are found in muscle. Carnosine has been reported (Krause, 1936) as the only other peptide in lens apart from those already mentioned. The presence of carnosine has been confirmed and its concentration is about 5×10^{-5} M. It is not yet clear whether anserine is present.

Ergothioneine

The concentration of reducing substances in lens, glutathione, ascorbic acid, and (probably) cysteine is extremely high and may be of significance for maintenance of transparency. This concentration is further increased by the discovery of ergothioneine, thiol-histidine betaine, as a normal constituent of lens. The concentration in calf lens is of the order of 0.1 mM. It has been shown that rats fed on [^{35}S]ergothioneine have some detectable radioactivity in the eye (Heath, 1953), but the amount could be explained by blood remaining in the tissue. Since ergothioneine is a normal constituent of blood, it is probably also present in the aqueous humour. The concentration in lens is comparable with that in blood. The function of ergothioneine is unknown.

Free amino acids

Individual fractions obtained from lens extracts have been examined in detail by electrophoresis and chromatography and the use of a number of locating agents specific for particular groups in the molecule. This has permitted a considerable concentration of material so that substances originally present in lens at 0.01 mM or lower concentration can be detected.

The amino acids commonly found in proteins are also found free in most tissues. All are present in lens. The concentration of cystine in lens is greater than that of methionine. This is the reverse of the situation found in the aqueous humour (François, Rabaey and Recoulès, 1960) where the concentration of methionine is high. Asparagine appears to be more abundant in cow lens than in calf. Large amounts of glutamic acid and glutamine are present, the amount of glutamine diminishes on storing as it slowly cyclizes to 5-oxopyrrolidine-2-carboxylic acid which is ninhydrin-negative.

A number of non-protein amino acids is also found free in lens. The most prominent is α-aminobutyric acid, which is found bound in ophthalmic acid, and which is at higher concentration free than many protein amino acids. Taurine, methylhistidine, β-alanine, γ-aminobutyric acid and ethanolamine have been detected. The presence of cystathionine, ornithine and citrulline is suspected but is not yet definitely established. The esters, phosphoryl-ethanolamine and glyceryl-phosphoryl-ethanolamine have been reported previously (van Heyningen and Pirie, 1958). One unidentified ninhydrin-positive compound occurs in the fraction which

contains basic amino acids. It appears to be an amine since its reaction with ninhydrin is destroyed by the action of nitrous acid but not by hydrochloric acid. It travels near the solvent front in all chromatographic solvents investigated.

Investigation of whole lens extracts

The preparation and investigation of lens extracts is a rather lengthy procedure and requires comparatively large amounts of tissue. This is a major disadvantage in attempting to apply similar methods to the study of changes which occur on cataract formation. In an attempt to overcome this difficulty a method of studying individual fractions of lens extract has been extended to extracts of whole lens. This is a variation of a procedure for investigating amino acids and peptides (Richmond and Hartley, 1959) and consists of low voltage paper electrophoresis at pH 1.6 in the first direction, followed by chromatography in butanol–acetic acid–water (40:9:20, by vol) in the second (S. G. Waley, *unpublished*). The advantage of this method over two-dimensional paper chromatography is that electrophoresis acts as a de-salting process thus giving a more reproducible pattern.

The extract is applied to paper near the anode. At the low pH employed, only strongly acidic groups remain charged, most of the carboxyl groups are unionized. This means that nucleotides and phosphorus compounds, such as phosphoryl-ethanolamine, also taurine with a sulphonic acid group, barely migrate from the origin, while basic amino acids and ethanolamine travel furthest with neutral amino acids in an intermediate position. Although the differences in migration of a group of mono-amino, mono-carboxylic acids, such as glycine, alanine and valine, are small, they are well separated by chromatography.

The pattern obtained is extremely reproducible and the position of each compound can be determined by separating mixtures of known compounds to give reference patterns. Semi-quantitative estimations of concentration are obtained by running markers of known strength. Figure 1 is the paper obtained from a trichloracetic acid extract of fresh calf lens. The amount of dry solid in the extract is about 15 mg/g lens. Solid is dissolved in 20 μl water to give a rather syrupy solution and half of this analysed on Whatman No. 3 paper. At this concentration about 55–60 ninhydrin-positive spots are detectable. Despite the number of minor constituents already identified in the bulk fractions, about 15 spots are still unidentified. Marker spots are run at the side as a check on each paper. Some of the spots are very concentrated and an equally satisfactory pattern is obtained by using half this amount of material, that is, equivalent to 0.25 g lens, except that some of the faintest spots cannot be seen so easily. Glutathione is largely observed in the oxidized form. One surprising finding from this paper was the presence of large amounts of a spot attributed to ascorbic acid which gives a purplish colour with ninhydrin, and quenches in ultra-violet light. The identification has been confirmed with a marker and with specific reagents.

A number of different extracts has been prepared and patterns obtained for comparison purposes. Since the bulk extracts originally studied were prepared from stored calf lenses, trichloracetic acid extracts of stored and fresh calf lenses were compared. The major findings were that in stored lens ascorbic acid decomposes, glutamine cyclizes and some breakdown of peptides to amino acids occurs. No significant difference exists between extracts of fresh calf lens prepared by removal of protein with trichloracetic acid and with phenol. Cow lens has been studied by the same method, one quarter of the extract (equivalent to about 0.5 g lens) being

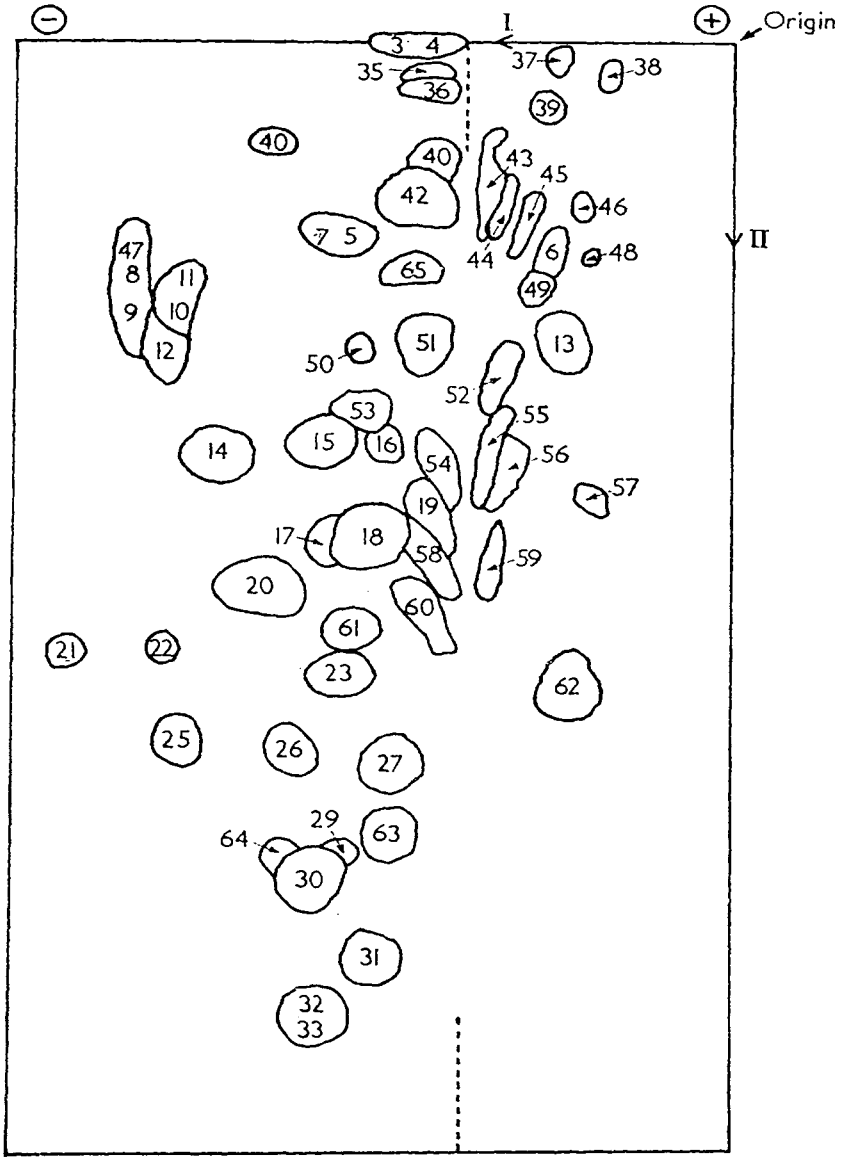


FIG. 1. Pattern of ninhydrin-positive spots in trichloracetic acid extract of fresh calf lens. The procedure is outlined in the text and will be published in detail elsewhere. The size of the spots is not indicative of their relative intensity. Some streaking of spots occurs over the apex of the paper which is indicated by the discontinuous dotted line. A key to the identification of compounds is given.

Key to Figs. 1 and 2

1. Unidentified
2. Hydroxylysine
3. Unidentified
4. Unidentified
5. Cystine
6. Phosphoryl-ethanolamine
7. Cystathionine
8. Ornithine
9. Lysine
10. Histidine
11. Methylhistidine
12. Arginine
13. Taurine
14. Glycine
15. Serine
16. Aspartic acid
17. Threonine
18. Glutamic acid
19. *S*-(α,β-dicarboxyethyl)-cysteine
20. Alanine
21. Ethanolamine
22. β-Alanine
23. Proline
24. Unidentified
25. γ-Aminobutyric acid
26. α-Aminobutyric acid
27. Tyrosine
28. Unidentified
29. Methionine
30. Valine
31. Phenylalanine
32. Leucine
33. Isoleucine
34. Unidentified
35. Unidentified
36. Unidentified
37. Unidentified
38. Unidentified
39. Unidentified
40. Arginyllysylglycine
41. Hydroxylysine phosphate
42. GSSG
43. Unidentified
44. Unidentified
45. Unidentified
46. Cysteic acid (?)
47. Carnosine

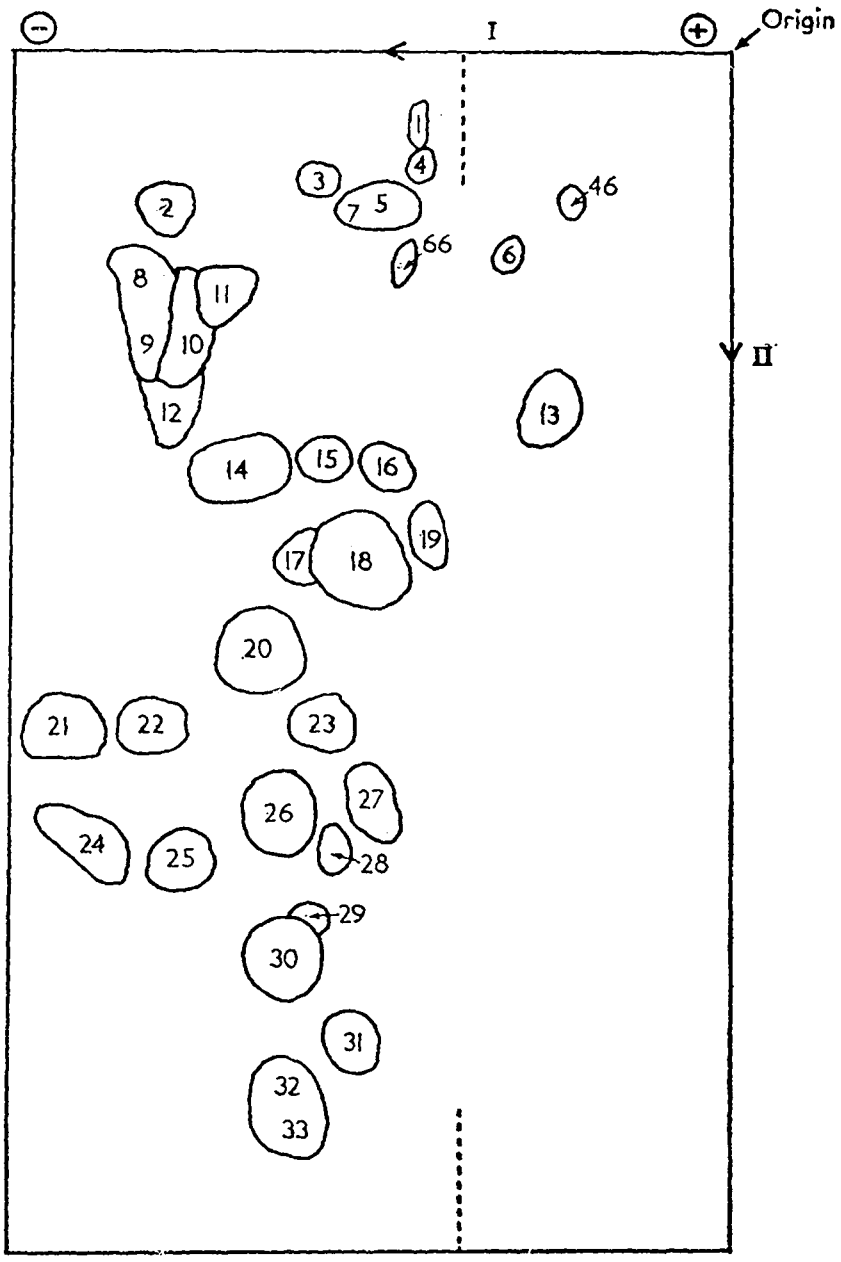


FIG. 2. Pattern of ninhydrin-positive spots in the hydrolysate of a trichloracetic acid extract of calf lens. This was obtained in the same manner as Fig. 1 and the compounds are identified in the key.

Key to Figs. 1 and 2 (continued)

48. *S*-Sulphoglutathione
49. Glyceryl-phosphoryl-ethanolamine
50. Asparagine
51. Unidentified
52. Unidentified
53. Glutamine
54. *S*-(α,β-dicarboxyethyl)-glutathione
55. Unidentified
56. Unidentified
57. Unidentified
58. Ophthalmic acid
59. Unidentified
60. Unidentified
61. Unidentified
62. Ascorbic acid
63. Tryptophan
64. Unidentified
65. Unidentified
66. Unidentified

The pairs of spots 42–43, 54–55, 58–59 may result from a single compound streaking over the apex of the paper. Many of the unidentified compounds are present only in trace amounts and are destroyed by hydrolysis.

sufficient. There is little difference compared with calf lens. Both *S*-(α,β-dicarboxyethyl)-cysteine and *S*-(α,β-dicarboxyethyl)-glutathione are present in fresh calf and cow lens. The concentration of many of the compounds is similar in both types of lens. The fluorescent peptide detected in the lens of human and primates (François, Rabaey and Recoulès, 1961) is not present.

The pattern of spots is considerably simplified by hydrolysis (Fig. 2). Because of the large increase in concentration of glutamic acid, glycine and other spots the equivalent of one quarter calf lens is quite sufficient for a paper. Taurine is now the only strong spot on the right of the apex of the paper. Only a few amino acids are present in lens in bound form as the concentration of many of them is unaffected by hydrolysis.

Other separations have been obtained by the same method and the papers developed with locating agents which reveal different series of compounds. Phosphorus-containing compounds mostly coincide with spots visible under ultra-violet light by quenching or fluorescence and are concentrated round the origin. Other ninhydrin-negative compounds detected by specific reagents are urea, ergothioneine, creatine, glucose and inositol, also ascorbic acid. Care is always necessary in the identification of spots since compounds present at high concentration have been found to give false positive reactions with location reagents. This is especially true of oxidized glutathione which is visible on most papers, and of ascorbic acid with ninhydrin.

The success of the method as applied so far suggests that satisfactory patterns could well be obtained from single human lenses. The distribution of low molecular weight compounds in human lens is completely unknown, as is the change in distribution on cataract formation. If the material were available, an appreciable amount of semi-quantitative information could be obtained by a relatively simple technique.

ACKNOWLEDGMENTS

I should like to thank Dr. S. G. Waley for his advice and encouragement, the National Council to Combat Blindness Inc., New York, for a Fight For Sight grant-in-aid for the purchase of equipment, the Distillers Co. Ltd. for a gift of glutathione and the University of Oxford for a Thornton Bursary.

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