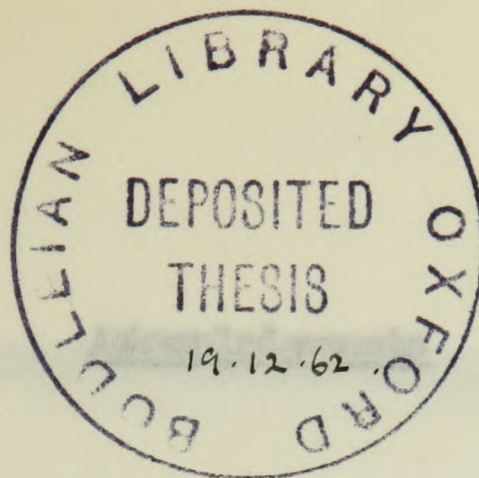




I am deeply indebted to Dr. Stephen Walley who suggested this subject. Some constituents of tissues of the eye has been a source of advice and encouragement at all times.

by

I am also very grateful to the following people: to Dr. A. Pirie for allowing me to work in this laboratory; to Dr. Pirie and Dr. A. Derek Harold Calam, B.A. for their great interest in my work and for such helpful discussions; and to the technical staff of the laboratory for their willing assistance.

The work described in this thesis submitted for the degree of Doctor of Philosophy in the University of Oxford since September 1960 during the tenure of a Research Scholarship from the University of Oxford.

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June 1962.

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The work described in this thesis has been performed since September 1960 during the tenure of a Thornton Bursary from the University of Oxford.

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Muffield Laboratory of Ophthalmology,
Oxford.

June 1962.

Previously published work

A summary of the main findings was presented as a paper at the Symposium on Lens Metabolism in Relation to Cataract, Oxford, 21 - 26 May 1962 (Calam 1962).

The constitution of the lens is described and discussed in the next two sections. Part III contains the work on new acidic compounds, Part IV is concerned with other constituents. The results are discussed in more general terms in Part V.

Method of presentation

List of figures

List of tables Method of presentation

Abbreviations

Part I. An account of the general background of this work is given in Part I. The experimental work was based on the analytical techniques described in Part II. The identification of some constituents of the lens is described and discussed in the next two sections: Part III contains the work on new acidic compounds, Part IV is concerned with other constituents. The results are discussed in more general terms in Part V.

Part III. New acidic compounds

Introduction

Part III. New acidic compounds

1. Identification of the acids

2. Acidic compounds

3. Identification of the acids

Experimental and results

1. Identification of the acids

2. Acidic compounds

3. Identification of the acids

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Abbreviations

Acetic acid	Glacial acetic acid
DNP	Dinitrophenyl
GSH and GSSG	Reduced and oxidized glutathione
M and mM	Molar and millimolar concentrations
N	Normal (concentration)
NH ₃ solution	Ammonia solution sp. gr. 0.890
Oph. acid	Ophthalmic acid
Water	Demineralized water

All temperatures are in °Centigrade and room temperature has been taken as 20°.

Standard abbreviations have been used throughout for amino-acids. It is assumed that all are in the L-form and this is not specified.

GENERAL INTRODUCTION

The lens of the eye is a unique structure. It is formed entirely from one layer of epithelial cells which live on the front surface of the lens, beneath the lens capsule. As the cells grow and divide, they are pushed toward the center of the lens. Old cells are not shed as growth proceeds. Until lens fibers mature and form a closely interlocking core in the lens. Cells elongate to form fibres which give the lens a layer structure similar to that of an onion (Figure 1). The ability of the lens to focus a sharp image on the retina depends upon its flexibility which the lens capsule and upon its transparency, which is near that of pure water. Lens contains approximately 65% water, 30% protein, 3% organic material, and 1% salts.

Part I

General Introduction

A large proportion of the organic matter in the lens is protein. With increasing age, a core, which contains comparatively insoluble protein, develops at the center of the lens. Eventually an opaque area may form and the lens is said to have developed a cataract. This is a nuclei cataract. Other cataracts arise from congenital abnormalities, drug metabolic defects such as diabetes, from radiation damage, and can be induced by the action of certain chemicals, for example cuprous ions (see Parts II and Supplement, 1955 for a complete account).

Figure 1 cataractous lens can be removed by a simple surgical operation but it is of obvious interest to discover what structural chemical and biochemical changes occur in cataract formation and if

GENERAL INTRODUCTION

The lens of the eye is a unique structure. It is formed entirely from one layer of epithelial cells which lie on the front surface of the lens, beneath the lens capsule. As the cells grow and divide, they are pushed towards the centre of the lens. Old cells are not shed as growth proceeds. Most lose their nucleus and form a slowly hardening core in the lens. Cells elongate to form fibres which give the lens a layer structure similar to that of an onion (Figure 1). The ability of the lens to focus a sharp image on the retina depends upon its flexibility within the lens capsule and upon its transparency, which is near that of pure water. Lens contains approximately 65% water, rather less than in most body tissues, 34% organic material, and 1% salts.

A large proportion of the organic matter is soluble protein. With increasing age, a core, which contains comparatively insoluble protein, develops at the centre of the lens. Eventually an opaque area may form and the lens is said to have developed a cataract. This is a senile cataract. Other cataracts arise from congenital abnormalities, from metabolic defects such as diabetes, from radiation damage, and can be induced by the action of certain chemicals, for example naphthalene (see Pirie & van Heyningen, 1956 for a complete account).

A cataractous lens can be removed by a simple surgical operation but it is of obvious interest to discover what detailed chemical and biochemical changes occur in cataract formation and, if

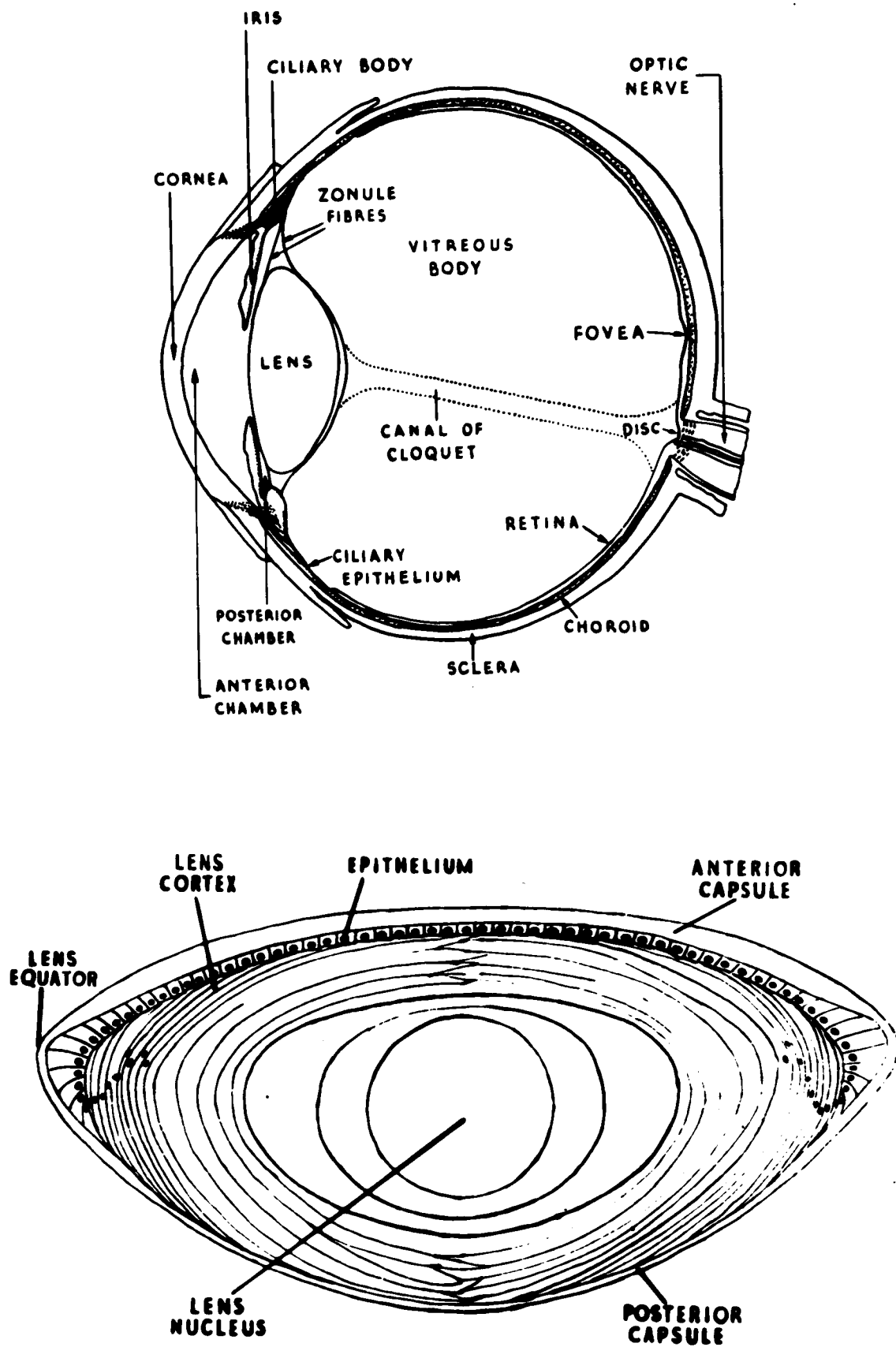


Figure 1. Anatomy of the eye and the lens.

Upper: Diagram of the human eye. The aqueous humour occupies the posterior and anterior chambers

Lower: Diagram of the dog lens.

possible, find ways of preventing them. Since senile cataract is one manifestation of the aging process, such changes may throw light on aging processes in general.

The lens is most homogeneous in young animals and the composition of young lens is one basis from which to study changes in mature lens. Sufficient normal human lenses are not available for detailed chemical analysis by most procedures. Calf lenses are suitable for this purpose because of their size and accessibility. An average calf lens weighs about 1 g., a cow lens about 2 g. and an adult human lens about 0.2 g.

One unique feature of the lens is its lack of blood supply. The lens is dependent upon a supply of nutrients and removal of waste material through the aqueous humour for its metabolism. The aqueous in turn is dependent through the ciliary body upon the blood stream. Some of the unusual features observed in the lens compared with other tissues may be a result of this means of nutrition.

The work described in this thesis has consisted of the fractionation of low molecular-weight organic constituents of calf lens and the characterization of a number of these. Identification has been limited to peptides, amino-acids, and related substances. Within this group, interest has centred upon compounds previously unobserved as tissue constituents, and upon those not recognised as constituents of the eye.

The remainder of this introduction is a survey of aspects of lens composition which are relevant to the experimental work described in later sections.

Peptides

Acidic. Glutathione (GSH), the tripeptide γ -glutamylcysteinylglycine, has long been recognized as a constituent of the lens. Glutathione is widely distributed in nature. In animals it is located mainly within tissue cells and largely absent from body fluids. It is found in whole blood but not in plasma. Only traces can be detected in the aqueous and vitreous humours. Glutathione is, therefore, synthesised in lens.

The concentration of glutathione in lens is probably higher than in any other tissue. Methods available for its estimation vary widely in their specificity. The most reliable values for lens are 1.51 g/kg wet weight for cow (Hockwin, Kleifeld & Hans 1955) and 2.2 g. for calf (Long 1961). Concentration of glutathione is highest at the lens cortex and decreases towards the nucleus. Glutathione must be important for correct maintenance of lens metabolism since its concentration shows a marked drop on formation of cataract. This concentration change is detectable before any clinical signs are observed. Turnover of glutathione has been studied in rabbit lens by incorporation of ^{14}C -glycine (McMillan, Ryerson & Mortensen 1959) and the turnover time estimated as four days.

Detection of oxidized glutathione (GSSG) is complicated by the ready oxidation of GSH in the presence of metal ions and at neutral pH. GSSG has only recently been recognized as a tissue constituent. Its concentration is low and falls within the variation (about 6%) from the mean of values obtained for GSH. Kinoshita and Masurat (1957)

estimated glutathione in lens before and after electrolytic reduction and found an increase of about 3% attributed to the oxidized form. This is in agreement with the value of 2 - 5% obtained for brain (Martin & McIlwain 1959) by reduction of GSSG with yeast apoglyoxalase.

The first naturally occurring analogues of glutathione were isolated from calf lens (Waley, 1956, 1957) by zone electrophoresis and preparative paper chromatography. One, ophthalmic acid, contains equimolar amounts of glutamic acid, α -amino-n-butyric acid, and glycine. Glutamic acid was shown to be N-terminal by the DNP method, and to be γ -linked by electrophoretic mobility of the peptide at pH 4. γ -Glutanyl peptides have greater net negative charges than the corresponding α -glutanyl peptides. Carboxypeptidase liberated glycine. The assigned structure: γ -glutanyl- α -amino-n-butyrylglycine was confirmed by chemical synthesis and by X-ray analysis of synthetic and natural material (Waley 1958).

By similar methods, the structure of the second peptide, norophthalmic acid, was found to be γ -glutamylalanylglycine. This peptide was obtained by the action of Raney nickel on glutathione. Further studies (Waley 1959) revealed a third peptide, S-sulphoglutathione. This could be formed in lens extracts by interaction of GSSG and sulphite but an experiment described later suggests that it is normally present in lens.

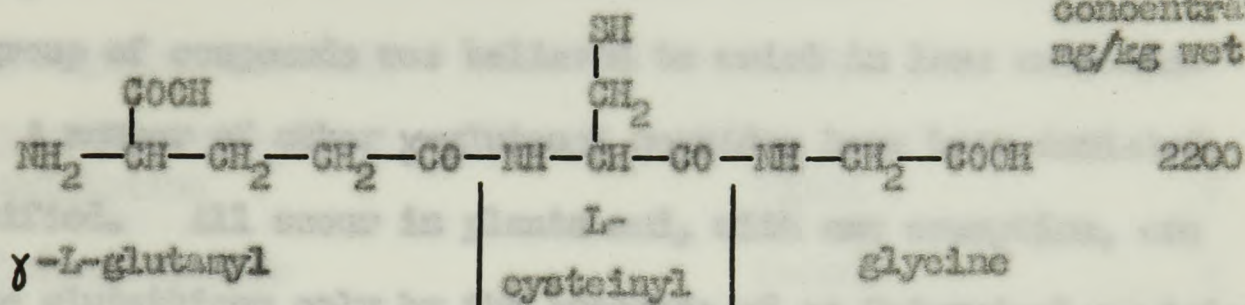
These peptides are related to glutathione by the general structure γ -glutanyl-X-glycine, where X is an amino-acid (Table 1). The enzymes in lens which synthesize glutathione from its constituent amino-

Table 1

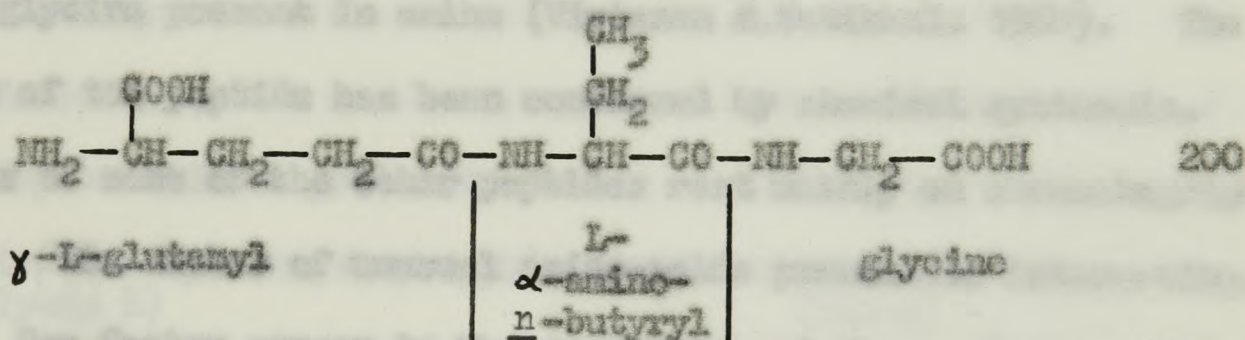
γ -glutamyl peptides in calf lens

Glutathione

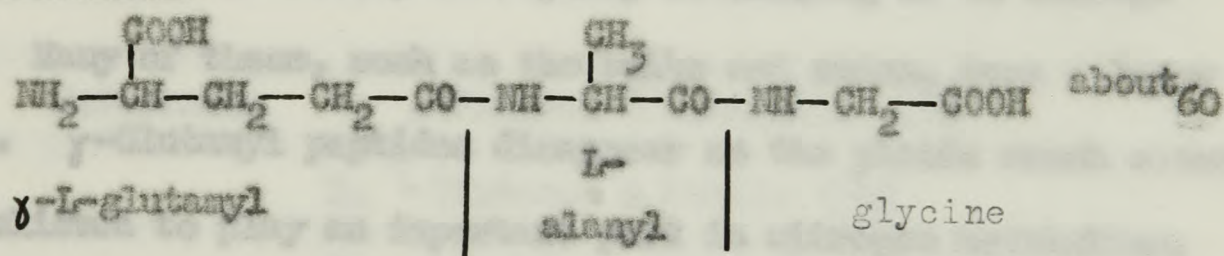
Approximate
concentration
ng/kg wet wt.



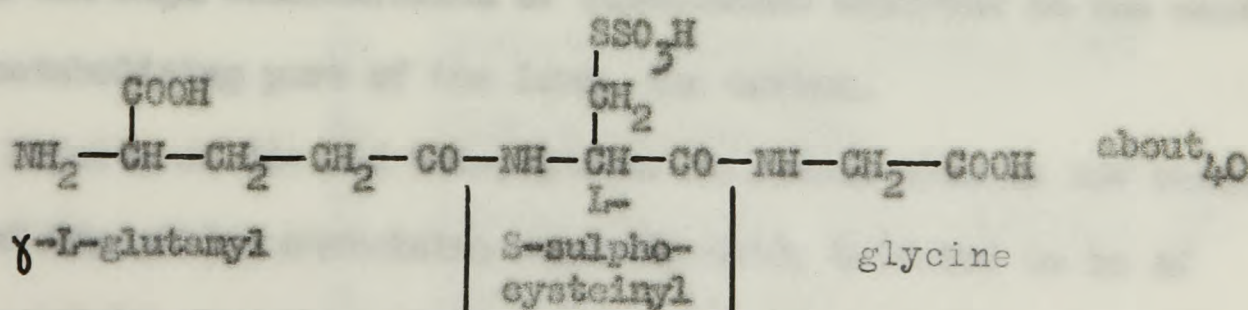
Ophthalmic acid



Norophthalmic acid



S-Sulphoglutathione



acids were shown to incorporate the appropriate amino-acid into the central position to form the other peptides (Cliffe & Waley 1958). Evidence was also obtained for the incorporation of threonine but the corresponding peptide has not been detected in lens. Another member of this group of compounds was believed to exist in lens extracts.

A number of other γ -glutamyl peptides have been isolated and identified. All occur in plants and, with one exception, are related to glutathione only by the presence of an N-terminal γ -glutamyl residue (Table 2). The exception is γ -glutamyl-S-(β -carboxy-n-propyl)-cysteinylglycine present in onion (Virtanen & Matildala 1960). The structure of the peptide has been confirmed by chemical synthesis. The structures of some of the other peptides rest mainly on chromatographic evidence. The number of unusual amino-acids present is interesting.

One factor common to the occurrence of these plant peptides is that they are found mainly in rapidly developing or in storage tissues. Many of these, such as the bulbs and onion, have a layer structure. γ -Glutamyl peptides disappear as the plants reach maturity and are believed to play an important part in nitrogen metabolism. The high concentration of γ -glutamyl peptides in developing plant tissue is similar to the high concentration of glutathione observed in the most actively metabolising part of the lens, the cortex.

Reports of di- and tri-peptides in animal tissues are rare. A number of dipeptides containing aspartic acid, believed to be of endogenous origin, have been isolated from urine. One has the structure β -aspartylhistidine, as shown by comparison with synthetic α - and β -linked

Table 2

γ -glutamyl peptides in plants

<u>Peptide</u>	<u>Origin</u>	<u>Reference</u>
γ -Glutamylvalylglutamic Acid	Rush	1
γ -Glutamyl-S-(β -carboxy-n-propyl)-cysteinylglycine	Onion	2
γ -Glutamylphenylalanine	Onion	2
γ -Glutamyl-S-methylcysteine) and its sulphoxide	Garlic	3
	Beans	4, 5
γ -Glutamylleucine	Beans	4
γ -Glutamylalanine	Pea seedlings	6
γ -Glutamyl- β -aminoisobutyric Acid	Iris bulbs	7
γ -Glutamyl- β -(methylenecyclopropyl)-alanine (Hypoglycin B)	Blighia sapida (Ackee)	8

References

1. Virtanen & Matikkala 1960
2. Virtanen & Ettala 1958
3. Suzuki, Sugii & Kakinoto 1961
4. Rinderknecht, Thomas & Aslin 1958
5. Zacharius, Morris & Thompson 1959
6. Virtanen & Berg 1954
7. Morris, Thompson, Asen & Irreverre 1961
8. Hassall & John 1960

peptides (Kakinoto & Armstrong 1961). This peptide might arise by decarboxylation of carnosine. The others, in which the nature of the peptide link was not reported, are aspartylglycine and aspartylserine (Haley, Buchanan & Markiw 1961).

Basic. The only other peptide known to be present in lens is carnosine, β -alanylhistidine. Krause (1936) isolated the copper salt of carnosine from several thousand bovine eyes and concluded that its concentration in lens is 54 mg/kg wet weight. No evidence was obtained for the presence of anserine, β -alanyl-3-methylhistidine. Carnosine and anserine are found in highest concentration in muscle. Snake muscle contains another peptide of this type, ophidine, β -alanyl-2-methylhistidine (Ono & Hirohata 1956). The presence of these peptides in tissue is usually inferred from liberation of β -alanine after hydrolysis.

A homologue of carnosine, homocarnosine (γ -amino-n-butyryl-histidine) has been isolated from extracts of cow brain (Pisano, Wilson, Cohen, Abraham & Udenfriend 1961). Cow brain also contains carnosine. Anserine is present in rat brain (Hosein & Smart 1960). These three peptides, together with ophidine and a number of other β -alanyl and γ -aminobutyryl peptides, can be synthesised from amino-acids by an enzyme system from chick muscle (Kalyankar & Meister 1959).

A basic tripeptide, leucylarginylleucine, has been isolated from pig pituitary gland (Gros, de Garilhe, Costopanagiotis & Schwyzer 1961). The structure was confirmed by chemical synthesis.

General. The absence of small peptides from animal tissues may be more apparent than real. Until recently, those which had been recognized

were either of general occurrence or at a high concentration in a specific tissue. This is similar to the situation in plants where some of the γ -glutamyl peptides represent a high proportion of the non-protein nitrogen. More careful examination of tissue extracts may well reveal small amounts of hitherto unrecognized peptides, as in the studies of bound amino-acids in grass (Synge & Wood, 1958, Carnegie 1961).

Amino-Acids

The development of techniques of chromatography gave rise to a rapid increase in information concerning free and bound amino acids in tissues. Most early studies were performed by two-dimensional paper chromatography with phenol-water in the first direction and collidine-lutidine in the second. A comprehensive investigation with known compounds (Dent 1948) was often used as a basis for the identification of spots on chromatograms. This combination of solvents was not entirely satisfactory and a number of other combinations were introduced. The major disadvantage of this method for the study of tissue extracts is interference by salts which affect positions of spots relative to marker solutions.

Introduction of ion-exchange chromatography (Moore & Stein 1951) was a considerable improvement. Tissues need only be de-proteinized before chromatography since salts no longer interfere with the elution pattern. With the use of standardized columns and buffers, the Moore and Stein method could be operated automatically. Amino-acids are identified by a peak in the ninhydrin colour reaction at a specific effluent volume. Quantitative results are obtained. The column chroma-

tography technique has been applied mainly to studies of known (mostly protein) amino-acids. Peaks in addition to those given by known compounds, have often been observed and, in some cases, substances yielding them have been identified (e.g. Tallan, Moore & Stein 1958). The specificity of the Moore and Stein method is such that it is very unlikely that an unidentified amino-acid would be eluted at the same position as one already recognized. Results obtained are probably more reliable than those based on two-dimensional paper chromatography. Both methods have been employed in the study of amino-acids in various tissues of the eye and the results will be considered separately.

Two-dimensional paper chromatography. The phenol-collidine method was used by Walker (1952) for a comparative study of tissues of the cow. Extracts were examined before and after hydrolysis, and after peroxide oxidation. Glutathione and phosphorylethanolamine were not detected in blood plasma but were present in all other tissues. The common protein amino-acids with the exception of arginine, asparagine, and tryptophan, were found in all tissues. Plasma also contained methionine sulphone (after oxidation), β -alanine and an unidentified spot (after hydrolysis), α -amino-n-butyric acid, γ -amino-n-butyric acid and taurine. These results are essentially in agreement with an earlier, less complete, analysis (Astrup, Carlström & Stage 1951) which also showed that little qualitative difference exists between tissues of calf embryo and cow.

Phenol-collidine chromatography was employed for the first study of eye tissues (Malatesta 1952). In this series of papers, which includes the only published work on the amino-acids of the lens, the

contents of bovine whole blood are compared with the lens and the aqueous and vitreous humours. Unfortunately, as in the paper by Walker (1952), the orientation of the two solvent systems seems to have been transposed on the published map.

Malatesta deproteinized the tissues with 10% trichloroacetic acid before chromatographing the extracts. The composition of whole blood, with minor exceptions, agrees with the findings of Walker. Most of the differences are probably explicable by the presence of a number of unidentified spots on the chromatogram published by Malatesta. One spot identified as β -alanine (observed by Walker only after hydrolysis) may be γ -amino-butyric acid.

Twelve compounds were identified in lens extract: leucine, valine, alanine, glutamine, serine, glycine, glutamic acid, aspartic acid, threonine (trace), taurine, phosphorylethanolamine, and 'phosphoserine'. Phosphoserine is an uncommon tissue constituent and the spot, which is the strongest on the paper, is more likely to be due to glutathione which has almost the same position on the map. The lens contained more compounds than the vitreous humour but less than the aqueous. Additional compounds in the aqueous were lysine, arginine, α -aminobutyric acid, and phenylalanine (trace) although no phosphorylethanolamine was detected. It is not possible to compare the relative amounts of material present in the different tissues.

More recently, a comparison of bovine aqueous with blood serum has been made (Francois, Rabaey & Recoules 1960). The solvent systems were butanol-acetic acid-water followed by phenol-water. In the aqueous,

all the amino-acids, except α -aminobutyric acid, detected by Malatesta were identified together with phenylalanine (quite strong), proline, methionine, tyrosine, histidine and cysteic acid (but not cystine). Markers were apparently not run and in this combination of solvents the positions of tyrosine and α -aminobutyric acid are similar. The concentration of some amino-acids was slightly higher in serum than in the aqueous. The composition of interstitial liquid in the cornea was also examined and resembled the aqueous. Cornea contained fewer amino-acids but a higher concentration of glutamic acid.

Bovine retina is notable for its high content of taurine (Kubiček & Dolenc 1958). Crystals of taurine to the extent of 1.27% dry weight of tissue have been isolated. Qualitative results obtained by one-dimensional chromatography indicated the presence of all protein amino-acids (except glutamine, asparagine, and tryptophan) together with γ -aminobutyric acid and traces of ornithine and α -aminobutyric acid. Glutamic acid and γ -aminobutyric acid are the most abundant of these.

Column chromatography. Free amino-acids of bovine vitreous humour have been investigated by the Moore and Stein method (Wootton, Young & Williams 1954). Positive identification was made by co-chromatography with known compounds. All the protein amino-acids were detected except cystine, glutamine, and possibly tryptophan. Glutamine and tryptophan are partly destroyed by this procedure (Moore & Stein 1954). The amount of arginine varied according to conditions employed for deproteinisation. It was highest when the extract was in contact with trichloroacetic acid for the shortest time. Taurine and urea were

detected and a number of unidentified peaks were also present. Two of these may be attributed to cysteic acid and phosphorylethanolamine.

Ion-exchange chromatography has also been used in a study of rabbit aqueous, vitreous and plasma (Reddy, Rosenberg & Kinsey 1961). The concentration of amino-acids in vitreous humour was about one-fifth that in aqueous. This difference is interpreted as an indication of material metabolized by intraocular tissues. The concentration of amino-acids within the aqueous appeared to be higher than in plasma, which suggests some form of active transport across the ciliary body. Data for bovine eyes are not sufficiently precise to give a definite comparison but are in general agreement with this finding. A number of unidentified peaks were observed in the amino-acid elution pattern.

Conclusions. Information about free amino-acids in bovine eye tissues is almost entirely limited to protein amino-acids. Data are largely qualitative. Only for the vitreous and aqueous humours have more than one set of results been published and these are not in complete agreement. The concentration of amino-acids may be higher in the aqueous than in plasma, by comparison with quantitative data for rabbit tissue.

No information is available about amino-acids in calf eyes although the general composition of calf tissues indicates that there are probably no qualitative differences compared with adult cow eyes.

Other constituents of lens

Certain groups of other low molecular-weight organic compounds are considered in this section. In a study of compounds present in the nitrogenous fraction of bovine eye tissues, Krause (1937) isolated the

amines, spermine and spermidine, as phosphates. Spermidine was detected only in the iris, but spermine was distributed throughout all the tissues examined. Concentration of spermine in lens was estimated as 39 mg/kg wet weight.

Urea gives a weak ninhydrin reaction and may be detected by column chromatography. Peaks at the appropriate elution volume were observed in the studies on bovine vitreous (Wootton et al 1954) and rabbit aqueous and vitreous (Reddy et al 1961) referred to in the previous section. Urea in bovine lens has been estimated with urease (O'Brien & Salit 1931) at a concentration of 208 mg/kg wet weight.

Creatine, (α -methylguanido)-acetic acid, and creatine phosphate have been estimated chemically in cow and calf lens (Pirie, van Heyningen & Flanders 1955). The amounts detected in cow, 7.9 mg/kg lens free and 19 mg phosphate, are considerably lower than those detected in calf (76 mg and 38 mg respectively).

A number of other non-nucleotide phosphates have been identified in trichloroacetic acid extracts of calf and cow lens (van Heyningen & Pirie 1958). Identity was established by co-chromatography and electrophoresis with authentic compounds. α -Glycerophosphate, inorganic phosphate, and a group of four esters: phosphorylethanolamine, glycerylphosphorylethanolamine, phosphorylcholine, and glycerylphosphorylcholine, were detected. Of the four esters, the first two were ninhydrin positive and liberated ethanolamine on hydrolysis. The presence of taurine in lens was confirmed. A number of nucleotides were also identified chromatographically.

Many phosphorus-containing compounds have also been estimated by incubation of calf lens in a medium containing ^{32}P and separation of the radioactive phosphates present in a perchloric acid lens extract by ion-exchange chromatography (Hockwin 1961).

Summary

The lens of the eye is a unique tissue. It is entirely derived from a single type of epithelial cell and, since no cells are shed, consists of cells at all stages of development. The lens has no blood supply and depends for its metabolism upon nutrients supplied from plasma through the aqueous humour. The distribution of low molecular-weight organic constituents seems to reflect the lens' unique character.

The concentration of glutathione is probably the highest observed in any tissue. Lens also contains a closely related group of peptides based on the structure γ -glutamylcysteinylglycine (glutathione) but with the side-chain thiol replaced by other groups: hydrogen, S-sulpho-, and methyl. The distribution of free amino-acids is not well characterized but, in general, compounds present in plasma are also present in the aqueous and in the lens, possibly at a higher concentration. The distribution of phosphorus-containing compounds has been examined in some detail but does not differ notably from that of other tissues. Few other small organic molecules have been detected. The only nitrogenous compounds which have been recognized are urea, spermine and creatine.

Analytical Techniques

Part II. Analytical techniques

This part is concerned with two main topics: the preparation and fractionation of lens extracts used for the detailed investigations described in Parts III and IV, and the analytical techniques employed in these investigations.

Discussion

1. Preparation and fractionation of extracts

An acidic peptide, in addition to those listed in Table I, had been detected in trichloroacetic acid extracts of calf lens (S.G. Waley, unpublished). This was observed as a new peak after anion-exchange chromatography, and separated from other acidic compounds on electrophoresis at pH 4. The first object of the work described in this thesis was the separation of the new peptide from other constituents of the lens (described here), followed by its identification (described in Part III).

The choice of methods for preparation and fractionation of lens extracts was based on the known properties of the peptide. Fractions which did not contain the peptide were used subsequently for the identification of other (mostly) ninhydrin-positive compounds in the lens (described in Part IV). Details of the fractionations are, therefore, given in this section on analytical techniques.

Preparation of extracts. Protein-free extracts were prepared with trichloroacetic acid which was removed by extraction with N-ethyldioctylamine in chloroform (Hughes & Williamson 1951). This procedure is based on the fact that partition of the amine salt is greatly in favour of the organic phase. An advantage of this method is that lipids

are extracted by the chloroform, and weak acids and amino-acids remain in the aqueous layer. Extracts de-proteinized with trichloroacetic acid and with phenol are compared in Part IV (p. 112).

The calf lenses which were used had been stored at -15° for between 4.5 and 5 years. Extracts prepared from stored and from fresh lenses have been compared (p. 113) and the main results are unaffected by the use of stored tissue.

The extraction procedure was also performed in the absence of lenses. The total amount of ninhydrin-positive material was less than 0.05% of that obtained from lens and consisted of traces of a number of substances. No attempt was made to identify these as none was present in significant amount.

The possibility exists that a small proportion of the amino-acids and peptides might be lost when protein is removed by precipitation. This loss has been observed with glutathione during ethanol precipitation (Walker 1952). The alternative method for removal of protein: dialysis, in which small molecules diffuse from a cellophane sac which retains protein, is unpractical for treatment of large amounts of tissue. Since quantitative recovery of material was not the prime consideration, any losses which occurred during preparation of the extracts were ignored.

Fractionation of extracts. A summary of the fractionations is given on a pull-out sheet at the end of the thesis. The first fractionation (1F) was based on the electrophoretic behaviour of the acidic peptide at pH 4. Separation of a large volume of extract was possible through the use of a continuous electrophoresis apparatus, with an automatic

fraction collector, which could be run for 72 hours. The technique of continuous electrophoresis has recently been reviewed (Pucar 1961). Although the method has been applied to the separation of amino-acids in a protein hydrolysate, it does not appear to have been used previously for a tissue extract. Satisfactory separation between basic, neutral and acidic compounds was achieved but resolution into smaller groups could not be obtained with a single run for so complex an extract. Some of the most mobile species migrated off the sides of the paper and were lost.

The second fractionation (2F) was performed by ion-exchange chromatography. Because purification of an acidic compound was required, chromatography was carried out on a strongly basic anion-exchange resin. This retains most of the acidic compounds but not basic or neutral materials. This method has been used previously for similar separations. The peptides S-sulphoglutathione (Waley 1959) and γ -glutamyl-S-(β -carboxy-propyl)-cysteinylglycine (Virtanen & Matikkala 1960) were both isolated by elution from Dowex 1 columns, while separation of N-acetylaspartic acid from brain extracts was achieved on the very similar Dowex 2 (Tallan, Moore & Stein 1956). The system normally used for chromatography of tissue extracts (Moore & Stein 1951) employs the strongly acidic Dowex 50 resin.

Since the fractions first obtained by chromatography were concentrated later, it was essential to develop the column with volatile buffers. For these fractionations, two pyridine-formate buffers were used. The fractions obtained by evaporation of pyridine-acetate in the

first (electrophoretic) fractionation were yellow to pale brown in colour depending upon the degree of concentration. For the chromatographic buffers, redistilled pyridine was used in place of ordinary pyridine in the hope that fewer non-volatile impurities would be present. The redistilled pyridine was not fresh, and the same observation was made with concentrated fractions. The impurities tend to affect the mobility of spots on paper electrophoresis and paper chromatography. Although traces of impurities can be liberated from the ion-exchange resin or from paper, including amino-acids (Steven & Tristram 1962), it seems more likely that improved results would be obtained by the use of freshly redistilled pyridine.

Difficulty arose during the second fractionation (2F) because of decomposition of some material on the resin. Gas was evolved which prevented elution. The resin bed was slightly disturbed when the gas bubbles were dislodged. The gas was probably CO_2 which was formed by decarboxylation of traces of trichloroacetic acid remaining in the extract. In the third fractionation (3F), acidic compounds were adsorbed directly onto a portion of resin and the mixture was degassed before the resin was placed as a band on the top of the column. No decomposition occurred. The elution peaks were quite sharp.

Basic and neutral compounds were not adsorbed on the resin and no separation between them was achieved by ion-exchange chromatography. They were separated, however, by electrophoresis and the fractions obtained in the first experiment were used for most of the investigations described in Part IV.

2. Analytical techniques

Considerable reliance has been placed throughout upon detection and identification of compounds on paper after electrophoresis and chromatography. This is reflected in the number of solvents and location reagents described, and has been necessary because of the small quantities of most of the compounds present in lens fractions, particularly the peptides which would have probably disappeared during attempts to isolate pure solids. For other compounds, use of these techniques has been dictated by the time otherwise necessary to obtain complete separation of individual substances from the fractions.

Paper electrophoresis. Pyridine-acetate, pH 4, has been of most value for separation of all compounds. When samples are applied at the cathode, division is achieved between neutral compounds, which all migrate to one position, and acidic compounds, which separate. Basic, neutral, and acidic compounds are separated on the same paper when samples are applied at the centre. First evidence for a new basic peptide in lens was obtained by this method.

Compounds with strong and with weak acid groups are separated at low pH; for example, taurine can be distinguished from other neutral amino-acids. The fractional charges of carboxyl groups at pH 4.6 are employed in the examination of crude extracts from lens on a single paper by electrophoresis in the first direction and chromatography in the second (see Part IV).

Many of the basic compounds can be separated at pH 9.

Paper chromatography. The standard solvent was butanol-acetic acid.

Most groups of compounds are well distributed on the chromatograms. Substances with low R_F were separated on fast paper to give a long solvent run. Good separation of these substances is also obtained in butanol-acetic acid-water-pyridine. No solvent was entirely satisfactory for chromatography of organic acids, different sets of acids were separated in the systems used. Most of the solvents are volatile and did not interfere with location reagents.

Location of compounds on paper. Investigations have mainly involved amino compounds and ninhydrin has been the most useful reagent. Permanent colours are given by ninhydrin-cobalt chloride (Wiggins & Williams 1952). This had to be replaced by acid ninhydrin after electrophoresis in alkaline buffer. Specific groups in the molecule were detected by other reagents whose specificity is given in the experimental section. Of these reagents, only the Pauly and acridine were as sensitive as ninhydrin. Location of organic acids was complicated by the reaction of amino-acids with the reagents.

A number of multiple-dip sequences were investigated, all were less sensitive than reagents applied singly, and none was used. Duplicate papers were run when information was required from two reagents.

Semi-quantitative estimation of measured samples was made by comparison with markers of known strength run on the same paper. These estimates are accurate to about 25%. In certain instances, peptide hydrolysates were analyzed by quantitative measurement of ninhydrin colour.

Experimental

1. Preparation and fractionation of extracts

Materials. Lenses. Eyes were removed at the slaughterhouse, brought to the laboratory, and the lenses removed within two hours of death. Lenses had been stored at -15° for about $4\frac{1}{2}$ years.

Buffers. Pyridine-acetate, pH 4 was the standard buffer (p. 27).

0.5 M Pyridine-formate, pH 5.3. Redistilled pyridine (81ml) and formic acid (90%, 21.2 ml) diluted with water (to 1 l).

M Pyridine-formate, pH 5.25. Redistilled pyridine (162 ml) and formic acid (90%, 42.4 ml) diluted with water (to 1 l).

Pyridine had been redistilled from bromine.

Water and buffers for ion-exchange chromatography were degassed at the water-pump immediately before use.

Methods

Preparation of lens extracts. Calf lenses (206.4 g), suspended in ice-cold 7% (w/v) trichloroacetic acid (800 ml), were disintegrated for 90 seconds with a top-drive macerator (Townson & Mercer, Ltd., Croydon) in chilled apparatus. The suspension was left at 4° for 30 mins. then centrifuged for 15 mins. at 10,000 rev/min. and 0° (M.S.E. refrigerated centrifuge). Some material remained on the surface of the supernatant. The liquid was decanted, stirred with a few drops of octanol, and centrifuged again under the same conditions. The slightly opalescent supernatant was stirred with diatomaceous silica (Celite 535, L. Light & Co.) and filtered under suction. Trichloroacetic acid was removed from the resultant clear solution by extraction

with N-methyl di-n-octylamine (80 ml, British Hydrological Corporation) in chloroform (1800 ml) in three portions (1050, 525, and 315 ml). The turbid aqueous layer had pH 5.5 - 6 (indicator paper) and, after treatment with Celite, gave a clear solution (620 ml). This was concentrated (to 200 ml) by evaporation below 45° in vacuo and stored at -15° . Half was fractionated by continuous electrophoresis (1F) and half by ion-exchange chromatography (2F).

The second extract was prepared similarly. All containers were washed with water and the washings (total 70 ml) added before extraction. The clear solution (720 ml) from 187.3 g lenses, had pH 6.7 and was stored over a weekend at -15° before concentration below 40° . This extract was fractionated in halves by ion-exchange chromatography (3F and 4F).

The extraction procedure was also carried out with $\frac{1}{10}$ quantities and with no tissue. The final solution was dried in vacuo, dissolved in water (50 μ l) and examined by electrophoresis and chromatography. At least 4 ninhydrin-positive compounds were present, of which at least two were strongly acidic. None was present at a significant concentration, the total concentration was estimated as 5mM, equivalent in a full extract to less than 0.05% of the lens material.

Fractionation of lens extracts

a). Continuous electrophoresis. 1F. (Table 3). This fractionation was performed in a Beckman continuous-flow paper-electrophoresis apparatus (Model CP) with pyridine-acetate buffer, pH 4, and at constant current (94mA). The potential fell from 390v to 360v over the course of the

Table 3

Fractionation (IV) of lens extract (100ml)
by continuous electrophoresis

Fraction	Main ninhydrin- positive constituents	Tube numbers	Initially	Volume ml after concentration
1F1	Ethanolamine	1 - 4	332	4.0
1F2	Basic Amino-Acids Garcosine Anine	5 - 8	223	1.6
1F3	Basic Peptide β -Alanine, γ -aminobutyric acid	9 - 16	340	3.7
1F4	Neutral Amino-Acids	17 - 20	163	2.6
1F5	Neutral Amino-Acids	21 - 23	126	3.0
1F6	Glutamic Acid Ophthalmic Acid G33G	24 - 26	132	10.0
1F7	G33G Acidic Peptide (Acidic Amino-Acid)	27 - 29	171	4.2
1F8	G330.H Acidic Peptide (and Amino-Acid)	30 - 32	242	2.4

experiment. The apparatus was equilibrated for 5 hours then the lens extract (100 ml) applied to the centre of the paper curtain at approximately 1.4 ml/hr. Collection of fractions was started 3 hours later, when material had almost reached the bottom of the paper. The fraction collector changed at intervals of 12 hours and 6 sets of 32 fractions were collected. Fractions were numbers from cathode (1) to anode (32). At the end of the run, the paper curtain was removed, and developed with ninhydrin (Figure 2). Positions of bands on the curtain do not match exactly the fractions in which material was collected. The band at fractions 30 and 31 was initially mauve. Three faint bands of basic material ran off the edge of the paper.

Drops of each tube in the first set of fractions were dried on paper and treated with ninhydrin. Fractions 18, 19, 20, 24 and 25 gave the strongest colours, weaker colours were obtained with fractions 6, 7, 23, 26 and 27. The sixth set of fractions was examined in the same way. Peak colours had moved towards the anode by one tube (e.g. fraction 25 instead of 24) and weak colours were given by fractions 29 and 30. Fractions were examined by electrophoresis (pyridine-acetate) and chromatography (phenol-ammonia), pooled according to their content, concentrated by evaporation in vacuo at about 45° , and stored at -15° .

b). Anion-exchange chromatography. The three fractionations (2F, 3F, 4F) were performed on the same column of Dowex 1 (4% cross-linked, 200-400 mesh) prepared as follows. Dowex 1 chloride (about 120 ml) was stirred with water (100 ml) and allowed to settle. Fine

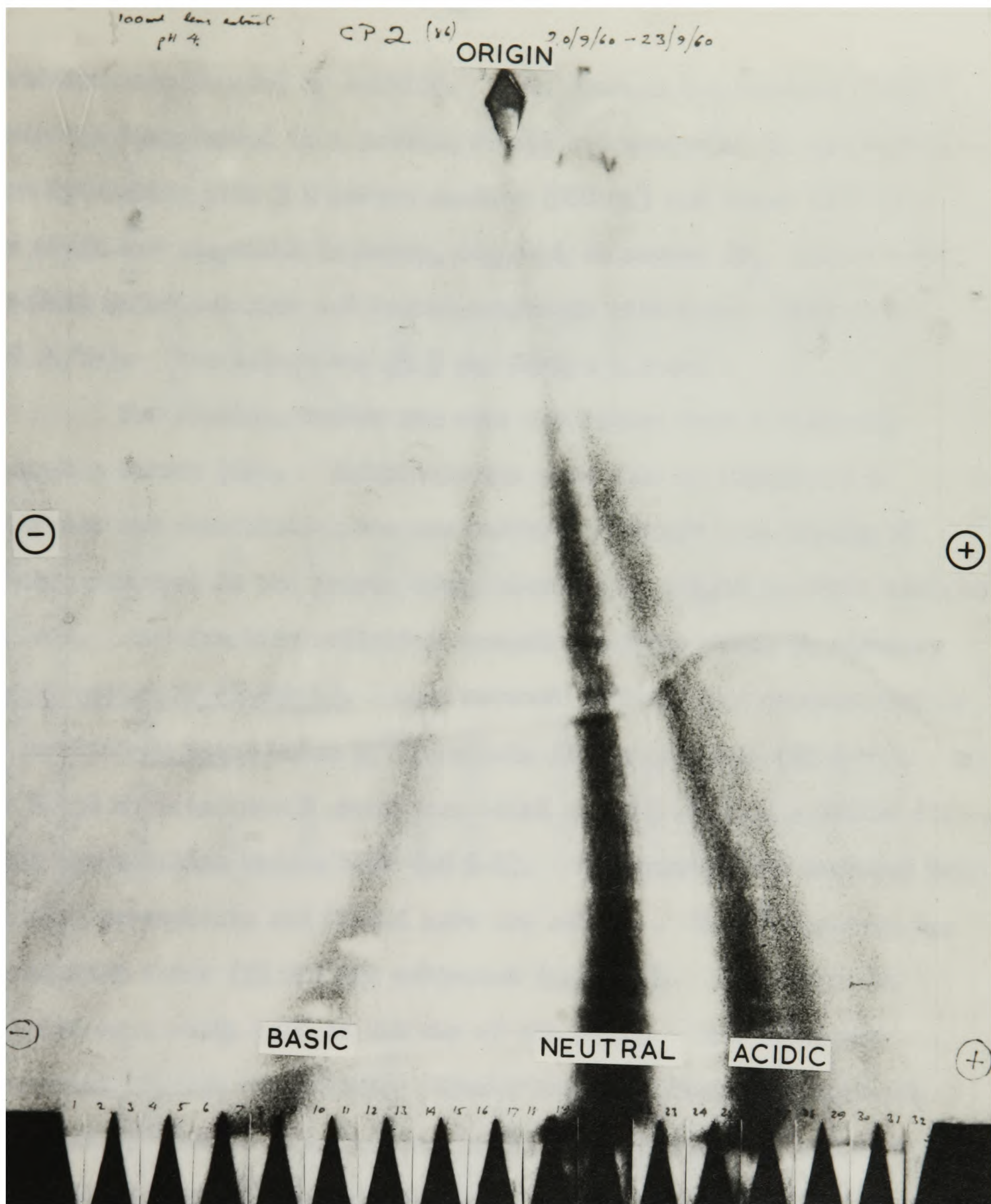


Figure 2. Fractionation (1F) of lens extract by continuous electrophoresis in pyridine-acetate buffer pH 4 at constant current (9mA). Paper curtain developed with ninhydrin.

particles were removed by suction. This process was repeated twice. Resin was transferred to a Buchner funnel and converted to the acetate form by washing with 3 M sodium acetate (500 ml) and water (200 ml). The resin was suspended in water, degassed to remove CO_2 , packed into a column under pressure and washed overnight with water (1725 ml at 138 ml/hr). The column was 29.5 cm. long x 1.2 cm.

For elution, buffer ran onto the column from a reservoir through a narrow tube. Buffer changes were made by emptying the reservoir and substituting the new buffer. Because some mixing of buffers occurred in the narrow tube, there was a slight gradient between buffers. The fraction collector changed fractions every $\frac{1}{4}$ minutes.

Fractionation 2F (Table 4). Lens extract (100 ml) was concentrated by evaporation in vacuo below 35° to a pale yellow solution (20.2 ml). A few drops of bromocresol green were added and 2 M aqueous pyridine (1.5 ml) until the solution became blue (pH 5.2). The extract was decanted from a slight precipitate and flowed onto the column. The precipitate was washed with water (18 ml) and collected (4.67 mg). Indicator was adsorbed as a sharp band at the top of the resin. The water was percolated through the column. Eluate which had been collected was stored at -45° (2F1). Elution was begun with water for 16 hours at 16 ml/hr and 4 hours at about 10 ml/hr. to give the second fraction (2F2). The change was made to 0.5 M pyridine-formate buffer and the third fraction (2F3) collected for 1.5 hours when it was judged that the buffer had emerged so the fraction collector was started.

Decomposition occurred during the night and the resin bed

Table 4

Fractionation (2F) of lens extract (100ml)
by anion-exchange chromatography

Fraction	Ninhydrin-positive constituents	Elution agent	Fraction numbers	Initially	Volume ml after concentration
2F1	Basic and Neutral Amino-Acids	Water	Bulk	37.5	2.0
2F2	Basic and Neutral Amino-Acids	Water	Bulk	304.0	8.0
2F3	Neutral Amino-Acids	Change to 0.5M Buffer	Bulk	44.6	2.1
2F4	Glutamic Acid Ophthalmic Acid	0.5M Buffer	Bulk + 1 - 4	66.3	4.6
2F5	GSSG	0.5M Buffer	5 - 14	29.1	3.8
2F6	GSSG Acidic Amino-Acid (Trace)	Change to M Buffer	15 - 41	76.9	3.6
2F7	Acidic Peptide Acidic Amino-Acid	M Buffer	42 - 53	36.8	3.5
2F8	Acidic Peptide (Trace)	M Buffer	54 - 70	47.6	2.6
2F9	Acidic Peptide (Trace)	M Buffer	71 - 95	52.2	2.8
2F10	GSSG _{3H}	M Buffer	96 - 140	102.5	2.3

Total volume collected 794.5 ml. Fractions of 2.5 ml. collected at 11 ml/hr

broke up with gas. The small volume of eluate was added to 2F3. The fourth fraction (2F4) was collected while bubbles of gas were carefully dislodged with a piece of wire without too much disturbance of the resin. Elution was continued with weak buffer for 31 fractions. The change to M buffer was made and a further 73 fractions (numbers 32 - 104) collected.

Spots from odd-numbered fractions were dried on paper and developed with ninhydrin. Peak colours were obtained with fractions 7, 9, 11, 45 and 47. The breakthrough of the stronger buffer at fraction 42 was indicated by an increase in background of the spots. Fractions were investigated by chromatography (phenol-ammonia). After 44 hours, elution was resumed with M buffer for 36 fractions (numbers 105 - 140), none of which reacted with ninhydrin. Fractions were pooled, concentrated by evaporation in vacuo below 42° to yellowish solutions, and stored at -15° .

Fractionation 3F (Table 5). The resin was regenerated in the acetate form by washing with 3 M sodium acetate (400 ml at 10 ml/hr) and water (100 ml). The band of resin with adsorbed indicator was scraped off the column and discarded. Lens extract (100 ml) was concentrated in vacuo below 40° to 25 ml, stored at 4° for one hour, and solid (6.78 mg) removed by centrifuging. The solution (pH about 7, indicator paper) was transferred to a small Buchner flask and two drops of bromocresol green added to give a blue solution. Resin in the acetate form (3ml), as a slurry in water was added. The solution became green (pH about 4.5) and indicator was adsorbed on the resin. An hour later, the mixture was degassed for 30 minutes. Quite a lot of gas was evolved. A

Table 5

Fractionation (3F) of lens extract (100ml)
by anion-exchange chromatography

Fraction	Ninhydrin-positive constituents	Elution agent	Fraction numbers	Initially	Volume ml after concentration
3F1	Basic and Neutral Amino-acids	Water	Bulk	46.0	3.2
3F2	Neutral Amino-Acids	Water	Bulk	224.0	1.7
3F3	Neutral Amino-Acids (Trace)	Change to 0.5M Buffer	Bulk + 1 - 7	64.5	0.75
3F4	Glutamic Acid Ophthalmic Acid	0.5M Buffer	8 - 20	28.6	0.4
3F5	GSSG	0.5M Buffer	21 - 36	30.0	0.85
3F6	GSSG	0.5M Buffer	37 - 64	42.0	2.2
3F7	Acidic Amino-Acid	Change to M Buffer	65 - 83	43.5	1.5
3F8	Glutamic Acid	M Buffer	84 - 97	26.0	1.0
3F9	Acidic Peptide	M Buffer	98 - 110	30.2	2.5
3F10	GSSO ₂ H (Trace)	M Buffer	111 - 187	43.8	6.9

Total volume collected 683.6 ml. Fractions of 1.95 ml collected at 6.5 ml/hr

further portion of resin (2 ml) was added and degassing continued for another 30 minutes. Most of the extract was decanted and the resin, as a slurry in the remainder, carefully placed as a band on the top of the column (final height 29 cm). The flask was washed with water (20 ml).

The first bulk fraction (3F1) was collected as the extract, followed by the washings, was flowed rapidly onto the column and elution begun with water. When the rate of elution had been adjusted, the second fraction (3F2) was collected (20 hours at 11.2 ml/hr). The change to 0.5 M pyridine-formate was made and the third fraction (3F3) collected until buffer had clearly emerged from the column (4.5 hr). A few small bubbles of gas appeared on the resin and elution was stopped. Since no change was observed after one hour, the fraction collector was started and elution continued for 64 fractions. The buffer was changed and elution effected with M pyridine-formate for 40 fractions (numbers 65 - 104). Spots of odd-numbered fractions on paper were developed with ninhydrin. Colours were obtained with fractions 5 - 9, 13 - 31, 43 - 49, 87 - 103 and possibly 69 - 79, with peaks at 7, 25, 45 and 103. Elution with M buffer was continued for a further 83 fractions (105 - 187) but only 105 and 107 gave a ninhydrin reaction. Fractions were examined by electrophoresis in pyridine-acetate, concentrated by evaporation below 40° in vacuo, and stored at -15°. Concentrated fractions were yellow, the depth of colour increasing with the extent of concentration.

Fractionation 4F (Table 6). This was essentially the same as fractionation 3F. Larger volumes of water and of 0.5 M pyridine-formate buffer were used for elution than in 3F.

Table 6

Fractionation (AF) of lens extract (100ml)
by anion-exchange chromatography

Fraction	Ninhydrin-positive constituents	Elution agent	Fraction numbers	Initially	Volume ml after concentration
4F1	Basic and Neutral Amino-Acids	Water	Bulk	40.0	2.5
4F2	Neutral Amino-Acids	Water	Bulk	305.0	2.3
4F3	Neutral Amino-Acids Glutamic Acid	Change to 0.5M Buffer	Bulk	71.5	1.6
4F4	Glutamic Acid Ophthalmic Acid	0.5M Buffer	1 - 28	53.5	1.5
4F5	GSSG	0.5M Buffer	29 - 50	29.0	1.4
4F6	GSSG (Trace) Acidic Amino-Acid (Trace)	0.5M Buffer	51 - 64	16.0	0.7
4F7	Acidic Amino-Acid	0.5M Buffer	65 - 79	39.0	1.1
4F8	Glutamic Acid	Change to M Buffer	80 - 89	30.2	1.2
4F9	Acidic Peptide	M Buffer	90 - 105	42.0	1.45
4F10	GSSO ₃ H	M Buffer	106 - 179	188.0	7.8

Total volume collected 844.2 ml. Fractions of 2.22 ml. collected at 9.65 ml/hr

2. Analytical techniques

Materials. AnalaR chemicals were used where available.

Electrophoresis buffers were prepared as stock solutions (2.5 l).

Solvents for paper chromatography were prepared as required and used once, or twice on successive days.

Location reagents were stable unless indicated.

Potassium bromide for infra-red spectroscopy was prepared from Anala R crystals by grinding in a vibrator mill for 15 minutes, drying at 130° for 6 hours, regrinding, and drying again overnight. The fine powder was stored over silica gel.

Methods

Paper electrophoresis. A ridge-pole apparatus (Durrum 1950), made of Perspex, was used. The centre of the paper rests on a polythene rod, apex height 11.5 cm., with the ends of the paper dipping into the electrode compartments. These compartments are divided to prevent the paper touching the platinum electrodes, and to allow free circulation of buffer.

The paper, 25 cm. long, was soaked in buffer, blotted to remove excess liquid, and placed between two thin polythene sheets with a line exposed for application of samples. This prevents evaporation of buffer while spots are applied to the paper. Approximately 5 μ l spots of samples and 10mM marker solutions were placed at 3 cm. intervals along a line either 5 cm. from one end or along the centre of the paper. Measured spots were applied from 20 μ l graduated pipettes for accurate work. The width of the paper varied according to the number of samples

run. For small-scale isolation, the solution was applied as a strip with a spot at one end as a guide to the position on the paper of the required band.

Normally, Whatman No. 52 paper was used as it remains strong when wet. The thicker No. 3 paper was employed when concentrated samples were run, and for papers on which chromatography was performed after electrophoresis.

Papers were run at 200 volts d.c. (8 v/cm.) supplied from the mains through a transformer (Locarte Co. London). The current varied with the nature and width of the paper and with the buffer, and no values are given. In most cases, adequate separation was obtained after 5 hours; with No. 3 paper, 3.5 hours was usually sufficient. Details of the various buffers are given below. References to buffers elsewhere are by the short description.

Pyridine-acetate (Grassmann, Hannig, and Plöckl 1955). Pyridine (15 ml), acetic acid (50 ml), and water (2.5 l). pH 4. Samples applied at the cathode, or in the centre of the paper for basic compounds.

10% acetic acid. 10% (v/v) acetic acid in water. pH 2.3. Samples applied at the anode.

Formic-acetic acid. Formic acid (90%, 125 ml) and acetic acid (375 ml) diluted with water (to 2.5 l). pH 1.6. Samples applied at the anode.

Sodium borate (van Heyningen and Pirie 1953). Sodium tetraborate (7.63 g) and boric acid (0.62 g) in water (1 l). pH 9. Samples applied at the cathode for phosphorus compounds, and at the anode for basic compounds.

Sodium carbonate. 0.05 M sodium carbonate in water. pH about 11.5. Samples applied at the cathode.

Paper chromatography. Descending chromatography on Whatman No. 1 paper was used. When greater resolution was needed, the faster Whatman papers, No. 4 and No. 3 and, in one series of experiments Schleicher and Schüll No. 2043 b paper, were employed. Approximately 5 μ l spots (accurately measured for semi-quantitative work) of samples and 10mM marker solutions were placed 2.5 cm. apart on the starting line. Chromatograms were normally run overnight. When the solvent front stayed on the paper, chromatograms were removed from the tank when the solvent had run about 30 cm. (6 cm. from the end of the paper). With the faster papers, the solvent was allowed to run off. Under these conditions the chromatograms were run for a definite time and some variation was observed in the distance travelled by a particular compound.

Solvents are listed below and referred to elsewhere by the short description. The upper phase was placed in the trough at the top, and the lower phase in an open dish at the bottom, of the tank. Solvent was always left for a number of hours to equilibrate before a tank was used for the first time. Particular care was taken with solvents for which no lower phase was used, the equilibrating solvent was discarded and fresh solvent used to develop the chromatogram.

Butanol-acetic acid (Waley 1956). Upper and lower phases: butan-1-ol-acetic acid-water (40:9:20, by vol.).

Phenol-ammonia (Waley 1957). Upper phase: 72% (W/V) phenol - 3% (W/V) NH_3 solution (50:0.3, by vol.). This was discarded as soon as

a pink colour developed. Lower phase: 0.3% (w/v) NH_3 solution.

Butanol-acetic acid-water-pyridine (Waley & Watson 1953). Upper phase: butan-1-ol-acetic acid-water-pyridine (30:6:24:20, by vol.).

AnalaR pyridine is essential, otherwise a heavy background appears when the paper is developed with ninhydrin. No lower phase.

Ethanol-ammonia (Smith 1960 p.273). Upper phase: ethanol- NH_3 solution-water (16:1:3, by vol.). No lower phase.

Butanol-ethanol (Smith 1960 p.263) Upper phase: butan-1-ol-ethanol-water (7:1:2, by vol.). Addition of a few drops of ethanol was usually necessary to give complete solution. No lower phase.

Butanol-propanol-ammonia (Reddy & Hegsted 1962). Upper phase: butan-1-ol-propan-1-ol-20% ammonium hydroxide (2:2:1, by vol.). The ammonium hydroxide was prepared by dilution of NH_3 solution (2 vols.) with water (1 vol.). No lower phase.

Isopropanol-hydrochloric acid (Wyatt 1951). Upper phase: propan-2-ol-(3 ml), conc. HCl (8.8 ml), and water (to 50 ml). No lower phase.

tert-Pentanol-phthalate (Blackburn & Leather 1951). Upper phase: tert-pentanol saturated with 0.05 M phthalate buffer pH 6. Buffer is prepared from 0.1 M potassium hydrogen phthalate (50 ml) and 0.1 N sodium hydroxide (45.45 ml) diluted with water (to 100 ml). No lower phase.

Paper must be soaked in buffer and dried thoroughly before use. Papers were run in the dark to prevent decomposition of DNP-amino-acids.

Location of compounds on paper. The chromatogram or electrophoresis paper was thoroughly dried in a current of air before any reagent was applied. With dip reagents, the paper was dipped three times and excess

of reagent allowed to drain off. Spray reagents were applied from a glass atomiser by an electric blower. Reagents are referred to by the abbreviated name and all spots are stable unless otherwise indicated.

Ultraviolet light. Papers were viewed at 254 m μ , using a 'Chromatolite' (Hanovia Ltd. London), for evidence of fluorescence and quenching. This cannot be used after solvents containing phenol or pyridine.

Ninhydrin (Wiggins & Williams 1952). 0.4% (w/v) ninhydrin and 0.2% (w/v) cobalt chloride in propan-2-ol. The paper was dipped, dried in a hot oven for 5 minutes, heated in steam for 3 minutes, dried, and heated again. Amino compounds give mauve or red colours on an almost colourless background. Intensity of the colours increases for about 24 hours. Sensitivity is 1 μ g or less. This reagent cannot be used on alkaline paper.

Acid ninhydrin. Water (2.5 ml) and acetic acid (2.5 ml) added to 0.5% (w/v) ninhydrin in acetone (45 ml). The paper was dipped, and dried for two minutes in a hot oven. Amino compounds give mauve-blue colours on a colourless background. This reagent was only used after alkaline electrophoresis. The spots slowly fade but are preserved as follows (Smith 1960 p.96). The paper is dipped in a solution containing saturated aqueous copper nitrate (1 ml), 10% (v/v) nitric acid (0.2 ml), and ethanol (100 ml). Pink-red copper complexes are given by the ninhydrin spots, without loss of intensity, on a pale blue background.

Acridine (Smith 1960 p.279). 0.1% (w/v) acridine in ethanol.

The paper was dipped, dried in a hot oven for two minutes, and viewed under ultraviolet light. Acids give green-yellow fluorescent spots on a mauve background. Strong spots are also visible in normal light as yellow on a white background.

Aniline-xylose (Smith 1960 p.279). Aniline (1 ml) was added to xylose (1 g) in water (3 ml) and dissolved in methanol (to 100 ml). The paper was dipped, dried in air, and heated in a hot oven for about five minutes. Acids give brown spots on a colourless background. The background slowly becomes brown.

Iodine-azide a). 3% (w/v) potassium iodide and 0.6% (w/v) iodine in water.

b). 1% (w/v) sodium azide in 1% (w/v) soluble starch solution. The two solutions are mixed immediately before use in the ratio 1 vol. iodine to 10 vols. azide. The paper was sprayed. Compounds containing divalent sulphur give white spots on a blue background. Thiocethers react very slowly.

Pauly reagent (Smith 1960 p.218). a). 5% (w/v) sodium nitrite in water.

b). Sulphanilic acid (1 g) in water (100 ml) and conc. HCl (10 ml). This is a saturated solution at 4°C.

c). 10% (w/v) anhydrous sodium carbonate in water. The stock solutions were stored at 4°C. Immediately before use, sulphanilic acid (1 vol) and sodium nitrite (1 vol) were mixed. Sodium carbonate (2 vols) was added after 5 minutes. The paper was heated, then sprayed. Phenols and imidazoles give red or orange spots on a pale yellow background. Pauly reagent cannot be used after phenol-ammonia.

Sakaguchi reagent (Smith 1960 p.226). a). 0.1% (w/v) 8-hydroxyquinoline in acetone (100 ml) to which is added urea (0.1 g) in water (0.2 ml).

b). Bromine (0.3 ml) in 2% (w/v) sodium hydroxide (100 ml). The paper was dipped in solution a) and dried, then sprayed with solution b). Mono-substituted guanidines give orange-red colours on a fawn background. Urea is incorporated to stabilize the spots. The second solution is only effective when yellow in colour. Sensitivity is decreased after phenol-ammonia.

Pentacyanoaquoferrate (Smith 1960 p.227). One volume each of 10% (w/v) potassium ferricyanide and sodium nitroprusside solutions (prepared from freshly washed crystals) is added to 10% (w/v) sodium hydroxide (1 vol, freshly prepared). This mixture changes from a deep red to a yellow colour in about 20 minutes and is then diluted with water (9 vols). Immediately before use, the mixture is diluted with an equal volume of acetone. The paper was sprayed. Mono- and di-substituted guanidines give red or orange colours on a yellow background. This reagent is less sensitive than the Sakaguchi reagent but will detect creatine.

Molybdate (Smith 1960 p.241). Perchloric acid (70%, 3 ml) added to ammonium molybdate (1 g) in water (8 ml) and conc. HCl (3 ml) is diluted with acetone (to 100 ml). The paper was dipped, dried in air, and exposed to daylight (preferably sunlight) for two or three hours. Phosphorus-containing compounds give blue colours (inorganic phosphate yellow) on a background which slowly becomes blue. The reagent must

be fresh. Small papers could also be developed conveniently under ultraviolet light for 30 minutes.

Alkaline silver. a). Silver nitrate (4.2 g) in water (4 ml) diluted with ethanol (to 250 ml).

b). 0.05 N potassium hydroxide in ethanol. The paper was dipped in silver nitrate, dried, and sprayed lightly with potassium hydroxide. Sugars, polyols, and some phenols, give brown or black spots on a light background. The background darkens fairly rapidly but the chromatogram can be preserved by dipping in a solution of X-ray fixer (Kodak Ltd.).

Ehrlich reagent (Dalgleish 1955). 2% (w/v) p-dimethylaminobenzaldehyde in 1.3 N hydrochloric acid. The paper was sprayed, and dried in air. Indoles, urea and citrulline give coloured spots fairly quickly and amino-acids give yellow spots slowly on a pale background.

Quantitative ninhydrin estimation. Samples (5 μ l) and markers of known concentration were run on the same electrophoresis paper. The dry paper was dipped 6 times in ninhydrin and excess of reagent drained off. The paper was heated at 90° for five minutes and steamed evenly on both sides for three minutes. It was then left in the dark for 48 hours to allow maximum colour development.

Strips, 1.3 cm. wide, were cut from the paper, parallel to the direction of electrophoresis, so as to include the centres of the coloured spots. The maximum intensity of each spot was measured at 540 m μ by winding the strip, in the apparatus of Tennent, Whitla and Florey (1951), past the photocell of a Beckman spectrophotometer, model DU.

Optical densities were measured against a blank area of the paper on the strip and three readings (agreeing within 5%) were obtained

for each spot. The mean reading was used. Concentrations were obtained from a calibration curve constructed for each compound from marker spots, a typical curve is shown in Figure 3.

Other standard procedures

Hydrolyses were performed in sealed Pyrex tubes for 16 hours at an average 110° . Dry material was dissolved in 6 N HCl, and solutions were diluted with an equal volume of conc. HCl for hydrolysis. Hydrolysates were transferred quantitatively to pieces of thick polythene, taken to dryness in vacuo, and redissolved in water and dried three times in order to remove hydrochloric acid.

Infra-red spectra were obtained from solids in potassium bromide windows with a Perkin Elmer spectrophotometer, model 137 (Infracord). A mixture of the dry sample (about 0.3 mg) in potassium bromide (about 100 mg) was ground in a vibrator mill for 15 minutes. The powder was packed evenly into a slot 14 mm x 2 mm cut in a brass support, and compressed in a hand press for 20 minutes. The brass disc fitted into a clip on the spectrometer so that the transparent window was entirely within one beam from the light source.

Dinitrophenylation for end-group determination was performed by the method of Waley (1956). The peptide in triethylamine carbonate buffer (0.1 ml) was treated with excess 1-fluoro-2, 4-dinitrobenzene in ethanol (0.25 ml). The buffer was prepared by treating 6% (v/v) triethylamine in water with solid CO_2 until the solution had pH about 9 (acid to thymol phthalein, alkaline to phenol red). The mixture was kept for about 3 hours in the dark and the ethanol removed by

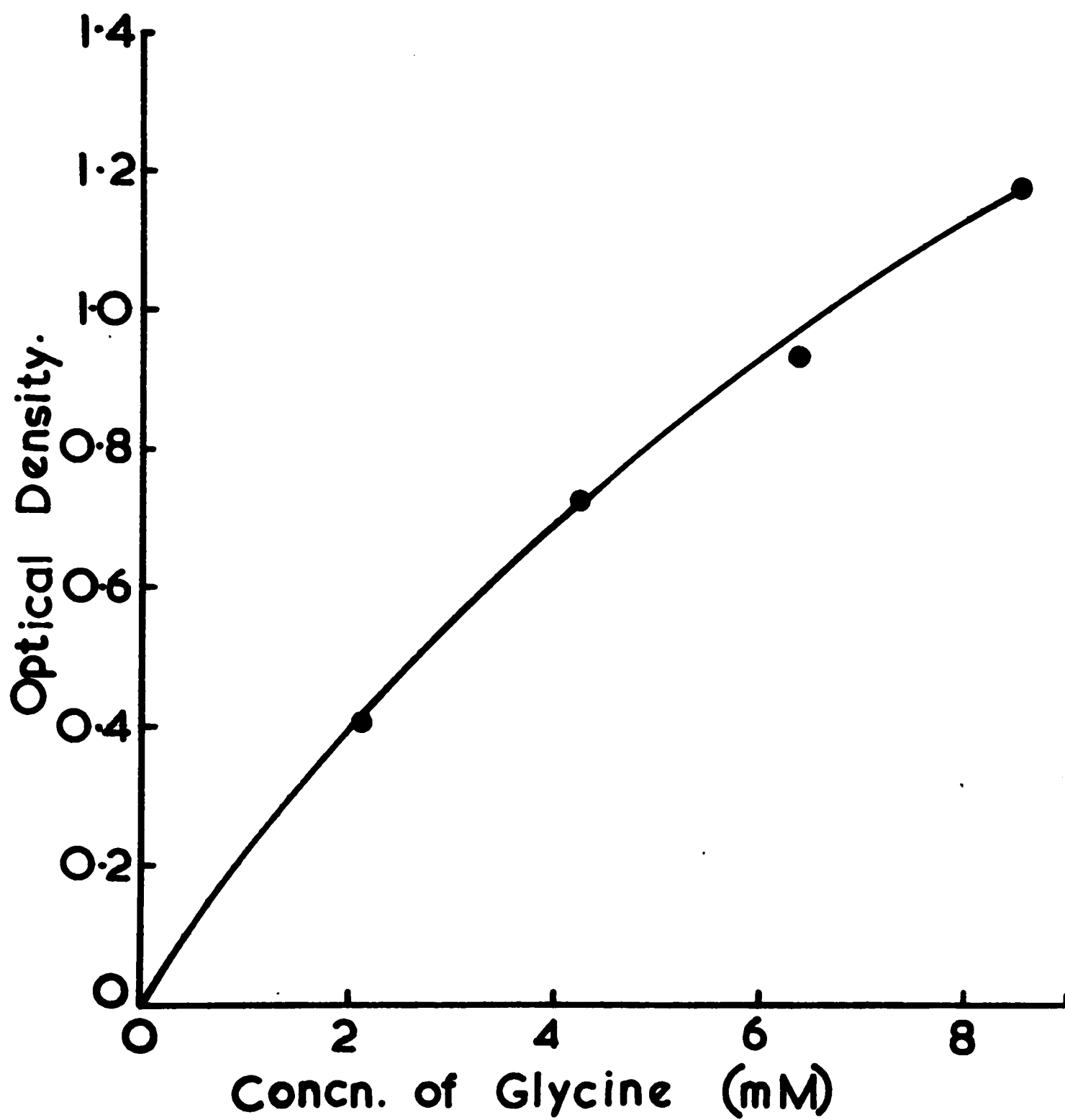


Figure 3. Calibration curve for quantitative estimation of glycine. Maximum optical density of ninhydrin spots was measured on paper.

evaporation under reduced pressure. The yellow solution was extracted with ether (three portions of 0.1 ml) and taken to dryness in vacuo.

Oxidation was carried out with performic acid. Hydrogen peroxide (30%, 0.16 ml) was added to formic acid (98%, 0.5 ml) and left at room temperature for 20 minutes. A portion of this solution was added to an equal volume of solution, containing the compound, on polythene sheet and left for one hour at room temperature under a glass cover. The oxidised solution was taken to dryness in vacuo, and redissolved in water and dried twice to remove excess acid.

Material was eluted from paper strips under a Perspex cover in an atmosphere of water vapour. The dry strip was developed with ninhydrin after elution and gave a negative reaction.

Solutions were dried in vacuo over phosphorus pentoxide and sodium hydroxide. Bulky solutions were concentrated in vacuo on a water bath in a rotary evaporator. The temperature given is the maximum attained by the water bath.

The pH of buffers were measured with a Pye pH meter standardised immediately before use with freshly prepared buffer solutions at pH 4 and pH 9.2.

Elementary analyses are by Weiler and Strauss, Oxford.

Melting points are uncorrected.

EXPERIMENTAL

Introduction

This part is devoted to the identification of the new acidic peptide in lens extracts. Several attempts have been made to purify the lens extracts (by column chromatography and by paper chromatography) applied to the identification of the new acidic peptide. The results of these attempts are shown in Table I. During the course of these attempts, it became clear that the extracts contained two related compounds. These could not be separated by column chromatography at pH 4. The present work was continued after the fourth fractionation (also by chromatography), and the results are shown in Table II.

Part III

The present work was continued after the fourth fractionation (also by chromatography), and the results are shown in Table II.

New acidic compounds in the lens

At this point, a new acidic peptide was isolated, and found to be identical with the lens peptide. The structure of the new acidic peptide is shown in Table III.

The work described in this part, therefore, deals with the isolation and identification of the new acidic peptide. In the first section, studies are devoted to the identification of the new acidic peptide by chromatography of lens extracts, and studies are devoted to the identification of the new acidic peptide by chromatography of lens extracts. The results of these studies are shown in Table I. The new acidic peptide is shown to be identical with the lens peptide. The structure of the new acidic peptide is shown in Table II.

Part III

New acidic compounds in the lens

Introduction

This part is concerned with the identification of the new acidic peptide in lens extracts. Material obtained from the first two fractionations of lens extracts (by electrophoresis and by ion-exchange chromatography) appeared to be homogeneous on the basis of electrophoretic mobility at pH 4. During examination of material obtained from the third fractionation (by column chromatography), it became clear that the extracts contained two related compounds. These could not be separated by electrophoresis at pH 4. The presence of two substances was confirmed after the fourth fractionation (also by chromatography), and the second substance was shown to be a new amino-acid liberated upon hydrolysis of the peptide. At this point, a model peptide was synthesized, and found to be identical with the lens peptide. The structure of the amino-acid could be deduced from that of the peptide.

The work described in this part, therefore, falls into two main sections and is treated accordingly. In the first section, studies are described on the identification of material obtained by fractionation of lens extracts, and evidence is presented for the presence of two new acidic compounds. The second section is concerned with the preparation of model compounds, and comparison of the new peptide: S-(α , β -dicarboxyethyl)-glutathione, and the corresponding amino-acid, S-(α , β -dicarboxyethyl)-cysteine, with the compounds present in lens extracts.

All experimental results are given after the discussion, and the main findings are summarized at the end of this part.

Discussion

1. Investigation of lens material

Table 7 outlines the stages towards identification of the two lens compounds.

First fractionation (Table 3). The acidic peptide was in fraction IF 7 which also contained glutamic acid, ophthalmic acid, and GSSG. This material was refractionated by continuous electrophoresis and the new peptide obtained in a fraction with traces of aspartic acid and fuchsin. Neither of these compounds interfered in subsequent experiments: fuchsin did not liberate ninhydrin-positive artifacts, and the amount of aspartic acid was small compared with that of the peptide. Hydrolysis yielded glutamic acid, glycine, an unidentified acidic amino-acid, and a little cystine. Dinitrophenylation, although incomplete, indicated that glutamic acid was N-terminal.

These results suggested that the peptide was probably another member of the group of γ -glutamyl peptides in lens (Table 1) and might contain a modified cysteine residue. Neither the peptide, nor the amino-acid liberated on hydrolysis, reacted with iodine-azide but thioethers, such as methionine, do not always give positive results. The presence of sulphur was confirmed by the behaviour of the peptide after oxidation.

The oxidation of sulphur compounds proceeds by two routes. Thioethers form sulfoxides in the presence of limiting amounts of oxidizing agents but give sulphones with excess reagent.

Table 7

Stages in identification of
new acidic compounds in lens

Stage in experiments

Suspected structure

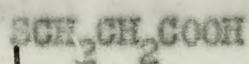
First lens fractionation 1F

Single substance γ -Glu-Cy-Gly

X acidic

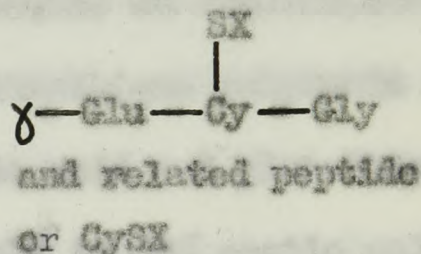
Second lens fractionation 2F

Attempted synthesis of γ -Glu-Cy-Gly



Third lens fractionation 3F

Two substances



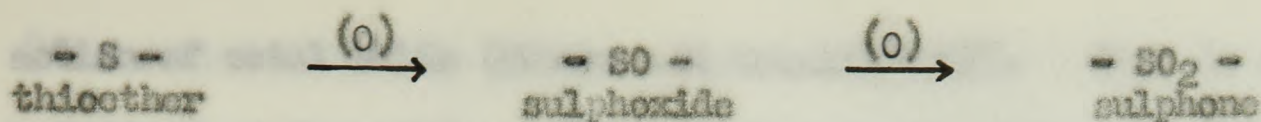
Fourth lens fractionation 4F

Second substance is CySX

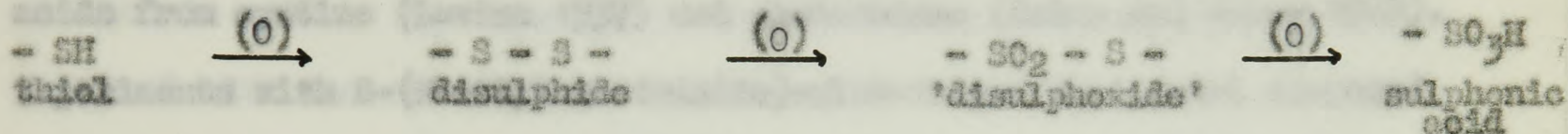
Synthesis of γ -Glu-Cy-Gly



and CySCH(COOH)CH₂COOH



Thiols are readily oxidized to disulphides and oxidation then proceeds via the unstable 'disulphoxide' to sulphonic acid. With limited amounts of reagent only sulphonic acid and unchanged disulphide are normally observed.



Oxidation of lens material gave one ninhydrin-positive spot on electrophoresis at pH 4, which moved to the same position as glutathione sulphonic acid. Two compounds were, however, detected on electrophoresis in 10% acetic acid. One did not separate from glutathione sulphonic acid, the other separated from both glutathione sulphonic acid and glutathione sulphonic acid. The mobility of the peptide itself in 10% acetic acid indicated a partly ionized acid group in the side chain. Behaviour on oxidation was thought to indicate formation of a sulphone and partial breakdown to a sulphonic acid (CSO_3H). The products were separated in 10% acetic acid and examined individually. Results of dinitrophenylation and hydrolysis were consistent with a sulphone structure for one product but the presence of CSO_3H was not confirmed as only traces of cysteic acid appeared after hydrolysis.

Second fractionation (Table 4). The peptide was obtained as a single broad peak, mostly in fraction 2F7, on breakthrough of the stronger buffer. The broad peak was attributed to disturbance of the resin by gas (p 17). In order to obtain more information about the side-chain attached to the sulphur atom, an attempt was made to cleave the thio-ether bridge by the

action of metal salts (Peters and Wakelin 1947). This is essentially a hydrolysis under mild conditions which is aided by formation of a metal complex at the sulphur atom. It has been used to liberate the porphyrin moiety of cytochrome C (Paul 1951). A similar reaction is observed with disulphides (e.g. Cecil 1950) and was used in the preparation of sulphinic acids from cystine (Lavine 1937) and glutathione (Calan and Waley 1962). Experiments with S-(N-ethylsuccinimido)-glutathione as a model compound indicated some fission, but these results could not be repeated with the lens peptide.

Third fractionation (Table 5). This was performed in order to obtain more material for the next experiment with the peptide. Elution was carried out with a greater volume of 0.5M pyridine-formate buffer than in the second fractionation. Two sharp peaks, containing material with the electrophoretic mobility of the peptide, were obtained. One was eluted during the buffer change (3F7) and also contained GSSG and traces of other compounds, the other was eluted with strong buffer (3F9) and contained traces of S-sulphoglutathione. These peaks presumably merged in the second fractionation as a result of the disturbance of the resin bed by decomposition of material. On hydrolysis, the material in fraction 3F7 yielded more of the sulphur amino-acid relative to other amino-acids than that in fraction 3F9. It was not clear at this point whether the compound in the first peak was a new peptide or the free amino-acid contaminated with other γ -glutamyl peptides.

The peptide S-(β -carboxypropyl)-glutathione, from onion, was characterized by the action of Raney nickel and separation of the products

(Virtanen & Matikala 1960). This method was chosen for the next study on the lens peptide. Small amounts of material are desulphurized by passage through a column of Raney nickel (Keil & Sorn 1960), and little loss of material occurs. Experiments with S-(N-ethylsuccinimido)-glutathione were easy to perform and yielded the expected product, nor-phthalnic acid. Fraction 3F9 seemed to contain the peptide, and was combined with the remainder of fraction 2F7. The experiment was more difficult with the lens material probably because of impurities in the solution, which was rather syrupy and smelt of pyridine. These impurities may have formed salts or nickel complexes. The method was, nevertheless, quite successful: norphthalnic acid and a ninhydrin-negative acidic substance were the main products.

Fourth fractionation (Table 6). This was performed with the objects of confirming the separation obtained in the third fractionation, and of characterizing the compounds in the two peaks. The change from weak to strong buffer was made later than in the previous fractionation so as to elute each peak with a single buffer. The amount of ninhydrin-positive contaminants in each peak (fractions 4F7 and 4F9) was small, and more definite conclusions concerning the two acidic substances could be drawn than from the previous experiments.

The two-dimensional separation achieved by electrophoresis in pyridine-acetate followed by chromatography in butanol-acetic acid proved very satisfactory (Figure 4). Results clearly indicated that the compound in fraction 4F7 was the sulphur amino-acid liberated by hydrolysis of the peptide in fraction 4F9. The slight differences in

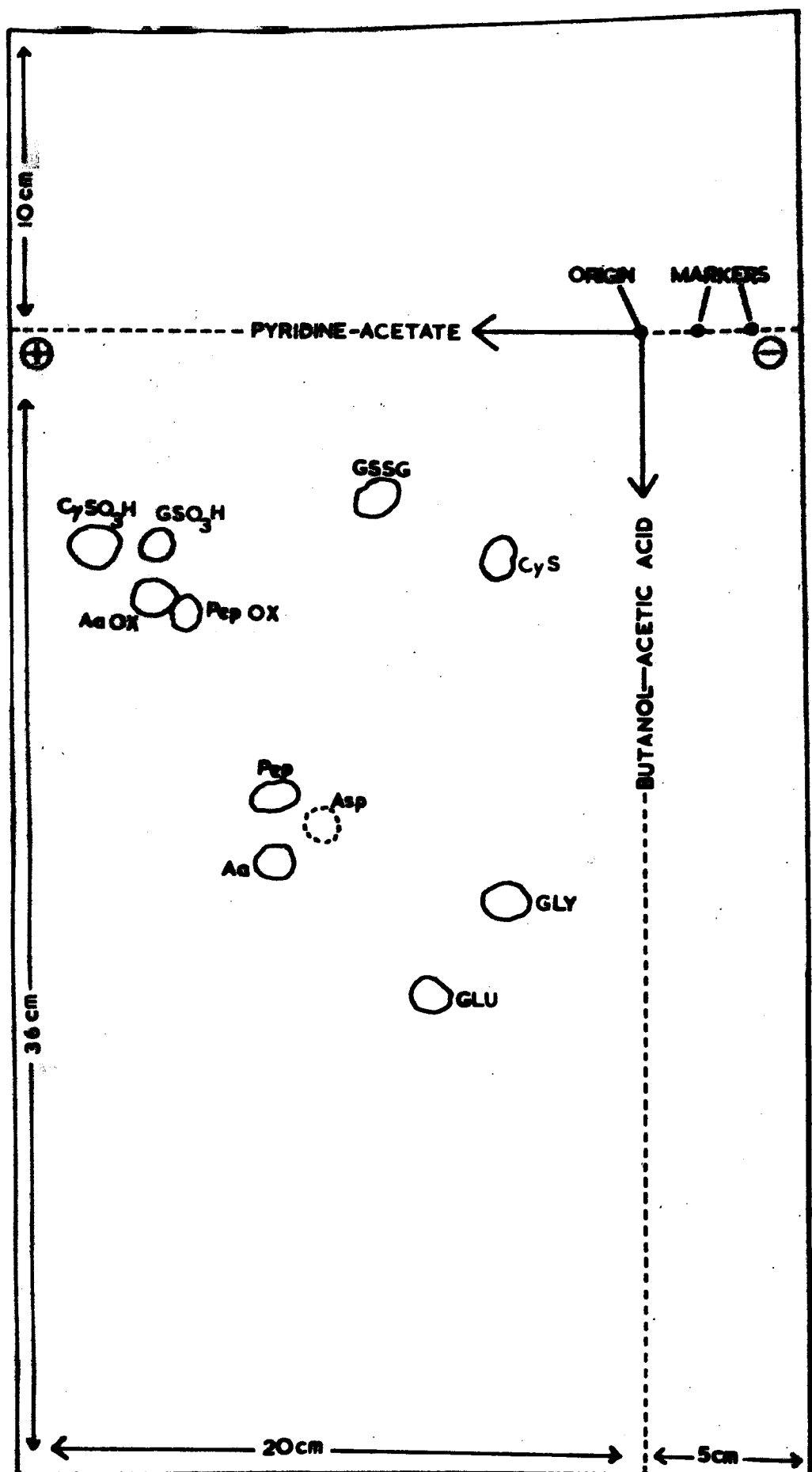


Figure 4. Separation of new acidic compounds in lens and related substances by electrophoresis at pH 4 followed by chromatography. The method is explained on p. 65. Relative positions of compounds are shown. As: new amino-acid, Pep: new peptide, Ox: positions after oxidation.

mobility of the substances before and after oxidation suggested that mixtures of the two were obtained from the first two fractionations (1F and 2F). Behaviour of the oxidized peptide and amino-acid on electrophoresis in 10% acetic acid explained the observations made with oxidized material from fractionation 1F. Both compounds appeared to form sulphones on oxidation. The amino-acid sulphone was not separated from glutathione sulphonic acid by electrophoresis, and the peptide sulphone could only be separated from glutathione sulphonic acid by electrophoresis in 10% acetic acid.

Fraction 4F7 was refractionated by chromatography on a cation-exchange resin at low pH in order to purify the amino-acid. Most material was eluted as a sharp peak but some was eluted at the solvent front probably because of the high concentration of acid added to adjust the pH initially. Solid which was isolated was not pure.

Before the Raney nickel experiment was repeated with the lens peptide, it was decided that experiments to identify the ninhydrin-negative product should be performed with another model compound. Choice of this peptide, which turned out subsequently to be identical with the lens peptide, is described in the next section.

2. Model compounds

Preliminary choices. A model compound was first used in preliminary studies on thioether fission. S-(N-ethylsuccinimido)-glutathione was chosen as the model because it is a thioether derived from glutathione and is readily prepared by addition of N-ethylmaleimide to glutathione (Kernack and Matheson 1957). This choice was not entirely satisfactory

since the electrophoretic mobility of the lens peptide at pH 4 suggested that at least one acidic group was present in the modified cysteine side-chain. The series of peptides derived from glutathione containing sulphur acids: S-sulpho GSSO_3H (Waley 1959), sulphonic acid GSO_3H , and sulphinic acid GSO_2H (Calam & Waley 1962) is known. These acids migrated further at pH 4 than the lens peptide, while the only other naturally-occurring analogue of glutathione, S-(β -carboxypropyl)-glutathione (Virtanen & Matikala 1960), did not travel as far as the lens peptide.

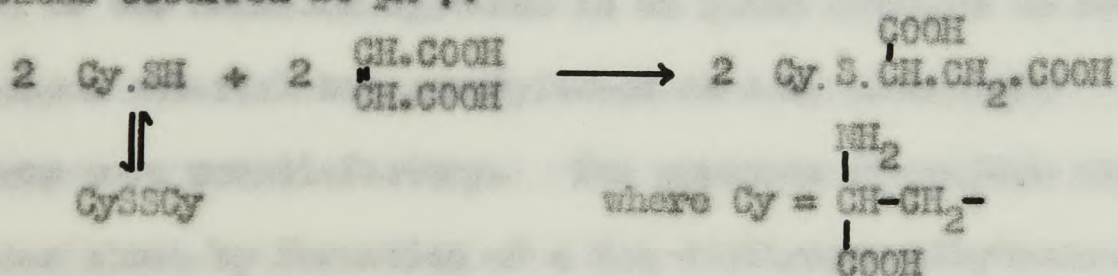
The preparation was attempted of another model which could be used to determine optimum conditions for the Ramey nickel experiment. S-(β -carboxyethyl)-glutathione was selected because of its suspected similarity to the lens peptide and in order to study its electrophoretic behaviour. The corresponding cysteine derivative, S-(β -carboxyethyl)-cysteine had been characterized in seeds of Acacia Willardiana (Guelin 1959). No compound was obtained, under conditions used in the synthesis of Virtanen's peptide and of S-substituted cysteines (Armstrong & Lewis 1951), which separated from known substances by the methods employed. The preparation was abandoned and S-(N-ethylsuccinimido)-glutathione used successfully as the model substance (see above).

At this point the fourth fractionation of lens material was carried out which established the presence of the new sulphur amino-acid as well as the peptide. The electrophoretic mobility of aspartic acid, the lower homologue of glutamic acid, is greater at pH 4 than that of glutamic acid. The sulphur amino-acid, probably an S-substituted cysteine, was more mobile than aspartic acid, which suggested that the

substituent contained two acid groups. The simplest compound with the required structure is S-(α, β -dicarboxyethyl)-cysteine. The peptide, S-(α, β -dicarboxyethyl)-glutathione was, therefore, chosen as the next model compound. Both the peptide and amino-acid had been obtained by addition of the thiol to maleic acid (Morgan & Friedmann 1938), and the cysteine compound had been prepared from fumaric acid (Pine & Peacock 1955).

S-(α, β -dicarboxyethyl)-glutathione and S-(α, β -dicarboxyethyl)-cysteine

Synthesis. These compounds were synthesized by addition of the appropriate thiol to either maleic or fumaric acid. The extent of reaction, however, depends on a number of factors. Maleic acid reacted with the thiols about four times as rapidly as fumaric acid, and both acids reacted more rapidly with cysteine than with glutathione. Thiols, such as cysteine, are oxidized in aqueous solution to disulphides. Oxidation is quite rapid in alkaline solution but proceeds more slowly at lower pH. Addition of thiol to the unsaturated acids was pH-dependent since competing reactions occurred at pH 7:



Negligible amounts of disulphide formed at low pH and the addition reaction went practically to completion. Fumaric acid is less soluble than maleic acid so the latter was used for much of the preparative work.

Syntheses were performed in salt-free solutions, rather than under conditions used by Morgan and Friedmann. This method facilitated

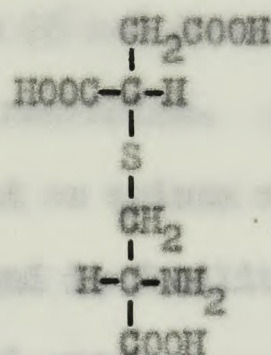
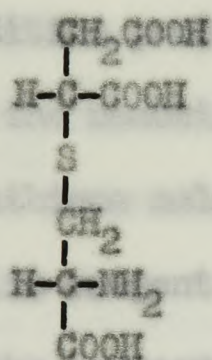
isolation of the product because only traces of unwanted material were present. Thiol and acid were dissolved in water to give an acid solution. The course of the reaction could be followed by electrophoresis. Disappearance of glutathione or cysteine was matched by a corresponding increase in a more acidic ninhydrin-positive compound, and by disappearance of the unsaturated acid. The product was precipitated or crystallized. Properties of the product (see below) indicate that only the cysteine residue is affected in the reaction.

The best sample of S-(α,β -dicarboxyethyl)-glutathione was obtained by decomposition of the blue copper salt. This method had been used previously for the purification of ophthalmic acid (Waley 1957). The amorphous nature of the solid was indicated by its featureless infrared spectrum, and elementary analysis was not performed.

S-(α,β -dicarboxyethyl)-cysteine was characterized as a highly crystalline di-diethylamine salt. The free amino-acid was obtained solid by precipitation from water with acetone. The amount of acetone added to the solution appeared to be quite critical as rather gummy amorphous material was precipitated on some occasions. Analytical figures were unsatisfactory. The presence of acetone was indicated, and was shown by formation of a 2:4-dinitrophenylhydrazone which co-chromatographed with an authentic sample. Portions of the amino-acid were dried at 100° in vacuo and gave a residue after combustion. Since AnalaR unsaturated acids were unobtainable and amino-acids often contain ash, it seems probable that the residue was present in the starting materials and was not removed during purification. Reasonably satis-

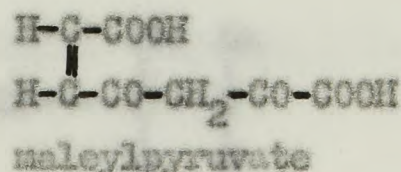
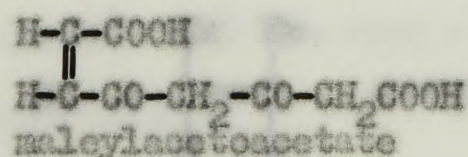
factory analytical figures for both the amino-acid and peptide were obtained by Morgan and Friedmann (1938).

Products obtained from maleic acid and from fumaric acid appeared to be identical. The free amino-acids gave identical infra-red spectra, the di-diethylhexylamine salts gave identical infra-red spectra and mixed melting-point, the peptides were indistinguishable on hydrolysis, and none of the pairs of compounds were separated by electrophoresis or chromatography. Since the carbon atom attached to sulphur is asymmetric:



it seems probable that both products are mixtures of diastereoisomers. No rotation studies were performed.

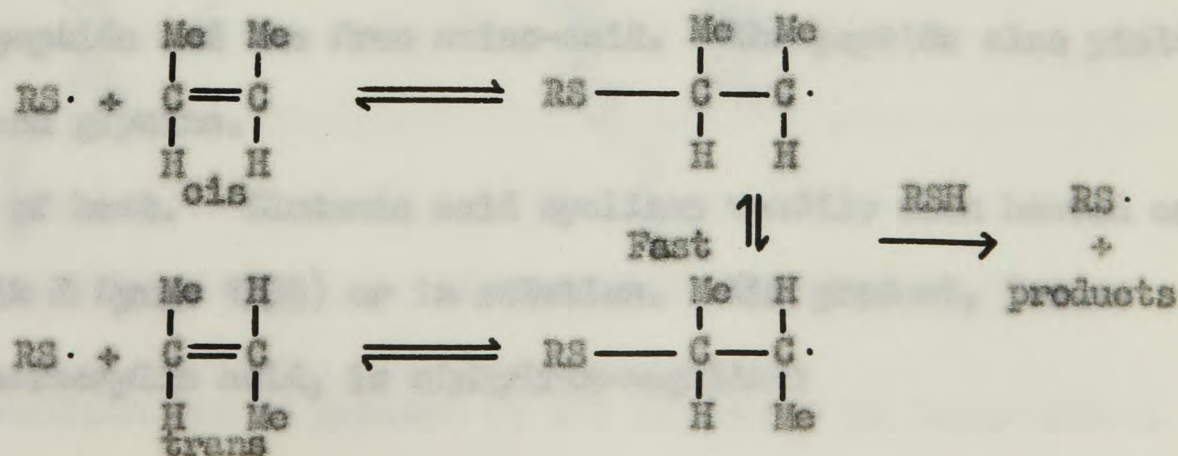
Isomerization of maleic acid during the reaction with glutathione, which was observed by Morgan and Friedmann (1938), was confirmed on paper. Similar cis-trans rearrangements have been observed with compounds related to maleic acid.



Both maleylacetoacetate (Edwards & Knox 1956) and maleylpyruvate (Lack 1961) can be converted to the corresponding fumaryl

compound either enzymically or non-enzymically. The enzymes which catalyze the isomerization require glutathione as co-factor. Rearrangement of maleylacetoacetate is involved in the metabolism of tyrosine and is catalyzed by an enzyme from liver; rearrangement of maleylpyruvate is catalyzed by a bacterial enzyme. The non-enzymic isomerizations are less rapid and occur in the presence of glutathione. Stable adducts have been detected. Lack has suggested that the reaction mechanism involves addition of the anion GS^- to the double bond to give an unstable transition complex which either takes up a proton to form a stable addition product or eliminates GS^- and isomerises. Certain features of the reactions support this mechanism. At pH 7 a proportion of the glutathione molecules are present as anions and addition of such a nucleophilic reagent to the double bond is facilitated in both substrates by the presence of an α -carbonyl group.

An alternative mechanism has been proposed for the isomerization which accompanies addition of methyl mercaptan to cis-2-butene in solution at 60° (Walling & Helmarich 1959). This involves free-radical addition of thiol to the double bond. The complex equilibrates rapidly then either decomposes or reacts further to give the addition product:



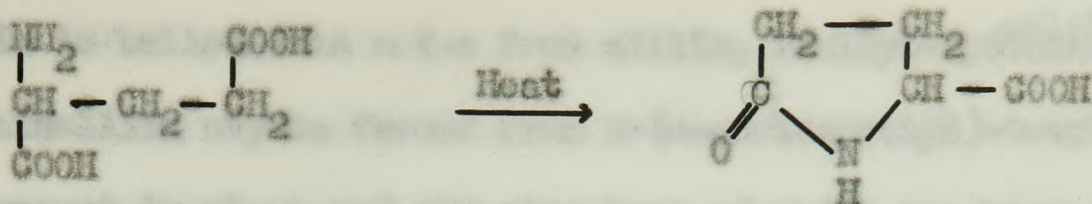
No evidence is available as to which mechanism operates in the case of maleic acid and glutathione. Both mechanisms postulate a two-stage reaction, the second stage of which is irreversible. The stability of S-(α , β -dicarboxyethyl)-glutathione is consistent with this. Since trans isomers are thermodynamically more stable than cis isomers, indicated by free energy values, the difference in rates of reaction of maleic and fumaric acids may be explained by the relative ease of formation of the transition complex.

Properties.

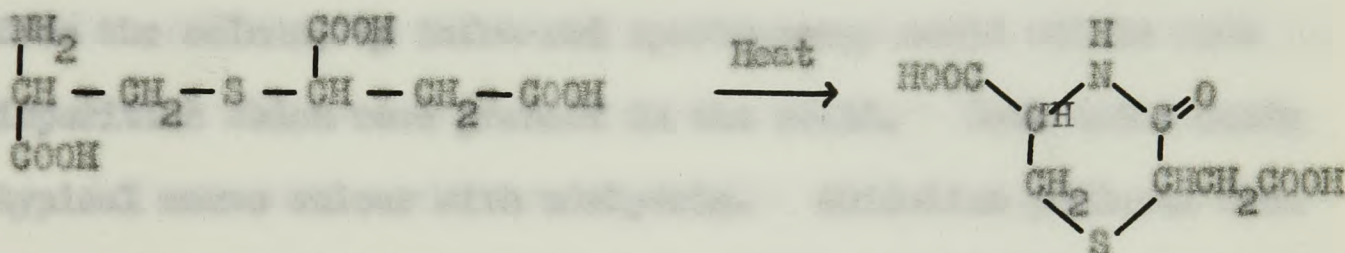
i) Oxidation. S-(α , β -dicarboxyethyl)-cysteine and S-(α , β -dicarboxyethyl)-glutathione both yielded oxidation products which are probably sulphones, as expected from a thio-ether. Cysteic acid was not obtained from the amino-acid, nor glutathione sulphonic acid from the peptide. The pattern shown in Figure 4 was confirmed with synthetic markers and indicates the small differences in mobility of these compounds on paper. The amino-acid sulphone could only be separated from GSO₃H by chromatography on fast paper, not by electrophoresis. The peptide sulphone was separated from GSO₃H by electrophoresis in 10% acetic acid.

ii) Hydrolysis. Slight decomposition of the amino-acid occurred on hydrolysis and about 10% of cystine was formed. This was observed with both the peptide and the free amino-acid. The peptide also yielded glutamic acid and glycine.

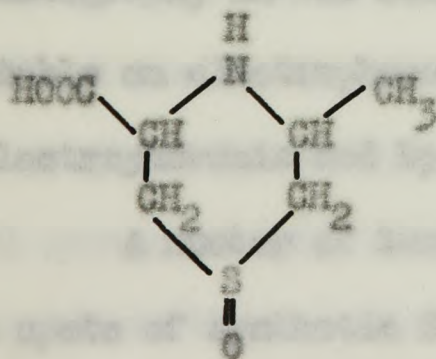
iii) Effect of heat. Glutamic acid cyclizes readily when heated as a solid (Hilfolt & Synges 1955) or in solution. The product, 5-oxo-pyrrolidine-2-carboxylic acid, is ninhydrin-negative:



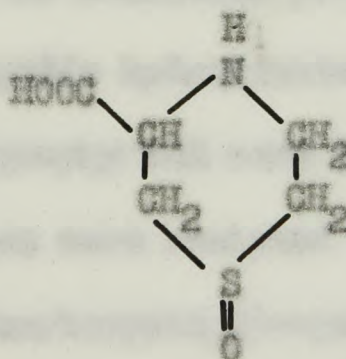
The effect of heat on S-(α, β -dicarboxyethyl)-cysteine was studied in solution and with the solid. Some desulphurization to alanine occurred but the main product was more mobile than the amino-acid on electrophoresis at pH 4 and reacted very slowly with ninhydrin. Although it has not yet been characterized, it is probably 2-carboxymethyl-3-oxo-1,4-thiazane-5-carboxylic acid:



The formation of this compound is of interest since it is closely related to two natural products which contain the same heterocyclic ring system. One is cycloalliin, 3-methyl-1,4-thiazane-5-carboxylic acid-1-oxide, which was found in onion (Virtanen & Matikala 1959). The other, which can be considered as de-methyl-cycloalliin, was isolated from algae (Kuriyama 1961).



cycloalliin



de-methyl-cycloalliin

Cycloalliin may be reduced to the thioether by Raney nickel.

Cycloalliin is believed to arise from alliin, S-allyl-cysteine^{sulphoxide}. De-methyl-cycloalliin may be formed from H-(α -carboxyethyl)-taurine which is also present in algae and the structure of which has been confirmed by synthesis (Kuriyama 1961).

Comparison of lens with synthetic compounds

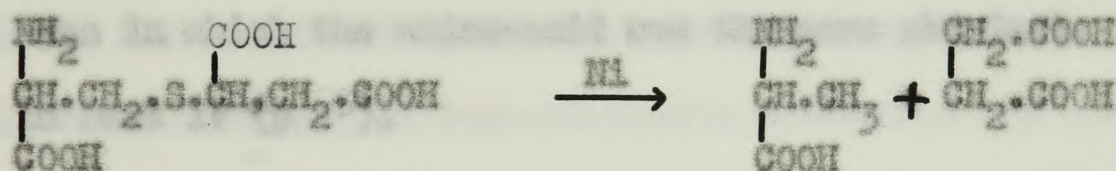
The most rigorous identification was made with the amino-acids. They were eluted at essentially the same position from a Dowex 50 column and could not be separated by electrophoresis in two buffers nor by chromatography in two solvents. Comparison of material, obtained from the column, by infra-red spectroscopy could not be made owing to impurities which were present in the solid. Both amino-acids gave an atypical mauve colour with ninhydrin. Oxidation products from the synthetic and natural amino-acids could not be separated by electrophoresis. Both substances cyclized when heated in aqueous solution and the solutions gave identical patterns with acridine and with ninhydrin after chromatography.

These findings were supported by the behaviour of the peptides which could not be separated by electrophoresis in two buffers nor by chromatography in two solvents. The oxidation products were indistinguishable on electrophoresis. Peptide hydrolysates were also examined by electrophoresis and by chromatography and were identical.

A number of lens fractions were analyzed by electrophoresis with spots of synthetic S-(α, β -dicarboxyethyl)-cysteine or S-(α, β -dicarboxyethyl)-glutathione superimposed. This clearly showed the effect of impurities in concentrated fractions on the mobility of the

compounds. Some separation was achieved by continuous electrophoresis (fractionation 1F) since fraction 1F3 was examined after oxidation and contained more of the amino-acid than the peptide. A two-dimensional separation of the remaining material from fraction 1F7 showed that both substances were present. Some separation of material was also achieved in fractionation 2F since fraction 2F6 appeared to contain mostly the amino-acid and fraction 2F8 mostly the peptide.

Products from desulphurization by Raney nickel (p₄₀) were also re-examined and the ninhydrin-negative acid was identified as succinic acid. Malic acid, which might have been formed by hydrolysis, was not detected. The presence of alanine indicated that this experiment was carried out with a mixture of peptide and amino-acid.



Experiments with lens

The possibility exists that the S-(α, β -dicarboxyethyl)-cysteine and S-(α, β -dicarboxyethyl)-glutathione detected in extracts of stored lens are artifacts. This possibility was tested in two ways. The first was to try to detect the two compounds in an extract of fresh lens prepared in the presence of N-ethylmaleimide which reacts with free thiol groups. This method has been used for the study of free sulphur compounds in blood and serum (Smith & Tuller 1955). The second was to determine the amount of adduct formed in a solution of glutathione and fumaric acid taken through the normal extraction procedure. Trichloro-

acetic acid extracts of stored and of fresh lens were also compared but this experiment and its implications are described in Part IV (p⁹³).

i) Extraction with N-ethylmaleimide. Cysteine reacts completely with N-ethylmaleimide at equimolar concentration within 5 minutes (Roberts & Rouser 1958); protein thiols react more slowly. The concentration of N-ethylmaleimide in this experiment was 30mM. As the concentration of glutathione in lens is about 10mM, and only traces of cysteine were likely to be present, there was a considerable excess of N-ethylmaleimide which could react with protein thiol. Both S-(α, β -dicarboxyethyl)-cysteine and S-(α, β -dicarboxyethyl)-glutathione were detected in the extract. The concentration of the amino-acid (about 0.06mM) was considerably lower than that of the peptide (about 0.27mM), unlike extracts of stored lens in which the amino-acid was the more abundant. This is discussed in Part IV (p⁹³).

Information concerning the distribution of glutathione and cysteine was also obtained from this experiment. A large amount of S-(N-ethylsuccinimido)-glutathione was present in the extract, together with some GSSG (about 3-5% of total glutathione). The presence of GSSG is in agreement with the findings of Kinoshita and Masurat (1957) but contrary to an earlier report that all glutathione in lens is in the reduced form (Herrmann & Moses 1945). The amount of GSSG indicated by this method is similar to the 3% obtained by electrolytic reduction (Kinoshita & Masurat 1957) and the 2-5% detected in brain by a method using yeast apoglyoxalase (Martin & Mollwain 1959). Herrmann and Moses found that added GSSG is rapidly reduced by various tissues of the eye,

including the lens. It seems probable that about 3-5% of glutathione in tissue is normally present in the oxidised form.

2.3.4. The extract also appeared to contain both S-(N-ethylsuccinimido)-cysteine and cystine. The amounts of the two compounds were roughly comparable and the total concentration of free cysteine, both oxidised and reduced, was about the same as that of GSSG: 3-5% of total glutathione. It has normally been assumed that lens contains no free cysteine and the total indicated by this experiment would not be sufficiently significant to alter the amount of glutathione determined by other methods. Free cysteine is usually found in only trace amounts in tissues (e.g. Tallan, Moore, & Stein 1954).

2.3.5. Separation of the compounds in the extract obtained in this experiment was best achieved by electrophoresis in pyridine-acetate followed by chromatography in butanol-acetic acid-water-pyridine. This gave a better separation of S-substituted compounds from other substances than the system used for whole extracts (p110): electrophoresis in formic-acetic acid then chromatography in butanol-acetic acid.

2.3.6. A competition experiment was also carried out with the thiols in solutions containing both fumarate and N-ethylmaleimide; control solutions contained only thiol and fumarate. Reaction of glutathione with N-ethylmaleimide was complete under conditions in which the control gave 4% of S-(α, β -dicarboxyethyl)-glutathione. The cysteine solution contained 2% of S-(α, β -dicarboxyethyl)-cysteine, about one-tenth of the amount in the control. Initial concentrations of reactants were 50mM. It seems unlikely that any proportion of the acidic compounds detected

in the lens extract experiment could have arisen during extraction.

It is probable, therefore, that both S-(α , β -dicarboxyethyl)-glutathione and S-(α , β -dicarboxyethyl)-cysteine are normal constituents of calf lens.

ii) Extraction without lens. Concentrations of reactants were chosen to resemble those in lens, that of glutathione was 10mM. Some of the citric acid cycle intermediates have been detected in lens and fumarate is presumably present. The citric acid cycle is rather unimportant in lens metabolism but the concentration of fumarate in rat kidney, where the cycle is very active, is about 0.7mM (Frohman, Orten & Smith 1951). No figures appear to be available for calf. For this experiment, the concentration of fumaric acid was 3mM in order to give a maximum value for the extent of reaction. About 2% of the glutathione reacted during the extraction which suggested that a small proportion of the material found in lens extracts could have formed by this method. The total amount in lens extracts, however, represented a greater amount of bound fumarate than was likely to be present free at any one time, which implies that the peptide is normally present in lens.

iii) Biosynthesis. A preliminary attempt has been made to study the biosynthesis of the two S-substituted compounds. The purpose of the experiment was to discover whether crude homogenates of lens catalyzed addition of thiol to fumarate when compared with a control in the absence of lens. For the reaction between cysteine and fumarate, no difference in the extent of reaction was observed after 2 hours at 37°, by which time both solutions contained about 60%^{of} _{λ} cysteine as S-(α , β -dicarboxyethyl)-cysteine.

The total concentration of glutathione in the lens mixture fell relative to the control, and there appeared to be relatively slightly less S-(α , β -dicarboxyethyl)-glutathione in the lens mixture. The control contained about 10 - 15% of glutathione as adduct after 4 hours at 37°. The probable explanation of these findings is that glutathione was bound to lens protein either by hydrogen-bonding or by formation of mixed disulphides with protein thiol. Experiments with S-(α , β -dicarboxyethyl)-glutathione and S-(α , β -dicarboxyethyl)-cysteine suggest that relatively little low molecular-weight material was removed from solution by the 90% (w/v) phenol used to precipitate protein. Kinoshita and Masurat (1957) have shown that the amount of glutathione, which can be extracted from bovine lens, by incubation in phosphate buffer, increases markedly if the buffer contains either urea or guanidine. Both these reagents are known to assist in the cleavage of hydrogen bonds.

Occurrence.

The significance of the occurrence of S-(α , β -dicarboxyethyl)-cysteine and S-(α , β -dicarboxyethyl)-glutathione in lens is discussed in Part V. It can be mentioned that this is the first S-substituted glutathione to be detected in animal tissue and probably the first S-substituted cysteine.

The formation of S-(α , β -dicarboxyethyl)-cysteine from cysteine and maleate has been implicated in the detoxification of maleic acid by kidney (Angielski & Rogulski 1961). Kidney homogenates contain glutathione^{ase} which degrades glutathione to amino-acids. The cysteine liberated in this way then reacts rapidly with maleate.

Methods and results

Experimental and results

1. Investigation of lens material

Materials. Dilute pyridine-acetate buffer used for continuous electrophoresis was prepared by dilution of stock pyridine-acetate pH 4 (1 vol.) with water (4 vols.).

Pyridine-formate buffer pH 2.5 for column chromatography. Formic acid (90%, 59 ml) and pyridine (16.2 ml) diluted with water (to 1l).

Glutathione was a gift from The Distillers' Co., Ltd., batch G.29; S-sulphoglutathione, ophthalmic acid, and norophthalmic acid were supplied by Dr. S. G. Waley; S-(β -carboxypropyl)-glutathione was a gift from Professor A. I. Virtanen.

S-(N-ethylsuccinimido)-glutathione was prepared by the method of Kermack and Matheson (1957); preparation of glutathione sulphonic and sulphinic acids has been described elsewhere (Calam & Waley 1962).

Preparation of Raney nickel was based on the method of Mezingo (1941) and one-quarter quantities were used. After addition of all the alloy, the flask was removed from the ice-bath and the temperature allowed to rise to 45° for 2.5 hours. The flask was heated on a water bath at 50° for 1 hour (Pavlic & Adkins 1946) and then cooled. The supernatant was decanted, and the residue treated with sodium hydroxide (8g) in water (80 ml) for 30 minutes. The supernatant was discarded, and the residual powder suspended in water (500 ml) and washed with water (7.5 l) at 3.0 ml/min until the washings were pH 7. Raney nickel was stored under water at 4°.

Methods and results

Methods were described in Part II (general techniques) unless otherwise indicated.

Material from the first fractionation. Fraction 1F7 was examined by electrophoresis in pyridine-acetate and contained the acidic peptide with traces of glutamic acid, ophthalmic acid, GSSG and aspartic acid. It was refractionated (Table 8) by continuous electrophoresis in the Beckman apparatus with dilute pyridine-acetate buffer, and at constant current (40mA). The apparatus was equilibrated, then the fraction (4.2 ml), diluted with water (7.8 ml) and fuchsin (0.3 ml of solution containing 3 mg/ml), applied to the cathode side of the paper curtain at 1.1 ml/hour. Fuchsin indicated the position of material as it travelled down the curtain. Collection of fractions was started 1.5 hours after application of the sample and was continued for 20 hours. The fraction collector changed after 12 hours. Fractions were numbered from cathode to anode, examined by electrophoresis in pyridine-acetate, pooled, concentrated in vacuo and stored at -15°. The paper curtain is shown in Figure 5.

Fraction 1F7D was examined by electrophoresis in pyridine-acetate and in 10% acetic acid, and by chromatography in phenol-ammonia. Papers were developed with ninhydrin. One main spot, attributed to the peptide, was observed together with aspartic acid and fuchsin. The concentration of aspartic acid was about 10% that of the peptide. Fuchsin was visible on papers before ninhydrin treatment. The spots were rather weak so the fraction was concentrated in vacuo to 0.5 ml.

Table 8

Refractionation of fraction W7 by continuous electrophoresis

Fraction	Pooled fractions	Chief ninhydrin-positive constituents	Initially	Volume ml after concentration
W7A	1 - 13	Glutamic Acid	240	9.3
W7B	14 - 19	Ophthalmic Acid GSSG	76	4.8
W7C	20 - 23	Ophthalmic Acid (Trace) GSSG	54	3.7
W7D	24 - 29	Aspartic Acid (Trace) Acidic Peptide Aspartic Acid (Trace) (Fuchsin)	82	2.7
W7E	30 - 32	Blank (Fuchsin)	63	1.8

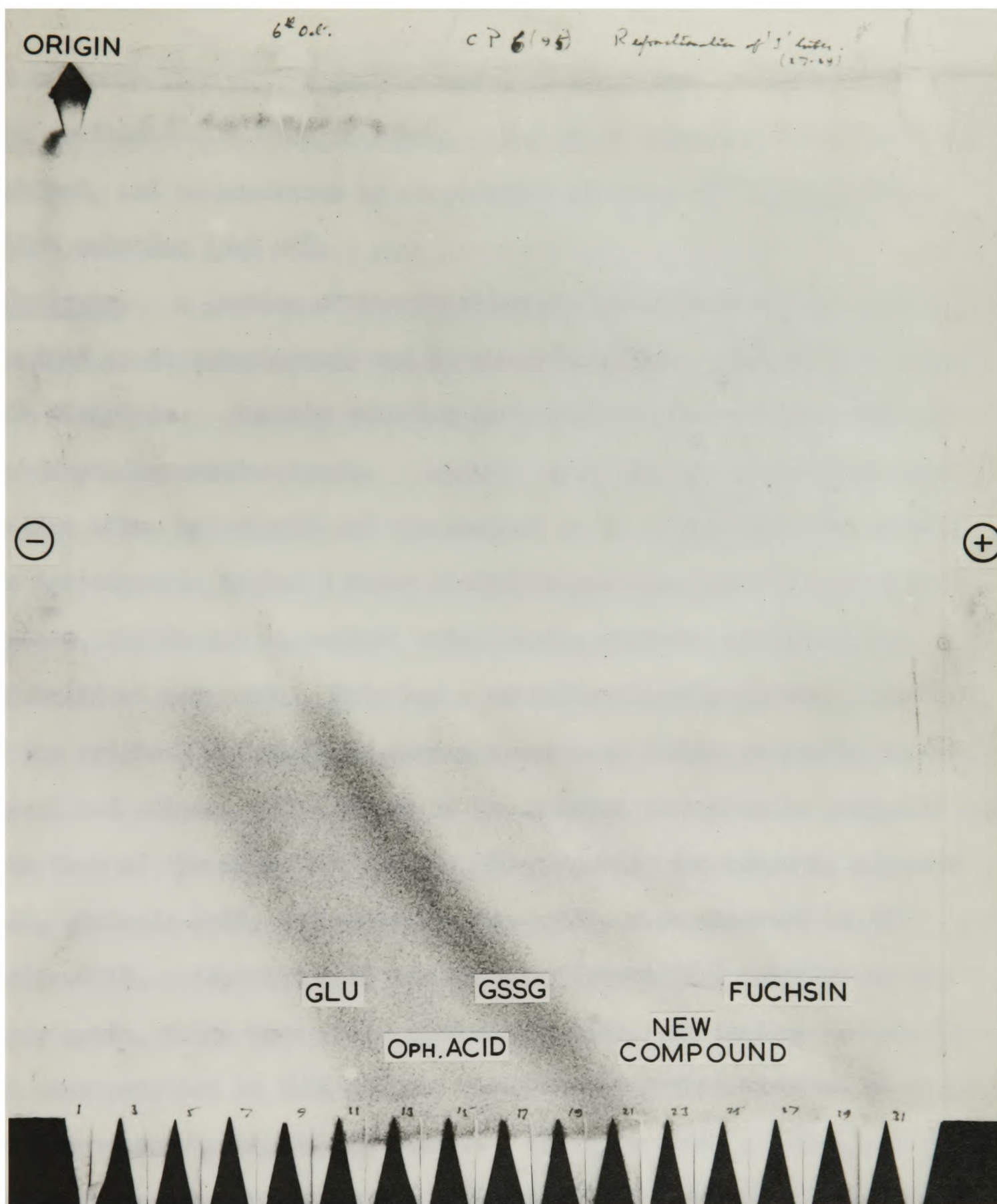


Figure 5. Refractionation of fraction 1F7 by continuous electrophoresis in dilute pyridine-acetate buffer at constant current (4.0mA). Paper curtain developed with ninhydrin. Fuchsin visible before curtain was developed.

The concentration of the peptide was then about 5mM. Initial studies were performed with this fraction. The other fractions were later recombined, and concentrated by evaporation at below 47° in vacuo to a yellow solution (3.3 ml).

Hydrolysis. A portion of the fraction was hydrolysed and the hydrolysate examined by electrophoresis and by chromatography. Papers were developed with ninhydrin. Fuchsin solution submitted to the same procedure gave no ninhydrin-positive spots. Aspartic acid did not increase in concentration after hydrolysis and was assumed to be absent from the peptide. The hydrolysate yielded 3 other ninhydrin-positive spots in pyridine-acetate, attributed to neutral amino-acids, glutamic acid, and one unidentified compound. This had a mobility slightly greater than that of the original material and gave a mauve spot fading gradually to the normal red colour. The amount of the unknown seemed to be slightly less than that of the other two spots. Four spots: the unknown, aspartic acid, glutamic acid, and neutral amino-acids, were observed in 10% acetic acid. Aspartic acid was stronger (about 25%) relative to the other spots, which were of comparable strength, but cystine travels to the same position in this buffer. Additional information was obtained by chromatography in phenol-ammonia. Glutamic acid, glycine, and cystine were confirmed by co-chromatographing a hydrolysate with markers on the same position. A purple-mauve spot was given by the unidentified acid which travelled in front of the peptide (no longer present in hydrolysates) and near cysteine acid. Cysteine acid could be differentiated by its normal ninhydrin colour. Amino-acid composition of the peptide was

obtained by measuring the optical densities of spots in a hydrolysate run in 10% acetic acid, and comparing with a hydrolysate of glutathione sulphonic acid on the same paper. The ratio glycine : glutamic acid was 1 : 1.04. Since the colour obtained with the unknown acid was atypical, and cystine and aspartic acid gave a single spot, direct comparison with these is not possible. The optical densities obtained: 0.445 for the unknown, 0.432 for cystine/aspartic acid, and 0.885 for glycine suggested that a small amount of the unknown is broken down to cystine on hydrolysis.

Qualitation Hydrolysis was also carried out in 0.5N. HCl (N acid with an equal volume of the fraction). Duplicate papers were analyzed by electrophoresis in pyridine-acetate. One paper was developed with ninhydrin and gave the same pattern as a normal hydrolysate. The other paper was treated with iodine-azide reagent. One spot was obtained from the fraction and two from the hydrolysate. The peptide and the new amino-acid did not react. The position of the spot in the fraction indicated weakly basic material which was probably an impurity from pyridine in the buffer used for fractionation. This spot was unaffected by hydrolysis and the second spot was at the position of neutral amino-acids (probably cystine).

N-terminal amino-acid. A small volume of fraction 1F7D was dinitrophenylated under the usual conditions (p 34). Dry material was dissolved in water (60 μ l) and concentrated HCl (60 μ l), and hydrolysed. A sample of glutathione sulphonic acid was treated similarly. The yellow hydrolysates were examined by electrophoresis (pyridine-acetate) and by chroma-

tography (phenol-ammonia). A yellow spot was visible before treatment with ninhydrin. This spot migrated faster than glutamic acid at pH 4, and was attributed to DNP-glutamic acid since it was in the same position as a marker. The concentration of free glutamic acid decreased relative to glycine but dinitrophenylation was not complete. Glycine and the unidentified acid were also present. The same compounds were identified after chromatography. Some loss of material occurred during dinitrophenylation because the spots observed on paper were less intense than was expected.

Oxidation. The fraction (1 vol.) was treated with hydrogen peroxide (30%, 1 vol.) and formic acid (98%, 1 vol.) for 40 minutes, taken to dryness in vacuo, and hydrolysed. A fuchsin solution treated similarly gave no interfering spots. The hydrolysate was analyzed with ninhydrin on paper and contained glycine, glutamic acid, aspartic acid, and a new compound. The unidentified amine-acid normally present in hydrolysates had disappeared. The new compound travelled further than the original material on electrophoresis at pH 4 and was slightly less mobile than cysteic acid. It separated from cysteic acid on chromatography in phenol-ammonia.

Oxidation products were also examined directly. On electrophoresis in pyridine-acetate, the peptide spot disappeared and a spot of more mobile material, fractionally slower than glutathione sulphonic acid, was obtained. Aspartic acid was unaffected by oxidation. Electrophoresis in 10% acetic acid gave a fresh pattern of ninhydrin spots. The peptide again disappeared but two new compounds were formed. One

had the same mobility as glutathione sulphonic acid, the other was less acidic and gave a spot between the first and the peptide. The less acidic substance separated from glutathione sulphonic acid. Two new spots were also detected by chromatography in butanol-acetic acid-water-pyridine for 42 hours on No. 4 paper. One was in the same position as GSO_3H , the other separated from GSO_2H .

Investigation of the oxidation products

Fraction 1F7D (50 μ l) was treated with a portion (0.2 ml) of solution containing hydrogen peroxide (30%, 0.5 ml) and formic acid (98%, 0.16 ml). Oxidised material was applied to paper as a band 6 cm. long, and subjected to electrophoresis in 10% acetic acid. Strips (2.7 cm. wide) containing each of the oxidation products were cut from the paper and the compounds eluted with water.

a) Less acid oxidation product. A portion of the eluate gave a single spot with ninhydrin after electrophoresis in 10% acetic acid. The remainder was dinitrophenylated and hydrolysed. DNP-glutamic acid (shown by a marker), glutamic acid (streak), glycine, alanine (trace) and another compound were detected after chromatography in phenol-ammonia. The unidentified compound gave a purple colour with ninhydrin which changed to red. It separated from GSO_3H and had a lower R_f than the acid present in un-oxidised hydrolysates. Electrophoresis in pyridine-acetate gave a set of spots attributed to neutral amino-acids, glutamic acid, DNP-glutamic acid (trace) and another compound with the mobility of GSO_3H . A similar pattern was observed after electrophoresis in 10% acetic acid, the unidentified compound travelled to the same position as

CSO_3H , which was also that of the more acid oxidation product.

b) More acid oxidation product. The compound eluted from paper had the same mobility as CSO_3H in 10% acetic acid. The solution was dinitrophenylated. One portion gave a yellow spot which seemed to separate from DNP- CSO_3H (streak) in phenol-ammonia. The remainder was hydrolysed in N. HCl and the hydrolysate was examined by electrophoresis. Traces of glutamic acid and neutral amino-acids appeared to be present but the amounts were too small for positive identification.

Material from the second fractionation. Fractions (Table 4) were analysed by electrophoresis in pyridine-acetate. Spots in the position of the peptide were observed in fractions 2F6, 2F7, 2F8 and 2F9. Only traces of material were present in fractions 2F8 and 2F9, fraction 2F6 also contained aspartic acid. Fraction 2F7 was dried in vacuo to a brown gum, dissolved in water (1.0 ml) and stored at -15° . Traces of aspartic acid were detected on electrophoresis. The identity of the peptide material was checked by hydrolysis. The pattern of amino-acids obtained was identical with that from fraction 1F7D on electrophoresis in pyridine-acetate.

Part of fraction 2F7 was used in attempts to cleave the suspected thioether linkage by the action of metal salts (Peters & Wakelin 1947). In a typical experiment, the fraction ($50\ \mu\text{l}$) and 0.2 M silver nitrate ($50\ \mu\text{l}$) were left for 4 hours in darkness then centrifuged. Creamy solid was suspended in water ($50\ \mu\text{l}$) and treated with 0.02 M potassium iodide ($50\ \mu\text{l}$). The contents of this solution were unchanged from the original fraction when examined by chromatography

(phenol-ammonia) and by electrophoresis (pyridine-acetate). Some glutathione was obtained from S-(N-ethylsuccinimide)-glutathione under similar conditions. The effect of mercury salts was also studied. Fraction (50 μ l) and 0.02 M mercuric acetate (50 μ l) were incubated at 37° for 6 hours and examined by chromatography in phenol-ammonia on No. 4 paper for 16 hours. Two spots near the position of GSSG, one of which was purple and the other mauve with ninhydrin, were present in the fraction but not in the incubated solution. Traces of solid were found after incubation and the compounds were probably precipitated. The remainder of the fraction was treated with Raney nickel (see below).

Material from the third fractionation (Table 5). Material with the electrophoretic mobility of the peptide in pyridine-acetate was eluted from the column in two peaks which were combined to give fractions 3F7 and 3F9. Traces of glutamic acid and GSSG were present in 3F7. The substance in fraction 3F7 gave a more mauve colour with ninhydrin than that in 3F9. The two substances were not separated by chromatography overnight in phenol-ammonia. Estimated concentrations were 10 - 15mM for the compound in fraction 3F7 and about 2mM for that in 3F9.

Hydrolysis. Portions of the fractions were compared by hydrolysis. Hydrolysates were examined by electrophoresis in pyridine-acetate and papers were developed with ninhydrin. The pattern of spots was the same for both fractions: neutral amino-acids, glutamic acid, and the unidentified compound which gave a mauve colour. This colour was similar to that given by the compound in fraction 3F7 but the substance

had travelled further in the hydrolysate. Positions of spots in 3F7 suggested that the mobility was affected by impurities in the fraction. Hydrolysis of fraction 3F9 gave the amino-acids in much the same relative proportions as had been observed previously. For fraction 3F7, the amount of the unidentified amino-acid was greater relative to the other acids. On the basis of these observations, fraction 3F9 was used for the next experiment. A hydrolysate of fraction 3F7 was also analyzed on No. 52 paper by electrophoresis in pyridine-acetate for 5 hours in the first direction followed by chromatography in butanol-acetic acid for 48 hours, and the paper developed with ninhydrin. The unidentified amino-acid gave the most intense spot, others were identified as glutamic acid, cystine, and glycine with traces of alanine and α -aminobutyric acid. The compounds did not move far in butanol-acetic acid but were identified by markers placed on the paper after electrophoresis.

Desulphurization with Raney nickel. Preliminary experiments with S-(N-ethylsuccinimido)-glutathione indicated that formation of norophthalmic acid (Table 1) could be achieved in about 50% yield by single passage of a solution of the thioether through a Raney nickel column over 48 hours. Loss of material by adsorption was minimized by using a small amount of nickel (about twice the weight of the sulphur compound).

Fraction 3F9 and the remainder of fraction 2F7 (total 3.3 ml) were combined, and concentrated in vacuo to a brown solution (0.6 ml) smelling of pyridine. A column, 6 mm high X 2.5 mm, of Raney nickel (20 - 25 mg) was prepared in the tapering portion of a Pasteur pipette. The solution was left on the column at 20°, and became green. Evolution

of gas was still detectable after 16 hours. The solution was flowed onto a second, freshly-prepared Raney nickel column then collected. All solution was on the second column after three days. During an attempt to clear a fine powder which formed and blocked the second column, some nickel passed into the eluate. The suspension was recycled and a green solution collected. This stage occupied more than two weeks, during which the first column was washed with water (0.4 ml) which passed straight onto the second column. Finally, the second column was washed. Water (0.5 ml), left on the nickel overnight, remained colourless, and was collected in a separate tube.

The eluate was analyzed during the course of the experiment. Three ninhydrin-positive compounds: unchanged material, norophthalmic acid, and neutral amino-acids were detected by electrophoresis in both 10% acetic acid and pyridine-acetate. The concentration of norophthalmic acid was about one-third that of unchanged peptide and about three times that of neutral amino-acids. Streaking occurred on chromatography in phenol-ammonia but unreacted peptide and norophthalmic acid were identified. Some material was held at the origin. One portion of eluate (10 μ l) was oxidized with performic acid. Duplicate papers were examined by electrophoresis in pyridine-acetate. One was developed with aniline-xylose and showed a spot in front of unchanged peptide. The other was developed with ninhydrin and showed that the unchanged material was oxidized to a compound which travelled almost to the position of GSO_3H . Norophthalmic acid was unaffected by oxidation.

The eluate was stored at -15° and re-examined when the structure

of the peptide had been established (p 80).

Material from the fourth fractionation (Table 6). Elution with weaker buffer was continued longer than in fractionation 3F to ensure that the two compounds with electrophoretic mobility of the peptide at pH 4 were eluted with different buffers. Fractions were examined by electrophoresis in pyridine-acetate. The compound in fraction 4F7 (mauve with ninhydrin) migrated fractionally further than the compound in fraction 4F9 (purple with ninhydrin) but combined fractions would have given a single spot. Fraction 4F7 also contained a trace of GSSG and possibly aspartic acid, and fraction 4F9 contained 5 - 10% of S-sulphoglutathione compared with the peptide. Concentration of the compound in 4F7, about 15mM, was greater than that in 4F9, about 5mM. Identical colours with ninhydrin were obtained from material in fractions 3F7 and 4F7.

Comparison of fractions 4F7 and 4F9. This was carried out by two-dimensional separation (Figure 4). Portions of each fraction (10 μ l) were placed 5 cm. from one end and 10 cm. from one side of a sheet of No. 3 paper 25 cm. X 46 cm. Electrophoresis in pyridine-acetate was carried out for 3.5 hours in the first direction; the paper was thoroughly dried, markers added, and chromatography in butanol-acetic acid at right-angles performed for 16 hours. Solvent ran off. Papers were developed with ninhydrin. Portions were also examined after hydrolysis and after oxidation, and the results are compared in turn.

Untreated fractions. The main spot in fraction 4F7 was a mauve colour. The main spot in fraction 4F9 was also mauve - purple at first but faded to a normal red colour. The compound had the same mobility in pyridine-

the original substance but slowly converted to the 2,4-dinitrophenyl derivative.

acetate as that in fraction 4F7 but was slightly less mobile in butanol-acetic acid.

Hydrolysis. The main compound in fraction 4F7 was unaffected by hydrolysis and was about 20 times the concentration of glutamic acid, glycine and cystine which appeared. In fraction 4F9, the position of the main compound was slightly altered and comparable amounts of glutamic acid and glycine, with less cystine appeared. The unidentified acid gave a mauve spot with ninhydrin.

Similar results were obtained when portions of fractions and hydrolysates were separated by electrophoresis in 10% acetic acid.

Oxidation. Little difference was observed between the fractions. The oxidized compound in 4F7 had the same electrophoretic mobility as GSO_3H (trace present), but travelled faster in butanol-acetic acid. In fraction 4F9, the oxidized peptide was slightly less mobile than GSO_3H in pyridine-acetate but only separated from it because of its higher mobility in butanol-acetic acid. Traces of cysteic acid appeared to be present in 4F7.

Oxidised samples were also examined by electrophoresis only. Both fractions contained a compound with the same mobility as GSO_3H in pyridine-acetate and which gave a pale mauve spot with ninhydrin. That in 4F9 was fractionally less mobile but the two would not have separated if the fractions had been combined. Different patterns were observed in 10% acetic acid. Fraction 4F7 yielded two ninhydrin spots, a trace of cysteic acid, and a compound travelling to the position of GSO_3H . The main spot detected in fraction 4F9 was more acidic than the original substance but clearly separated from GSO_3H .

Refractionation of 4F7. This was performed in order to try and isolate the sulphur amino-acid.

Dowex 50 (4% cross-linked, 200 - 400 mesh) was stirred with 4N HCl (120 ml) for 30 minutes, suspended in 2 N HCl (2 l) overnight, then washed with water until washings were pH 4. Resin was collected, stirred with 4 M pyridine (300 ml) for 30 minutes and washed with water (600 ml). It was suspended in 4 M pyridine (150 ml) overnight, washed with water (300 ml) and pyridine-formate buffer until washings had pH 2.5. Resin was packed under pressure into a column 96 cm. long X 1.2 cm.

Fraction 4F7 was brought to pH 2 with 90% formic acid and placed on the resin bed with a bent-tipped pipette. Elution was performed with degassed pyridine-formate buffer and 96 fractions (average 2.5 ml) were collected. Spots of fractions dried on paper were developed with ninhydrin, and fractions 20-23, 58-66, and 53-57 (weak) reacted. Fractions were pooled, and concentrated by evaporation below 40° in vacuo. Impurities in concentrated fractions caused material to travel slowly on electrophoresis in pyridine-acetate. The two main peaks were examined.

Fraction 20 was dried in vacuo and the residue dissolved in water (1.0 ml). A single ninhydrin-positive spot in the position of the sulphur amino-acid was detected after chromatography in butanol-acetic acid. On electrophoresis in 10% acetic acid, a compound was observed near the position of $\text{C}_6\text{H}_5\text{SO}_3\text{H}$ which gave a blue fluorescence in ultra-violet light and reacted with the molybdate reagent for phosphorus (see p 32). The sulphur amino-acid did not react with molybdate, and had a net positive charge at this pH by comparison with the mobility of

glucose (detected with alkaline silver) on the same paper. Mobility of the sulphur amino-acid on electrophoresis in borate buffer was greater than that of GSO_3H .

Pooled fractions 58-66 (21.0 ml) were concentrated to 0.6 ml. by evaporation in vacuo. Two ninhydrin-positive spots were detected on electrophoresis in 10% acetic acid: the sulphur amino-acid and a trace (about 5%) of S-sulphoglutathione. One portion was hydrolyzed and the hydrolysate examined on No. 3 paper by electrophoresis in pyridine-acetate for 3.5 hours then chromatography in butanol-acetic acid for 16 hours. One main ninhydrin-positive spot was observed which was mauve and in the position of the sulphur amino-acid. Traces of glutamic acid, glycine and cystine were also present.

Pooled fractions 58-66 were used for most of the other experiments.

Attempted isolation of the sulphur amino-acid. The concentrated solution (0.6 ml) was dried in vacuo, the residue redissolved in water and dried. Water (50 μl) was added and the solution transferred to a small centrifuge tube. A few specks of insoluble material settled out, the yellow supernatant was transferred to another tube and diluted with acetone (0.2 ml). The oily precipitate which separated overnight was triturated under acetone and the resultant solid dried in vacuo. A rather featureless infra-red spectrum was obtained from one portion. The remainder was dissolved in water and used for comparison with synthetic material.

2. Model compounds

Materials. Cysteine. Free base was prepared from the hydrochloride

as required. Cysteine hydrochloride (1 g) in ethanol (25 ml) was filtered after 30 minutes. Triethylamine (0.84 ml) was added slowly to the filtrate. Cysteine was collected, washed with ethanol and dried in vacuo.

Solutions of glutathione, cysteine, maleic acid, and fumaric acid at pH 7 were prepared immediately before use. Solid was dissolved in water, adjusted to pH 7 by addition of N sodium hydroxide, and diluted with water to the required concentration.

Methods

Attempted synthesis of S-(β -carboxyethyl)-glutathione (compare Virtanen & Matikkala 1960). In a typical experiment, β -bromopropionic acid (2.25 mg) was added to glutathione (3.81 mg) in 0.5 N lithium hydroxide (0.05 ml) and ethanol (0.1 ml) and left for 2 hours at 20°. The solution was examined by chromatography in phenol-ammonia for 16 hours on No. 4 paper. Ninhydrin-positive compounds were identified as GSSG, glutamic acid and probably GSH (no marker). Ethanol (0.1 ml) was added to the solution after 19 hours but only traces of solid were detected. The solution was also analyzed by electrophoresis. In pyridine-acetate, glutamic acid and glutathione (oxidized and reduced) were identified by their mobility; the sample studied in 10% acetic acid was rather weak and only GSSG, which appeared to have travelled rather slowly, was detected. S-(β -carboxypropyl)-glutathione (Virtanen & Matikkala 1960) had the same mobility as GSSG on electrophoresis in pyridine-acetate.

... (33.0 mg). This gave a single ...
... in pyridine-acetate. The ...
... broad absorption bands.

S-(α,β -dicarboxyethyl)-glutathione and S-(α,β -dicarboxyethyl)-cysteine

Synthesis of S-(α,β -dicarboxyethyl)-glutathione

Glutathione (151.7 mg) was added to maleic acid (57.85 mg) in water (1.5 ml) and the suspension warmed at 50° for 5 minutes to give a clear solution which was left at 20° for 25 hours. A sample (5 μ l) examined by electrophoresis in pyridine-acetate by ninhydrin contained one main spot which was purple, fading to red, attributed to S-(α,β -dicarboxyethyl) glutathione, with traces of glutathione and GSSG. The solution was treated with 0.3 M copper acetate (1.65 ml) and diluted with ethanol. An oil separated which slowly solidified. This was collected, washed with 50% (v/v) ethanol (until the washings were colourless) and ethanol, and dried (161.35 mg). A portion, examined by electrophoresis in pyridine-acetate, gave a single spot with ninhydrin.

The copper salt (151.7 mg), suspended in water (7.5 ml), was extracted with 0.1% (w/v) 8-hydroxyquinoline in chloroform (12 X 5 ml portions). The copper salt slowly dissolved and left an oily residue. The aqueous solution was transferred to a ^{fresh} separating funnel and extracted further (8 X 5 ml portions) until the chloroform layer was colourless. Each portion of chloroform solution was extracted successively with water (0.5 ml). The combined aqueous solutions were extracted with chloroform (5 X 5 ml) and the slightly yellow solution dried in vacuo. The resultant glass (31.3 mg) in water (0.1 ml) was treated with acetone (1.1 ml in portions). An oil separated which slowly solidified and was collected (24.0 mg). This gave a single ninhydrin-positive spot on electrophoresis in pyridine-acetate. The infra-red spectrum contained only broad absorption bands.

Comparison of rates and products of reaction. Reaction mixtures were prepared from fresh 100mM stock solutions pH 7. One mixture contained glutathione (0.5 ml) and fumarate (0.5 ml), the other glutathione (0.5 ml) and maleate (0.5 ml). Both were left at 20° and examined by electrophoresis after 4 and 22.5 hours (pyridine-acetate), and 47.5 hours (10% acetic acid). Papers were treated with ninhydrin. About four times as much S-(α,β -dicarboxyethyl)-glutathione was detected in the solution containing maleate as in that containing fumarate. In the maleate solution, the adduct represented about 50% of total ninhydrin-positive material after 4 hours and about 75% after 22.5 hours. Corresponding values for the fumarate solution were 10% and 20%. After 22.5 hours the fumarate solution contained about twice as much GSSG as the maleate solution. Little further change was observed in either mixture after 47.5 hours.

S-(α,β -dicarboxyethyl)-glutathione in the two solutions had the same mobility in two electrophoretic systems employed. Material obtained from each acid was hydrolysed. That from maleic acid was obtained by decomposition of the copper salt; that from fumaric acid was eluted from paper after electrophoresis in pyridine-acetate. Identical patterns were observed by electrophoresis (pyridine-acetate and 10% acetic acid) and chromatography (butanol-acetic acid).

Isonerisation (compare Morgan & Friedmann 1938). Maleic acid (11.65 mg) was dissolved in phosphate buffer pH 7.4 (1.0 ml) containing glutathione (30.75 mg) and left at 20°. Aliquots (0.1 ml) were removed, at intervals over one hour, diluted with water (0.9 ml) and stored at 4°. Portions

(5 μ l) of diluted samples were examined by electrophoresis in pyridine-acetate. S-(α, β -dicarboxyethyl)-glutathione was detectable after 5 minutes and 15-20% of reaction had occurred after 60 minutes. No increase in GSSG was detected during this time. Samples of the original mixture were also examined after 47.5 hours (electrophoresis in pyridine-acetate for 4 hours) and 78 hours (chromatography in butanol-acetic acid). Papers were developed with acridine and both showed a spot in the position of fumaric acid. Maleic acid and fumaric acid are separated in both the systems employed.

Synthesis of S-(α, β -dicarboxyethyl)-cysteine.

Fumaric acid (213.8 mg) was added to cysteine (220.15 mg) in water (3 ml) and left overnight at 20°. The suspension was heated at 60° for a few minutes to dissolve the remaining fumaric acid. Paper electrophoresis in pyridine-acetate gave two ninhydrin-positive spots, one for S-(α, β -dicarboxyethyl)-cysteine and one for cystine, and indicated about 90% reaction. The solution was diluted with acetone and the solid collected, washed twice with acetone, and dried (406.2 mg). Solid was dissolved in hot water (2 ml) and the cold solution filtered. It gave a single ninhydrin spot after electrophoresis, indicating removal of cystine. The solution was diluted with acetone and the product collected as before. Yield 374.4 mg, 87%. This material had an infrared spectrum identical with that obtained from the product derived from maleic acid by the same method (Figure 6). The latter was re-precipitated twice and had M.P. 125-126°.

The equivalent weight was obtained by titrating portions (1 ml)

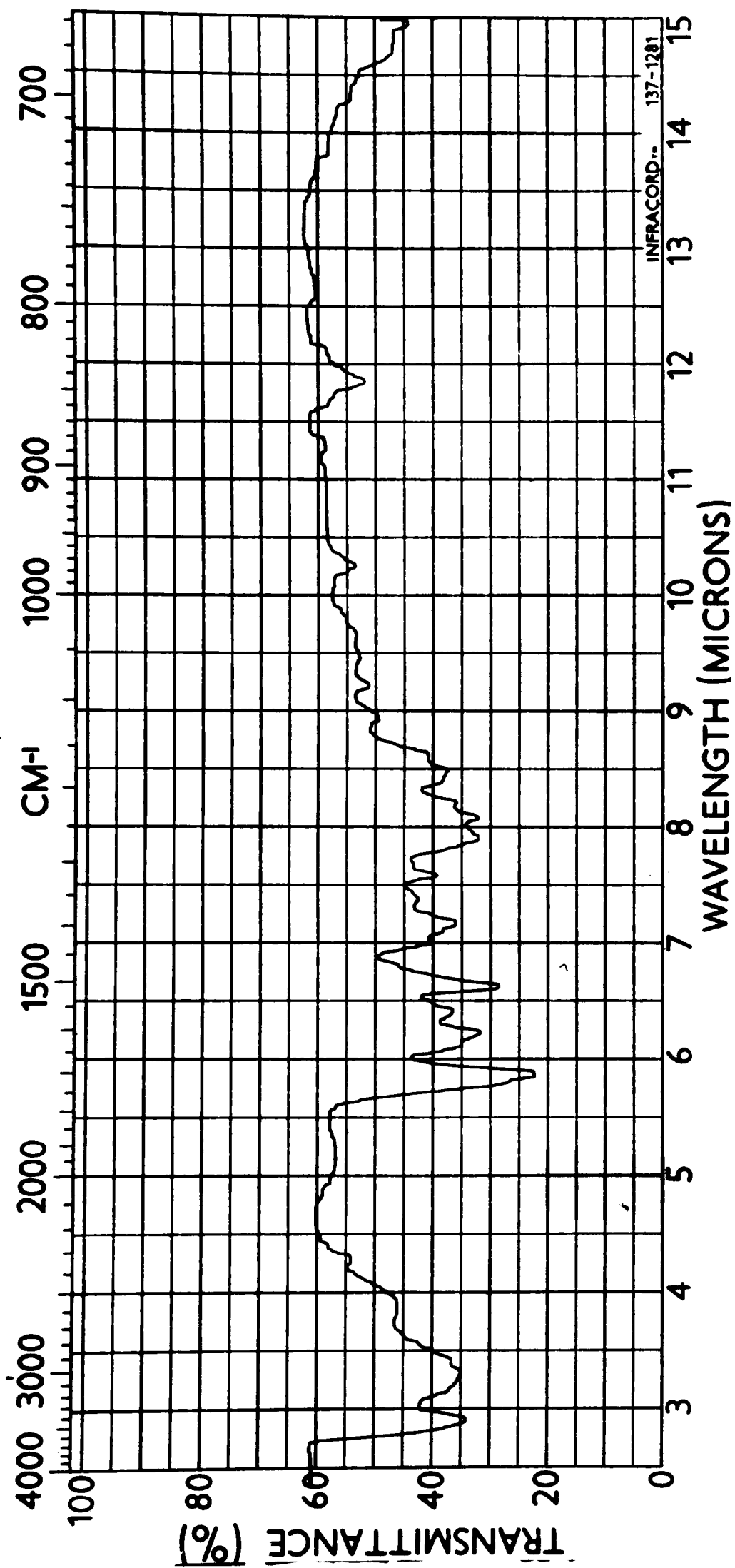


Figure 6.

INFRA-RED SPECTRUM OF
S-(α / β -DICARBOXYETHYL)-CYSTEINE

of a solution of S-(α, β -dicarboxyethyl)-cysteine (11.57 mg) in water (5 ml) with 0.011 N sodium hydroxide, using bromothymol blue as indicator. The average titre, 1.76 ml, indicated a molecular weight of 244 titrating as a dibasic acid (required 237).

Presence of acetone. S-(α, β -dicarboxyethyl)-cysteine (11.4 mg) in water (0.1 ml) was treated with 1% (w/v) 2 : 4-dinitrophenylhydrazine in 2 N HCl. The yellow precipitate was collected and dried (0.6 mg). The filtrate was examined by electrophoresis (pyridine-acetate) and contained all the amino-acid. A portion of solid (0.1 mg) in ethyl acetate (40 μ l) gave a single yellow spot which co-chromatographed with acetone dinitrophenylhydrazone in butanol-ethanol. The solid had M.P. 121-122° identical with that of acetone dinitrophenylhydrazone prepared by the same method and also not recrystallized (literature M.P. 123°).

Preparation of dicyclohexylamine salt. 1) From fumaric acid. Fumaric acid (214.4 mg) was added to cysteine (220.65 mg) in water (3 ml). The mixture was left at 20° overnight then heated briefly at 60° to dissolve the remaining fumaric acid. After a further 48 hours, dicyclohexylamine (0.725 ml) was added, and the resultant yellow solution diluted with acetone. The crystalline solid was collected, washed with acetone, and dried (305 mg). The filtrate yielded a further crop of crystals (460.6 mg). The salt was recrystallized from water by addition of acetone and had M.P. 118-119° which was not altered when mixed with the product from maleic acid.

11) From maleic acid. Maleic acid (582.45 mg) was dissolved in water (5 ml) containing cysteine hydrochloride (394 mg). Dicyclohexylamine (2 ml) was added and insoluble amine hydrochloride (592 mg) collected. The filtrate deposited more solid which was discarded. Another portion of dicyclohexylamine (0.5 ml) was added, and the solution diluted with acetone. Crystalline solid was collected in three crops (962.3 mg) and dried. The needles were recrystallized from water by dilution with acetone and had M.P. 118° . The infra-red spectrum is shown in Figure 7. Found: C 57.6, H 9.8, N 6.4, loss on drying in vacuo at 100° 15.1%. $C_{31}H_{57}N_3O_6S \cdot 2\frac{1}{2}H_2O$ requires C 57.7, H 9.7, N 6.5, loss on drying 9.7%. Analysis indicated the presence of water of crystallisation but this could not be confirmed because the loss in weight on drying, and analysis of dried material, showed loss of amine as well as water.

A solution of the salt (2.9 mg) in water (0.435 ml) was examined by electrophoresis in pyridine-acetate and compared with markers of the free amino-acid on the same paper. Strength of the ninhydrin colour indicated a 10mM solution as required for a di-amine salt.

Properties

Stability. S-(α, β -dicarboxyethyl)-cysteine is freely soluble in water to give an acid solution (pH about 2). A solution, adjusted to pH 8 with N sodium hydroxide and containing a drop of chloroform, showed no change after four weeks when examined by electrophoresis in pyridine-acetate with ninhydrin. No reaction was obtained with the iodine-azide reagent after electrophoresis in pyridine-acetate. Cystine, cystathionine, methionine, and glutathione on the same paper all gave positive reactions.

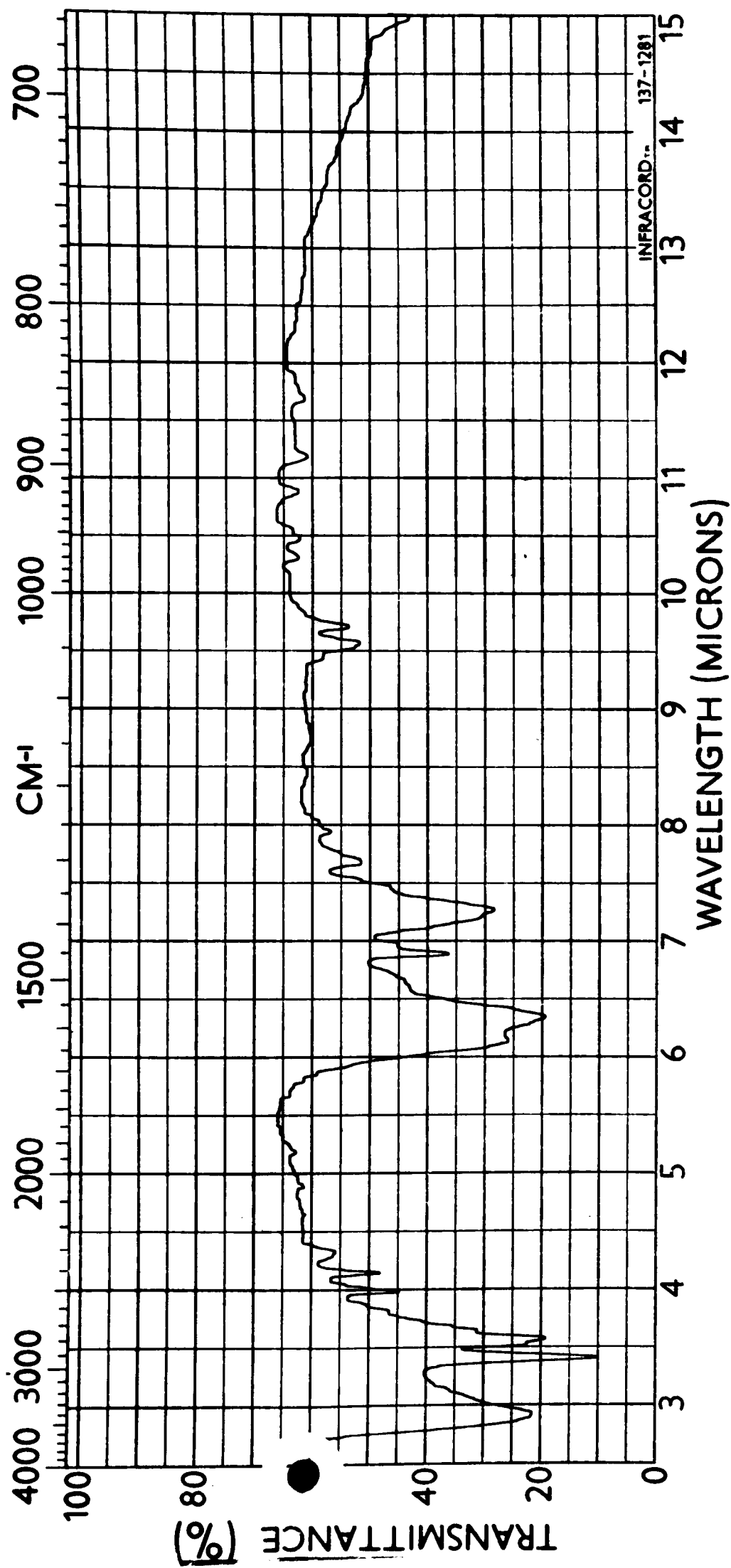


Figure 7.

INFRA-RED SPECTRUM OF

DI-DICYCLOHEXYLAMINE SALT OF

S-(α/β -DICARBOXYETHYL)-CYSTEINE

S-(α , β -dicarboxyethyl)-glutathione is readily soluble in water and gives an acid solution (pH about 2). It is stable in aqueous solution at pH 2, at pH 7, and at pH 7 in solutions which also contain fumarate or glutathione. No change was observed in the three solutions at pH 7 after one week when they were examined by electrophoresis in pyridine-acetate. A solution at pH 10 contained traces of neutral amino-acids and, probably, glutamic acid after 5 days.

Oxidation. Peptide (0.87 mg) in water (0.1 ml) was oxidized with performic acid (0.2 ml). S-(α , β -dicarboxyethyl)-cysteine (0.7 mg) was treated similarly. Oxidized material was examined by electrophoresis. In pyridine-acetate, both solutions gave a single ninhydrin-positive spot with the mobility of glutathione sulphonie acid. The oxidized peptide travelled slightly less rapidly than GSO₃H but would not have separated if the solutions had been superimposed. Oxidized amino-acid could not be separated from glutathione sulphonie acid in 10% acetic acid but the oxidized peptide was detected about halfway between GSO₃H and the unoxidized material. Traces of GSO₃H in the solution of S-(α , β -dicarboxyethyl)-glutathione sulphone observed in 10% acetic acid probably arose from traces of GSSG in the original solid.

The amino-acid sulphone was separated from glutathione sulphonie acid by chromatography in butanol-acetic acid on No. 3 paper for 46 hours.

The pattern observed on oxidation of fractions 4F7 and 4F9 (Figure 4) was confirmed by separation of marker solutions of GSO₃H, and oxidized samples of S-(α , β -dicarboxyethyl)-glutathione and S-(α , β -dicarboxyethyl)-cysteine on a single paper under the same experimental conditions.

Hydrolysis. Solutions of the peptide and of the amino-acid were hydrolysed. Peptide hydrolysate gave 3 ninhydrin-positive spots on electrophoresis in pyridine-acetate: neutral amino-acids, glutamic acid, and S-(α, β -dicarboxyethyl)-cysteine which gave a mauve colour. In 10% acetic acid, additional spots were observed: cystine (about 10% of total sulphur amino-acid), and a trace spot near the position of the oxidised peptide. Formation of cystine was confirmed by chromatography in butanol-acetic acid.

Hydrolysate of S-(α, β -dicarboxyethyl)-cysteine on electrophoresis in pyridine-acetate gave unchanged material and a weak ninhydrin-positive spot in the position of neutral amino-acids. The cyclic compound (see below) could not be detected on a duplicate paper which was developed with acridine. Chromatography in butanol-acetic acid showed that the hydrolysate contained 5-10% of cystine but no alanine. The same results were obtained after hydrolysis for 20 hours.

Action of heat on the amino-acid. 1) In aqueous solution. S-(α, β -dicarboxyethyl)-cysteine (4.6 mg) in water (0.2 ml) was sealed in two hydrolysis tubes. The tubes were heated at 110° , one for 20 hours, and one for 40 hours. No evolution of gas occurred and the pale yellow solutions had an unpleasant fleshy odour. Solutions were examined by electrophoresis and by chromatography. About 10% of the amino-acid remained after 40 hours and a further 10% of ninhydrin-positive material in the position of neutral amino-acids was detected in pyridine-acetate. A compound, more acidic than the amino-acid, slowly gave a weak atypical purple colour with ninhydrin. This substance was probably a cyclic

compound and was detected both with acridine and with aniline-xylose. The same results were obtained after chromatography in butanol-acetic acid and in phenol-ammonia. Papers were developed with ninhydrin and with acridine. Cystine was absent, and the presence of alanine was confirmed by co-chromatography with a marker.

ii) Solid phase. S-(α, β -dicarboxyethyl)-cysteine (33 mg) was heated on an oil-bath at 120-125° for 30 minutes. Gas was evolved, and the melt solidified to a yellow glass. The loss in weight was 14.6%. The glass was dissolved in water and examined by electrophoresis in pyridine-acetate. Small amounts of unchanged material and of neutral amino-acid (alanine) were detected with ninhydrin. The main product quenched ultra-violet light and slowly gave a weak purple colour with ninhydrin. This was assumed to be the cyclic compound, and was essentially uncharged in 10% acetic acid and did not migrate as far as the amino-acid. Similar results were obtained by chromatography in butanol-acetic acid and in phenol-ammonia. The cyclic compound could not be isolated using ethyl acetate, ethanol, or chloroform. Some purification was achieved by extracting the glass with boiling acetone.

Comparison with lens material. 1) Amino acid. Lens material and synthetic S-(α, β -dicarboxyethyl)-cysteine gave single spots when run together on electrophoresis in pyridine-acetate and in 10% acetic acid, and after chromatography for 16 hours on No. 3 paper in butanol-acetic acid and on No. 4 paper in butanol-acetic acid-water-pyridine. Both compounds gave the same mauve colour with ninhydrin when run on separate spots.

Solutions of lens compound and synthetic amino-acid were heated for 40 hours at 110° in sealed tubes. Products were identified by chromatography in butanol-acetic acid. Papers were developed with acridine and with ninhydrin. The change in pattern on heating and the patterns observed were identical for the two solutions.

Lens and synthetic amino-acid gave a single ninhydrin-positive spot after oxidation. The oxidized substance was not separated from glutathione sulphonic acid after electrophoresis in 10% acetic acid in each case.

Synthetic S-(α , β -dicarboxyethyl)-cysteine (1.7 ng) in water (0.7 ml) was eluted from the Dowex 50 column (p⁶⁷) with pyridine-formate buffer pH 2.5. Elution was carried out at 9.6 ml/hour and 72 fractions of about 2.2 ml collected. The amino-acid was eluted in fractions 59-66 with a peak at fractions 62-64. This is equivalent to approximately 3.5 column volumes of buffer. Lens material in fraction 4F7 was eluted in approximately 3.6 column volumes. An attempt to recover solid material was not satisfactory as the solid was contaminated with impurities and gave a poor infra-red spectrum.

41) Peptide. Synthetic S-(α , β -dicarboxyethyl)-glutathione and the lens peptide travelled to the same position on electrophoresis in both pyridine-acetate and 10% acetic acid. The lens peptide (fraction 4F9) had slightly lower mobility than the synthetic sample when run separately because of impurities in the fraction. Identity was established by superimposing spots of the two solutions before electrophoresis. Chromatography was carried out by the same method in butanol-acetic acid and

butanol-acetic acid-water-pyridine for 16 hours on No. 4 paper. In the latter solvent, the peptide just separated from glutathione sulphonie acid and was well separated from GSSG and S-(α,β -dicarboxyethyl)-cysteine.

Hydrolysates of lens and of synthetic peptide were identical when analyzed by electrophoresis in pyridine-acetate, and by chromatography in butanol-acetic acid for 16 hours on No. 4 paper. The two hydrolysates were run separately and superimposed on each paper. Amino-acids were identified by superimposing marker solutions of glycine, glutamic acid, cystine, and S-(α,β -dicarboxyethyl)-cysteine.

A portion (20 μ l) of fraction 4F9 was oxidized with performic acid and oxidized material submitted to electrophoresis in 10% acetic acid. A ninhydrin-positive spot in the position of the oxidized peptide was obtained which was not separated from a synthetic sample.

Re-examination of lens extract fractions.

A number of fractions from the lens extracts were analyzed by electrophoresis and by chromatography with markers of S-(α,β -dicarboxyethyl)-cysteine and S-(α,β -dicarboxyethyl)-glutathione on the same spot. Both substances were identified in fractions 1F7 by chromatography in butanol-acetic acid on No. 4 paper for 17 hours. The peptide was identified in fractions 2F8 and 2F9; the amino-acid was found in fractions 2F6 and 4F6. Fractions 3F8 and 4F8 contained traces of material which migrated to the position of the synthetic substances on electrophoresis in pyridine-acetate.

The presence of both compounds in fraction 1F8 was shown by

oxidation and examination of oxidized material in 10% acetic acid.

Desulphurisation with Raney nickel (p 63). The material obtained from this experiment was stored at -15° and re-examined when the sulphur compounds had been identified. About 40% of the material had reacted with Raney nickel. Norophthalmic acid and alanine were identified by electrophoresis in pyridine-acetate and in 10% acetic acid, and by chromatography in butanol-acetic acid and in ethanol-ammonia. The amount of alanine was about one-quarter that of norophthalmic acid as judged by the depth of colour with ninhydrin. Unreacted S-(α, β -dicarboxyethyl)-cysteine and S-(α, β -dicarboxyethyl)-glutathione were identified with synthetic markers.

Succinic acid was located with acridine. It was not well separated from norophthalmic acid by electrophoresis in pyridine-acetate, and malic acid travelled to a similar position on chromatography in ethanol-ammonia. Malic acid and succinic acid were separated in butanol-acetic acid. No malic acid was detected in the reaction products.

Examination of extraction procedures

Phenol extraction. Solutions (5mM) of S-(α, β -dicarboxyethyl)-cysteine and S-(α, β -dicarboxyethyl)-glutathione were extracted with 90% (v/v) phenol and examined by electrophoresis in pyridine-acetate. No decrease in concentration of amino-acid or of peptide was observed when compared with the strength of ninhydrin colour given by marker solutions of various concentrations on the same paper.

Extract prepared with N-ethylmaleimide 1) Competition reaction.

Glutathione in solution at pH 7 (150mM, 0.5ml) was added to fumarate at

cysteine (about 75%) and cysteine.

pH 7 (150mM, 0.5 ml) and N-ethylmaleimide (150mM, 0.5 ml). A second solution which contained water (0.5 ml) in place of N-ethylmaleimide was prepared as a control. Two other mixtures were prepared with cysteine in place of glutathione. The solutions were left at 20° and samples (5 μ l) examined after 1 hour by electrophoresis in pyridine-acetate, and after 4 hours by chromatography in butanol-acetic acid-water-pyridine for 16 hours on No. 4 paper. Both papers were developed with ninhydrin.

The glutathione competition solution contained only GSSG and S-(N-ethylsuccinimido)-glutathione after 1 hour, while the control contained about 4% of S-(α , β -dicarboxyethyl)-glutathione. The same result was obtained by chromatography. The amount of GSSG in the competition mixture (about 10% of total glutathione) probably indicated the amount present initially in the glutathione used for preparation of the solutions.

Cysteine, cystine and S-(N-ethylsuccinimido)-cysteine are not separated in pyridine-acetate. The competition solution contained about 2% of S-(α , β -dicarboxyethyl)-cysteine and the control about 20%. The compounds were readily identified by characteristic colours with ninhydrin following chromatography in butanol-acetic acid-water-pyridine: cystine yielded a brown spot and S-(α , β -dicarboxyethyl)-cysteine an indigo spot. The amount of adduct in the competition solution was unchanged after 4 hours, the other compounds present were identified as cystine (about 8% of total) and S-(N-ethylsuccinimido)-cysteine (90%). The control solution contained S-(α , β -dicarboxyethyl)-cysteine (about 75%) and cystine.

ii) Lens experiment. Fresh calf lens (1.04 g) was ground in a glass mortar with a little sand under a solution of N-ethylmaleimide (3.75 mg) in water (1 ml) for 10 minutes. Protein was precipitated by addition of 90% (^W/v) phenol (1 ml) and centrifuged down. The clear aqueous layer was extracted with chloroform (1.5, 1.0 and 0.5 ml successively) and the layers separated by centrifuging after each extraction. The final aqueous layer was dried in vacuo. Residue (14.1 mg) was dissolved in water and examined by electrophoresis in pyridine-acetate. Six spots were obtained with ninhydrin and were assigned to neutral amino-acids, glutamic acid, S-(N-ethylsuccinimido)-glutathione, GSSG, aspartic acid and S-(α , β -dicarboxyethyl)-glutathione. The amount of peptide and amino-acid detected indicated a concentration of about 0.3mM in the lens originally. One portion of residue (equivalent to 35% of the total) was examined on No. 3 paper by electrophoresis in pyridine-acetate for 4 hours in the first direction, followed by chromatography in butanol-acetic acid-water-pyridine in the second direction for 15 hours. The paper was developed with ninhydrin and the following substances identified: S-(α , β -dicarboxyethyl)-glutathione, S-(α , β -dicarboxyethyl)-cysteine (about 10-20% of peptide), S-(N-ethylsuccinimido)-cysteine, and cystine, S-(N-ethylsuccinimido)-glutathione, GSSG (about 5% of total glutathione) and S-sulphoglutathione. Another portion was examined on No. 3 paper by electrophoresis in formic-acetic acid for 3.5 hours then chromatography in butanol-acetic acid for 16 hours. The paper was developed with ninhydrin and the same series of compounds was identified. The total concentration of cysteine/cystine detected as S-(N-ethylsuccinimido)-

cysteine and cystine was about the same as that of GSSG.

Extraction procedure without lens (see p 20). Ice-cold 7% (^w/v) trichloroacetic acid (4.0 ml) was added to glutathione (30.7 mg) and fumaric acid (3.4 mg) in water (10 ml) at 4°. The solution was left for 4.5 hours then extracted with N-methyl dioctylamine (4.5 ml) in chloroform (90 ml) in three portions (52.5, 26.25, and 15.75 ml). The slightly opaque aqueous layer was stirred with Celite and filtered. A small volume of solution was sucked down the water pump. The clear filtrate (43 ml) had pH 2.5 (indicator paper) and was stored at 4° overnight. It was concentrated (to 2.1 ml) by evaporation below 39° in vacuo and examined by electrophoresis in pyridine-acetate. Three ninhydrin-positive spots were obtained, and identified as GSH, GSSG and S-(α , β -dicarboxyethyl)-glutathione. The concentration of the last, about 1mM, represented about 2% of the total glutathione.

Preliminary study of biosynthesis. Three reaction mixtures were prepared. The first was a control and contained fumarate (150mM, 1 ml) and cysteine (150mM, 1 ml) at pH 7, with water (1 ml). The second contained a fresh calf lens in place of water. The third was a lens control and contained a fresh calf lens in water (2 ml). At time 0, fumarate was added and the lenses were ground in glass mortars with the appropriate solution for 5 minutes in order to disintegrate the tissue. After being incubated at 37° for 2 hours, the three mixtures were each shaken thoroughly with 90% (^w/v) phenol (3 ml) and centrifuged for 15 minutes at 4,600 rev/min. A fourth mixture, which had the same composition as the first, was prepared when phenol had been added to the other three.

This was a control for the extent of reaction after incubation. Samples (5 μ l) of each mixture were placed on paper 10 minutes later and the fourth solution had then been mixed for 25 minutes. Electrophoresis was performed in pyridine-acetate and the paper developed with ninhydrin. The fourth solution contained about 5% of S-(α , β -dicarboxyethyl)-cysteine. No difference was detected between the mixture with the lens and the one without lens. Both contained approximately 60% of adduct, identified by a marker solution superimposed on a second set of samples on the same paper. A ninhydrin-positive spot attributed to the amino-acid or peptide could just be detected in the mixture which contained only lens material.

A similar experiment was performed with glutathione in place of cysteine. Solutions were incubated for 4 hours because the addition reaction is slower than that with cysteine. The incubated reaction mixture contained GSH, GSSG (about 25% of total glutathione), and S-(α , β -dicarboxyethyl)-glutathione (about 10-15% of total). The mixture incubated with lens homogenate contained only about two-thirds of the amount of glutathione present in the control, and S-(α , β -dicarboxyethyl)-glutathione represented about 5% of total ninhydrin-positive material. The fourth mixture contained a trace of adduct (about 1% of total).

Summary.

A new amino-acid and an acidic peptide containing this acid were separated by column chromatography of lens extracts. The relationship between the peptide and amino-acid was established by hydrolysis. The peptide also contained glutamic acid and glycine. The presence of sulphur was indicated by behaviour of the two compounds after oxidation, and an S-substituted cysteine residue indicated by desulphurization with Raney nickel to alanine.

The compounds: S-(α, β -dicarboxyethyl)-glutathione and S-(α, β -dicarboxyethyl)-cysteine were synthesized by addition of the thiol groups of cysteine and glutathione to the double bonds of either maleic or fumaric acids. The compounds were not separated from lens material by electrophoresis in two buffers and chromatography in two solvents, nor could they be separated after oxidation. Identical products were obtained on hydrolysis of lens and synthetic peptides, and after heating aqueous solutions of lens and synthetic amino-acids. The amino-acids were also eluted in the same place from a Dowex 50 column.

Both substances were present in a lens extract prepared in the presence of N-ethylmaleimide and do not appear to be artifacts of the extraction procedure. Lens homogenates do not appear to catalyze the addition of either cysteine or glutathione to fumarate.

Part IV. Other constituents of the lens

Introduction

This Part is concerned with the identification of a number of other less well-known constituents of the lens. These are mostly peptides and amino-acids but include a few other low-molecular-weight compounds. Work described here stems from a study of the chemical composition of the lens and the identification of the various constituents which have been previously described in Part III (see also Table at end of Chapter). It became clear that a complete picture of the lens was possible only by the separation and analysis of individual fractions and this is a lengthy process and required material in quantities which are not readily available. A technique used in the study Part IV which facilitates this, therefore, adopted for analysis of extracts of single lenses in order to obtain such non-quantitative information fairly easily.

Other constituents of the lens

The following section is devoted to the presentation of this Part. Compounds which may be previously mentioned these constituents are described in the first section. These are a basic tripeptide, which appears to be an enzyme, and a compound which appears to be a nucleoside. The second section is concerned with analysis of various fractions and extracts of lens. Identification of some compounds present in these extracts is described in detail in the third section.

Discussion for each section is given in detail before the complete experimental results are given. The results of the analysis of the various fractions are given in detail in the third section. The results of the analysis of the various extracts are given in detail in the fourth section.

Part IV. Other constituents of lens

Introduction

This Part is concerned with the identification of a number of other low molecular-weight constituents of the lens. These are mostly peptides and amino-acids but include a few ninhydrin-negative compounds. Work described here stemmed from a study of fractions obtained during separation of the new acidic compounds from lens extracts which was described in Part III (see also Table at end of thesis). It became clear that a complex mixture of substances was present in the extracts. Separation and analysis of individual fractions was quite a lengthy process and required material in quantities which are not readily available. A technique used in the study of individual fractions was, therefore, adapted for analysis of extracts of single lenses in order to obtain such semi-quantitative information fairly quickly.

The following method is employed for presentation of this Part. Compounds which may be previously unidentified tissue constituents are described in the first section: these are a basic tripeptide, what appears to be an amine, and a compound which contains guanine. The second section is concerned with analysis of various complete crude extracts of lens. Identification of some compounds present in these extracts is described in detail in the final section. Discussion for each section in turn is presented before the complete experimental results.

Discussion

1. New compounds

A number of unidentified substances were detected during examination of the fractions obtained from lens extracts. Many of these substances were present in trace quantities and only 3 compounds, discussed in this section, were studied in detail. The basic peptide had a characteristic electrophoretic mobility at pH 4 which facilitated its separation; the amine had somewhat unusual chromatographic properties; and the phosphorus-containing compound had been partly purified during the separation of S-(α , β -dicarboxyethyl)-cysteine.

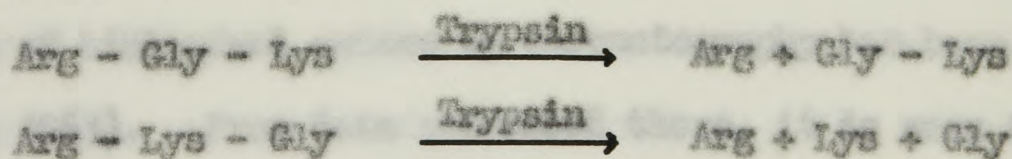
Arginyllysylglycine. The basic character of this compound was indicated by its electrophoretic mobility. A peptide structure was likely since the substance was destroyed by hydrolysis with an increase in concentration of amino-acids. For preparation of the peptide, advantage was taken of the separation which can be achieved by continuous electrophoresis even between compounds of similar mobility, in this case the peptide, β -alanine, and γ -aminobutyric acid. This method also yielded larger amounts of material than were obtained by elution from strips of paper after electrophoresis.

Hydrolysis of the peptide gave lysine, glycine, and arginine. Quantitative analysis of the hydrolysate, based on arginine as the sole basic amino-acid, gave a ratio glycine : arginine of 1 : 2.2. Dinitro-phenylation was not entirely successful, partly because of the small amounts of peptide available. DNP-arginine was identified by co-chromatography with a marker in butanol-acetic acid. ϵ -DNP-lysine was

not detected. It would not be confused with dinitrophenol, which travels to a different area on the paper, and, being an indicator, is decolourised by acid.

Gros et al (1961) deduced the structure of leucyl-arginyllousine from pig pituitary, on the basis of amino-acids liberated on hydrolysis, dinitrophenylation to indicate the N-terminal amino-acid, and the action of carboxypeptidase to identify the C-terminal amino-acid. For the lens peptide, information on the amino-acid sequence could be obtained by an alternative enzymic method. Trypsin specifically attacks peptide bonds on the carboxyl side of arginine and lysine. This affords a useful method for controlled degradation of polypeptide or protein chains (e.g. Ingram 1956).

The lens substance appeared to be a tripeptide containing lysine, glycine, and arginine, with arginine as N-terminal amino-acid so that only two structures were possible: arginylglycyllysine or arginyllysylglycine. These would be attacked by trypsin with different results. In the former, only arginine is linked by the carboxyl group in a peptide bond, and this is the only bond susceptible to the action of trypsin. In the latter, both basic amino-acids are linked by their carboxyl groups in peptide bonds and trypsin should degrade the molecule completely to free amino-acids:



The lens peptide was only slowly attacked by trypsin at room temperature, and the reaction was conveniently performed on a micro scale

with portions of the mixture in micro-pipettes. This prevented evaporation of the solution and permitted study of the reaction at various times with comparable volumes of solution. When digestion was carried out at higher temperatures, the results were complicated by self-digestion of the enzyme. Results obtained from reactions under various conditions studied by electrophoresis or chromatography in different solvents indicated that arginine, lysine, and glycine were liberated, not arginine and a peptide. It is tentatively concluded, therefore, that the probable structure of the lens tripeptide is arginyllysylglycine. Assuming that no degradation of the peptide occurs in stored lens (see below), the concentration in lens appears to be about 0.02mM or 7.5mg/kg fresh weight. The occurrence of this peptide is discussed in Part V.

Unidentified amine. This substance was first noted because of its high R_F in butanol-acetic acid. Since its mobility was essentially the same in 3 chromatographic solvents, it seemed unlikely that an acidic group was present. The presence of a single unsubstituted amino group was indicated by reaction with ninhydrin, and by the electrophoretic mobility which was similar to that of the basic amino-acids. The substance was unaffected by hydrolysis.

Spermine, diaminopropanetetramethylenediamine, has been identified in lens (Krause 1937) but travelled only very slowly on chromatography. Identification of biological amines by chromatography has been discussed recently (Blau 1961). From data presented there, it is seen that the R_F of aliphatic amines in butanol-acetic acid-water-pyridine increases with length of the side-chain. Pentylamine has an R_F 0.87. Aromatic amines,

such as aniline, have high R_F values but these decrease with increase in length of the side-chain.

The lens compound was not observed in the extract prepared in absence of tissue (p. 21), so does not appear to be an artifact. It yielded a picrate and probably a hydrochloride, and appeared to be attacked by nitrous acid. It seems possible that the lens compound is an unsubstituted aliphatic long-chain amine. Dodecylamine was found to have an R_F 0.91 in butanol-acetic acid, and from the data of Blau it seems unlikely that the carbon chain contains less than 6 atoms. No amines of this type have previously been observed in tissue.

Phosphorus-containing compound. Fraction 4F7 was refractionated by chromatography on Dowex 50 in order to purify S-(α , β -dicarboxyethyl)-cysteine (p. 67). A substance was detected in this fraction, which was not adsorbed on the column and which gave a blue fluorescence under ultraviolet light after electrophoresis in 10% acetic acid. A fluorescent peptide has been detected in lenses of primates but not of calf or cow (Francis, Rabay & Recoules 1961). The substance in fraction 4F7, however, did not react with ninhydrin before hydrolysis, but gave a positive reaction for phosphorus with molybdate.

Attempts to separate the substance by electrophoresis of portions of fraction 4F6 in 10% acetic acid yielded two compounds with the same mobility which were separated in pyridine-acetate. Ninhydrin-positive contaminants were removed by this method. The effect of ultraviolet light was pH - dependent, since quenching was observed after electrophoresis at pH 4 and at pH 9 (sodium borate). Since only traces

of ninhydrin-positive material were liberated on hydrolysis, much less than the amount of phosphorus detected, it seems unlikely that the compound contains amino-acids.

One portion of material was hydrolysed to see whether it contained a nucleotide base. Under the experimental conditions employed, nucleotides would be expected to yield purine bases (adenine and guanine) and pyrimidine nucleosides (cytidine and uridine) together with a sugar (ribose) and inorganic phosphate (see Chargaff & Davidson 1955 for a full discussion). Guanine was identified in the hydrolysate but the main phosphorus-containing product was not inorganic phosphate. Both guanosine monophosphate and guanosine triphosphate are probably present in lens (Hookwin 1961) but these would probably yield mostly inorganic phosphate on hydrolysis.

2. Investigation of whole extracts

Preparation of fractions of lens extracts (Part II) requires material in quantities not readily available, but a considerable degree of concentration was achieved in these fractions which enabled identification of a number of compounds in lens for the first time. Individual lens fractions were analysed by a combination of electrophoresis in formic-acetic acid at pH 1.6 in the first direction followed by chromatography in butanol-acetic acid in the second (S.G. Waley, unpublished). This is similar to a method employed for analysis of peptide hydrolysates but which used high-voltage electrophoresis (Richmond & Hartley 1959). Another version had previously been used for separation of peptides obtained by enzymic degradation of a protein since a better separation

of large molecules is obtained by electrophoresis than by chromatography (Ingram 1956).

Since compounds in the various lens fractions tended to occupy different regions of the paper, it seemed likely that the method could be extended to analysis of unfractionated lens extracts. A combination of electrophoresis and chromatography has one important advantage over two-dimensional chromatography for the examination of tissue extracts. Electrophoresis acts as a desalting process since many inorganic ions migrate off the paper and do not interfere subsequently with chromatography. This avoids the necessity of desalting the extract before analysis, and gives a reproducible pattern under standard conditions. Although some slight variation in position was observed, usually only in the direction of chromatography, the pattern was constant, which aided identification of compounds. Tryptophan, which fluoresced under ultraviolet light, proved to be a useful internal marker.

A reasonably good separation of all ninhydrin-positive compounds is obtained (Figure 8). The pattern is more congested in crude extracts than in hydrolysates. In a few cases a good separation is not achieved but this is confined to some of the basic amino-acids, the isomeric leucines, and to the γ -glutamyl peptides which travel near the apex of the paper. Many of the compounds were identified in lens fractions before two-dimensional analysis was performed and their identification is considered in the following section. Discussion is confined ^{here} to differences between various types of lens extract.

A comparison was made between trichloroacetic acid extracts

CALF LENS EXTRACT.

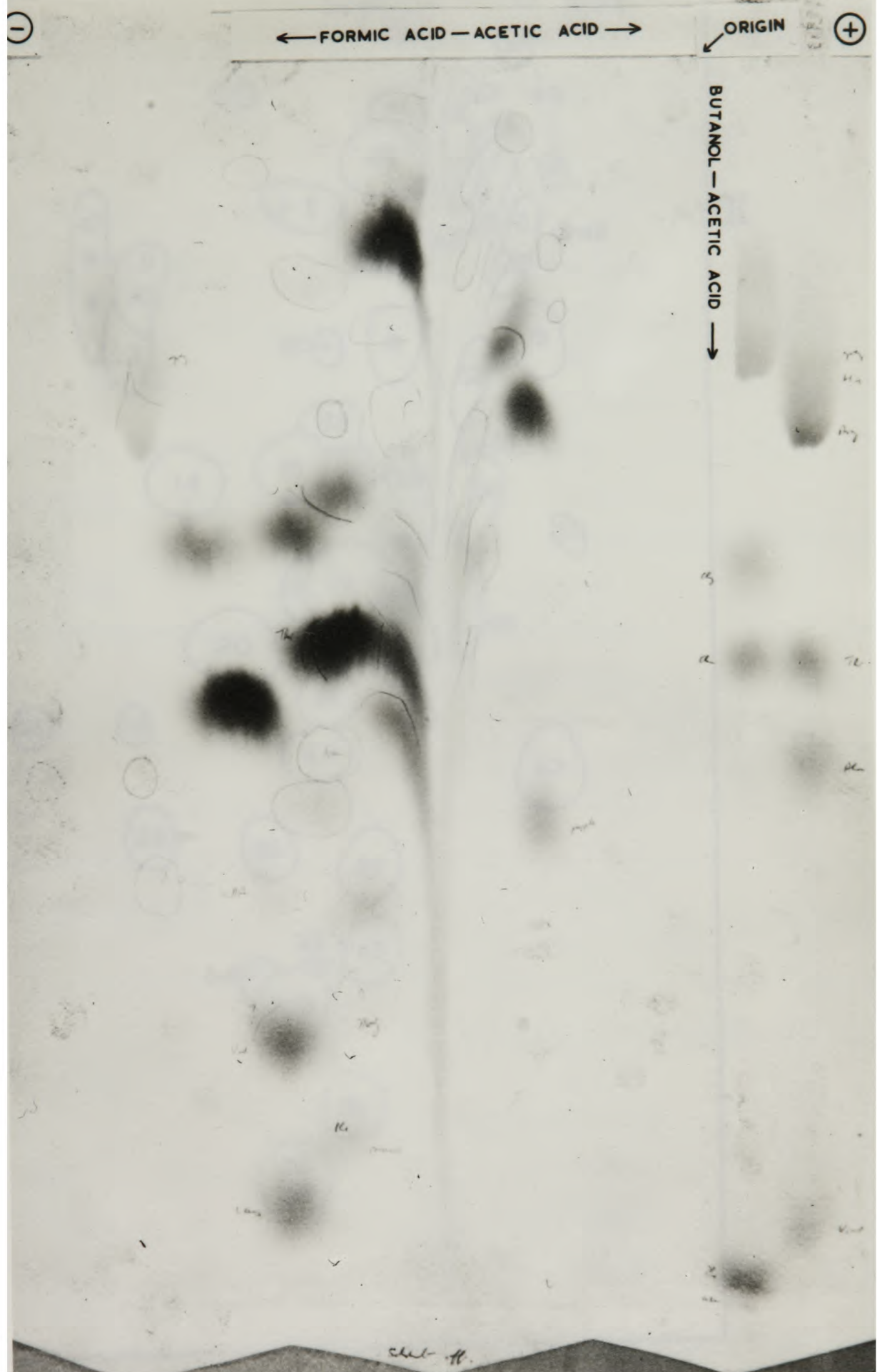


Figure 8. Separation of trichloroacetic acid extract of fresh calf lens by electrophoresis at pH 1.6 followed by chromatography. The method is explained on p.110, markers are on the extreme right. Paper was developed with ninhydrin and contains half of the extract from a single lens. Faintest spots do not show clearly. A key is given on following pages

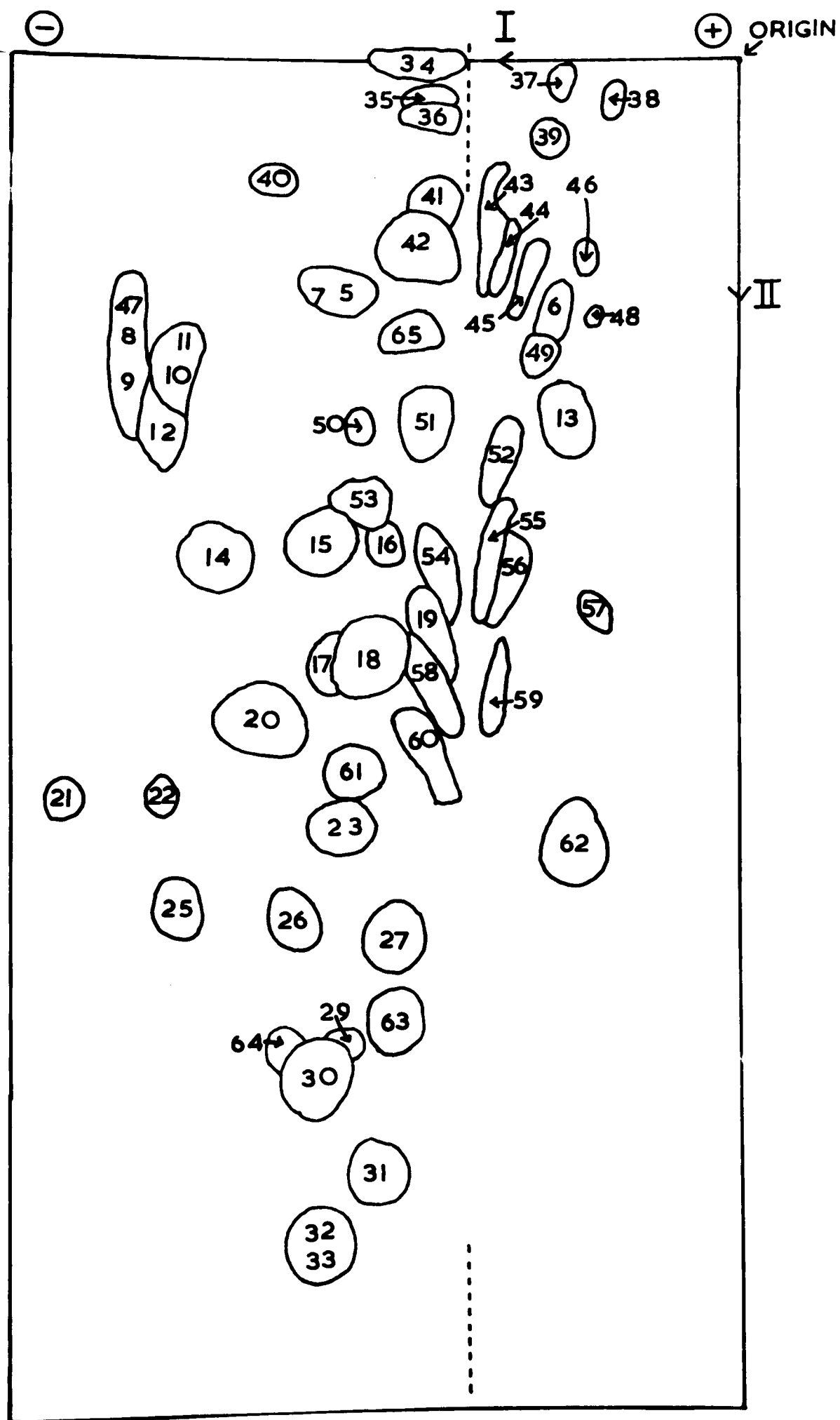


Diagram of ninhydrin-positive spots in Figure 3. Size of spots is not indicative of intensity of colour. I: direction of electrophoresis, II: direction of chromatography. Dotted line indicates apex of paper. A key to the numbers is given on the next page.

Key to Figure 8

- | | |
|--|---|
| 5. Cystine | 37. Unidentified |
| 6. Phosphorylethanolamine | 38. Unidentified |
| 7. Cystathionine | 39. S-sulphoglutathione |
| 8. Ornithine | 40. Arginyllysylglycine |
| 9. Lysine | 41. Unidentified |
| 10. Histidine | 42. GSSG |
| 11. 1-Methylhistidine | 43. Unidentified |
| 12. Arginine | 44. Unidentified |
| 13. Taurine | 45. Unidentified |
| 14. Glycine | 46. Unidentified |
| 15. Serine | 47. Carnosine |
| 16. Aspartic acid | 48. Unidentified |
| 17. Threonine | 49. Glycerylphosphorylethanolamine |
| 18. Glutamic acid | 50. Asparagine |
| 19. S-(α,β -dicarboxyethyl)-cysteine | 51. Unidentified |
| 20. Alanine | 52. Unidentified |
| 21. Ethanolamine | 53. Glutamine, citrulline? |
| 22. β -Alanine | 54. S-(α,β -dicarboxyethyl)-glutathione |
| 23. Proline | 55. Unidentified |
| 25. γ -Aminobutyric acid | 56. Unidentified |
| 26. α -Aminobutyric acid | 57. Unidentified |
| 27. Tyrosine | 58. Ophthalmic acid? |
| 29. Methionine | 59. Unidentified |
| 30. Valine | 60. Norophthalmic acid? |
| 31. Phenylalanine | 61. Unidentified |
| 32. Leucine | 62. Ascorbic acid |
| 33. Isoleucine | 63. Tryptophan |
| 34. Unidentified | 64. Unidentified |
| 35. Unidentified | 65. Unidentified |
| 36. Unidentified | |

As explained in the text, the pairs of spots 42-43, 54-55, and 58-59 may arise from a single compound streaking over the apex of the paper and (probably) tryptophan.

of stored and of fresh lens because fractions from extracts of stored lens were studied originally. One surprising feature was the apparent loss in weight of lens on storage. Although the container did not seem to have lost weight, the lenses in it had a dehydrated appearance and were surrounded by a mass of ice crystals. A stored lens was rather lighter than a fresh lens and gave a higher proportion of solid on a weight basis. These observations suggest that, over a considerable length of time at low temperature, a change occurs, similar to that observed with freeze-drying and water sublimed from the lens under the low vapour pressure.

These extracts were similar in most respects. There appeared to be a decrease in concentration of γ -glutamyl peptides on storage but the basic peptide did not seem to be affected. The concentration of S-(α, β -dicarboxyethyl)-cysteine was greater than that of S-(α, β -dicarboxyethyl)-glutathione in stored lens, while the peptide was the more abundant in the extract of fresh lens prepared in the presence of N-ethylmaleimide (p. 82). No breakdown of peptides is normally observed when lens is incubated at 37° for a few hours. Breakdown on storage may be influenced by rupture of cells, which is known to occur on freezing and thawing, and which might permit contact between enzymes and substrates. Enzyme activity persists in vegetable tissue stored at low temperature (Joalyn 1949) and may be responsible for changes in smell or flavour which take place in stored vegetables. These changes are avoided by scalding for a few minutes to inactivate the enzymes before freezing. Other minor effects of storage were the disappearance of ascorbic acid, most glutamine, and (probably) tryptophan.

Little qualitative difference was observed between extracts of fresh calf lens prepared with trichloroacetic acid and with phenol. The latter extract contained a slightly higher concentration of acidic compounds which were probably removed from the trichloroacetic acid extract during treatment with N-methyldiisobutylamine in chloroform. Trichloroacetic acid extracts of cow and of calf lens were also qualitatively very similar.

Quantitative studies were not performed. A more satisfactory comparison would probably be obtained if the same weight of solid was applied to each paper rather than a constant proportion of material obtained from lenses which are not of uniform weight.

The pattern of ninhydrin-positive spots is considerably simplified after hydrolysis (Figure 9) and most of the unidentified compounds are no longer present. Again, there was little or no difference between cow and calf lens extracts. The concentration of many amino-acids seems to be unaffected by hydrolysis which suggests that only a few are present in peptides at other than very low concentration. If some of the unidentified substances normally present are peptides, it is unlikely that small changes in concentration of amino-acids, occurring on hydrolysis, would be detected.

A number of compounds were also observed in whole extracts by use of specific reagents. In almost all cases these compounds were already known to be present in lens, the only exception being ergothioneine (see below).

3. Identification of compounds

Most of the compounds were identified in individual fractions

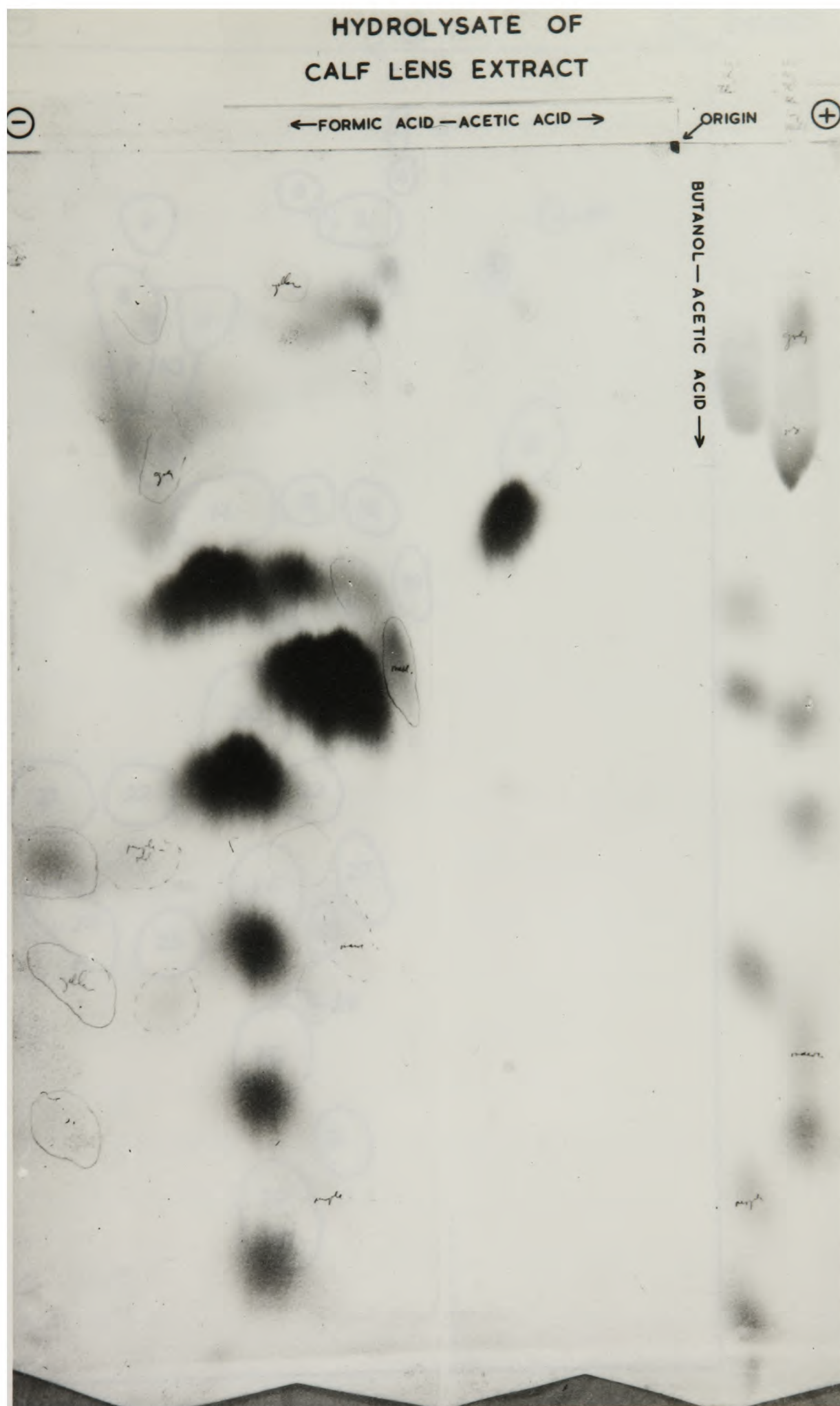


Figure 9. Separation of hydrolysate of trichloroacetic acid extract from stored calf lens. Obtained in the same way as Figure 8 with half of the extract from a single lens. Paper was developed with ninhydrin. A key is given on the following pages.

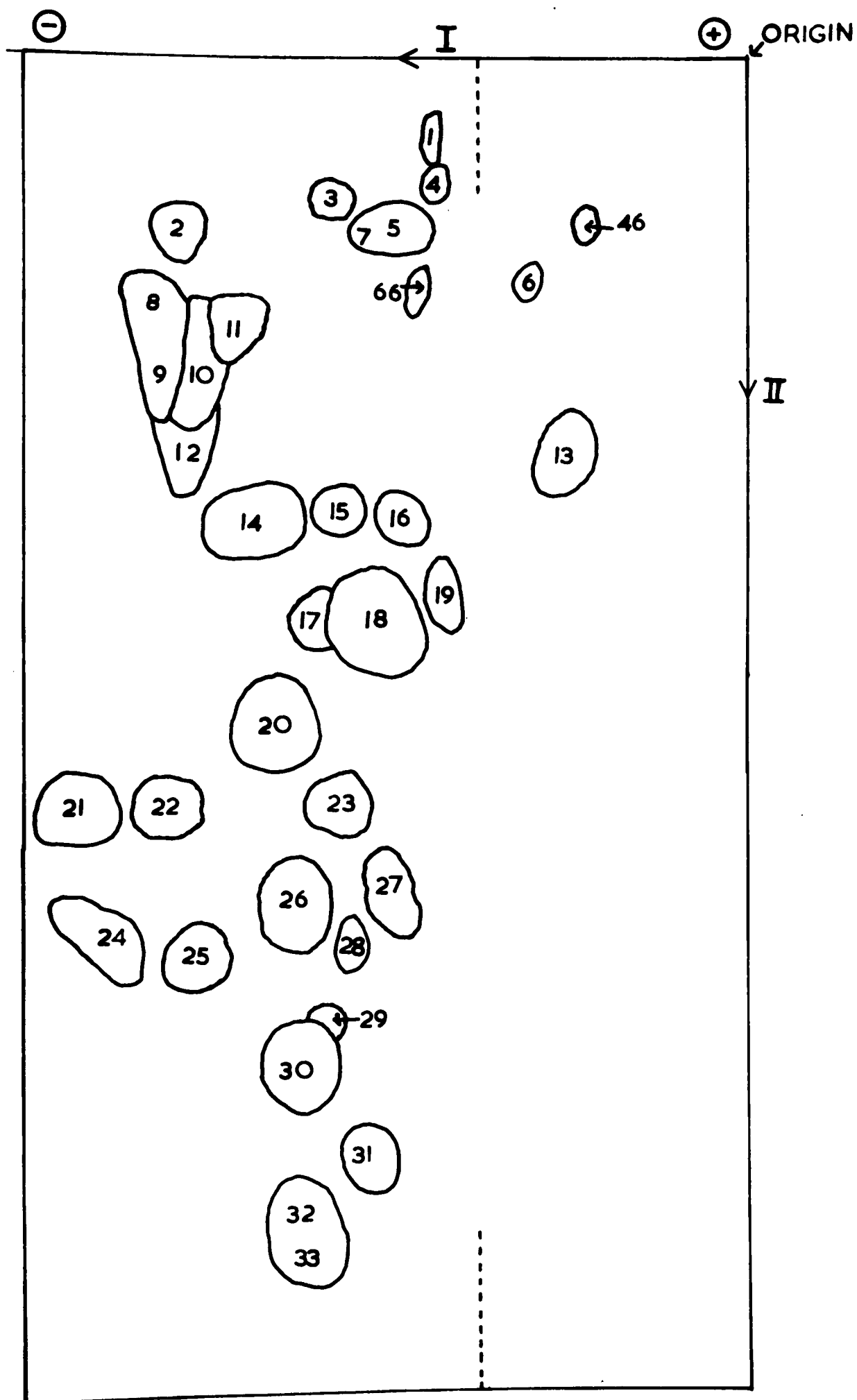


Diagram of ninhydrin-positive spots in Figure 9. Size of spots is not indicative of their intensity. Spot below no. 24 was absent from fresh lens and is omitted. I: direction of electrophoresis, II: direction of chromatography. Dotted line indicates apex of paper. A key to the numbers is given on the next page.

Key to Figure 9

1. Unidentified
2. Unidentified
3. Unidentified
4. Unidentified
5. Cystine
6. Phosphorylethanolamine
7. Cystathionine
8. Ornithine
9. Lysine
10. Histidine
11. 1-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
46. Unidentified
66. Unidentified

of lens extracts, and the results were combined to give information about whole extracts. Minhydrin-positive compounds were studied in greatest detail and the keys to Figures 8 and 9 show that the number identified in cow and calf lens is considerably greater than the 12 observed by Malatesta (1952). Identification is reasonably certain for compounds which were submitted to two-dimensional analysis (p.112) but is only tentative for the remainder. Since many of the compounds observed in extracts had already been recognized in lens and many of the others were known to be present in plasma, their identification is not discussed in detail. Similarly, since identity was established entirely by location on paper, the experimental part of this section is limited largely to a summary of results in order to avoid repetition.

Peptides. All the peptides which have been identified in calf lens were observed in the extracts. Glutathione is normally detected in whole extracts mostly in the oxidized form and was present only as GSSG in fractions from stored lens. The assignment of positions for the γ -glutamyl peptides except glutathione is only tentative. The presence of carnosine (Krause 1936) has been confirmed. No evidence has been obtained as yet for the presence of anserine but ϵ -methylhistidine, which is combined in the peptide, has been detected free. Many of the unidentified substances are probably peptides but their concentrations are such that it seems likely that their structures will remain unknown unless large amounts of material become available.

Protein amino-acids. All have been identified free in lens during this study. Malatesta identified 9 of these (p. 9) and of the remainder

only two, asparagine and tryptophan, which would be destroyed by hydrolysis, were not detected by Dardenne and Kirsten (1962). These workers analyzed hydrolysates of cow and calf lens extracts by the Moore and Stein technique of column chromatography (p. 7). Compounds were identified on the basis of the elution pattern from the column.

In stored lens, glutamine had cyclized to 5-oxopyrrolidine-2-carboxylic acid which was identified and which liberated glutamic acid on hydrolysis. Crystalline cystine was isolated merely by concentration of fraction IV₄. The solid, without further purification, gave an infra-red spectrum identical with that of an authentic sample. The concentration of tryptophan seemed to diminish on storage.

Non-protein amino-acids. Glycerylphosphorylethanolamine, phosphorylethanolamine, and taurine were identified by van Heyningen and Pirie (1958) and the last two also by Malatesta (1952). Ethanolamine, β -alanine, and α -aminobutyric acid were observed after hydrolysis by Dardenne and Kirsten (1962), together with two unidentified peaks which might be S-(α, β -dicarboxyethyl)-cysteine and cystathionine. A number of the uncommon amino-acids appear to be eluted near the buffer change during Moore and Stein analysis which makes their identification on the basis of an elution pattern rather difficult (Frimpter & Bass 1962).

The 3 compounds identified by Dardenne and Kirsten are also present free before hydrolysis together with S-(α, β -dicarboxyethyl)-cysteine, γ -aminobutyric acid, ornithine, 1-acetylhistidine and probably cystathionine. Citrulline is probably also present but it travels to the position of glutamine. A spot was detected in fractions where

glutamine had cyclized and a weak reaction in the same area of the paper was obtained with Ehrlich reagent. Both citrulline and glutamine are destroyed by hydrolysis.

Ascorbic acid. This compound is normally present in lens at high concentration but decomposes during storage. The discovery that it gives a colour with ninhydrin is not altogether surprising in view of the recent observation that levulinic acid, β -acetylpropionic acid, is a ninhydrin-positive artifact formed by hydrolysis of plant extracts and present in protein hydrolysates (Zacharius & Talley 1962). The sensitivity of ascorbic acid to ninhydrin on paper varies from 5% to 10% of that of glutamic acid. The position of ascorbic acid on two-dimensional separation has been confirmed with a marker, by quenching ultraviolet light, and by the more specific alkaline silver reagent. Ascorbic acid, in high concentration, also gives colours with reagents with which it would not be expected to react. It is destroyed by hydrolysis.

Ninhydrin-negative compounds. Ninhydrin-negative compounds identified in whole lens extracts or fractions of lens extracts were, with one exception, already known to be present in lens. These compounds are glycerylphosphorylcholine, phosphorylcholine and α -glycerophosphate (van Heyningen & Pirie 1958), creatine (Pirie, van Heyningen & Flanders 1955), and urea. Inositol and glucose have also been tentatively identified in whole extracts. The exception is ergothioneine (thio-histidine-betaine).

Ergothioneine. Ergothioneine does not react with ninhydrin but quenches

ultraviolet light and reacts with Pauly reagent. Its position on analysis of whole extracts has been deduced from these reactions. It was identified in fractions of stored lens with the aid of a marker. Ergothioneine is normally present at a rather low concentration in blood and its estimated concentration in lens, about 0.1mM, is similar. Rats fed ^{35}S -ergothioneine had some detectable radioactivity in the whole eye (Heath 1953) but it was thought that this might be due to blood remaining in the tissue. The occurrence of ergothioneine is discussed in Part V. It is possible that one of the unidentified Pauly-positive compounds in whole extracts of lens is hercynine (histidine-betaine).

Unidentified compounds. Most of the unidentified compounds in whole extracts are present only in trace amounts and the majority are destroyed by hydrolysis. Hydrolysed extracts also contain a few unidentified ninhydrin-positive substances. A larger number of trace constituents appeared to be present in the fractions of lens extracts studied individually. It is difficult to decide to what extent these have been observed because of the degree of concentration achieved rather than because they are artifacts formed during storage. In general, it has been found that traces of unidentified substances can always be observed near the limits of detection with ninhydrin, no matter to what extent the extracts are concentrated.

Experimental and results

Materials. Dilute pyridine-acetate buffer for electrophoresis was prepared by diluting stock pyridine-acetate pH 4 (1 vol) with water (1 vol).

Lenses. For preparation of crude lens extracts, fresh lenses were used. These had been stored at -15° for not more than one week in most cases.

90($\frac{w}{v}$) phenol. This was an aqueous solution stored in a dark bottle and used while practically colourless.

Trypsin (Armour lot 14,329) contained 50% magnesium sulphate. Solutions, 2% ($\frac{w}{v}$) with respect to the enzyme, were prepared in 0.001N hydrochloric acid immediately before use. Activity of the enzyme was tested with dilysine dihydrochloride as substrate (Waley & Watson 1954).

Dilysine dihydrochloride was supplied by Dr. S.G. Waley; phosphorylethanolamine, glycerylphosphorylethanolamine, phosphorylcholine and glycerylphosphorylcholine were supplied by Dr. R. van Heyningen.

Methods and results. Methods were described in Part II (general techniques) unless otherwise indicated.

1. New compounds

Arginyllysylglycine

Fraction 1F3 (Table 3) was concentrated in vacuo to a brown syrup (0.4 ml). Portions applied to the centre of the paper were examined by electrophoresis in pyridine-acetate for 3.5 hours and gave 5 ninhydrin-positive spots attributed to β -alanine, γ -aminobutyric acid, an unidentified spot between these, and traces of neutral and of basic amino-acids. The unidentified compound disappeared on hydrolysis and

the amounts of neutral and of basic amino-acids increased. Fraction 1F3 was also examined after separation of compounds in two dimensions (p. 110) and the ninhydrin spot attributed to the unidentified compound (Figure 8, 40) was no longer present after hydrolysis. Increases in the amounts of arginine, lysine, possibly histidine, glycine, and glutamic acid were observed after hydrolysis and a number of unidentified spots in the fraction disappeared. The new substance was believed to be a peptide on this evidence. Its concentration in fraction 1F3 indicated a concentration of about 0.02mM in lens.

Material from fraction 1F3. Initial studies were performed on material separated by electrophoresis. In a typical ^{experiment} ~~sept~~, a portion of fraction 1F3 (0.18 ml) was applied as a band (4 cm) to No. 3 paper and submitted to electrophoresis in pyridine-acetate for 5 hours. The strip (1.4 cm. wide) which contained the peptide, located by developing a marker spot of the fraction run on the same paper with ninhydrin, was cut out and material eluted with 1% (^v/v) acetic acid to give a colourless solution (0.18 ml). Material obtained by this method usually contained traces of β -alanine or γ -aminobutyric acid as shown by electrophoresis in pyridine-acetate.

Hydrolysis. One hydrolysate was analysed by chromatography in butanol-acetic acid and the paper developed with ninhydrin. The peptide, which barely moved from the origin disappeared and three main spots appeared, two of which were in the position of basic amino-acids, the position of the other, probably glycine, seemed to be affected by salts in the hydrolysate. Arginine was identified with the Sakaguchi reagent.

Another hydrolysate was separated by electrophoresis in pyridine-acetate for 5 hours. A quantitative estimation of the amounts of amino-acids liberated was obtained by comparison with markers of glycine, arginine, and glutamic acid on the same paper (p. 33). The ratio of glycine to basic amino-acids (estimated as arginine) was 1 : 2.2; the amount of colour given by other compounds present (glutamic acid, β -alanine) was no more than one-third of that given by glycine.

Dinitrophenylation. A portion of eluate was dried in vacuo and the residue dinitrophenylated then hydrolysed. The hydrolysate was examined by chromatography in tert-pentanol-phthalate for 24 hours on No. 3 paper. Two yellow spots were obtained, one of which streaked from the origin to the position of a marker of DNP-arginine, the other was identified as dinitrophenol by comparison with a marker and was decolourized by fumes of hydrochloric acid.

Material from fractions 2F1 and 3F1 (Tables 4 and 5). These fractions, obtained by anion-exchange chromatography, were examined by electrophoresis in pyridine-acetate and contained both basic and neutral amino-acids. The new peptide was detected in fraction 3F1 and was presumably present in fraction 2F1. It was separated from other constituents of these fractions by continuous electrophoresis (Table 9). Fraction 3F1 was centrifuged to remove traces of solid, combined with fraction 2F1, and diluted with water (to 18 ml). Bromocresol purple (about 0.2 ml) was added, and the solution adjusted to pH 4.5 with M acetic acid (about 2 ml). Fractionation was performed in the Beckman apparatus with dilute pyridine-acetate buffer, and at constant current (76mA). The potential fell from

Table 2

545 v to 465 v during the experiment. The experiment was continued until the sample was fractionated. Fractionation of basic and neutral compounds by continuous electrophoresis

Pooled Fractions	Chief ninhydrin-positive constituents	Volume ml	
		Initially	after concentration
1	Ethanolamine Amine (Trace)	78.3	0.9
2 - 5	Amine Basic Amino-Acids	169.0	0.5
6 - 9	Amine Basic Amino-Acids γ -Aminobutyric Acid Basic Peptide	115.0	0.4
10	γ -Aminobutyric Acid Basic Peptide	23.0	1.3
11	Basic Peptide β -Alanine	27.5	0.3
12 - 13		50.0	0.55
14 - 16		70.0	1.9
17 - 19	Neutral Amino-Acids	79.5	3.0
20 - 22	Neutral Amino-Acids	74.2	4.7
23 - 32		392.5	0.4

Rate of collection of fractions 0.8 ml/tube/hour

Total volume of fractions 1079 ml

515 v to 465 v during the experiment. The apparatus was equilibrated then the sample applied halfway towards the anode side of the paper at 0.5 ml/hour which was increased to approximately 0.65 ml/hour for the last 7 hours. The dye did not indicate the position of material on the curtain very satisfactorily and was collected in fractions 25-29, mostly in 27 and 28. Collection of fractions was begun 3 hours after first application of the sample, and continued for 42 hours. The fraction collector changed every 12 hours and 3.5 sets of fractions were collected. Spots of each set dried on paper and developed with ninhydrin showed that the elution pattern remained constant. Fractions were numbered from cathode to anode, pooled according to their content as shown by electrophoresis in pyridine-acetate, concentrated by evaporation below 44° in vacuo, and stored at -15° . Two bands of mobile basic material migrated off the side of the paper curtain.

The peptide was detected by electrophoresis in pyridine-acetate in pooled fractions 6-9 with larger amounts of basic amino-acids, in fraction 10 with a trace of γ -aminobutyric acid, and in fraction 11 with traces of γ -aminobutyric acid and β -alanine. The remaining experiments were performed with fraction 10 (1.3 ml).

Hydrolysis. A portion (0.15 ml) of the fraction was dried in vacuo, the residue dissolved in water and hydrolysed. The hydrolysate was examined by electrophoresis in sodium borate for 4 hours and the paper developed with acid ninhydrin. Three spots were observed and attributed to neutral amino-acids (probably glycine since a rather pink colour was obtained, as with a glycine marker), arginine (streaked slightly), and lysine. Arginine

and lysine are separated under these conditions and arginine was also detected on a duplicate paper developed with the Sakaguchi reagent.

Dinitrophenylation. Residue, from a portion of the fraction (0.2 ml) dried in vacuo, was dinitrophenylated then hydrolysed. The hydrolysate was analysed by chromatography in butanol-acetic acid on No. 3 paper for 24 hours. The paper was cut lengthwise to allow a longer time of run without loss of material from the end of the paper. DNP-arginine was identified by co-chromatography with a marker and gave a yellow spot, as did dinitrophenol. The paper was developed with ninhydrin, and lysine and glycine identified by markers superimposed on the hydrolysate. Traces of glutamic acid, of β -alanine and of a compound with R_F similar to that of the peptide were also present.

Digestion with trypsin. Trypsin, 8% (^w/v) enzyme in 0.001 N hydrochloric acid in Visking cellulose tubing (0.25" dia.) was dialyzed for 7 hours against three changes of 0.001 N hydrochloric acid in order to remove magnesium sulphate. A portion of the fraction (0.2 ml) was dried in vacuo. Residue was dissolved in 0.2 N potassium hydrogen carbonate (20 μ l) and dialyzed trypsin (20 μ l) to give a slightly brown solution with a final concentration 1.6% of enzyme. One portion of solution (about 5 μ l) was stored in a sealed tube at -15° . The remainder was sealed in a second tube and incubated at 37° together with another solution containing trypsin (6 μ l) and potassium hydrogen carbonate (6 μ l) as a control for digestion of the enzyme. Samples (5 μ l) of the three solutions were examined after 24 hours by chromatography in butanol-acetic acid on Schleicher and Schüll No. 2043 b paper for 24 hours, and the paper

was developed with ninhydrin. Breakdown of the enzyme occurred on incubation and spots appeared in the positions of arginine and lysine. The peptide had partly decomposed and glycine, arginine and lysine were identified with superimposed markers. The two incubated solutions were examined after 40 hours by electrophoresis for 4 hours in sodium borate and spots visualized with acid ninhydrin. The peptide mixture contained a streak from the origin to the position of neutral amino-acids which gave a spot, also spots at the positions of lysine and arginine.

Digestion with trypsin was also performed under slightly differing conditions with essentially the same results. One solution was incubated at 37° for 30 hours, and examined by chromatography in butanol-acetic acid as above. Enzyme concentration was 1% and this gave a clearer chromatogram but less breakdown of the peptide. Comparable amounts of glycine, arginine and lysine appeared to be liberated. In another experiment portions ($5 \mu\text{l}$) of the digestion mixture were drawn into micropipettes and left at 26° for 24 hours. They were examined by electrophoresis in pyridine-acetate for 5 hours. A paper, developed with ninhydrin, showed that about half of the peptide had been degraded with increase in concentration of basic and of neutral amino-acids. β -Alanine and γ -aminobutyric acid present as contaminants in the fraction did not increase in concentration. One spot was observed at the origin which was attributed to the enzyme and to large peptides formed by breakdown of the enzyme. Liberation of arginine was demonstrated by developing a duplicate paper with Sakaguchi reagent.

by electrophoresis in pyridine-acetate and in sodium borate, and by

Unidentified amine

Fraction 1F2 gave a single ninhydrin-positive spot in the position of basic amino-acids on electrophoresis in pyridine-acetate for 3.5 hours. A ninhydrin-positive spot was detected near the solvent front after chromatography in phenol-ammonia, butanol-acetic acid, and butanol-acetic acid-water-pyridine. This spot was not observed after chromatography (in butanol-acetic acid) of the extract prepared without tissue (p. 21), and was assumed to indicate a new compound present in lens. Fraction 1F2 only gave spots with acid ninhydrin in positions of basic amino-acids and of neutral material after electrophoresis in sodium borate for 2.5 hours.

The new compound was not detected when portions of fraction 1F2 were treated with Sakaguchi reagent, Pauly, or Ehrlich reagents after chromatography in butanol-acetic acid. Hydrolysates of fraction 1F2 contained this compound at unchanged concentration on chromatography in the same solvent.

The basic nature of the unidentified substance was also indicated by its position in fractions obtained by anion-exchange chromatography. Basic amino-acids were detected with ninhydrin in fractions 2F1 and 3F1 by electrophoresis in pyridine-acetate. The compound was detected in fraction 2F1 by chromatography in butanol-acetic acid, and in fraction 3F1 by chromatography in phenol-ammonia. When these fractions were submitted to continuous electrophoresis (p.101), the unknown was still obtained in the fractions containing basic amino-acids as detected by electrophoresis in pyridine-acetate and in sodium borate, and by

chromatography in butanol-acetic acid and in butanol-acetic acid-water-pyridine. Impurities in the concentrated fractions (Table 9) caused a decrease in R_F in butanol-acetic acid (0.80).

Average R_F values in the chromatographic solvents were: phenol-ammonia 0.94, butanol-acetic acid 0.90 and butanol-acetic acid-water-pyridine 0.89. Further studies were performed entirely with material separated from fraction 1F2.

Material from fraction 1F2. Separation of the compound was based on its high mobility on chromatography. Fraction 1F2 (0.5 ml) was applied as a band (15 cm. wide) to No. 3 paper and the paper developed by chromatography in butanol-acetic acid for 8 hours. The compound was eluted with water from a strip 4.5 cm. wide. Eluate was concentrated in vacuo to 0.2 ml. Subsequent separations were performed similarly but elution was carried out with 1% (v/v) acetic acid.

Eluates (about 10mM concentration) gave a single ninhydrin-positive spot on chromatography in butanol-acetic acid and in phenol-ammonia. After electrophoresis in pyridine-acetate for 3.5 hours the compound had travelled slightly further than lysine but would not have separated if the spots had been superimposed. A single ninhydrin-positive spot which did not migrate as far as lysine was obtained after electrophoresis in 10% acetic acid. The compound was well separated from spermine, which barely moved from the origin, by chromatography in butanol-acetic acid. Dodecylamine had an R_F 0.94 in this solvent.

A portion of eluate (0.1 ml) was treated with saturated aqueous picric acid (15 μ l) and left at 4°. The yellow crystals were dried in

vacuo. They gave a weak infrared spectrum which had some resemblance to that of dodecylamine picrate obtained for comparison but the strongest bands were attributed to the picrate ion, and so no useful conclusions could be drawn about the nature of the base.

An attempt was made to isolate some of the base as a hydrochloride but the infra-red spectrum of the solid was too featureless to provide any information.

A portion of eluate (5 μ l) was treated for 10 minutes with nitrous acid (20 μ l), prepared from 5% (w/v) sodium nitrite (0.05 ml) and N hydrochloric acid (0.1 ml), then dried in vacuo. The residue was examined by chromatography in butanol-acetic acid and compared with an equal volume of untreated eluate. The strength of colour with ninhydrin was considerably decreased in the nitrous acid sample. The concentration of the base in lens appears to be about 0.04mM judged by depth of colour with ninhydrin.

Phosphorus-containing compound

After refractionation of fraction 4F7 by cation-exchange chromatography (p.67), a compound was detected in fractions 20-23 which gave a blue fluorescence in ultraviolet light following electrophoresis in 10% acetic acid. The substance migrated further than glutathione sulphonic acid and to roughly the position of S-sulphoglutathione. Its position indicated a negative charge at pH 2.3 by comparison with glucose detected on the same paper with alkaline silver reagent. The substance was not tested with this reagent but gave a positive reaction for phosphorus with molybdate reagent. Pooled fractions were examined by

electrophoresis in pyridine-acetate and gave a spot, with the same mobility as glutathione, which quenched ultraviolet light and reacted with molybdate but not with ninhydrin. A compound with the same characteristics did not migrate as far as glutamic acid on electrophoresis in sodium borate for 2.5 hours. A ninhydrin-negative substance which reacted with molybdate was also observed after chromatography in butanol-acetic acid.

The substance was also detected by its fluorescence after electrophoresis in 10% acetic acid in fractions 1F6, 1F7, 1F8, 2F6, 3F7, 3F8 and 4F6. Its mobility in some fractions was affected by impurities which were present. It was also detected in a sample of fraction 1F8 which had been oxidised with performic acid. Fractions 1F6 and 1F7 were examined by electrophoresis in formic-acetic acid followed by chromatography in butanol-acetic acid (see below) and contained a ninhydrin-negative compound which moved to a position near S-sulphoglutathione and gave a blue fluorescence in ultraviolet light. This compound was no longer present after hydrolysis.

Separation from fraction 4F6. A portion of fraction 4F6 (0.2 ml) was applied as a band (4 cm. wide) to No. 3 paper and submitted to electrophoresis in 10% acetic acid. The compound was detected with ultraviolet light and eluted from a strip (2 cm. wide) with water to give a colourless eluate (0.4 ml). The strip was not fluorescent after elution. A portion of eluate was examined after electrophoresis in pyridine-acetate, and 3 phosphorus-containing spots were detected with molybdate reagent. One was near the position of glutathione, one near S-(α , β -dicarboxyethyl)-

cysteine, and the weakest was probably inorganic phosphate.

Eluate (50 μ l), as a band (2.5 cm. wide) was submitted to electrophoresis in pyridine-acetate. The substance which travelled near glutathione quenched ultraviolet light and was eluted with water from a strip 1.5 cm. wide. The eluate was taken to dryness in vacuo, the residue dissolved in N hydrochloric acid and hydrolysed at 100° for one hour. The hydrolysate was examined by chromatography in isopropanol-hydrochloric acid for 24 hours and gave a blue fluorescent spot in ultraviolet light which was indistinguishable from guanine and co-chromatographed with guanine. The compound in the hydrolysate was separated from xanthine but ^{not} guanine in this solvent after chromatography on No. 3 paper for 48 hours. Xanthine and guanine also gave slightly different fluorescent colours. Duplicate papers were run on electrophoresis in pyridine-acetate. The original substance disappeared on hydrolysis and gave two spots with molybdate reagent, one near S-(α , β -dicarboxyethyl)-cysteine and a trace of inorganic phosphate. The second paper was developed with ninhydrin and the hydrolysate gave a faint spot at the position of neutral compounds, presumably an impurity acquired during elution. Hydrolysates were not investigated with alkaline silver reagent.

2. Investigation of whole lens extracts

Preparation of extracts. Fresh calf lens (0.90 g) was ground in a glass mortar with a little sand under ice-cold 7% ($^w/v$) trichloroacetic acid (2 ml) for 10 minutes to disintegrate the tissue. The curdy suspension, together with water (0.5 ml) used to wash the mortar, was

centrifuged for 15 minutes at 4,600 rev/min. The supernatant was extracted with N-methyldioctylamine (0.15 ml) in chloroform (3 ml) in two lots (2.1 ml, 1.05 ml) and centrifuged after each extraction to separate the layers. The clear aqueous solution (pH 5, indicator paper) was taken to dryness in vacuo, and the residue (13.9 μ g) in water (0.2 ml) extracted a third time with N-methyldioctylamine (10 μ l) in chloroform (0.2 ml) and centrifuged for 15 minutes at 5,000 rev/min. A trace of insoluble material remained at the interface of the two layers. The aqueous layer was dried in vacuo and the residue dissolved in water (20 μ l) to give a rather viscous, pale yellow solution.

Extracts of cow lens were prepared similarly but, since a cow lens weighs about 2 g, twice the volume of trichloroacetic acid was used and an extra extraction with chloroform-amine solution (3.15 ml) was performed. The amount of solid obtained from calf lens was fairly constant; about 15.5 μ g/g fresh weight, cow lens gave more variable values.

In one instance, protein was precipitated with phenol. A calf lens (0.94 g) was ground under water (1 ml), the suspension shaken with 90% (w/v) phenol (1 ml) and centrifuged. The aqueous layer was extracted with chloroform (1 ml) and taken to dryness in vacuo. Residue (15.4 μ g) in water (0.4 ml) was extracted with chloroform (2 X 0.4 ml) and dried in vacuo.

Method. The sample (10 μ l) was applied 10 cm. from one end and 5 cm. from one side of a sheet of No. 3 paper, 46 cm. X 25 cm. Electrophoresis in formic-acetic acid was performed in the first (short) direction for

3.5 hours, and the paper thoroughly dried in air. Markers (5 μ l) were applied near the origin on the line of electrophoresis. Chromatography was carried out in the second (long) direction for 16 hours and the solvent ran off. The paper was viewed in ultraviolet light then developed, usually with ninhydrin.

Results. Although some variation was observed in the distance travelled from the origin on electrophoresis or, more usually, chromatography, the pattern of spots obtained was extremely reproducible. Compounds which moved to the apex of the paper tended to streak in a V-shape on chromatography and caused some difficulty in identification of spots. Phenol extracts were noticeably worse than trichloroacetic acid extracts in this respect. Hydrolysates contained dark material (remaining at the origin) but tended to give more discrete spots than crude extracts.

Satisfactory patterns were obtained from samples (10 μ l) of residues of calf lens in 20 μ l water and of cow lens in 40 μ l water, equivalent to 6.5-8 mg total solid on a single paper. Because the amount of solid varied from paper to paper, no estimates of concentration are given. Patterns were also obtained for each of the fractions 1F1 to 1F6 (10 μ l each except 1F4, 20 μ l) and their hydrolysates, and for fractions 2F2 (20 μ l) and 3F2 (10 μ l). Fractions 2F2 and 3F2 gave the same pattern as fraction 1F4 (neutral amino-acids), 2F2 also contained small amounts of basic amino-acids.

Identification of compounds (see keys to Figures 8 and 9) is based on the patterns obtained with fractions 1F1 to 1F6 and the studies on individual compounds in these (section 3 below), on the chromatographic

behaviour of known markers, and on two-dimensional patterns obtained by the same method with various mixtures of the following 28 compounds: arginine, histidine, cystine, alanine, glutamine, serine, proline, tyrosine, methionine, valine, asparagine, glutamic acid, aspartic acid, ornithine, 1-methylhistidine, β -alanine, cystathionine, citrulline, taurine, α -aminobutyric acid, S-(α,β -dicarboxyethyl)-cysteine, carnosine, phosphorylethanolamine, glycerylphosphorylethanolamine, glutathione, GSSG, S-(α,β -dicarboxyethyl)-glutathione, and ascorbic acid. All these compounds were located with ninhydrin. Tryptophan acted as an internal marker, and was run as a chromatographic marker, for all extracts because of its characteristic blue fluorescence in ultraviolet light.

Fresh calf lens. The pattern of ninhydrin-positive spots in a tri-chloroacetic acid extract of fresh ^{calf} lens is shown in Figure 8, more information on individual compounds is given in section 3 (p. 117). All the protein amino-acids appear to be present, together with a number of non-protein amino-acids and some peptides. Most of the unidentified compounds disappear on hydrolysis and are present in only trace amounts. Chromatography on one paper was performed for only 8 hours so that the solvent front had just remained on the paper. The only ninhydrin-positive spot which was detected in front of the leucines, which usually reach the end of the paper, was attributed to the unidentified amine described in section 1 (p. 105).

The pattern obtained from a phenol extract of calf lens was essentially the same as that observed with the trichloroacetic acid extract. Quantitatively, the phenol extract seemed to contain a slightly

higher concentration of acidic compounds on the right of the apex of the paper, most of which are unidentified and were no longer present after hydrolysis. Since more streaking of spots seemed to occur in phenol extracts, trichloroacetic acid was used in preparation of extracts for which the other results are described. The hydrolysate of the phenol extract also resembled that from a trichloroacetic acid extract but gave more diffuse spots.

Stored calf lens. The lens (0.53 g) had been stored at -15° for 6.25 years. It was smaller than a fresh lens and looked much less moist. On the basis of a normal value of 15.5 mg solid/g fresh weight lens, the amount of solid obtained, 12.9 mg indicated a loss in weight on storage of about 35%. The most striking observation on the pattern given by this extract was the apparent decrease in concentration of peptides relative to amino-acids, e.g. the concentration of GSSG was less than that of valine. Most of the compounds were present at similar concentrations to those observed in fresh lens but higher concentrations were observed of amino-acids, such as glutamic acid, cystine, glycine, α -aminobutyric acid, S-(α , β -dicarboxyethyl)-cysteine, and possibly alanine, known to be bound in peptides. The amount of cystine, for example, was greater than that of tyrosine. The concentration of arginyllysylglycine seemed to be unaltered. The spot in the position attributed to norophthalmic acid seemed to be increased in concentration. Glutamine, ascorbic acid, and tryptophan (largely) had disappeared. Traces of a few additional unidentified substances were present.

The pattern obtained with a hydrolysate of this extract is

shown in Figure 9, and is the same as that given by the hydrolysed phenol extract. Many of the unidentified compounds decompose on hydrolysis and a less complex mixture of ninhydrin-positive compounds is obtained. As with fresh lens, the concentration of most of the substances is largely unaffected by hydrolysis which indicates that few of the amino-acids are normally present in quantity in low molecular-weight peptides. Of the 8 unidentified compounds in this mixture, two (3 and 24) give yellow colours with ninhydrin and are probably not amino-acids, two (2 and 46) may be hydroxylysine and cysteic acid, and one (4) may be present before hydrolysis (36).

Fresh cow lens. This extract was examined before and after hydrolysis.

The pattern given by the crude extract was very similar to that given by calf extract (Figure 8) but seemed to contain larger amounts of aspartic acid, asparagine, and peptides. A few extra spots were probably only visible because the amount of solid was slightly greater than in the experiment with calf lens. Hydrolysates gave the same pattern as calf lens but appeared to yield larger amounts of the unidentified compound (66).

Studies with other location reagents. Since there appears to be little qualitative difference between the composition of calf and cow lenses, both have been used in initial studies with location reagents other than ninhydrin. These reagents detect a number of ninhydrin-negative compounds and positions of the more important of these are indicated in relation to the pattern in Figure 8.

All papers were viewed under ultraviolet light and no attempt

has been made to identify many of the compounds which quenched or fluoresced under these conditions. The blue fluorescence of tryptophan was a useful internal marker for unhydrolysed extracts which were not treated with ninhydrin.

Calf extracts were examined with Pauly, Sakaguchi, and pentacyanoaquoferriate reagents. Four compounds have been identified with Pauly reagent: carnosine (47) and histidine (10) which gave red spots, tyrosine (27) a purple spot turning to orange, and ergothioneine which quenched ultraviolet light and gave an orange spot with the reagent in the position of 56 and 59 near the apex of the paper (see p.124). Three other unidentified compounds seemed to give a genuine reaction, one was in the position of proline (23), the others gave orange colours and had low R_F values, one was near (41) and the other to the right of (38). High concentrations of amino-acids gave rise to pale yellow spots and ascorbic acid (62) inhibited the reagent. Guanidines were detected on a single paper by spraying one side with pentacyanoaquoferriate reagent and the other with Sakaguchi. The former reagent reacted with arginine (12) and creatine (near proline) to give red spots on a yellow background, and GSSG (42) gave a purple spot. The new basic peptide, arginyllysylglycine (40), and arginine reacted with Sakaguchi reagent.

Cow extracts have been examined with a wider variety of reagents. Sakaguchi reagent gave the same results as with calf extracts. With Ehrlich reagent, tryptophan gave a purple spot which slowly faded; a weak yellow spot at the position of glutamine (53) was probably given by traces of citrulline, which does not separate; urea gave an immediate

strong yellow spot which turned to turquoise, below ascorbic acid (62) on the line of uncharged compounds. Colours were also obtained with GSSG (yellow, quite rapidly), ascorbic acid (faint yellow which became pink) and many amino-acids (yellow, slowly). Ascorbic acid quenched ultraviolet light and gave a very strong reaction with alkaline silver reagent before application of the second part of the reagent. Strong spots were also obtained for, probably, inositol and glucose (no markers) on the line of uncharged substances. The reagent was inhibited by GSSG and colours were obtained with amino-acids at high concentration and, probably, inorganic phosphate. Many of the compounds detected with ultraviolet light also reacted with molybdate reagent for phosphorus and are grouped near the origin with low R_F values. No attempt has been made to identify these. A blue fluorescent spot in this area gave a positive reaction and may be the unidentified guanine compound (p107). Phosphorylethanolamine (6), glycerylphosphorylethanolamine (49), phosphorylcholine and glycerylphosphorylcholine (probably), together with α -glycerophosphate and inorganic phosphate were detected. An attempt to locate sulphur compounds with iodine-azide was largely unsuccessful, only GSSG among known compounds reacted, but a number of brown spots were obtained with urea (very strong) and some of the unidentified compounds. The reagent was inhibited by ascorbic acid.

In all cases, compounds were identified by means of chromatographic markers on the same paper.

3. Identification of individual compounds

Numbers refer to keys for Figures 8 and 9. Within each group the behaviour on two-dimensional separation is described before the results of other experiments.

Acidic peptides. The positions of glutathione (19), GSSG (42) and S-(α , β -dicarboxyethyl)-glutathione (54) were determined with markers. In most extracts glutathione was probably largely present in the oxidized form, the reduced form did not separate from S-(α , β -dicarboxyethyl)-cysteine. GSSG, S-(α , β -dicarboxyethyl)-glutathione, and ophthalmic acid (58) travelled near the apex of the paper and may have given double spots (Figure 8). GSSG, ophthalmic acid, and nurophthalmic acid (60) were detected in fractions 1F6 and 1F7; the new acidic peptide was also in fraction 1F7, and in fraction 1F8 together with S-sulphoglutathione (39) and a trace of GSSG. Identification of these compounds in fractions was limited largely to two-dimensional separation and to behaviour on electrophoresis in pyridine-acetate and in 10% acetic acid compared with markers. All spots attributed to these compounds were destroyed by hydrolysis and the appropriate amino-acids were liberated.

Basic peptides. Arginyllysylglycine (40) was only detected in fraction 1F3 and has been described earlier (p.99). The position of carnosine (47) was determined with a marker. Carnosine was detected in fraction 1F2 and gave a grey-brown colour with ninhydrin. It was separated from histidine by electrophoresis in sodium borate for 4 hours and detected with acid ninhydrin, also in sodium carbonate after 8.5 hours and detected with Pauly reagent. Separation was also obtained by chromato-

graphy in butanol-propanol-ammonia on No. 3 paper for 48.5 hours with both ninhydrin and Pauly reagents for location. These conditions gave more compact spots and a wider separation than on No. 4 paper for 60 hours (Reddy & Hegsted 1962). The presence of carnosine was also inferred by liberation of β -alanine by hydrolysis. β -Alanine was identified with ninhydrin after electrophoresis in pyridine-acetate and sodium borate, also after chromatography in phenol-ammonia, butanol-acetic acid and butanol-acetic acid-water-pyridine.

Protein amino-acids. The positions of 13 of these on two-dimensional patterns were determined with markers (p.112), and the remaining 7: lysine, glycine, threonine, tryptophan, phenylalanine, leucine and isoleucine were run as chromatographic markers on numerous occasions. Basic amino-acids were detected in fraction 1F2 with ninhydrin, neutral amino-acids in fractions 1F4 and 1F5, glutamic acid in fractions 1F6 and 1F7, aspartic acid in fractions 1F7 and 1F8.

Identification has also been obtained with markers for each acid by some of the following: electrophoresis in pyridine-acetate (acidic amino-acids), in 10% acetic acid (acidic and neutral), in sodium borate (basic), chromatography in phenol-ammonia, in butanol-acetic acid, in butanol-acetic acid-water-pyridine (mostly basic amino-acids), and in butanol-propanol-ammonia (basic). Tryptophan gave a characteristic blue fluorescence in ultraviolet light. All the compounds reacted with ninhydrin, some gave characteristic colours: tryptophan, tyrosine and phenylalanine mauve or purple, histidine grey, aspartic acid purple, asparagine grey-blue, proline brown (not always). Specific reagents were

also used for some of the acids: histidine and tyrosine reacted with Pauly reagent, arginine with Sakaguchi reagent, and tryptophan with Ehrlich reagent. Tryptophan (63), glutamine (53) and asparagine (50) were destroyed by hydrolysis.

~~histidine~~ Basic amino-acids were readily separated by electrophoresis in sodium borate for 4 hours, spots of arginine and lysine were just separated and the acids were differentiated by Sakaguchi reagent. Neutral amino-acids were only analyzed satisfactorily by two-dimensional separation but many could be identified by chromatography of fractions, particularly after hydrolysis. Acidic amino-acids were separated by electrophoresis at pH 4.

~~because~~ The presence of cystine was also indicated by the lens extract prepared in the solution containing N-ethylmaleimide (p. 82). Fraction 1F₄ deposited a white powder on storage at -15°. The fraction was centrifuged and the solid dried in vacuo (2.4 mg). A portion had an infra-red spectrum identical with that of authentic cystine.

~~containing~~ Glutamine was present in quite high concentration in extracts of fresh lens but not of stored lens. Fraction 1F₈ contained a compound which was detected with aniline-xylose but not ninhydrin. This compound migrated to the same position as 5-oxopyrrolidine-2-carboxylic acid on electrophoresis in pyridine-acetate and on chromatography in butanol-acetic acid. A portion of fraction 1F₈ (50 μ l) was applied as a band (5 cm. wide) to No. 1 paper and submitted to chromatography in butanol-acetic acid. The strip (3.2 cm. wide) believed to contain the cyclic compound was located with guide strips and material eluted with water.

A portion of eluate was hydrolysed and the hydrolysate contained a considerable amount of glutamic acid, detected by electrophoresis in pyridine-acetate.

Non-protein amino-acids. The positions of ornithine (8), 1-methyl-histidine (11), β -alanine (22), cystathionine (7), α -aminobutyric acid (26), citrulline (53), S-(α , β -dicarboxyethyl)-cysteine (19), taurine (13), phosphorylethanolamine (6), and glycerylphosphorylethanolamine (49) were determined with markers. Two other non-protein amino-acids, ethanolamine (21) and γ -aminobutyric acid (25) were identified by chromatographic markers only in the two-dimensional separations.

Taurine and the two phosphorylethanolamines were distinctive because they are the only compounds in the neutral fraction 1F4 obtained by electrophoresis at pH 4 which separate at lower pH by virtue of their strong acid groups. They also migrated as a separate group on electrophoresis in 10% acetic acid. Taurine and traces of phosphorylethanolamine were observed after hydrolysis of fraction 1F4. The phosphorus-containing substances were identified by electrophoretic mobility at pH 4 and reaction with molybdate, also by liberation of inorganic phosphate on hydrolysis. They were observed in extracts of cow lens treated with molybdate after two-dimensional analysis.

S-(α , β -dicarboxyethyl)-cysteine gave a mauve colour with ninhydrin, its identification was discussed in Part III.

Ornithine and 1-methylhistidine were identified in fraction 1F2 and in fractions containing basic amino-acids obtained by refractionation of 2F1 and 3F1 (p101). 1-Methylhistidine gave a brown-grey colour with

ninhydrin and did not react with Pauly reagent. Identity was established with markers by electrophoresis in pyridine-acetate and detection with ninhydrin; 1-methylhistidine was also observed after electrophoresis for 4 hours in sodium borate and gave a grey colour with acid ninhydrin. Both acids were recognized after chromatography in butanol-acetic acid after 16 hours on No. 3 paper, in phenol-ammonia, and in butanol-propanol-ammonia after 48.5 hours on No. 3 paper. Ornithine was identified after chromatography in butanol-acetic acid-water-pyridine on No. 3 paper for 15 hours since it is separated from lysine under these conditions. One-dimensional separations of mixtures of basic amino-acids were not very satisfactory in phenol-ammonia or in butanol-acetic acid.

Ethanolamine was found free in fraction 1F1 and was largely separated from basic amino-acids during refractionation of fractions 2F1 and 3F1. Its position on two-dimensional patterns was indicated by formation from the phosphorylethanolamines on hydrolysis of fraction 1F4. Ethanolamine was detected with ninhydrin and was identified with a marker after electrophoresis in pyridine-acetate, and in sodium borate for 4 hours (acid ninhydrin); also after chromatography in phenol-ammonia, in butanol-acetic acid, and in butanol-acetic acid-water-pyridine on No. 3 paper for 15 hours.

Fraction 1F3 contained both β -alanine and γ -aminobutyric acid. These compounds have characteristic mobilities on electrophoresis at pH 4, travelling between the basic and the neutral amino-acids. Both travel near the position of uncharged compounds on electrophoresis in sodium borate but β -alanine gives a distinctive royal blue colour with acid

ninhydrin when the paper has just been heated in ^{the}oven. Identification was also obtained after chromatography in phenol-ammonia, in butanol-acetic acid on No. 3 paper for 16 hours, and in butanol-acetic acid-water-pyridine on No. 3 paper for 15 hours. In the last solvent, γ -aminobutyric acid and ethanolamine travel to similar positions.

α -Aminobutyric acid was detected in fraction 1F₄ and behaved as a typical neutral amino-acid on electrophoresis in pyridine-acetate. It is well separated from other neutral amino-acids by chromatography in butanol-acetic acid. It was also detected after hydrolysis of fractions known to contain ophthalmic acid.

A weak reaction with Ehrlich reagent was obtained with fraction 1F₄ in the area of citrulline after chromatography in butanol-acetic acid. This compound also behaves as a neutral amino-acid on electrophoresis in pyridine-acetate and reacts with ninhydrin. Identification on two-dimensional analysis is complicated because it travels to a position near that of glutamine and both compounds are destroyed by hydrolysis.

The presence of cystathionine in fraction 1F₄ rests largely on identification after two-dimensional analysis. A marker behaved as a neutral amino-acid on electrophoresis in pyridine-acetate, and ninhydrin positive spots in the correct position were detected after chromatography of fraction 1F₄ in phenol-ammonia and in butanol-acetic acid. Chromatography in one dimension is, however, not satisfactory for identification of material in such a complex fraction. After analysis by the standard combination of electrophoresis and chromatography, 24 ninhydrin-positive spots were observed in fraction 1F₄.

Ascorbic acid. This compound was not detected in fractions of stored lens. Its position was identified by means of a 50mM marker which quenched ultraviolet light and gave a ninhydrin colour of roughly the same intensity as a 5mM marker of glutamic acid. The colour with ninhydrin is initially purple but slowly changes to a normal colour. Ascorbic acid was also detected with alkaline silver reagent. After chromatography in butanol-acetic acid, markers of ascorbic acid quenched ultraviolet light and reacted with ninhydrin, and with 0.2% (w/v) ninhydrin in acetone but not with acid ninhydrin. The mobility of ascorbic acid in this solvent is very similar to that of α -aminobutyric acid.

Ninhydrin-negative compounds. The identification of phosphorylcholine, glycerylphosphorylcholine, α -glycerophosphate, creatine and urea after two-dimensional analysis of extracts is based on behaviour of chromatographic markers on the same paper. Inositol and glucose were identified on the basis of their known high concentrations and previous behaviour of markers on chromatography. Phosphorylcholine and glycerylphosphorylcholine would be observed in fraction 1F₄ by reason of their electrophoretic mobility at pH 4. Spots in the appropriate region were obtained with molybdate reagent after chromatography in butanol-acetic acid but were not present after hydrolysis of the fraction. α -Glycerophosphate was detected with molybdate reagent in fraction 1F₈ after electrophoresis in pyridine-acetate and after chromatography in butanol-acetic acid.

Urea was present in fraction 1F₄. It was identified by means of a marker with Ehrlich reagent after electrophoresis in pyridine-acetate and chromatography in butanol-acetic acid.

Creatine has also been tentatively identified in fraction 1F4 and in fraction 4F1, which contains neutral compounds, by the pentacyanoaquoferriate reagent after chromatography in butanol-acetic acid.

Ergothioneine. Ergothioneine was not isolated from fractions. It was identified with a marker and by quenching ultraviolet light and formation of an orange or orange-red spot with Pauly reagent. It did not react with ninhydrin. Ergothioneine did not migrate quite as far as glutamic acid on electrophoresis in formic-acetic acid, and had a mobility similar to that of glutamic acid on chromatography in butanol-acetic acid. A spot, in a position consistent with these properties, and with the reactions of ergothioneine was observed in whole lens extracts and in fractions 1F4, 1F5 and 2F2 (detected in ultraviolet light only).

Ergothioneine travelled with neutral amino-acids on electrophoresis in pyridine-acetate. It was detected with Pauly reagent and under ultraviolet light after chromatography in butanol-acetic acid in fractions 1F4, 1F5, 2F2, 3F2, 4F1 and 4F2 and in the pooled fraction containing neutral amino-acids obtained by refractionation of fractions 2F1 and 3F1. Ergothioneine had an R_f of 0.12 in this solvent. The estimated concentration of ergothioneine in lens was 24-28 $\mu\text{g}/\text{mg}$ fresh weight, about 0.1mM, obtained by the intensity of colour given with the Pauly reagent and compared with a number of markers on the same paper. Fractions 1F4 also contained a compound with a lower R_f than ergothioneine which gave an orange colour with Pauly reagent after chromatography in

butanol-acetic acid for 16 hours on No. 3 paper.

Ergothioneine appeared to be affected by hydrolysis since it was no longer detected in two-dimensional patterns of hydrolysates of fresh lens or fractions 1F4 and 1F5. There also appeared to be a change in pattern of Pauly spots given by fraction 1F4 on hydrolysis after chromatography in butanol-acetic acid but their mobility was affected by chloride which was present.

Unidentified ninhydrin-positive compounds. Only a few of these were not observed during analysis of individual fractions. Two were present before hydrolysis: (34) about which no information has been obtained, and (64) which was not always present and may result from a slight streaking of valine (30). One (3) was only observed on hydrolysis of whole extracts and gave a yellow spot with ninhydrin, it may not be an amino compound. As explained above (p. 117) three spots (43, 55 and 59) are probably given by identified peptides which travel to the apex of the paper.

1) Present before hydrolysis. Fifteen unidentified compounds were observed in whole extracts of fresh lens. Although a number of others were present in individual fractions of stored lens they are not described as the amounts indicated were extremely small. Most of these substances appear to be acidic and may be peptides. Eight were probably present in acidic fractions: 41, 51 and 65 in fraction 1F6; 35, 41 and 65 in fraction 1F7; 38, 44, 45, 48 and 65 in fraction 1F8. One (61) gave a brown colour with ninhydrin and seemed to occur in fraction 1F5. This fraction also contained 36, 37, 56 and 57 while fraction 1F4 contained

36, 52, 56 and 57. Two substances (37 and 57) occupied regions of the paper which gave a positive reaction with molybdate and one of these may be hydroxylysine phosphate. It is not clear whether substance (36) is the same as (4) in hydrolysed extracts and which was observed in hydrolysates of fractions 1F4, 1F6 and possibly 1F7. One substance (46) was observed in higher concentrations in the stored extracts and appeared to be present before hydrolysis in fraction 1F7 and 1F8 and after hydrolysis in all the fractions 1F4 - 1F8; this is possibly cysteic acid.

11) Present after hydrolysis. Eight spots come within this category. One (3) is probably not an amino-acid, another (24) also gave a yellow colour with ninhydrin but was observed in a hydrolysate of fraction 1F4 which also contained (4). Two substances, (4) which may be the same as (36), and (46) which may be cysteic acid are mentioned above. Of the remaining three unidentified substances, one (66) appeared in hydrolysates of fractions 1F6, 1F7 and 1F8; one (28) seemed to be present in hydrolysates of fractions 1F2 and 1F5 only; and the third (2) which was probably present in hydrolysates of fractions 1F2, 1F3, 1F4 and 1F5, might possibly be hydroxylysine.

Part I

General discussion

In this Part, the general significance of the experimental results of Parts III and IV are discussed in relation to findings with other tissues.

General discussion

Less appears to be known about the effect of these changes in circulating levels of steroid hormones, particularly those of the adrenal cortex. The main results in Part III are the identification in terms of another member of this family, $3-(\alpha, \beta\text{-diene-20-one})$ -steroids, together with the new steroid $3-(\alpha, \beta\text{-diene-20-one})$ -steroids in animal form. Experiments indicate that Part V in these experiments are actually normally present in man. The purpose is to show 3 -ketosteroids, particularly to be identified which is of animal origin. They are other has as yet been found in man: $3-(\beta\text{-ketone-20-one})$ -steroids which is present in man (Koster & Winkler 1951).

General discussion

$3-(\alpha, \beta\text{-diene-20-one})$ -steroids is slightly less abundant as a diverse group of 3 -ketosteroids than the 3 -ketosteroids as a number of sources (Table 10). One of these sources are the present in human form (Table 2) and are also in adipose tissue, especially which is 3 -ketosteroids (Koster & Winkler 1951). It is not known whether the 3 -ketosteroids are in other tissues. Sallen et al (1954) have shown that 3 -ketosteroids is present in man in tissues in tissues of one and although the level is not higher in these tissues than of any other steroid. It is believed to be found in the

Part V

General Discussion

In this Part, the general significance of the experimental results of Parts III and IV are discussed in relation to findings with other tissues.

Acidic compounds

Lens appears to be unique among both animal and plant tissues in containing a family of closely-related γ -glutamyl peptides (Table 1). The main results in Part III are the identification in lens of another member of this family: S-(α , β -dicarboxyethyl)-glutathione, together with the new amino-acid S-(α , β -dicarboxyethyl)-cysteine in unbound form. Experiments indicate that both these compounds are probably normally present in lens. The peptide is the first S-substituted glutathione to be identified which is of animal origin. Only one other has as yet been found in nature: S-(β -carboxypropyl)-glutathione which is present in onion (Virtanen & Matikola 1960).

S-(α , β -dicarboxyethyl)-cysteine is slightly less remarkable as a diverse group of S-substituted cysteines have been identified from a number of sources (Table 10). Some of these compounds are also present in bound form (Table 2) and some are known as sulphoxides, e.g. alliin which is S-allylcysteine sulphoxide (Stoll & Seebach 1948). It is not known whether the urinary amino-acids occur in other tissues. Tallen et al (1954) have shown that felinine is present at most in traces in tissues of the cat although the level in cat urine is about 100 times that of any other amino-acid. Felinine is believed to be formed in the

Table 10

Naturally occurring
S-substituted cysteines

<u>Substituent</u>	<u>Occurrence</u>	<u>Reference</u>
Methyl	Kidney bean	1
Allyl	Garlic	2
β -Carboxyethyl	Acacia	3
α -Methyl, β -carboxyethyl	Acacia	4
α, α -dimethyl, γ -hydroxypropyl (Felinine)	Urine	5
β -Carboxypropyl	Urine	6
β -Methyl, α -carboxypropyl	Urine	7
α, β -Dicarboxyethyl	Lens	8

References

1. Thompson, Morris & Zacharius 1956
2. Suzuki, Sugii, Kakimoto & Tsuboi 1961
3. Gaelin 1959
4. Gaelin & Hietala 1960
5. Westall 1953
6. Mizuhara & Oosori 1961
7. Oosori & Mizuhara 1960
8. Present study

kidney and excreted directly. It is of interest that kidney contains quite a high concentration of glutathione, and the enzyme glutathionase. In the detoxication of maleic acid, the efficiency of kidney relative to other tissues for binding maleic acid with thiol groups is probably due to the enzyme which degrades glutathione to free amino-acids (Angielski & Rogulski 1961). Cysteine then reacts much more rapidly with maleic acid than does glutathione (p. 43). Under certain circumstances, excretion of S-(α,β -dicarboxyethyl)-cysteine might occur. The biosynthetic pathway and the functions of S-substituted cysteines are quite unknown. The lens compound appears to be unique among those in Table 10 in being located within animal tissue.

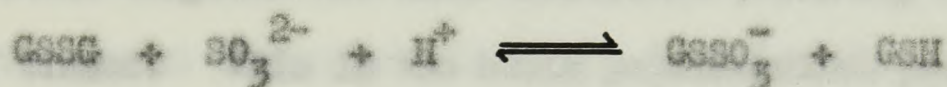
One important difference exists between plant and animal tissues. Plants normally synthesize all their own protein amino-acids, unlike animals which need a number of 'essential' amino-acids. For animals, certain nutritional requirements must therefore be satisfied in the diet and the metabolism of tissues is probably more specialized than in plants where a variety of metabolic pathways may operate. Evidence is accumulating which suggests that the increasing numbers of new amino-acids isolated from plants (see Fowden 1962) are a reflection of this metabolic difference since some at least appear to play an important part in the storage and metabolism of nitrogen.

The γ -glutamyl peptides in plants (Table 2) are thought to play a similar role in nitrogen metabolism but it is not known whether this is also true of the γ -glutamyl peptides in lens. It seems clear that biosynthesis of the lens peptides takes place by the same route

as that of glutathione (Cliffe & Waley 1958). At the moment it is not certain whether the peptides are another unique feature of the lens or whether they are merely as yet unobserved in other tissues. There is increasing evidence that the absence of peptides in tissue extracts is more apparent than real.

Characterization of substances which occur in small amounts in tissue presented formidable difficulties until the advent of techniques such as chromatography and electrophoresis. Even now, the situation is complicated by the very small quantities of some unidentified compounds, for example those which have been observed in lens (p.125). The presence of peptides or peptide-like material is indicated from a number of sources including plants, microorganisms, and animals. Bound amino-acids, some of which are probably combined in genuine peptides, were found in extracts of grass (Synge & Wood 1958, Carnegie 1961), and about 40 peptides appeared to present in yeast extracts (Turba & Esser 1957). At least 30 peptides were obtained from extracts of pig pituitary gland which had been fractionated by chromatography on silicic acid (Ramachandran & Winnick 1957). The peptides were partly characterized as to amino-acid composition and N-terminal acid but their concentration was estimated at only $\frac{1}{3}$ that of the total peptide material. It does not necessarily follow that the lens peptides will be among those identified elsewhere since the occurrence of this family within one tissue seems to be analogous with the presence of a group of γ -substituted glutamic acids in tulips and peanuts (Fowden 1962) and a group of ω -guanido acids in brain (Blass 1960).

One question, which arises following identification of a new compound in a tissue extract, is whether or not the compound is an artifact from the isolation procedure. Experiments with N-ethylmaleimide (p. 80) clearly indicate that the two new acidic compounds in lens are normally present and do not form during the extraction. A similar situation occurred with S-sulphoglutathione (Waley 1959). This peptide is also present after preparation of the lens extract with N-ethylmaleimide. Formation of S-sulphoglutathione during extraction could probably only take place by the reaction:



Under the extraction conditions, GSH was removed with N-ethylmaleimide which should therefore aid the formation of S-sulphoglutathione. The concentration observed was, however, certainly no greater and possibly slightly less than that observed previously which was equivalent to about 0.1mM in lens. The concentration of GSSG was about 5 times that of S-sulphoglutathione and about 5% of the total glutathione. The concentration of sulphite in lens is unknown. Waley prepared protein-free lens extracts by dialysis and by precipitation of protein with heat or with trichloroacetic acid. Since S-sulphoglutathione has been detected at similar concentration in extracts obtained by a variety of methods, it seems probable that it is a genuine constituent of lens.

The N-ethylmaleimide experiment also supported previous results for the presence of GSSG in lens (Kinoshita & Masurat 1957) and provided the first evidence for the presence of free cystine and cysteine.

Because of the normal variation in amount of glutathione in lens, the small contribution (about 5% of total) from cysteine thiol is not sufficient to alter the values obtained by different methods. The presence of cysteine is not surprising in view of the large amounts of glutathione synthesized in lens. The concentration of free cysteine, although probably higher than in most other animal tissues, represents only a small portion of the total bound in glutathione.

Basic peptides

One other compound has been identified which may be a new constituent of tissue. This is the basic peptide which probably has the structure arginyllysylglycine (Part IV). Di- and tri-peptides containing lysine do not appear to have been observed previously in nature. Arginine has been identified in a dipeptide arginylglutamine from green algae (Makimura 1959) and a tripeptide leucylarginylleucine from pig pituitary (Gros et al 1961). It is also present in a nucleotide-peptide anhydride, arginylalanylarginylalanyl-5'-uridyate identified in brewer's yeast (Davies & Harris 1960). As with the acidic peptides, the scarcity of basic peptides in tissue may be more apparent than real. It seems unlikely that the new tripeptide enters the lens through the aqueous humour and so it is presumably synthesized in lens by an unidentified enzyme system and may perform some specific function.

Lens may be considered as a type of muscle since its contraction is mostly, but not completely, controlled by the zonule fibres (Figure 1). The presence of carnosine (Krause 1936), which was confirmed

on paper, is consistent with this concept. Anserine, which is frequently detected in muscle, may also be present in lens. Separation of carnosine and anserine, together with their hydrolysis products histidine and 1-methylhistidine, is rather difficult by paper chromatography and only carnosine and histidine can be detected with Pauly reagent. 1-Methylhistidine has now been identified free in lens and if carnosine is synthesised there, anserine might be synthesised as well since the same enzymes are involved (Kalyanekar & Holster 1959). The possibility also exists that traces of homocarnosine, recently isolated from cow brain (Pisano et al 1961), may be present. This peptide shares the same biosynthetic pathway as carnosine and, although it would react with Pauly reagent, it would probably not separate from known compounds in two-dimensional analysis of lens extracts.

Other compounds

Analysis of protein-free extracts of cow and calf lens (Part IV) before and after hydrolysis has revealed the presence of many amino-acids and related compounds. A number of these have been identified in lens for the first time although they are recognised constituents of tissues. Results show that the qualitative composition of lens closely resembles that of blood with respect to many low molecular-weight substances.

Since the lens is dependant upon the blood stream via the aqueous humour for a supply of nutrients, it is reasonable to expect that many compounds which are present in plasma will be transported into the lens. In fact, a number of compounds, such as 1-methylhistidine,

ornithine and ethanolamine, were identified in lens which have not been detected in cow plasma (Walker 1952) but which by comparison with other species (e.g. Tallan et al 1954, Stein & Moore 1954) are probably present. The detection of ergothioneine (thiohistidine-betaine) in lens is of interest in this connection since ergothioneine is present in blood but, being ninhydrin-negative, is not observed in analyses performed by the Moore and Stein (1954) method of ion-exchange chromatography. Ergothioneine and mercynine (histidine-betaine) have been identified in cattle blood (Ackermann, List & Hensen 1959). Mercynine may well be present in lens since a number of unidentified substances were detected with Pauly reagent. The function of ergothioneine is unknown.

A number of compounds, including glutathione, are located within blood cells and are not found in plasma. Some of these compounds were observed in lens extracts and since it seems unlikely that they are carried in the supply of nutrients, they are presumably synthesised within the lens. Many of the unidentified substances in the extracts which are no longer present after hydrolysis may also be synthesised in lens. Studies on rabbit tissues indicate an increase in concentration of free amino-acids from blood to aqueous humour (Reddy et al 1961) and from aqueous to lens (Reddy & Kinsey 1962) and suggest that some form of active transport is involved. It is probable that the same situation occurs in calf and cow.

A few compounds were also identified in lens extracts which have not yet been identified with certainty in blood. These include β -alanine, tryptophan which is partly destroyed by column chromatography

and cystathionine which is present in brain extracts (Tallan et al 1958). One other amino-acid, β -hydroxyglutamic acid, has recently been observed in cattle brain (Ohara, Michimura, Kaneko & Taketomo 1962) but has not been identified in lens.

In general, therefore, it appears that the distribution of amino-acids in lens is similar to that in other tissues and is not affected by the lack of a direct blood supply. Lens also contains small quantities of a number of unidentified substances which may be peptides or amino-acids. It is not known, however, whether some or all of these are present in other tissues.

During the study of lens extracts, a method of analysis has been developed which may help to supply answers to some of the questions regarding the relationship of the lens to other tissues. Comparatively large amounts of tissue are required for analysis by column chromatography. For most purposes this is not particularly important but where only small amounts of material are available, analysis by this method may no longer be possible. Analysis by a combination of electrophoresis and chromatography has not been applied previously to tissue extracts, and will yield satisfactory results with the equivalent of 0.25 g tissue. Furthermore, the technique is relatively simple and quick, and does not require very complex apparatus. Its disadvantage is that only qualitative or at most semi-quantitative results can be obtained.

However, results obtained so far suggest that a method is now available which is potentially capable of giving a considerable amount of information on the distribution of various classes of low molecular-

weight compounds in tissue. Although it has wider applications, the particular importance of this technique of tissue analysis is that it could be applied to single human lenses (average weight about 0.2 g). The distribution of low molecular-weight compounds in human lens, and the human eye in general, is completely unknown, as are the changes in distribution which take place in cataract formation. Thus it should be possible to add to our knowledge of the eye and the lens and to our understanding of the unique character of the lens as compared with other tissues.

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Part VI

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Summary of fractionations of lens extract

This table summarizes information given in Tables 3 - 6. Division of material into fractions is shown, together with the major ninhydrin-positive constituents, and the elution agent in column chromatography.

Fractionation 1F by continuous electrophoresis

Cathode	1F1	1F2	1F3	1F4	1F5	1F6	1F7	1F8
Tube nos.	1-4	5-8	9-16	17-20	21-23	24-26	27-29	30-32
	Ethanolamine	Basic Amino-acids	β -Ala, γ -AB, Basic Peptide	Neutral Amino-Acids		Glu, GSSG, Oph. Acid	New Acidic compounds, GSSG, Asp.	GSSO ₃ H, New Acidic compounds

Fractionations 2F, 3F, 4F by anion-exchange chromatography

2F1	2F2	2F3	2F4	2F5	2F6	2F7	2F8	2F9	2F10
Water → 0.5 M Pyridine - Formate			→ M Pyridine - Formate			→ M Pyridine - Formate			
Basic & Neutral Amino-Acids	Neutral Amino-Acids		GLU	GSSG	Acidic Amino-Acid	Acidic Compounds	Acidic Peptide	GSSO ₃ H	
3F1	3F2	3F3	3F4	3F5	3F6	3F7	3F8	3F9	3F10
Water → 0.5 M Pyridine - Formate			→ M Pyridine - Formate			→ M Pyridine - Formate			
Basic and Neutral Amino-Acids	Neutral Amino-Acids		Glu	GSSG	GSSG	Acidic Amino-Acid	GLU	Acidic Peptide	GSSO ₃ H
4F1	4F2	4F3	4F4	4F5	4F6	4F7	4F8	4F9	4F10
Water → 0.5 M Pyridine - Formate			→ M Pyridine - Formate			→ M Pyridine - Formate			
Basic & Neutral Amino-Acids	Neutral Amino-Acids		Glu	GSSG	GSSG	Acidic Amino-Acid	GLU	Acidic Peptide	

Abstract

Introduction

The lens of the eye is a unique tissue in several respects. It is located entirely from one layer of epithelial cells and, since no cells are shed during growth, its constituent cells are all of the same age. It has no blood supply and is dependent upon the aqueous humor for supply of nutrients and removal of waste. This lens contains a family of γ -glutamyl peptides which is unique to this tissue. These peptides are related to glutathione, γ -glutamylcysteinylglycine, by replacement of the central amino acid with alanine in γ -glutamylalanine, with γ -glutamylcysteine in γ -glutamylcysteinylglycine, and with γ -glutamylcysteine in γ -glutamylcysteinylglycine. Gillette and Selig have shown that the unique system by which these peptides are synthesized will also

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incorporate other amino acids into the central position, but the corresponding peptides have not been detected in lens. The function of this family of compounds is unknown and only one other member has been identified in nature: γ -glutamyl-L-homocysteine, which is present in milk. γ -glutamyl-L-homocysteine

At least one other amino acid tripeptide was shown to be present in calf lens and the main object of this work was to isolate and identify it. Isolation involved fractionation of lens extracts, and the fractions obtained were subsequently analyzed for (mostly) dipeptide-positive substances. Approximately 50 compounds were detected, some of which are known tissue constituents that have not been identified before in lens, and some of which may be previously unrecognized tissue

Abstract

Introduction

The lens of the eye is a unique tissue in several respects. It is derived entirely from one layer of epithelial cells and, since no cells are shed during growth, it contains cells at all stages of development. It has no blood supply and is dependent upon the aqueous humour for supply of nutrients and removal of waste. Calf lens contains a family of γ -glutamyl peptides which is unique among tissues. These peptides are related to glutathione, γ -glutamylcysteinylglycine, by replacement of the central amino-acid with alanine in norophthalmic acid, with α -aminobutyric acid in ophthalmic acid, and with S-sulphocysteine in S-sulphoglutathione. Cliffe and Waley have shown that the enzyme system by which these peptides are synthesized will also incorporate other amino-acids into the central position, but the corresponding peptides have not been detected in lens. The function of this family of compounds is unknown and only one other member has been identified in nature: S-(β -carboxypropyl)-glutathione, which is present in onion.

At least one other acidic tripeptide was known to be present in calf lens and the main object of this work was to isolate and identify it. Isolation involved fractionation of lens extracts, and the fractions obtained were subsequently analyzed for (mostly) ninhydrin-positive substances. Approximately 60 compounds were detected, some of which are known tissue constituents that have not been identified before in lens, and some of which may be previously unrecognized tissue

constituents. A method has been developed for analysis of most of the low molecular-weight substances in small amounts of tissue by a combination of electrophoresis and chromatography on a single paper.

New acidic compounds

Fractionation of trichloroacetic acid extracts of lens was performed by two methods. The first, which had not been applied previously to tissue extracts, was by continuous-flow paper electrophoresis; the second was by anion-exchange chromatography. A better gross fractionation was obtained by continuous electrophoresis at pH 4 than by chromatography since basic and neutral substances were not adsorbed on the resin and were not separated. Acidic compounds were fractionated more satisfactorily by chromatography and the material obtained by the first method, which was homogenous on electrophoresis at pH 2.3 and pH 4, was split into two peaks by the ion-exchange column. These peaks were given by two different compounds which have been identified, by degradation and by comparison with synthetic material, as the peptide S-(α , β -dicarboxyethyl)-glutathione and the related amino-acid S-(α , β -dicarboxyethyl)-cysteine. Contaminants, present in the concentrated solutions after fractionation, prevented isolation of natural material as pure solids.

The peptide, on hydrolysis, yielded equimolar amounts of glycine and glutamic acid, together with a small amount of cysteine and an unidentified amino-acid. This amino-acid gave an atypical mauve colour with ninhydrin-cobalt chloride and contained sulphur. Dinitrophenylation indicated that glutamic acid was N-terminal in the

peptide. Both the peptide and the amino-acid were oxidized with performic acid to what appeared to be sulphones. The oxidation products were separated from glutathione sulphonie acid by chromatography but not by electrophoresis at pH 4, nor was the amino-acid sulphone separated from it at pH 2.3. The sulphur atom was assumed to be present as a thioether in the new compounds since neither yielded a sulphonie acid on oxidation. Satisfactory separation of the amino-acid, the peptide, their oxidation products, cysteic acid, and glutathione sulphonie acid, was achieved on a single paper by a combination of electrophoresis at pH 4 and chromatography in butanol-acetic acid-water. The presence of a modified cysteine residue in the amino-acid was indicated by desulphurization of the peptide with Raney nickel to norophthalmic acid.

The compounds S-(α, β -dicarboxyethyl)-cysteine and S-(α, β -dicarboxyethyl)-glutathione were known and had been prepared by addition of thiol to the double bond of maleic acid. The amino-acid had also been obtained by addition of cysteine to fumaric acid. These syntheses were repeated under salt-free conditions which facilitated isolation of the products, and the peptide was also obtained by addition of glutathione to fumaric acid. Corresponding products synthesized from each of the unsaturated acids appear to be identical and are probably mixtures of diastereoisomers. The amino-acid was conveniently isolated as a crystalline di-dicyclohexylamine salt.

Identity between the natural and the synthetic compounds was established in several ways. The amino-acids were eluted at the same position from a cation-exchange column. They gave identical mauve

colours with ninhydrin-cobalt chloride and could not be separated by electrophoresis at two pH values nor by chromatography in two solvents. Products of oxidation with performic acid were indistinguishable on electrophoresis. Both natural and synthetic S-(α , β -dicarboxyethyl)-cysteine yielded approximately 10% of cystine on hydrolysis, and largely cyclized on heating in aqueous solution to form more acidic substances, indistinguishable by chromatography, which gave strong reactions for organic acids but only weak colours with ninhydrin. Identical patterns of products were obtained on electrophoresis and on chromatography of peptide hydrolysates. Both natural and synthetic S-(α , β -dicarboxyethyl)-glutathione were oxidized to the same substance which was separated from glutathione sulphonic acid by electrophoresis at pH 2.3 but not at pH 4.

Thiols react with N-ethylmaleimide much more rapidly than with either maleate or fumarate. An extract of fresh calf lens was prepared in the presence of N-ethylmaleimide in order to establish whether or not the new acidic compounds are artifacts. This extract contained both S-(α , β -dicarboxyethyl)-cysteine and S-(α , β -dicarboxyethyl)-glutathione and it seems unlikely that either compound was formed during the extraction procedure. Both compounds are, therefore, probably genuine constituents of calf lens and the concentrations in lens are about 0.05mM for the amino-acid and 0.2mM for the peptide, compared with 7-10mM for glutathione.

Lens homogenates do not appear to catalyse the addition of either glutathione or cysteine to fumarate. The amount of free glutathione seemed to decrease in the lens mixture relative to a control

solution containing no lens homogenate. Added glutathione may become bound to protein in some way, possibly by hydrogen bonding. The amount of diffusible glutathione is known to increase in the presence of urea or guanidine which rupture hydrogen bonds.

Other sulphur compounds

The lens extract prepared in the presence of N-ethylmaleimide also contained GSSG, cystine, and S-(N-ethylsuccinimide)-cysteine. The concentration of GSSG, about 3-5% of total glutathione, is in agreement with other values for lens and brain obtained by different methods. Free cysteine has previously been assumed to be absent from lens and is present only in traces in other tissues. The total concentration of cysteine/cystine indicated by this experiment is about the same as that of GSSG, 3-5% of glutathione, which is not sufficient to alter appreciably the amount of glutathione determined by various techniques.

New tissue constituents

Analysis of individual fractions of lens extracts by electrophoresis and by chromatography before and after hydrolysis indicated the presence of a number of unidentified substances. Three of these have been studied and are believed to be new constituents of tissue.

One compound is a basic tripeptide, with the probable structure arginyl-lysylglycine, which is present in lens at a concentration of about 0.02M. Lysine, arginine and glycine were liberated on hydrolysis, and quantitative analysis of the hydrolysate with ninhydrin gave a ratio of 1 : 2.2 for neutral to basic amino-acids. Dinitrophenylation indicated N-terminal arginine. The complete amino-acid sequence was obtained by

digestion of the peptide with trypsin which yielded the three amino-acids. A peptide with the alternative structure would have yielded arginine and the dipeptide glycyllysine. One other arginine tripeptide, leucylarginylleucine, has been isolated from pig pituitary gland.

The other two substances have been only partly characterized. One may be an aliphatic amine with a single unsubstituted amino group and possibly between 6 and 12 carbon atoms. No substance of this type has been identified in tissue. Its concentration in lens is about 0.04 mM. This substance travels near the solvent front on chromatography in three solvents; it is unaffected by hydrolysis but appears to be attacked by nitrous acid. It yields a picrate and probably a hydrochloride. The second substance contains phosphorus and does not react with ninhydrin. It has a characteristic blue fluorescence in ultra-violet light after electrophoresis at pH 2.3 but not at pH 4 or pH 9. Guanine is liberated on mild acid hydrolysis but since the hydrolysate contains only traces of inorganic phosphate, it seems unlikely that this substance is guanosine mono- or tri-phosphate, both of which are believed to be present in lens.

Other tissue constituents

In general, little is known about the distribution of low molecular-weight compounds in lens. Many of the compounds known to be present in lens were found in individual fractions of extracts, and a number of other compounds have been identified by electrophoresis and by chromatography. These are mostly present in plasma and are transported through the aqueous humour into the lens. No quantitative studies have

been carried out but it seems likely that some at least of the compounds are at a higher concentration in lens than in plasma.

All the common protein amino-acids were found free in calf lens. Previous data (on cow lens) had indicated the presence only of alanine, glycine, glutamine, serine, threonine, valine, leucine, aspartic and glutamic acids. The concentration of cystine is greater than in most other tissues and is probably connected with the high concentration of glutathione in lens. Lens, unlike aqueous humour, contains more free cystine than methionine. The amino-acids were identified with markers and detected with ninhydrin, also with reagents specific for particular groups in the molecule wherever possible.

A number of non-protein amino-acids have been identified in the free state. α -Aminobutyric acid, which is present bound as ophthalmic acid, is also found free at a higher concentration than many protein amino-acids. Other compounds which have been detected are ornithine, 1-methylhistidine, β -alanine, γ -aminobutyric acid, and ethanolamine. Citrulline and cystathionine are probably present but the evidence for these is not as firm as with the others. Taurine had been detected previously.

Only a few ninhydrin-negative compounds were characterized and one, ergothioneine, was detected in lens for the first time. It was identified with a marker by its electrophoretic and chromatographic behaviour, and was located under ultraviolet light and with Pauly reagent. The apparent concentration of ergothioneine in lens, about 0.1mM, is similar to that in plasma.

Investigation of whole extracts

Preparation and investigation of fractions of lens extracts required comparatively large amounts of tissue and was a lengthy procedure. A considerable concentration of material was obtained which permitted identification of some compounds present in only small amounts. Electrophoresis or chromatography in a single direction did not give a sufficiently good separation for analysis of complex fractions. Satisfactory separations were achieved on single papers by low-voltage electrophoresis at pH 4.6 in the first direction followed by chromatography in butanol-acetic acid-water in the second. Since compounds in individual fractions tended to occupy different areas of the paper, the method was extended to analysis of unfractionated extracts. Much information could be gained quite quickly from extracts of single lenses.

A combination of electrophoresis and chromatography has one main advantage over two-dimensional chromatography for the study of tissue extracts. Electrophoresis acts as a desalting process by removing most inorganic ions and a more reproducible pattern is obtained without treatment of the deproteinized extract before analysis. This method does not seem to have been applied previously to analysis of tissue extracts although protein hydrolysates and enzymic digests of proteins have been studied by similar systems. The relative pattern of spots was constant but actual positions varied slightly in the direction of chromatography. Many compounds were identified by comparison with markers. Papers were viewed under ultraviolet light then treated with ninhydrin or with another location reagent. The blue fluorescence of

tryptophan, present in unhydrolysed extracts acted as a useful internal marker. A number of different extracts were compared.

Satisfactory results were obtained with half of a calf lens extract or quarter of a cow lens extract on a single paper, that is about 7 mg total solid. About 60 spots were detected with ninhydrin before hydrolysis, of which about half have been identified with some certainty, another 10 have been identified tentatively, and the remainder are unidentified. Most of the unidentified substances are present in only trace amounts and virtually no information has been obtained about them. About 35 spots were detected in hydrolysed extracts, and only a few of these substances are unidentified. Some compounds have also been identified in extracts which were examined with reagents other than ninhydrin. Coloured spots were sometimes given by compounds present in high concentration which do not normally react with the reagents.

Since lens extracts were obtained initially from stored tissue, a comparison was made between trichloroacetic acid extracts of stored and of fresh lens. The extracts were almost the same with the exception that in stored lens ascorbic acid had decomposed, glutamine had cyclised, and some breakdown of peptides seemed to have occurred. The last is probably a consequence of enzyme activity which is known to persist in vegetables stored at low temperature. No significant difference was observed between extracts of fresh calf lens prepared by removal of protein with phenol or with trichloroacetic acid. Cow and calf lenses gave almost identical patterns and the concentrations

of many compounds were similar in both extracts. S-(α , β -dicarboxyethyl)-cysteine, S-(α , β -dicarboxyethyl)-glutathione, and the new basic peptide were all detected in fresh cow and calf lenses. The unidentified ninhydrin-positive spots may be peptides, but their concentration is low relative to that of the amino-acids, and most of the amino-acid spots are not noticeably more intense after hydrolysis.