49(638)-764], from the National Institutes of Health, United States Public Health Service (No. A2650) and from the Foundation for the study of Diabetes and Related Metabolic Disorders. The authors would like to express their gratitude to Dr Ned Redgate of the Dept. of Physiology for the performance of the operative procedure involving the infusion of [³⁵S]cystine into the third ventricle of the dog.

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Biochem. J. (1961) **79**, 669

Acidic Peptides of the Lens

7. THE PREPARATIVE ENZYMIC SYNTHESIS OF OPHTHALMIC ACID*

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(Received 14 December 1960)

Ophthalmic acid (γ -glutamyl- α -amino-*n*-butyryl-glycine) is a peptide isolated from calf lens (Waley, 1958). This analogue of glutathione has been used in studies on *Hydra* (Cliffe & Waley, 1958a), on the metabolism of γ -glutamyl peptides (Cliffe & Waley, 1961a), on the glyoxalase reaction (Cliffe & Waley, 1959, 1961b) and on embryological development (Lallier, 1959). The need for a method of producing ophthalmic acid is not altogether met by the synthesis reported by Waley (1958), which is fairly lengthy and requires the expensive L- α -amino-*n*-butyric acid. The enzymic synthesis of ophthalmic acid from the constituent amino acids (Cliffe & Waley, 1958b; 1961a), in which DL- α -amino-*n*-butyric acid is used, has now been adapted to the production of this tripeptide.

The source of enzyme for the synthesis is rabbit liver, supplemented by lens (Cliffe & Waley, 1961a). The main problem is the isolation of the ophthalmic

acid. This has been accomplished by ion-exchange chromatography. The method was based on the carrier-displacement technique (Buchanan, 1957). The separation of aspartic acid and glutamic acid was effected by Buchanan by adsorption on an anion-exchange resin in the acetate form; this column was then fitted over another column that contained the resin in the lactate form. When both columns were eluted with aqueous citric acid, the glutamic acid and aspartic acid emerged in that order, separated by a band of lactic acid ('the carrier'). When the same method was tested on a mixture of glutamic acid and ophthalmic acid, separation was effected, although lactic acid did not intervene between the amino acid and the peptide. Evidently ophthalmic acid is not acidic enough to be strongly retained by the lactate column. This trial experiment was carried out with about 5 mg. of ophthalmic acid and 50 mg. of glutamic acid on a column of 0.25 cm.² cross-sectional area. The fractions that contained the ophthalmic acid were extracted with amyl alcohol, and with ethyl

* Part 6: Cliffe & Waley (1961a).

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acetate, to remove as much as possible of the lactic acid and citric acid; the peptide was then precipitated from solution with acetone. This material was free from glutamic acid, so that the resolution is adequate even with high loading of the column. The method was applied to the isolation of ophthalmic acid from the enzymic reaction mixture. The solution (after removal of protein, and of trichloroacetic acid) was freed from inorganic salts (and organic phosphates) by adsorption on a cation-exchange resin, which was washed with water and eluted with aqueous pyridine. Tests showed that there was no hydrolysis of ophthalmic acid during this procedure, which was carried out expeditiously. The method used for desalting was also useful for the final separation of ophthalmic acid (after the ion-exchange chromatography) from the lactic acid and citric acid in the eluate: the peptide crystallized readily from a concentrated aqueous solution on dilution with acetone.

The identity of the specimens of ophthalmic acid (a) isolated from calf lens, (b) synthesized non-enzymically and (c) synthesized enzymically, has already been shown by X-ray crystallography and has now been confirmed by infrared spectroscopy. The enzymic synthesis evidently utilizes only the L-isomer of DL- α -aminobutyric acid, since the product has the same specific rotation as the specimen synthesized (non-enzymically) from L- α -aminobutyric acid. We had earlier inferred that this was so, because the rate of synthesis of ophthalmic acid was the same whether DL- α -aminobutyric acid was used at one concentration or the L-isomer at half the concentration (Cliffe & Waley, 1958b).

The overall yield of ophthalmic acid in the enzymic synthesis was about 20% (based on L- α -aminobutyric acid): this yield could probably be improved if purified enzymes were used and, in particular, if γ -glutamyl lactamase (which diverts some of the intermediate, γ -glutamyl- α -aminobutyric acid, to 5-oxopyrrolidine-2-carboxylic acid) could be removed from the enzyme preparation.

MATERIALS

Enzyme preparation from liver. Rabbit liver was macerated under acetone at -10° (10 ml./g. of liver), collected at the pump, washed with cooled acetone and dried. The enzyme preparation was made from the powder by the procedure of Snoko & Bloch (1955); 24 g. of powder gave 210 ml. of enzyme preparation. The preparation may be stored at -15° for 3 weeks.

Dialysed lens extract. Calf lenses (20 g.) (stored at -15° for up to 4 weeks) were ground with cooled water (20 ml.) and a little sand. The mixture was dialysed for 48 hr. at 4° and then centrifuged at 3000g for 10 min. The supernatant may be stored frozen for up to 1 week without appreciable loss of activity.

Potassium salt of 3-phosphoglyceric acid. The barium salt

(L. Light and Co. Ltd, Colnbrook, Bucks.) (14 g.) was triturated with water and Amberlite IR-120 (H^+) (200 ml.) was added. Portions of 5N-KOH were added until the pH rose to 6.4; after each addition of alkali, 3 min. was allowed for equilibration (Wade & Morgan, 1955). The mixture was transferred to a column, the solution run off and the resin washed twice with water (100 ml.). The combined solution and washings were concentrated under reduced pressure to 35 ml.

Solution of amino acids. A solution of glycine (2.25 g.), L-glutamic acid (2.942 g.) and DL- α -aminobutyric acid (4.124 g.) in water was neutralized with N-KOH (20 ml.) and made up to 100 ml.

METHODS

Incubation mixture. The components were: sodium salt of adenosine triphosphate (Sigma Chemical Co.) (1 g.) in water (40 ml.); 3-phosphoglyceric acid (potassium salt) soln. (12 ml.); M-KCl (36 ml.); M-MgSO₄ (4 ml.); amino acid soln. (20 ml.); M-KCN (6 ml.); 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (44 ml.) [the buffer contained tris (12.1 g.) and N-HCl (60 ml.) in 100 ml.]; dialysed lens extract (32 ml.); enzyme preparation from liver (200 ml.); water (6 ml.). The mixture (total volume 400 ml.) was incubated at 37° for 6 hr. at pH 7.8. Potassium glutamate [from L-glutamic acid (0.588 g.) and N-KOH (4 ml.) in 10 ml.] was then added, and the incubation continued for a further 3 hr.

Removal of protein. At the end of the incubation, cooled 15% (w/v) trichloroacetic acid (400 ml.) was added; the mixture was kept at 4° for 1 hr. and then centrifuged at 3000g for 10 min. The precipitate was washed with 7.5% (w/v) trichloroacetic acid (400 ml.). The combined supernatant solutions were extracted with N-methyl diethylamine (British Hydrological Corp., Merton, Surrey) in CHCl₃ (10%, w/v; 1.5 l.) to remove trichloroacetic acid, and then with CHCl₃ to remove excess of base. The volume of the aqueous layer was then decreased to 300 ml. by distillation under reduced pressure.

Removal of salt. The aqueous solution was made to flow through a column 4.1 cm. $\times$ 10 cm. long of Dowex 50 (H^+) (4% cross-linked; minus 400 mesh; Bio-Rad Laboratories, Berkeley, Calif., U.S.A.; analytical grade). The column was then washed with water (about 150 ml.) until the pH of the effluent rose to about 3. The application of the sample to the column, and the washing, could be hastened by applying pressure to the top of the column. The amino compounds were eluted with 2M-pyridine; they started to come off just before the yellow band on the column, and continued rather after it. The ninhydrin-positive fractions were collected and concentrated; water was added, and removed by distillation several times, to remove pyridine. After drying *in vacuo*, a solid residue (2.5 g.) was obtained.

Anion-exchange chromatography. The anion-exchange resin, Dowex 1 (4% cross-linked; 200–400 mesh; Bio-Rad Laboratories; analytical grade) was converted from the chloride into the lactate form as follows. The resin was washed with water by decantation until the supernatant was clear, and then transferred to a Büchner funnel and washed with 2M-sodium lactate until the effluent no longer contained appreciable amounts of Cl⁻ ion, and finally was washed thoroughly with water. The acetate form of the resin was prepared by an analogous method.

The columns were packed with aqueous suspensions of the resins.

The desalted sample (2.5 g.) in water (100 ml.) was treated with pyridine until green to bromocresol green, and added to a column 1.6 cm. $\times$ 21 cm. long of Dowex 1 acetate during $\frac{3}{4}$ hr. The column was then washed with water (150 ml.); the effluents were rejected. This column, which contained the acidic amino compounds (glutamic acid and ophthalmic acid) was then fitted over a second column, 1.6 cm. $\times$ 65 cm. long, of Dowex 1 lactate. The tip of the bottom of the acetate column was slipped into a piece of plastic tubing fitted to the top of the lactate column, and filled with water so that there were no air bubbles between the columns. The columns were eluted overnight with citric acid (63 g.) in water (1500 ml.); the flow rate was 80 ml./hr. and fractions of 20 ml. were collected.

The ninhydrin-positive fractions were examined by paper electrophoresis at pH 4 (Cliffe & Waley, 1958b); tube 36 contained glutamic acid and tubes 37 and 38 contained ophthalmic acid. The effluent volume at which glutamic acid and ophthalmic acid are eluted varied in different experiments, but glutamic acid always emerged just before ophthalmic acid and there was never appreciable contamination.

Isolation of ophthalmic acid. Cation-exchange resin (Dowex 50) was washed with 2M-pyridine and then with water; it was converted into the acid form by washing with 3N-HCl and then with water. This resin was then made into a column 0.56 cm. $\times$ 12 cm. high. The fractions that contained ophthalmic acid were made to flow through the column (under pressure) at a rate of 1 ml./min. The column was washed with water during $\frac{3}{4}$ hr. and then eluted with 2M-pyridine. Fractions of 3 ml. were collected. The ninhydrin-positive fractions were evaporated to dryness *in vacuo*. The residue in water (0.8 ml.) was diluted to 3.5 ml. with acetone and the solution kept at room temperature for 48 hr. Ophthalmic acid (0.2 g.) separated; a further quantity (0.04 g.) was obtained from the mother liquors after dilution with more acetone (total yield 20%, based on α -aminobutyric acid); $[\alpha]_D^{25}$ in 10% (v/v) acetic acid $-26 \pm 1^\circ$ (c, 3). Under the same conditions, a synthetic

specimen (Waley, 1958) had $[\alpha]_D^{25} -27 \pm 1^\circ$. The infrared spectrum of the specimen isolated from the enzymic synthesis was the same as the spectrum of the authentic specimen.

SUMMARY

1. The enzymic synthesis of ophthalmic acid from L-glutamic acid, DL- α -amino-*n*-butyric acid and glycine has been carried out on a preparative scale. Batches of about 400 ml. of reaction mixture yield about 200 mg. of ophthalmic acid.

2. The product has been isolated by ion-exchange chromatography and purified by recrystallization.

3. The enzymic synthesis is stereospecific and this enables racemic α -aminobutyric acid to be used.

We should like to thank our colleagues at this Laboratory for advice and encouragement, the staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their co-operation and the National Council to Combat Blindness Inc., New York, for a Fight for Sight Grant for the purchase of chromatographic equipment. One of us (E. E. C.) was in receipt of a Thornton Bursary.

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