Acidic Peptides of the Lens

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Discussion of the important problem of protein synthesis is often concerned with the apparent paucity of peptides (other than glutathione) in cells. That this paucity may be no more than apparent is suggested by the recent work of Turba & Esser (1955) on yeast, of Westall (1955) on urine, and the work described below on the lens of the calf. Nearly all the solid in the lens is protein, and a cow lens has more than twice the weight of a calf lens (and is no less dense), so the absolute amount of protein in the lens of a young animal is increasing. Moreover, the lens is an exceptional tissue in two ways: there is no flux in the cellular population, and the organ is enclosed in a capsule believed to be impermeable to proteins. These characteristics formed the basis of the idea that peptides, if involved in protein metabolism, might well be present in the lens of young animals. It is also noteworthy that glutathione is present at a relatively high concentration in the lens (Kleifeld & Hockwin, 1956). Carnosine is the only other peptide whose presence has been reported in the lens (Krause 1936).

As the predominant diffusible ninhydrin-positive species are amino acids, it is an advantage to use a method of fractionation which separates peptides from most of them. With acidic (or basic) peptides, this separation can be effected by electrical-transport methods or ion-exchange resins; in the work described below zone electrophoresis (Tiselius & Flodin, 1953) has been used. Preliminary experiments showed that it was the acidic fraction in which hydrolysis brought about the greatest change in chromatographic pattern, and so the work described below deals only with acidic peptides. One such peptide (for which the name ophthalmic acid is proposed) is particularly abundant, and is being investigated in detail.

METHODS

Analytical techniques

Separation of peptides from proteins. Dialysis was the main method of removing high-molecular-weight material used in this work. A clear-cut separation between small peptides and proteins can thus be effected in homogeneous solution. The use of protein precipitants has been mostly avoided (cf. Gordon, 1949; Synge, 1951), as precipitated proteins may adsorb peptides. Thus Walker (1952) has noted losses of glutathione in ethanol precipitation of proteins, and the presence of peptides adsorbed on trypsin, even after repeated recrystallization, has been reported (Desnuelle, 1953).

Dialysis in buffered solutions. Enzymic reactions during the dialysis must be minimized, otherwise any peptides present may be hydrolysed to amino acids, and proteolysis will give rise to peptides which are artifacts. Thus it is desirable to inactivate enzymes without precipitating them. Hence the tissue was disintegrated, and dialysis carried out, in a buffered solution. The buffer must also be volatile, or dissolve in solvents which are not likely to dissolve peptides, and it must not precipitate proteins. The alkaline buffer used was triethylamine half neutralized with formic acid; at this point the pH (10·8) is so high that enzymic reactions are unlikely to take place, but not so high that peptide bonds are likely to split. In a comparative experiment, dilute formic acid was used as an acidic buffer. Both buffers were good solvents for the lens proteins, so that practically all the cellular constituents remained in solution during the dialysis. The main results were qualitatively the same whether dialysis took place in acidic or alkaline solution.

Phenol–water partition. In the earlier work, the next step in the fractionation was phenol–water partition. Grassmann & Deffner (1953) showed that proteins were extracted from water by phenol, and phenol–water partition has been used in the study of the peptides of the licheniformin group (Callow & Work, 1952), corticotrophin (Dixon & Stack-Dunne, 1955) and the bound amino acids of grass (Synge & Wood, 1954). An important advantage of this step is that inorganic salts which interfere with paper chromatography are thereby removed. It has since been found that salts do not interfere in paper electrophoresis, so that in the later work this step has been omitted; in fact, some of the peptides encountered do have favourable partition coefficients for extraction by phenol, but this may not be so for all of them. The partition coefficient of glutathione was found to be increased nearly 20-fold in favour of the non-aqueous phase when the system phenol–2% trichloroacetic acid replaced the

phenol–water system. A comparable change was brought about in paper chromatography: thus the R_F value of γ-glutamylglutamic acid was raised from 0·08 in phenol–water (ammoniacal atmosphere) (LeQuesne & Young, 1950) to 0·6 in phenol–1% trichloroacetic acid (with paper which had previously been soaked in the solvent and dried).

Paper electrophoresis. This technique has proved invaluable. It can be carried out on protein-free tissue extracts, thus obviating the need to remove salts, and it has the great advantage that electrophoretic mobility is much more easily correlated with chemical structure than is chromatographic mobility. This point is particularly important when compounds of unknown structure are present in complex mixtures. Larger amounts can be handled by paper electrophoresis than by paper chromatography, and nearly a gram of material can be fractionated in one run when slabs of cellulose powder replace sheets of filter paper. The use of high voltages adds resolution (Michl, 1951; Werner & Westphal, 1955; Wieland & Pfleiderer, 1955). After paper electrophoresis, further fractionation by paper chromatography has proved useful.

General

The eyes, which were removed from the calves soon after death, were cooled in ice while being taken to the laboratory; the lenses (average weight approx. 1 g.) were stored frozen. The tissues were disintegrated in a top-drive macerator (Townson and Mercer, Ltd., Croydon).

Descending paper chromatography on Whatman no. 1 or Whatman no. 4 paper was generally used; the solvent mixture *n*-butanol–acetic acid–water (40:9:20, by vol.) is referred to as butanol–acetic acid; freshly prepared solvent was employed.

The several different procedures for paper electrophoresis are described below; Whatman no. 3 and Whatman no. 3 MM papers were used.

Paper blanks. In the earlier work, chromatographically washed paper was used for preparative chromatography and electrophoresis; however, there is little contamination when unwashed Whatman no. 3 paper is used. The amount of impurity extracted from the paper depends on the chromatographic solvent (or on the buffer for electrophoresis) and the solvent afterwards used for elution. Paper electrophoresis in the acid (pH 2) buffer and elution with dilute acid appeared to give relatively little impurity; presumably much of the inorganic contaminant is removed during the electrophoresis. More impurity is obtained after chromatography, but even here paper 'blanks' (i.e. extraction of the paper where no spots were), which were normally carried out at each stage of the fractionations, yielded little ninhydrin-positive material after hydrolysis.

Detection of amino acids and peptides. Amino compounds were detected by dipping the papers in ninhydrin (0·4%, w/v) and cobalt chloride (0·2%, w/v) in propan-2-ol (Wiggins & Williams, 1952), heating until the colours started to appear, and then holding the papers in a jet of steam for a short time; maximum colour development was attained after about 24 hr. Compounds containing an amino or imino group, and amides (including some nucleotides), were detected by chlorination followed by treatment with *o*-tolidine (Reindel & Hoppe, 1954); the colours fade fairly fast and especially fast if phenol was used as a chromatographic solvent, even if the paper has been washed or subsequently developed in another solvent. When the chromatographic solvent contained pyridine, the dried paper must be chromatographically washed with acetone, or held in a vigorous jet of steam for about 20 min.; the small amount of pyridine in the pyridine–acetate buffer used for electrophoresis does not interfere.

Hydrolysis of peptides. This was effected with redistilled 6N-HCl in sealed Pyrex-glass tubes for 16 hr. at 110°. The hydrolysates were transferred to a polythene sheet and evaporated to dryness in a vacuum desiccator; a drop of water was added, the mixture rubbed with a fine glass rod to dissolve the residue, and the solution evaporated again.

End-group determination. The peptide (about 50 μg.) in water (10 μl.) and triethylamine carbonate buffer [0·1 ml.; prepared by treating a 6% (v/v) solution of triethylamine with CO_2 until the solution was alkaline to phenol red but neutral to phenolphthalein] was treated with 1-fluoro-2:4-dinitrobenzene (10 mg.) in ethanol (0·25 ml.). The solution was kept for about 3 hr. in the dark, and the ethanol distilled at room temperature; more buffer (0·2 ml.) was added, and the solution extracted three times with ether and then evaporated under reduced pressure. The residue was hydrolysed under the conditions described above. The hydrolysate was diluted with 5 vol. of water, and extracted three times with ether. The combined ether extracts were extracted with a little water (which was added to the aqueous layer of the hydrolysate), dried with Na_2SO_4 and evaporated. The residue was dissolved in acetone and examined by paper chromatography in the *tert.*-pentanol–phthalate system of Blackburn & Lowther (1951), modified by using *tert.*-pentanol containing 10% (v/v) of propan-2-ol (R. Gregory & G. T. Young, unpublished observation). The amino acids in the aqueous phase of the hydrolysate were identified in the usual way. This method of dinitrophenylation is similar to the modification of Lockhart & Abraham (1954) of the method of Sanger & Thompson (1953), but here the reaction is carried out at a lower pH; little dinitrophenol is formed.

RESULTS

Three experiments on calf lenses are described separately as different methods of extraction and fractionation were used.

Experiment L-I

The scheme of fractionation in this experiment is set out in Fig. 1.

Dialysis in acid solution. Twenty calf lenses were macerated with 18% (w/v) formic acid (70 ml.), and the solution was dialysed against $CHCl_3$–water (500 ml.) for 16 hr. at 4°; the membrane was cellulose dialysis tubing, 0·75 in. in diameter (Gallenkamp and Co., London). The diffusate (which had a pH of 2·5) was evaporated under reduced pressure, and finally kept *in vacuo* for a week.

Phenol–water partition. The syrup (325 mg.) in water (3 ml.) (pH about 5·8) was submitted to a three-tube countercurrent distribution between phenol and water, with the 'diamond' procedure of Bush & Densen (1948) and

3 ml. of each phase. The combined phenol solution (9 ml.) was diluted with light petroleum (30 ml.) and ether (20 ml.) and extracted with water (3 × 3·5 ml.), and the combined aqueous extracts were washed with ether and evaporated *in vacuo*, giving 34 mg. (fraction L-I-P). The aqueous fractions from the phenol–water partition were combined, washed with ether, desalted electrolytically and evaporated *in vacuo*, giving 90 mg. (fraction L-I-A).

Electrophoretic fractionation of fraction L-I-P. Fraction L-I-P in 10% (v/v) propan-2-ol (0·5 ml.) was applied along the starting line of Whatman no. 3 MM paper [57 cm. × 19 cm.; previously washed chromatographically with 1% (v/v) acetic acid] and the paper moistened with a pyridine–acetate buffer (50 ml. of acetic acid, 15 ml. of pyridine and 2·5 l. of water; Grassmann, Hannig & Plöckl, 1955) up to the starting line from each side, and electrophoresis then carried out at 240 v (8 mA) for 5 hr. in the apparatus of Cannon & Gilson (1954). After drying at 40°, the paper was heated at 110° for 15 min. (Phillips, 1948); three fluorescent bands were revealed in u.v. light (Fig. 2). Much buffer siphons down the sheet, so that all the substances move downwards. More satisfactory separations are effected with thicker paper (see Expt. L-III). An earlier run under these conditions (but with only a drop of the solution L-I-P) had shown three ninhydrin-positive spots in positions corresponding both to the fluorescent bands and approximately to the positions occupied by acidic, neutral and basic amino acids. The three fractions were eluted with 1% (v/v) acetic acid, and the eluates evaporated *in vacuo*; the residues weighed: 6 mg. (fraction 1); 22 mg. (fraction 2); 4·6 mg. (fraction 3).

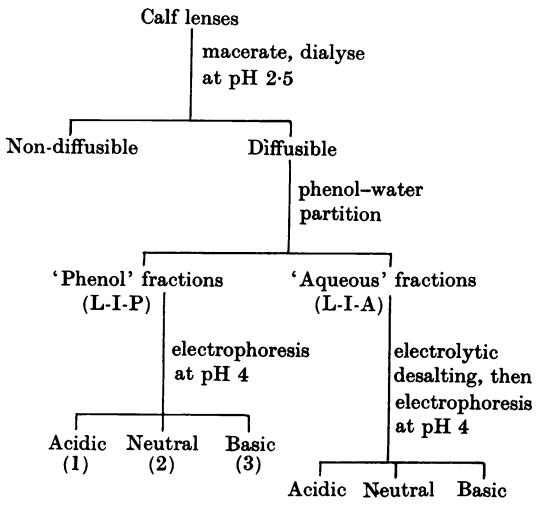

Fig. 1. Scheme of fractionation in Expt. L-I.

Chromatography before and after hydrolysis. The fractions were dissolved in sufficient 10% (v/v) propan-2-ol to give 5% (w/v) solutions of the acidic and basic fractions and a 10% (w/v) solution of the neutral fraction. Half of the acidic and basic fractions and one-third of the neutral fraction were hydrolysed with an equal volume of conc. HCl at 105° for 28 hr. in sealed tubes. The hydrolysates were transferred to polythene, evaporated, water was added and the solution again evaporated. Portions of the hydrolysates and the original solutions were examined by paper chromatography, with butanol–acetic acid as solvent; the acidic fraction (L-I-P-1) clearly contained ninhydrin-positive species other than glutathione and glutamic acid, although these appeared to be the main products present. The hydrolysate of this fraction showed the expected spots of cystine, glycine and glutamic acid, and also a spot in the position of α-amino-*n*-butyric acid; a fainter alanine spot was also observed. On some chromatograms faint valine or methionine spots, or both, and leucine or isoleucine spots, or both, could be seen. Paper electrophoresis (in the pyridine–acetate buffer) of the hydrolysate showed two strong spots: one in the position of glutamic acid, one in the position of neutral amino acids; there was also a faint spot in the position of the basic amino acids. Under these conditions, γ-aminobutyric acid and β-amino*iso*butyric acid travel further towards the cathode than other neutral amino acids; they were not present in the hydrolysate. The chlorine–tolidine reagent also showed that fraction L-I-P-1 contained several substances which were unstable to acid hydrolysis. The marked changes in the chromatographic pattern brought about by hydrolysis of the acid fraction were not shown by the neutral or basic fractions.

Electrophoretic fractionation of L-I-A. Fraction L-I-A (80 mg.) in 10% (v/v) propan-2-ol (0·36 ml.) was fractionated electrophoretically by the procedure described for fraction L-I-P. The results are not illustrated as they were essentially similar to those described for the phenol extract, but the separation between the slower-moving acidic and the neutral species was not clear-cut. The faster-moving acidic fraction on hydrolysis showed a strong spot of the same R_F as alanine, and a faint one of the same R_F as α-amino-*n*-butyric acid; the solution before hydrolysis did not show the presence of these substances.

Experiment L-II

The scheme of fractionation in this experiment is set out in Fig. 3.

Dialysis in alkaline solution. Calf lenses (117 lenses, 111 g.), stored frozen (for approx. 6 weeks) were added to

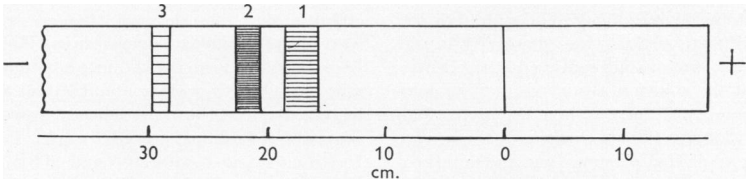

Fig. 2. Electrophoretic fractionation of fraction L-I-P: no. 3MM paper; pH 3·9; 4·2 v/cm.; 5 hr. The depth of shading represents the strength of the ninhydrin colour reaction.

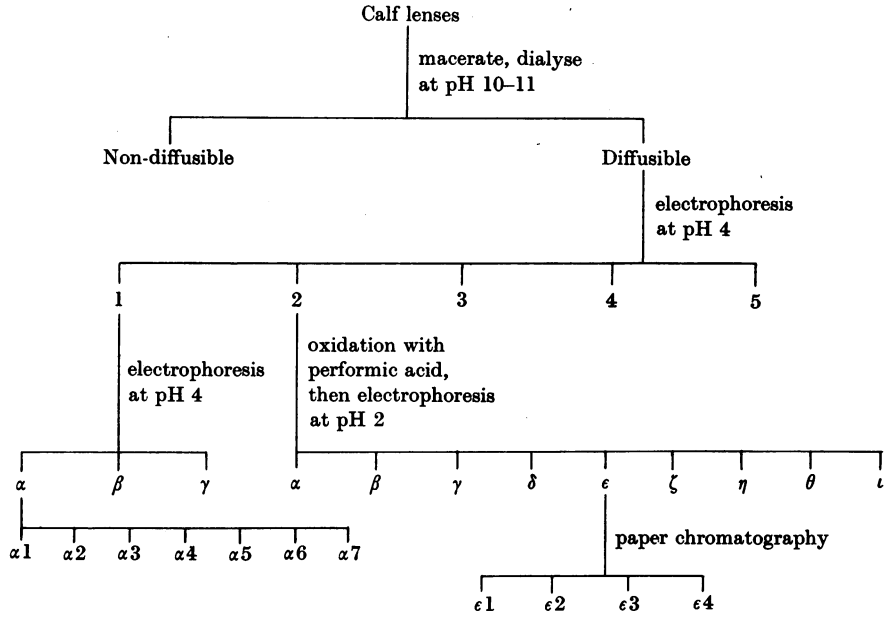

Fig. 3. Scheme of fractionation in Expt. L-II.

cooled buffer (275 ml. of buffer prepared from 50 ml. of triethylamine, 8·9 g. of 90% formic acid and 360 ml. of water; pH about 11), stirred by hand till the frozen tissue thawed, and then macerated for a few minutes after the addition of a few drops of capryl alcohol. The solution was dialysed against 1·8 l. of $CHCl_3$–water at 4° for 3 days. The diffusate was concentrated to a syrup on a rotary evaporator at 30° and finally left for a week in a high vacuum (in the presence of P_2O_5 and NaOH). From about four-fifths of the diffusate, 5·8 g. of a syrup was obtained; this was shaken with $CHCl_3$ (50 ml.) to remove triethylammonium formate (and lipids) and dried, giving 1·4 g. of a gum.

Electrophoresis on cellulose powder. Whatman cellulose powder (ashless, standard grade; 190 g.) was stirred into a paste with 500 ml. of pyridine–acetate buffer (Grassmann *et al.* 1955), and poured on to a piece of 0·002 in. thick polythene film draped over a block of marble at the bottom and glass strips at the side, so as to form a slab 43 cm. × 20 cm. × 0·4 cm.; the ends were blocked with folded filter paper. Excess liquid was removed by blotting the surface, contact was made with the electrode vessels by strips of Whatman seed-test paper (1/16 in. thick) and the slab was left several hours to equilibrate. The sample was introduced by absorbing it on filter paper and inserting this in the slab; a similar arrangement has been used by Smithies (1955) in electrophoresis of proteins on starch gels. The gum obtained from the alkaline diffusate (0·7 g.) was dissolved in the buffer (1 ml.), a small amount of solid removed at the centrifuge and the whole of the solution absorbed on Whatman no. 3 MM paper (20 cm. × 5·7 cm.). When the paper had dried it was cut into 13 strips (20 cm. long) and these were inserted into a trough made just previously in the middle of the cellulose-powder slab; then the powder was pressed back against the paper, the whole slab covered with a piece of Whatman no. 1 paper, and this covered with polythene film. Electrophoresis was carried out for 15 hr. at 200 v (current 35 mA); the mobility is much lower in cellulose powder than on sheets of filter paper. At the end of the run, the covering piece of paper was dried and developed with ninhydrin. This print (Fig. 4) showed five bands on the cellulose slab. The slab was then cut up, and the fractions were dried in air for several days. The combined fractions from two runs were packed into a column (diameter 1·8 cm.) and eluted with water; about 40 ml. of solution was collected, although the ninhydrin reaction was no longer positive after about 5 ml. had flowed through. The yields of the acidic fractions 1 and 2 were 156 and 203 mg. respectively.

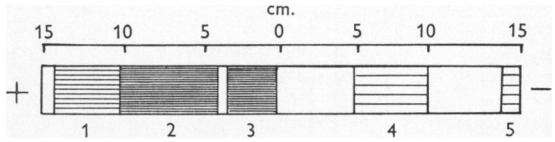

Fig. 4. Electrophoretic fractionation of diffusate, Expt. L-II: cellulose powder; pH 3·9; 4·7 v/cm.; 15 hr.

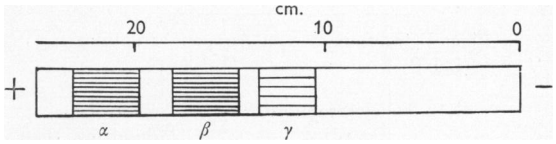

Fig. 5. Electrophoretic fractionation of fraction L-II-1: cellulose powder; pH 3·9; 4·7 v/cm.; 19 hr.

Fraction 1 was taken up in buffer (0·5 ml.) and fractionated again on an 8 cm. wide cellulose-powder slab (200 v for 19 hr.); the print (Fig. 5) showed three fractions (1α, 1β, 1γ).

The cellulose-powder fractions were dried, packed into a column (diameter 1 cm.), and eluted with water. The yield of 1α was 35 mg., and of 1β, 23 mg.

Chromatographic examination of fractions 1α *and* 1β. Small spots of 10% (w/v) aqueous solutions of 1α and 1β were run on paper chromatograms to gauge the complexity of the mixtures and test various solvents. The solvents investigated were butanol–acetic acid, *n*-butanol–pyridine–water (1:1:1, by vol.), (72%, w/w) phenol–3% (w/v) NH_3 soln., phenol–cresol–borate, pH 9·3 (Levy & Chung, 1953) and *n*-butanol–acetic acid–water–pyridine (30:6:24:20). In the phenol and phenol–cresol solvents, the R_F values were too low; of the other solvents, the butanol–acetic acid–water–pyridine one was preferred, as the more regular shape of the spots suggested that adsorption was not interfering; in this solvent, about six ninhydrin-positive spots were obtained from both 1α and 1β; in 1α one spot was much stronger than the others, but in 1β there were several spots of much the same strength.

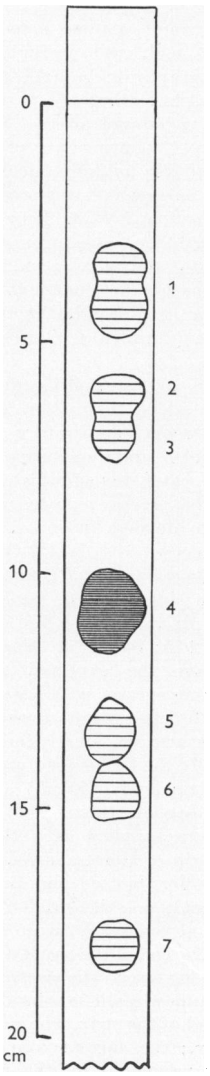

Fig. 6. Chromatographic fractionation of fraction L-II-1α: no. 3 paper; *n*-butanol–acetic acid–water–pyridine (30:6:24:20, by vol.); 22 hr.

Chromatographic fractionation of 1α. The whole of 1α was applied as a 10 cm. band (with guide spots each side) on Whatman no. 3 paper, and run in *n*-butanol–acetic acid–pyridine–water [the pyridine (B.D.H. 'redistilled') was redistilled under N_2 after adding Br_2 (2 ml./l.); the *n*-butanol was also redistilled] for 22 hr. One guide strip was treated with ninhydrin (Fig. 6); the other was rinsed in acetone, dried and then chromatographically washed with acetone, to remove the pyridine. It showed several spots which quenched the fluorescence of the paper in u.v. light (probably nucleotides), and after treatment with chlorine, followed by *o*-tolidine, showed several spots mostly in the same positions as the ninhydrin-positive ones. The fractions were eluted with water and the eluates evaporated. As there were only small amounts of the fractions (other than fraction 4), they were hydrolysed and the amino acids liberated on hydrolysis identified by paper chromatography in butanol–acetic acid and phenol–0·3% (w/v) NH_3 soln.; the first two fractions were examined only in the former solvent, which does not separate cystine and cysteic acid. The results are given in Table 1. The main peptide, 1α4, had *N*-terminal glutamic acid (Table 5); it had zero net charge at pH 2 (Fig. 7).

*Fraction L-II-*1β. This fraction is obviously quite complex, judging by paper chromatography and paper electrophoresis at pH 2 (Fig. 7); some of the components had zero net charge at pH 2. The main amino acids liberated on hydrolysis were cysteic acid, glycine and glutamic acid (Table 2).

Table 1. *Peptides from fraction L-II-*1α

Spot no. (Fig. 6)	Main amino acids liberated on hydrolysis	Strength	Minor amino acids
1	Cys and/or $CySO_3H$	+ + + +	? Ala
	Gly	+ + +	
	Glu	+ + +	
2	Cys and/or $CySO_3H$	+ + + +	
	Gly	+ +	
	Glu	+ + + +	
3	Cys + $CySO_3H$	+ +	? Lys
	Gly	+ + + +	
	Glu	+ +	
4	Cys	+ + + +	*X**
	Gly	+ + + +	
	Glu	+ + +	
5	Cys	+ +	*X**, ? Lys
	Gly	+ + +	
	Glu	+ + +	
6	$CySO_3H$	+ +	
	Gly	+ +	
	Glu	+ + + +	
7	Cys	+ + +	? Arg, *X**
	Gly	+ +	
	Glu	+ + + +	
	Ala	+	

* An unidentified compound, travelling just in front of arginine in butanol–acetic acid.

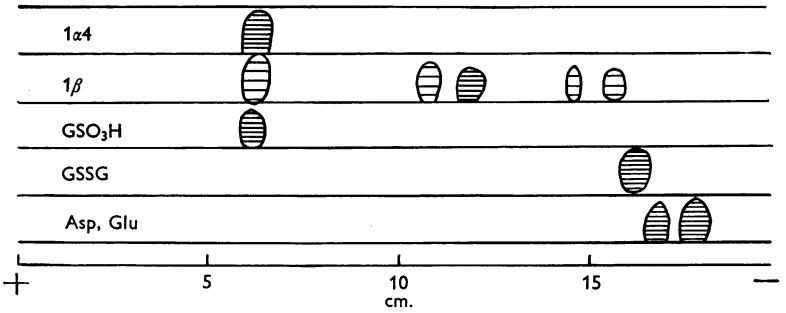

Fig. 7. Electrophoresis of fractions L-II-1-α4 and L-II-1β, and acidic markers; no. 3MM paper; pH 2; 29v/cm.; 1·5 hr.

Table 2. *Amino acid composition of fraction L-II-1β*

Main amino acids liberated on hydrolysis	Strength	Minor amino acids
$CySO_3H$	+ +	Asp, Ala, α-Amino-*n*-butyric acid
Gly	+ +	? Cys, ? Ser
Glu	+ + + +	

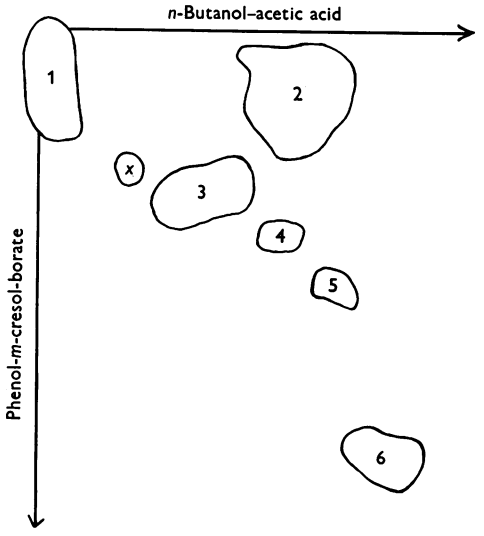

Fig. 8. Chromatography of amino acids liberated on hydrolysis of fraction L-II-2: no. 52 paper; butanol–acetic acid, followed by phenol–*m*-cresol–borate buffer. The identities of the spots are as follows: 1, cystine; 2, glutamic acid; 3, glycine; 4, threonine; 5, alanine; 6, α-amino-*n*-butyric acid. Spot *X* has not been identified.

Fraction L-II-2. The amino acids present in a hydrolysate of this fraction were identified by paper chromatography with the procedure of Levy & Chung (1953) on Whatman no. 52 paper; development in the first direction was carried out for 20 hr., and in the second direction for 40 hr. The results (Fig. 8) show the presence of cystine, glycine, glutamic acid, threonine, alanine, a spot in the position of α-amino-*n*-butyric acid, and an unidentified spot (*X*). Further chromatography in butanol–acetic acid, phenol–0·3% (w/v) NH_3 soln., *n*-butanol–pyridine–water (1:1:1, by vol.), and *n*-butanol–acetic acid–water–pyridine (30:6:24:20, by vol.) confirmed the presence of the amino acids mentioned above; no amino acid with an R_F above that of α-amino-*n*-butyric acid was present.

Oxidation of fraction L-II-2. This fraction is mainly composed of glutathione. Oxidation with performic acid converts this into the corresponding sulphonic acid (GSO_3H), which has zero net charge at low pH values; this facilitates the separation of glutathione from peptides not containing cystine (Consden & Gordon, 1950; Sanger & Thompson, 1953).

Formic acid (98–100%; 7 ml.) containing H_2O_2 (30%, w/v; 0·7 ml.) was added to fraction 2 (180 mg.) and the solution kept at room temperature for 30 min. Water (8 ml.) was then added, and the solution concentrated under reduced pressure; water was added and the solution again evaporated, and this process repeated; finally, the solution was evaporated to dryness *in vacuo*. The residue was a colourless solid (referred to as L-II-2-Ox).

Electrophoretic fractionation of L-II-2-Ox at pH 2. Three electrolytes were investigated for electrophoresis of L-II-2-Ox at low pH values. The first was 0·2N acetic acid (1·1%, v/v) (pH 2·75; Sanger & Thompson, 1953), and, although this gave the expected separation between GSO_3H and the other species, with heavy loading the bands were diffuse. Much better results were obtained if the concentration of acetic acid was greatly increased (e.g. to 30%, v/v), and eventually the buffer used was 90% (w/v) formic acid (50 ml.), acetic acid (150 ml.) and water (800 ml.) (Kickhöfen & Westphal, 1952). The sample (18 mg.) in water (0·05 ml.) was applied as a thin streak across a 57 cm. × 10 cm. strip of Whatman no. 3 paper which had been soaked in buffer, blotted and laid on a marble slab covered with a sheet of polythene (0·002 in. thick). A film of water ensured good contact between the marble and the polythene sheet. The end of the paper dipped into the buffer in Perspex electrode vessels; the electrodes were 24 in. long (27 S.W.G.) platinum wires. The solution was put on 12 cm. from the anode end of the strip. The run was carried out at 2000v for 1·75 hr.; the current rose from 34 to 50 mA. Overheating was prevented by the periodic addition of powdered solid CO_2 to a glass plate lying on top of the polythene sheet covering the paper. At the end of the run, the moist strip of the no. 3 paper was placed between two pieces

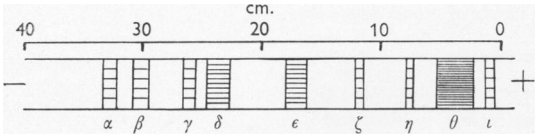

Fig. 9. Electrophoretic fractionation of fraction L-II-2-Ox: no. 3 paper; pH 2; 36 v/cm.; 1·75 hr.

Table 3. *Amino acid composition of peptide fractions of L-II-2-Ox*

Band no. (Fig. 9)	Main amino acids liberated on hydrolysis	Strength	Minor amino acids
α	$CySO_3H$	+	
β*	Glu	+	
	Ala	+ +	
γ†	—	—	
δ	Glu	+ + +	Asp
ε	Gly	+ + +	
	Glu	+ + +	
	α-Aminobutyric acid	+ + +	
	Ala	+	
ζ	$CySO_3H$	+	*X*
	Gly	+ + +	
	Glu	+ + +	
η‡	$CySO_3H$	+ +	*X*, α-amino-*n*-butyric acid
	Gly	+ +	
	Glu	+ +	
θ§			
ι	$CySO_3H$	+ + +	
	Gly	+	
	Glu	+	

* Faint by ninhydrin, strong by chlorine–tolidine method.

† A very faint band, not giving sufficient amino acids to be identified.

‡ Negative by ninhydrin, strong by chlorine–tolidine method.

§ See Table 5.

of Whatman no. 1 paper, and pressure applied with a photographic roller (Ryle, Sanger, Smith & Kitai, 1955). The strip and the prints were dried; the top print was treated with ninhydrin (Fig. 9) and the bottom one with chlorine, followed by *o*-tolidine. The fractions were eluted with acetic acid (10%, v/v), and about 3 ml. was collected from the major fractions (δ, ε and θ) and 0·3 ml. from the minor ones; the eluates were dried *in vacuo*, giving 2 mg. of fractions δ and ε, and 12·2 mg. of fraction θ. The amino acids present in hydrolysates of the fractions were identified by chromatography in butanol–acetic acid and phenol–0·3% (w/v) NH_3 soln.; the results are given in Table 3. Fraction δ is mainly glutamic acid together with a little aspartic acid, and fraction θ is the sulphonic acid derived from glutathione. Fraction ε consisted of several peptides, and was further fractionated.

Chromatographic fractionation of L-II-2-Ox-ε. Fraction ε (2 mg.) in water (0·1 ml.) was spread as a narrow band 17 cm. long on Whatman no. 3 paper, with guide spots on each side. The chromatogram was developed with butanol–acetic acid for 48 hr. The guide spots developed with ninhydrin showed four spots, of which the fastest (ε1: ophthalmic acid) was the most intense (Fig. 10). The bands were eluted with 10% (v/v) acetic acid, and the eluates dried *in vacuo*.

The amino acid composition of the fractions is given in Table 4; fractions 1 and 2 appear to be homogeneous. This is confirmed by the results of the *N*-terminal end-group determination given in Table 5.

Experiment L-III

Extraction with 60% (v/v) ethanol. Eleven calf lenses were added to boiling ethanol (10 ml.), and after several minutes the solid was separated at the centrifuge and extracted with four portions of boiling 60% (v/v) ethanol. Ethanol was removed by distillation from the combined extracts, and the aqueous solution extracted with ether, concentrated, and finally dried in the frozen state. The residual resin weighed 143 mg.

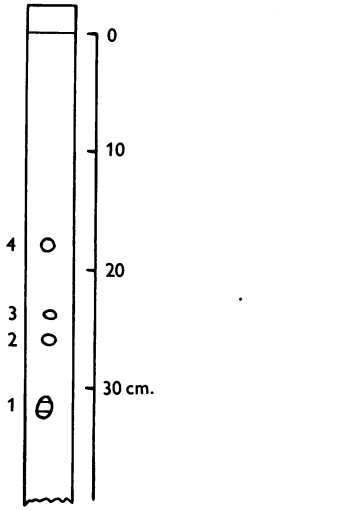

Fig. 10. Chromatographic fractionation of fraction L-II-2-Ox-ε: no. 3 paper; butanol–acetic acid; 48 hr.

Table 4. *Peptides from fraction L-II-2-Ox-ε*

Spot no. (Fig. 10)	Main amino acids liberated on hydrolysis	Strength	Minor amino acids
1	Gly	+ + +	
	Glu	+ + +	
	α-Amino-*n*-butyric acid	+ + +	
2	Gly	+ +	
	Glu	+ +	
	Ala	+ +	
3	Gly	+	$CySO_3H$, *X*
	Glu	+	
4*	Glu	+	$CySO_3H$

* Present only in traces; too little for the amino acid composition to be determined.

Table 5. N-*Terminal amino acids of some peptides*

Peptide	Amino acids present	Strength of amino acid after Hydrolysis	Strength of amino acid after DNP* treatment	DNP* amino acid identified	Structure
1 α4	Cys	+ + +	+ + +	Glu	
	Gly	+ + +	+ + +		
	Glu	+ + +	−		
	X	+			
2-Ox-θ	$CySO_3H$	+ + + +	+ + + +	Glu	Glu [$CySO_3H$, Gly]
	Gly	+ + + +	+ + + +		
	Glu	+ + + +	+		
2-Ox-ε-1	Gly	+ + +	+ + +	Glu	Glu [Gly, α-amino-*n*-butyric acid]
	Glu	+ + +	−		
	α-Amino-*n*-butyric acid	+ + +	+ + +		

* DNP = 2:4-dinitrophenyl

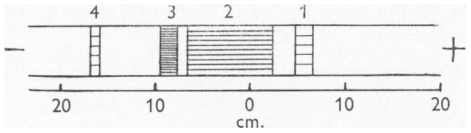

Fig. 11. Electrophoretic fraction of extract, Expt. L-III: seed-test paper; pH 6·8; 3·5 v/cm.; 7·25 hr.

Table 6. *Amino acid composition of fractions L-III-1 and L-III-2*

Fraction	Main amino acids liberated on hydrolysis	Minor amino acids liberated on hydrolysis
1	Gly Glu Ala	Cys
2	Cys Gly Glu α-Amino-*n*-butyric acid	Ala

Electrophoretic fractionation. The resin in 0·5 ml. of collidine–acetate buffer (Newton & Abraham, 1954) was applied along the starting line of Whatman seed-test paper (57 cm. × 15 cm.) (chromatographically washed in 10% (v/v) acetic acid). The electrophoresis was then carried out at 200 v (16 mA initially; 27 mA finally) for 7·25 hr., as described for fraction L-I-P. The 'print' (Fig. 11) taken from the paper before drying shows that there is much less washing of the neutral species down the thick seed-test paper than occurred with the 3MM paper. The main acid fraction (2) is very broad, possibly owing to adsorption. Fractions 1 and 2 and a 'blank' cut from above fraction 1, were eluted with 10% (v/v) acetic acid (5 ml.), and the eluates evaporated. When the fractions were tested by electrophoresis in pyridine–acetate buffer, only traces of neutral amino acids were present, and no ninhydrin-positive spot was detected from the 'blank'; fraction 1 showed only one spot, but fraction 2 gave two spots, the smaller corresponding in position to fraction 1. The amino acids liberated on hydrolysis are listed in Table 6.

Experiments on aqueous humour

Collection, concentration and dialysis. Aqueous humour was withdrawn from cattle eyes at the slaughterhouse, and stored frozen; 500 ml. was concentrated in the frozen state to 80 ml., and then dialysed against 200 ml. of $CHCl_3$–water for 4 days at 4°. The diffusate was freeze-dried. The solid residue (3·2 g.) was dissolved in water (8 ml.); the solution had a pH of about 9·8, although the pH of aqueous humour is about 7·6. This rise in pH may be brought about by the action of urease on urea.

Phenol–water partition. The aqueous solution was extracted twice with an equal volume of phenol, and the combined phenol extracts were diluted with ether (15 ml.) and light petroleum (15 ml.), and extracted with water. This aqueous solution still contained sufficient salts to interfere with chromatography, so it was concentrated to a volume of 1·3 ml. and submitted to further phenol–water partition, with three tubes, 1 ml. of each phase, with two extra ('withdrawal') tubes for the mobile (aqueous) phase (Craig, 1952). The contents of the two tubes, in which the most phenol-soluble constituents would be concentrated, were combined, diluted with ether (4 ml.) and light petroleum (4 ml.), extracted with water, and the solution evaporated. The residue (5·4 mg.) was dissolved in 10% propan-2-ol (0·4 ml.) (fraction Aq-P).

Chromatographic analysis of fraction Aq-P. Fraction Aq-P was now free from salts, and the main ninhydrin-positive species were tyrosine, proline, arginine, lysine, valine, phenylalanine, leucine and isoleucine. As expected, these are most of the amino acids which have a high R_F in paper chromatography with phenol as solvent (in an alkaline atmosphere). The presence of tryptophan, which was confirmed with Ehrlich's reagent (Dalgliesh, 1952; Smith, 1953), has not been reported in earlier analyses of aqueous humour (Blaha, Wewalka & Zwiauer, 1953; Malatesta, 1952). Two-dimensional chromatography in *n*-butanol–pyridine–water (60:40:35, by vol.), and butanol–acetic acid, followed by colour development with chlorine and *o*-tolidine (after steaming the paper), showed about seventeen spots: the amino acids, and urea, were the main substances present. After acid hydrolysis, as well as the amino acids already mentioned, alanine, glycine, glutamic acid and probably α-amino-*n*-butyric acid were present, but in low concentrations.

Chromatographic fractionation of fraction Aq-P. Fraction Aq-P was then fractionated as a 12 cm. long band on Whatman no. 3MM paper in the butanol–pyridine solvent for 18 hr. Nine bands were revealed (by fluorescence in u.v. light, and from guide spots developed with chlorine and *o*-tolidine), and eluted with 1% (v/v) acetic acid. The eluates from the four slowest bands were again fractionated (as 6 cm. bands) in butanol–acetic acid: the fractions were eluted and the eluates hydrolysed, but too little material was obtained to identify the hydrolysis products. So the eluates from the other bands of the butanol–pyridine fractionation were hydrolysed without any further fractionation. In only one fraction (7) did hydrolysis liberate much of an amino acid; in the others the main amino acids were already present before hydrolysis. Fraction 7, which contains phenylalanine, leucine and isoleucine, liberated alanine on hydrolysis. The amino acid arising from hydrolysis is believed to be alanine, on the basis of its R_F in butanol–acetic acid and phenol–3% (w/v) NH_3 soln.; paper electrophoresis at pH 4 showed the absence of β-alanine from the hydrolysate.

Phenol–trichloroacetic acid–water partition. The partition coefficient of glutathione between phenol and water was found to be about 7 (in favour of aqueous phase), but the value for phenol–2% (w/v) trichloroacetic acid was reduced to 0·4; the partition coefficient of NaCl in the latter system was about 20. From these values, by the method of Bush & Densen (1948), the optimum ratio of volumes of phases is 3·7 ml. of aqueous trichloroacetic acid:10 ml. of phenol for the separation of glutathione and NaCl.

The aqueous layers after the phenol–water partition of aqueous humour described above were combined and seven-tenths was evaporated; the residue was dissolved in 6·3 ml. of 2% (w/v) trichloroacetic acid (saturated with phenol) and a three-tube partition carried out (Bush & Densen, 1948) with the ratio of solvent volumes given above. The contents of the two tubes, in which the most phenol-soluble constituents would be concentrated, were combined, diluted with ether (50 ml.) and light petroleum (50 ml.) and extracted with water. The aqueous solution was washed with ether (3 × 70 ml.), evaporated, and the residue (179 mg.) dissolved in 0·6 ml. of 10% propan-2-ol. The small amount of trichloroacetic acid remaining affected paper chromatography in butanol–acetic acid, displacing leucine and isoleucine to positions ahead of their normal positions; this did not happen in phenol–3% (w/v) NH_3 soln., or *n*-butanol–acetic acid–water–pyridine (30:6:24:20, by vol.). Acid hydrolysis caused little change in the chromatographic pattern, apart from the hydrolysis of glutamine to glutamic acid; the mixture was obviously complex, and has not been further investigated.

Experiments on liver and blood

Alkaline dialysis. Calf liver (40 g.) was stirred with triethylamine formate buffer, pH 10·8 (100 ml.), and dialysed at 4° against 150 ml. of $CHCl_3$–water for 3 days. The diffusate was dried in the frozen state, and the product (2·4 g.) extracted with $CHCl_3$ (2 × 15 ml.); the residue (0·64 g.) was dissolved in collidine–acetate buffer.

Electrophoretic fractionation. The fractionation was carried out on acid-washed Whatman seed-test paper, as described in Expt. L-III. The acid fraction was eluted with 10% (v/v) acetic acid, and a portion hydrolysed with acid. Calf erythrocytes (40 ml.) were investigated by the same procedure as described for liver.

Results. The hydrolysates of the acid fractions were examined by two-dimensional chromatography by the method of Levy & Chung (1953) and also by chromatography in phenol–3% (w/v) NH_3 soln. and butanol–acetic acid. The major amino acids were cystine, glycine and glutamic acid; smaller amounts of alanine and aspartic acid were also present. Electrophoretic comparison of the acid fractions before and after acid hydrolysis showed a relatively strong ninhydrin-positive fraction (of mobility between those of aspartic and cysteic acids) disappearing on hydrolysis.

DISCUSSION

Peptides in the lens

Preliminary experiments. Two fractionations were carried out; they differed only in that in one (Expt. L-I; see Fig. 1) an acid buffer was used for extraction and dialysis, and in the other preliminary experiment (not described in detail) an alkaline buffer was used. In both experiments, the most striking result was that hydrolysis of the acidic fraction liberated a ninhydrin-positive compound, chromatographically indistinguishable from α-amino-*n*-butyric acid. A smaller amount of alanine, and much smaller amounts of valine (or methionine or both) and leucine (or isoleucine or both) were also formed. Paper electrophoresis showed the absence of β-amino-*iso*butyric acid and γ-aminobutyric acid. These are the three isomers of aminobutyric acid which have so far been found to occur naturally. α-Amino-*iso*butyric acid gives a relatively faint colour with ninhydrin, and β-amino-*n*-butyric acid would probably have nearly the same electrophoretic mobility as β-amino-*iso*butyric acid. Hence, I tentatively conclude that the hydrolysis product is α-amino-*n*-butyric acid. A separate experiment showed that the proteins of calf lens do not contain α-amino-*n*-butyric acid, at least in amounts comparable with the other amino acids. α-Amino-*n*-butyric acid has frequently been found in mammalian tissues in the free state (Gordon, 1949; Walker, 1952); peptides of this amino acid, on the other hand, have not hitherto been detected.

The next step was to identify the acidic peptide containing α-amino-*n*-butyric acid. Because a larger amount of the acid fraction was obtained when dialysis was carried out in alkaline solution, this procedure was adopted in the next fractionation (Expt. L-II).

Main experiment. In this experiment, the phenol–water partition step was omitted (see Fig. 3). The electrophoretic fractionation showed that there were three acidic fractions (Figs. 4 and 5); for convenience, two (Fig. 5, 1α and 1β) will be called the 'fast-acid' fraction, and the other (Fig. 4, fraction 2) the 'slow-acid' fraction. The fast-acid fraction was not detected in the preliminary experiments but it

was observed in the confirmatory one (Expt. L-III). It was quite complex, containing at least a dozen ninhydrin-positive components, yet the main amino acids in the hydrolysate were glutamic acid, glycine, cystine and cysteic acid. Two factors probably contribute to the complexity of this fraction: disulphide interchange (Ryle & Sanger, 1955) (probably occurring during the dialysis in alkaline solution) and autoxidation of cystine residues to cysteic acid ones. Nevertheless, the peptides appeared to contain differing molar ratios of glycine to glutamic acid, so that there probably is a class of peptides containing glutamic acid, glycine and cystine. It is to be expected that peptides containing the strongly acidic cysteic acid residue should be found in the fast-acid fraction; but further fractionation showed that several peptides contained cystine but no cysteic acid (Table 1), so their high electrophoretic mobility at pH 4 suggests the presence of some unidentified strongly acidic group. This has been confirmed for the main component of fraction 1α (1α4), which was found to have zero net charge at pH 2 (Fig. 7); the amino group, bearing a positive charge, must be balanced by a group with pK about 1, carrying a negative charge. The acidic group might be a phosphate; the presence of phosphorus in this fraction is inconclusive, as nucleotides (i.e. phosphorus-containing spots which also absorb ultraviolet light) are also present. Further fractionation is necessary to decide whether the nucleotides are combined with the peptide. Keir & Davidson (1956) have isolated a fraction from rabbit tissues which on hydrolysis yields glutamic acid, cystine, glycine and an ultraviolet light-absorbing base, and it is possible that this substance, or class of substances, is present in the lens of the calf. Fraction 1α4 resembles glutathione in that equimolecular proportions of the three amino acids appear to be present, in that the N-terminal amino acid is glutamic acid, and all the glutamic acid is N-terminal, so that it is a tripeptide.

Acid hydrolysis of the slow-acid fraction liberated cystine, glycine, glutamic acid, threonine, alanine, α-amino-n-butyric acid and an unidentified substance (X) (Fig. 8). This unidentified substance has approximately the same R_F as the unidentified substance obtained on hydrolysis of some of the components of the fast-acid fraction, and so they are both referred to as X in Tables 1 and 3. Larger amounts of one of the fractions yielding X on hydrolysis will have to be obtained before further work on its structure can be undertaken. Although threonine seems to be one of the amino acids in the hydrolysate of the slow-acid fraction, it is apparently present in too low a concentration to be detected in any of the hydrolysates of the individual fractions (Table 3). Leucine (and/or isoleucine) and valine (and/or methionine), which were present in low concentration in the hydrolysate of the acid fraction in the preliminary experiments, were not detected in the main experiment. Extraction with phenol, used only in the preliminary experiments, is probably particularly efficient in concentrating peptides of the amino acids with large non-polar side chains.

Glutathione was expected to be the major component of the slow-acid fraction, so the mixture was oxidized so as to convert cystine peptides into cysteic acid ones and fractionated by electrophoresis at pH 2, where the sulphonic acid (GSO_3H) derived from glutathione has zero net charge (Fig. 7). The three main fractions consisted of glutamic acid, GSO_3H, and a mixture of peptides, which was fractionated further by paper chromatography (Fig. 10). The main fraction (ϵ-1) proved to be the acidic peptide (called ophthalmic acid) containing α-amino-n-butyric acid. Ophthalmic acid probably contains equimolecular amounts of glutamic acid, α-amino-n-butyric acid and glycine; there is only one glutamic acid residue, which is N-terminal, so the structure of ophthalmic acid is probably either glutamyl-α-amino-n-butyrylglycine or glutamylglycyl-α-amino-n-butyric acid. The number of possible structures is raised to four by the possibility that either the α- or γ-carboxyl group of the glutamic acid may be combined in the peptide bond:

$H_2N.CH(CO_2H).[CH_2]_2.CO.NH.CH_2.CO.NH.CHEt.CO_2H$
$H_2N.CH(CO_2H).[CH_2]_2.CO.NH.CHEt.CO.NH.CH_2.CO_2H$
$H_2N.CH(CH_2.CH_2.CO_2H).CO.NH.CH_2.CO.NH.CHEt.CO_2H$
$H_2N.CH(CH_2.CH_2.CO_2H).CO.NH.CHEt.CO.NH.CH_2.CO_2H$

These structures must be considered tentative. For one thing, α-amino-n-butyric acid has not been rigorously identified as a hydrolysis product; for another, α-amino-n-butyric acid might arise from the decomposition of another amino acid. Heyns & Walter (1953) found that threonine was converted into α-amino-n-butyric acid under more rigorous conditions than are normally used for the hydrolysis of peptide bonds; it is clearly possible that some unusual structural feature renders a threonine residue especially labile. Ophthalmic acid occurs at a relatively high concentration in calf lens, of the same order as glutamic acid and approximately one-tenth the concentration of glutathione; these estimates are based on the weights of the fractions obtained. Another peptide, separated in the last fractionation (ϵ-2), probably contained equimolecular amounts of glutamic acid, glycine and alanine. Further work is necessary to gauge the complexity of the minor fractions.

Confirmatory experiment. In another experiment (L-III), the proteins, after precipitation with ethanol, were extracted repeatedly with hot aqueous ethanol. This procedure was adopted as by its use Turba & Esser (1955) had obtained numerous peptides from yeast, and it seemed desirable to compare the results obtained by extraction in neutral solution with those obtained in acid and alkaline solution. Two acid fractions were again detected, and hydrolysis of the slow-acid fraction gave cystine, glycine, glutamic acid, α-amino-*n*-butyric acid and alanine.

Peptides in the aqueous humour

The work on the lens described above was preceded by a search for peptides in the aqueous humour of cattle eyes. Phenol–water partition was used to remove the large amounts of inorganic salts, and was followed by preparative paper chromatography; electrophoresis was not used here. One fraction contained phenylalanine, leucine and isoleucine, and liberated alanine on hydrolysis. It is not possible to say whether this fraction contained a peptide built up from alanine and one (or more) of the three amino acids present before hydrolysis, or whether 'bound' alanine was present in some other form. Further investigation of this material was precluded by its low concentration; it is probably present at a concentration of less than 0·1 mg./100 ml. of aqueous humour, a concentration comparable with that of the unidentified ampholytes in urine, for whose investigation Westall (1955) starts with 100 l. of urine. It is possible that electrophoretic methods of fractionation would enable other peptides or bound amino acids to be detected, but it should be borne in mind that the concentration of glutathione in aqueous humour is only about one-thousandth of that in the lens (K. W. Daisley, unpublished observation).

Peptides in other tissues

The search for peptides in the erythrocytes and liver of the calf was not carried out as thoroughly as in the main experiment described above; the hydrolysate of the acid fractions contained cystine, glycine, glutamic acid, aspartic acid and alanine, but no α-amino-*n*-butyric acid; thus there is no evidence for the presence of ophthalmic acid in the liver or blood. The only evidence of the presence of peptides was that a ninhydrin-positive acid fraction (electrophoretically distinct from glutathione) was unstable to hydrolysis. Turba & Esser (1955) have mentioned the presence of peptides in liver, and Heilmeyer (1954) has observed unidentified ninhydrin-positive acidic compounds in erythrocytes. (Earlier work on peptides in tissues has been reviewed by Synge, 1953; see also Bricas & Fromageot, 1953.) Thus the balance of evidence is in favour of the idea that certain kinds of peptides, at least, may occur generally in animal tissues.

Metabolic role of peptides

There is but little definite knowledge to hamper speculation about the metabolic role of peptides in higher organisms. Campbell & Work (1953) have pointed out that the general lack of success in detecting peptides (other than glutathione) in tissues may only reflect the inadequacy of our techniques and cannot justifiably be used as an argument against peptides mediating in protein synthesis. Peptides may be present at very low concentrations and yet be intermediates in the synthesis of proteins, just as monomer and high polymer are the only abundant species during the course of a free-radical vinyl polymerization. The peptides encountered in the work described above all contain glutamic acid, possibly γ-linked. This recalls the views put forward by Waelsch (1954) that transpeptidation of γ-glutamyl peptides may be important in protein synthesis. Peptides could, of course, also figure as intermediates in protein breakdown. The extent to which protein breakdown occurs generally in animal tissues is not known (see Stanier, 1955; Tarver, 1954); it is not likely that ophthalmic acid, in particular, is an intermediate in either the synthesis or the breakdown of any of the main proteins of the lens, as α-amino-*n*-butyric acid was not detected in a hydrolysate of calf-lens protein. In yeast, Turba & Esser (1955) have found numerous acidic peptides; when the yeast is grown with radioactive acetate as a source of carbon, the peptides are labelled before the proteins. This is a necessary but not sufficient condition for these peptides being precursors of the proteins, and certainly rules out the possibility that the peptides in yeast are breakdown products of the proteins.

SUMMARY

1. Calf lenses were disintegrated in an acid or alkaline buffer, and the solution was dialysed. The diffusible compounds were fractionated by electrophoresis on cellulose powder, and on filter paper, followed by paper chromatography.

2. Two ninhydrin-positive acidic fractions have been examined in detail: both contain several peptides.

3. Hydrolysis of one of these acidic fractions liberates α-amino-*n*-butyric acid, arising from a tripeptide containing also glutamic and glycine residues; the glutamic acid is *N*-terminal. The tripeptide ('ophthalmic acid') is present at a relatively high concentration, of the order of 20 mg./100 g. of calf lens, but it has not been detected in the liver or erythrocytes of the calf.

4. The other acidic fraction contains peptides (other than glutathione) built up from glutamic acid, cystine (or cysteine) and glycine. Some of these peptides contain a strongly acidic group (so far unidentified), so that the molecule has zero net charge at pH 2. One of these peptides is a tripeptide containing an *N*-terminal glutamic acid residue.

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The Metabolism of Propionic Acid

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One pathway of propionic acid metabolism has been shown by Flavin, Ortiz & Ochoa (1956) to be: propionic acid → methylmalonic acid → succinic acid → citric acid cycle. That this is not the only pathway is indicated by the work of Gray, Pilgrim, Rodda & Weller (1951) who demonstrated the synthesis of *n*-valeric acid from propionate and acetate in the rumen. Recently *n*-undecanoic, *n*-tridecanoic *n*-pentadecanoic and *n*-heptadecanoic acids have been isolated from some natural fats by Hansen, Shorland & Cooke (1954*a*, *b*, 1955) and by Shorland, Gerson & Hansen (1955). James & Martin (1956), James & Wheatley (1956) and Wheatley & James (1957) have demonstrated by means of gas–liquid chromatography the widespread occurrence of these straight-chain odd-numbered acids in natural