attributed to error in the chemical method. The evidence further suggests that the normal values for serum calcium are some 3–4 % higher than the figures given by King (1951).

SUMMARY

1. A flame-spectrophotometric method of estimating calcium in biological fluids is described.

2. Spectral interference due to sodium is mostly eliminated by measuring the calcium emission at 423 mμ.

3. Phosphate interference is overcome by diluting the samples with excess of phosphate.

4. Studies with radioactive calcium (^{45}Ca) show that about 7 % of calcium in serum is lost during washing and precipitation in the procedure of Kramer & Tisdall (Tisdall, 1923).

5. Magnesium is coprecipitated in this procedure in a variable amount (range 0–11 %), averaging 3 %.

6. It is concluded that the accepted normal serum-calcium figures are approximately 4 % too low.

The author wishes to express his thanks to Professor King for suggesting this investigation, and for subsequent advice and criticism.

The flame spectrophotometer was constructed for me by Mr S. Roberts.

Mr A. Asatoor and Miss Doreen Ailward gave much technical assistance.

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Acidic Peptides of the Lens

2. THE USE OF ION-EXCHANGE RESINS AS MOLECULAR SIEVES*

BY S. G. WALEY
Nuffield Laboratory of Ophthalmology, University of Oxford

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A tripeptide, named ophthalmic acid, has recently been isolated from calf lens (Waley, 1956). Further work on the structure of ophthalmic acid needed more material than was available from the isolation already carried out. The original method used dialysis, followed by zone electrophoresis, to obtain acidic compounds of low molecular weight; large volumes of solution have to be concentrated to isolate the diffusible compounds, and zone electrophoresis is limited to handling fairly small amounts of material. The procedure described below avoids these drawbacks, has permitted the isolation of ophthalmic acid in the crystalline state, and has allowed the identification of another peptide, norophthalmic acid. This procedure utilizes ion-exchange resins to effect separations based on molecular size as well as ionic charge.

* The paper by Waley (1956) is to be regarded as Part 1.

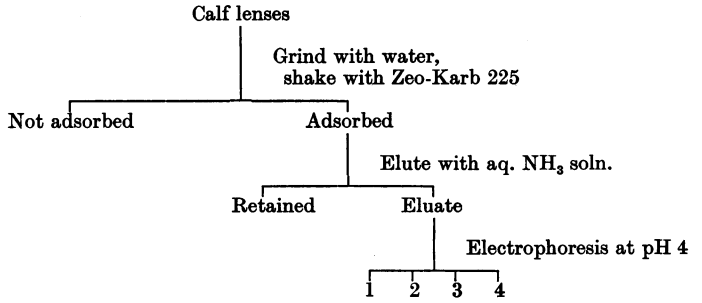

Fig. 1. Scheme of fractionation in Expt. I.

Richardson (1949, 1951) and Kunin (1949) have shown that large ions may not be adsorbed on ion-exchange resins, and Partridge (1952) has pointed out the usefulness of this 'molecular sieve' effect for the separation of small and large ions; amino acids were separated from a protein by using a cation exchange resin (Thompson, 1952). Similarly, Deuel, Solms & Anyas-Weisz (1950) used an anion-exchange resin to separate galacturonic acid from polygalacturonic acid.

METHODS AND RESULTS

General

The methods used were generally the same as those previously described (Waley, 1956). The chromatographic solvent mixture butanol–acetic acid–water (40:9:20, by vol.) is referred to as butanol–acetic acid, and 72% (w/w) phenol–3% (w/v) NH_3 soln. as phenol. Numerical R_F values are not recorded, as the solvent was allowed to drip off the edge during an overnight run on Whatman no. 4 paper; in both these solvents ophthalmic acid had the same R_F as alanine, and norophthalmic acid the same as glycine.

Amino acid estimations on paper were carried out after chromatography on Whatman no. 52 paper; the ninhydrin reagent was that described previously. The same volume (5 μl.) of both the solution and standard solutions was run on the same sheet. Finally, the sheet was cut into strips, parallel to the direction of the solvent flow, and the maximum intensity of the spots measured at 510 mμ, with the apparatus of Tennant, Whitla & Florey (1951) (obtained from K. Kniazuk, 614 Franklin Street, Elizabeth, New Jersey, U.S.A.) in a Beckman Spectrophotometer, Model DU.

Experiment I

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

Adsorption on cation-exchange resin. The resin used was that recommended by Partridge (1952): Zeo-Karb 225 (Permutit Co., London), 15–30 mesh, water-regain 0·75 g./g. Calf lenses (11 g.; stored in a frozen condition), and a little sand, were ground with water (30 ml.), the resin (4·2 g.) was added and the mixture stirred for 1 hr. The supernatant was decanted and the resin washed with water, transferred to a burette and eluted with aq. 3% (w/v) NH_3 soln. until the ninhydrin reaction (tested on filter paper, after removal of the NH_3 in a current of warm air) was no longer positive. The eluate was evaporated to give a yellow syrup (38 mg.).

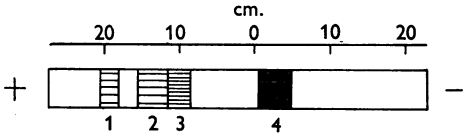

Fig. 2. Electrophoretic fractionation of aq. NH_3 eluate, Expt. I. No. 3 paper; pH 4; 29 v/cm.; 1·7 hr. The depth of shading represents the strength of the ninhydrin colour reaction.

Electrophoretic fractionation. The syrup (38 mg.) in water (0·2 ml.) was applied as a narrow band (14 cm. long) along the starting line (equidistant from the ends and parallel to the shorter side) of a sheet (59 cm. × 20 cm.) of Whatman no. 3 paper. Electrophoresis was carried out at pH 4, with the polythene-covered marble slab previously described; the initial voltage was 2200, but had to be lowered during the run to prevent overheating; after 100 min. the run was stopped (at this stage the voltage was 1100, and the current 115 mA) and a print taken (Fig. 2). The three acidic bands were eluted with water and the eluates evaporated; the residues weighed 1·7 mg. (fraction 1), 1·3 mg. (fraction 2), 4·5 mg. (partly crystalline, fraction 3).

In Fig. 2 band 4 contains neutral species, and the basic compounds have migrated off the paper. Fraction 1 is glutathione (in the disulphide form; oxidation occurs in the alkaline solution used to elute the Zeo-Karb 225 column), fraction 2 consists mainly of ophthalmic acid, and fraction 3 is glutamic acid. These assignations, based on experiments in which these compounds were run alone and in mixtures, were confirmed by paper chromatography in phenol; in this solvent the ratios of the R_F values to the R_F of alanine are, approximately: glutathione, 0·25; glutamic acid, 0·5; ophthalmic acid, 1. As calf lens is reported to contain about 200 mg. of glutathione/100 g. of lens (Kleifeld & Hockwin, 1956), the losses of glutathione in this method are high.

Experiment II

Preliminary experiments were carried out to measure the recovery of ophthalmic acid after adsorption by an anion-exchange resin. The resin [Amberlite IR-4B(OH), analytical

grade, British Drug Houses Ltd.] was converted into the acetate form by stirring with an excess of 5% (v/v) acetic acid for 1 hr., and then washing thoroughly with water. Ophthalmic acid (1 mg.) in water (5 ml.) was added to Amberlite IR-4B acetate (0·5 g., wet wt.), the mixture shaken for 3 min., and the supernatant then shaken again successively with two further batches of resin. The resin was washed with water and then each batch shaken in turn with N-HCl (3 ml.), 2N-HCl (4·5 ml.) and 3N-HCl (5 ml.). The opthalmic acid recovered in the acid extracts, measured by the ninhydrin reaction (Troll & Cannan, 1953), was 0·7 mg., and more might have been recovered on further extraction with acid. This was essentially the method used in the fractionation shown in Fig. 3.

Adsorption on anion-exchange resin (IR-4B). Calf lenses (100 g.) were macerated with water (250 ml.) for 2 min. Amberlite IR-4B acetate (40 g.) was added, the mixture stirred for 10 min., the supernatant decanted on a second lot of resin and stirred, and the supernatant again stirred with a third lot of resin and then rejected. The first lot of resin was stirred with water (250 ml.), the supernatant decanted on the second lot and stirred, and the supernatant again stirred with the third lot of resin before being rejected. This washing was repeated twice more. The third wash was no longer turbid. The third lot of resin was transferred to a tube (diam. 4 cm.), followed by the second lot, and then the first lot, which was thus at the top of the column. The column was eluted with 2N-HCl at 250 ml./hr. for 3 hr., by which time the ninhydrin reaction had become faint. Portions (250 ml.) of the eluate were shaken with *N*-methyldioctylamine in $CHCl_3$ (5% w/v; 2·5 l.) to remove HCl (Smith & Page, 1948; Hughes & Williamson, 1951), and then with $CHCl_3$ to remove excess of base. The eluate had pH of about 5 and was conveniently stored at this stage in the refrigerator (bacterial growth was prevented by the solution's being saturated with $CHCl_3$).

When the solutions were examined by paper electrophoresis at pH 4 and paper chromatography in phenol, the main ninhydrin-positive components seemed to be glutamic acid, glutathione and ophthalmic acid. Other acidic species would, however, be expected to be present. The next step in the fractionation was designed to separate amino compounds by adsorption on a cation-exchange resin; several phosphorus-containing compounds (which are not adsorbed) were thus removed.

Adsorption on cation-exchange resin. The resin (Dowex 50, 200–400 mesh, 12% cross-linked, Microchemical Specialties Inc., California) (70 g.) was washed with 4N-HCl (600 ml.), then with water (3 l.) and made into a column 6·5 cm. long × 4 cm. The eluate (750 ml.) from the anion-exchange column (freed from HCl as described above) was percolated through the column at 80 ml./hr., and the column then washed with water (750 ml.). Elution was effected with 3% (w/v) NH_3 solution, the eluate being collected in 35 ml. fractions; the rate of elution was 180 ml./hr. The ninhydrin-positive material (tested on paper, as described in Expt. I) was found in fractions 5–8, which were combined and concentrated to a volume of about 3 ml., and filtered to remove a brown solid.

The chief advantage gained by this stage of the fractionation was that the paper chromatography gave a much cleaner chromatogram than previously, presumably owing to the removal of other acidic species. There were no spots in positions corresponding to glutamic acid, glutathione or ophthalmic acid in the aqueous solution flowing through the cation-exchange column; so these compounds had, as expected, been quantitatively retained by the column.

Chromatographic fractionation. Whatman no. 3 paper was washed chromatographically with acid and alkali as described by Fowden (1956); a final wash with ethanol was also carried out to hasten drying.

The solution described above (3 ml.) was spread along the full width of two washed no. 3 papers (22½ in. × 18¼ in.);

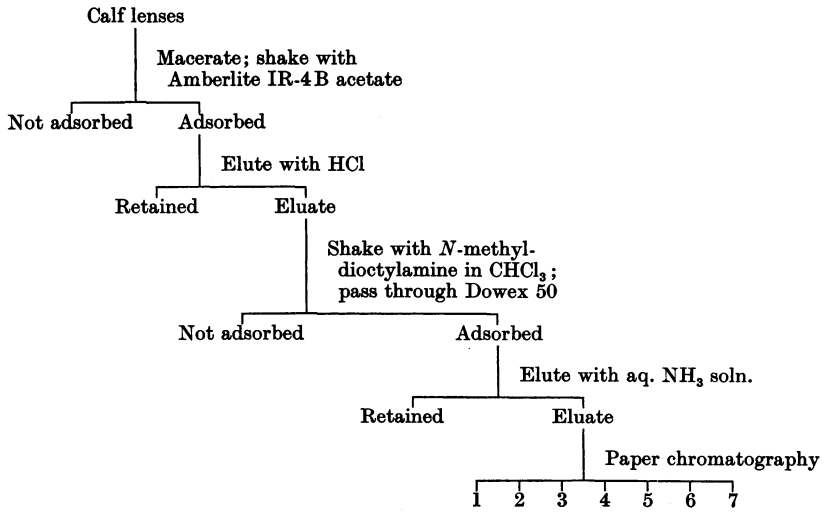

Fig. 3. Scheme of fractionation in Expt. II.

repeated applications (drying in between) ensured a narrow band. The chromatograms were run with phenol as solvent for 17 hr., and prints then taken; narrow strips (their long directions parallel with the direction of flow) were also cut out of the middle of both sheets, and these strips and the prints were developed with ninhydrin. The fraction with the same R_F as alanine (fraction 2 in Fig. 3) was cut out and eluted with water; evaporation of the eluate gave a brown syrup (19·8 mg.). The syrup was dissolved in water (0·15 ml.) and some contaminating solid spun down. A portion (10 μl.) of the supernatant was diluted with 10% (v/v) propan-2-ol (40 μl.) and this solution examined by paper chromatography in butanol–acetic acid and electrophoresis at pH 4 and pH 2. These tests showed a strong ninhydrin-positive spot, not separated from ophthalmic acid in mixed chromatograms; a faint spot was also seen, travelling further in paper electrophoresis at both pH values.

The bulk of the solution of ophthalmic acid was treated with 0·3 M-copper acetate (0·1 ml.) and kept in the refrigerator overnight. The crystalline copper salt of ophthalmic acid separated (6 mg.).

In another experiment the anion-exchange resin Amberlite IR-45 was used in the acetate form. The rest of the isolation was carried out as described above and the copper salt of ophthalmic acid (4 mg.) isolated.

Conversion of the copper salt of ophthalmic acid into the free tripeptide. A warm solution of 8-hydroxyquinoline in $CHCl_3$ (5 ml. of 0·1%, w/v) was added to the powdered copper salt (4 mg.) suspended in water (0·5 ml.); the $CHCl_3$ layer was separated and the extraction repeated until the $CHCl_3$ layer was colourless. Each lot of the $CHCl_3$ solution was extracted successively with water (0·3 ml.); the combined aqueous layers were then extracted three times with $CHCl_3$, concentrated to a volume of about 0·05 ml., and cautiously diluted with acetone till turbid. After a short time, the initial precipitate was spun down; crystalline ophthalmic acid slowly separated from the supernatant.

Action of carboxypeptidase on ophthalmic acid. Potassium carbonate (2·4 mg.) was added to carboxypeptidase (0·3 mg., Armour Laboratories) in water (0·03 ml.); as soon as the enzyme had dissolved, ophthalmic acid (approximately 0·2 mg.) was added, and the pH of the solution lowered by passing CO_2 over the surface. The reaction was followed by paper chromatography in butanol–acetic acid. After 8 hr. decomposition of ophthalmic acid was nearly complete, and a strong spot with the same R_F (and colour) as glycine had appeared; the other product had the same R_F as γ-L-glutamyl-DL-α-amino-*n*-butyric acid, applied as a superimposed spot. It was observed that the R_F of this dipeptide was lowered by the other constituents of the reaction mixture. An authentic specimen of the dipeptide was prepared by condensing benzyloxycarbonyl-γ-L-glutamyl azide with α-amino-*n*-butyric acid, followed by hydrogenation (S. G. Waley, paper in preparation).

Norophthalmic acid. The ninhydrin-positive band (fraction 3 in Fig. 3) travelling just behind ophthalmic acid was eluted and the eluate evaporated. The experiment using Amberlite IR-4B gave 6·8 mg., and that using Amberlite IR-45 gave 5·8 mg.; the samples were united. Further fractionation was necessary, as ophthalmic acid was still present. The solution was spread as a band, 30 cm. long, on the washed no. 3 paper, and run for 36 hr. in butanol–acetic acid. Strips cut out and developed with ninhydrin showed two bands: the faster was ophthalmic acid and the slower one the new peptide.

Table 1. *Structure of norophthalmic acid*

The peptide was treated with 1-fluoro-2:4-dinitrobenzene in aqueous ethanol containing triethylamine carbonate, and the dinitrophenyl derivative isolated, by the general method previously described (Waley, 1956).

Amino acid	Molar ratio amino acid:Ala	Strength of amino acid in hydrolysate of dinitrophenyl-norophthalmic acid	2:4-Dinitrophenyl amino acid identified
Gly	1·2	+ + +	—
Glu	1·3	–	Glu
Ala	1·0	+ + +	—

Amino acid analysis carried out, as described in the *General* section, on a portion of the hydrolysate gave the results shown in Table 1; glutamic acid was also found to be the *N*-terminal amino acid.

The reaction with carboxypeptidase was carried out as described for ophthalmic acid. The amino acid liberated was isolated by the method of Thompson (1952) and identified by paper chromatography in butan-2-ol–aq. 3% (w/v) NH_3 soln. (5:2, v/v) (Hausmann, 1952) as glycine.

The glutamic acid residue in norophthalmic acid is believed to be γ-linked (on the basis of the electrophoretic mobility; this evidence will be discussed in detail in a later paper).

DISCUSSION

Isolation of ophthalmic acid

Both the cation-exchange resin (Zeo-Karb 225) and the anion-exchange resins (Amberlite IR-4B or IR-45) could be used to adsorb ophthalmic acid directly from the disintegrated lens, although there is about a thousand times as much protein present as there is ophthalmic acid. Acidic amino acids and peptides were isolated by the successive use of the anion-exchange resin (as its acetate) and the cation-exchange resin (in the acid form) (Fig. 3); the first adsorbs acidic species and the second amino compounds, provided that they do not contain an acidic group comparable in strength with that of the acidic resin. A detailed discussion of the relationship between the ionic charge of a solute and its adsorption by ion-exchange resins has been given by Partridge & Brimley (1952). The main ninhydrin-positive constituents of the fraction obtained by the use of these resins were glutamic acid, glutathione and ophthalmic acid, and this mixture can be directly resolved by paper chromatography. This fraction, then, resembles the fraction L-II-2 previously obtained by electrophoresis (Waley, 1956); this was expected, as both methods of fractionation depend mainly on the net ionic charge.

The ophthalmic acid obtained from the calf lens was obtained in a crystalline form, and comparison with synthetic material established the structure (Waley, 1957); a detailed account of the synthesis will be published later. Since the peptide is more readily available by synthesis than by isolation from calf lens, no investigation has been made of the losses incurred during the isolation.

Norophthalmic acid

One of the minor constituents of the mixture of acidic ampholytes separated by paper chromatography has also been identified. Evidence from degradation (with the same methods as were applied to ophthalmic acid) leads to the structure γ-glutamylalanylglycine; thus the peptide is a lower homologue of ophthalmic acid, and has been named norophthalmic acid; it was also detected in the earlier work (Waley, 1956; fraction L-II-2-Ox-ε-2). This structure is confirmed by the observation that norophthalmic acid from the lens is chromatographically indistinguishable from γ-glutamylalanylglycine obtained by the action of Raney nickel on glutathione; this synthesis has also been described by Kermack & Matheson (1957*a*). The optically inactive tripeptide, γ-DL-glutamyl-DL-alanylglycine, was found to act as an inhibitor of glutathione in the glyoxalase reaction (Kermack & Matheson, 1957*b*); the configuration of the norophthalmic acid from the lens is unknown, but it seems likely that both the glutamic acid and alanine residues will have the L-configuration; so presumably the norophthalmic acid in the lens is a somewhat more active inhibitor than the material studied by Kermack & Matheson. The effect of ophthalmic acid itself in the glyoxalase reaction will be investigated soon.

Occurrence of γ-glutamyl peptides

The γ-linked peptides of glutamic acid at present known are a diverse class of metabolites; bacterial polyglutamic acid (Waley, 1955) is of restricted occurrence; a peptide, believed to be γ-glutamylalanine, has been found in pea seeds (Virtanen & Berg, 1954); the pteroylglutamic acids and glutathione are widely distributed. Of these compounds, it is glutathione that ophthalmic acid and norophthalmic acid resemble most closely, and it will be interesting to see whether they are at all widely distributed; ophthalmic acid has not been detected in the liver or erythrocytes of the calf (Waley, 1956), and there are several general reports on the failure to detect peptides (other than glutathione) in tissues (Askonas, Campbell, Godin & Work, 1955; Christensen & Riggs, 1953). As ophthalmic acid has the same R_F values as alanine, and norophthalmic acid as glycine, in both phenol–ammonia and butanol–acetic acid, paper chromatography of unfractionated tissue extracts will not readily reveal these peptides.

SUMMARY

1. The tripeptide, ophthalmic acid, has been isolated in the crystalline state from calf lens.

2. The isolation depends on the use of ion-exchange resins as molecular sieves: the peptide is selectively adsorbed from dilute solution without interference from proteins. Further fractionation has been effected with ion-exchange resins and paper chromatography.

3. Another tripeptide (norophthalmic acid) has been identified: it is γ-glutamylalanylglycine.

I should like to thank my colleagues at this Laboratory for advice and encouragement, Miss Joyce Blackwell for the collection of calves' eyes, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation, the National Council to Combat Blindness, Inc., New York, for a grant for chromatographic equipment, and The Distillers Co. Ltd. for a gift of glutathione.

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