Acidic Peptides of the Lens

3. THE STRUCTURE OF OPHTHALMIC ACID

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

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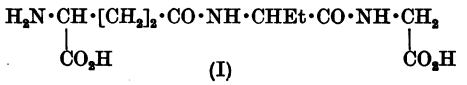
The investigation of the peptides in the lens has led to the discovery of a new tripeptide named ophthalmic acid. Earlier papers (Waley, 1956, 1957*b*) have described the isolation of this peptide, and provided some evidence for its structure. Acid hydrolysis gave glutamic acid, α-amino-*n*-butyric acid and glycine; glutamic acid provided the *N*-terminal amino acid residue, and glycine the *C*-terminal one. Assuming that the amino acids present in the hydrolysate are also those present in the tripeptide, these results establish the sequence of amino acid residues, and the only other question is whether it is the α- or the γ-carboxyl group of glutamic acid which is engaged in peptide-bond formation. Evidence on this point has been provided by measurements of mobilities in paper electrophoresis at pH 4. From the values given in Table 1 for several peptides of known structure it is seen that the γ-linked peptides of glutamic acid have mobilities over twice as large as the α-linked ones, and that ophthalmic acid has the mobility expected for a γ-linked peptide. This leads to the structure (I)



(I)

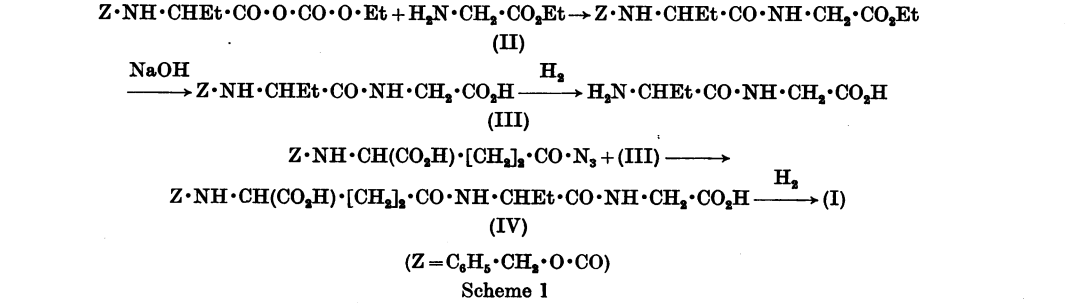
for ophthalmic acid. As there was insufficient material isolated from the lens for elementary analysis, the structure (I) is based on the assumptions that no undetected groups are present, that the amino acids liberated on hydrolysis have been correctly identified (by paper chromatography) and that hydrolysis has only liberated and not altered the amino acids forming the peptide. It was to test these assumptions that the synthesis of ophthalmic acid was undertaken; a preliminary account of this work has been given (Waley, 1957*a*). The preparation of γ-glutamyl-α-amino-*n*-butyric acid is also described below; this dipeptide was used in identifying the products from the action of carboxypeptidase on ophthalmic acid (Waley, 1957*b*).

In the synthesis of a γ-linked tripeptide of glutamic acid it is convenient to avoid protecting the α-carboxyl group, and this means that the glutamic acid residue must be incorporated last. So the synthetic route chosen was the one outlined in Scheme 1.

Table 1. *Electrophoretic mobility* (μ) *of peptides of glutamic acid*

Electrophoresis was carried out on the polythene-covered marble slab previously described (Waley, 1956) on Whatman no. 3 MM paper at a potential gradient of 11·1 v/cm. for 6·25 hr. in the pyridine–acetic acid buffer, pH 4. The mobilities were corrected for electroendosmosis with *N*-(2:4-dinitrophenyl)ethanolamine as marker; the physicochemical interpretation of mobility measurement in paper electrophoresis has been discussed by Svensson (1956) and by Synge (1957).

Peptide	$10^5 \mu$ ($cm.^2 v^{-1} sec.^{-1}$)
α-L-Glutamyl-L-valine	2·8
α-L-Glutamyl-L-tyrosine	3·3
Glutamic acid	5·3
γ-L-Glutamyl-L-tyrosine	6·9
Ophthalmic acid	7·2
γ-L-Glutamyl-L-valine	7·5
Glutathione (disulphide form)	7·6
γ-L-Glutamylglycine	8·6



Scheme 1

Benzyloxycarbonyl-L-α-amino-*n*-butyrylglycine ethyl ester (II) was prepared in satisfactory yield by the mixed-anhydride method; although racemization may attend the use of mixed anhydrides from benzyloxycarbonyldipeptides, no case of racemization has been reported with the mixed anhydrides from benzyloxycarbonylamino acids (Waley & Watson, 1954; North & Young, 1955). The original description of the preparation of mixed anhydrides with ethyl hydrogen carbonate recommends allowing the reaction mixture to stand for about 10 min. at 0° to ensure formation of the anhydride (Boissonnas, 1951). Recently it has been suggested that this step should generally be omitted (Hooper, Rydon, Schofield & Heaton, 1956), as sometimes the mixed anhydrides are formed rapidly and rapidly decompose. But the protected dipeptide (II) was obtained in low yield, and much benzyloxycarbonyl-α-amino-*n*-butyric acid was recovered, when insufficient time was allowed for the formation of the mixed anhydride.

Saponification of (II), followed by hydrogenation, gave L-α-amino-*n*-butyrylglycine (III), which crystallized readily. Benzyloxycarbonyl-γ-L-glutamyl hydrazide was converted into the azide and condensed with the dipeptide (III) under conditions similar to those used in the synthesis of other γ-linked peptides of glutamic acid (Waley, 1955). The crude product, which was clearly impure, was probably contaminated with the isomeric α-glutamyl peptide; some isomerization of the γ-azide normally occurs (Sachs & Brand, 1954; Rowlands & Young, 1957). Fortunately, the isomers can usually be separated by fractional extraction from an organic solvent by aqueous alkali (LeQuesne & Young, 1950*a*), and the γ-linked compound was readily obtained pure by this method. The by-products, separated at this stage, were hydrogenated: the main constituent was glutamic acid, but there was also a compound with an electrophoretic mobility about half that of glutamic acid, and this suggests (see Table 1) that this compound is the α-linked isomer of (I). The main product was hydrogenated to give γ-L-glutamyl-L-α-amino-*n*-butyrylglycine (I); in the earlier experiments this was isolated as the copper salt, which crystallizes very readily, and reconverted into the tripeptide which could then be obtained crystalline; this was also the procedure used in the later stages of the isolation of ophthalmic acid from the lens (Waley, 1957*b*). With crystalline material to act as seeds, however, the copper-salt stage can be omitted.

The synthetic tripeptide was compared with, and found indistinguishable from, the ophthalmic acid from the lens, by the use of paper chromatography and paper electrophoresis. Final proof of identity was provided by comparison of the X-ray powder diagrams of the free tripeptides (for which I am indebted to Mrs D. M. Hodgkin, F.R.S., and Dr Beryl Oughton, whose report is given in the Experimental section). Ophthalmic acid from the lens has not been isolated in sufficient quantity to determine its optical rotation; it is assumed that the glutamic acid and the α-amino-*n*-butyric acid have the L-configuration.

The structure of ophthalmic acid (I) is clearly very similar to that of glutathione. Indeed, the only difference is the replacement of the thiol group in glutathione by a methyl group, and the thiol and methyl groups are nearly the same size. Thus whereas ophthalmic acid is devoid of the special reactivity of glutathione associated with the thiol group, the two tripeptides are probably very similar in shape. Their R_F values in paper chromatography, and mobilities in paper electrophoresis, are also very similar, and their specific rotations are not very different.

The biological role of ophthalmic acid may well be connected with its similarity to glutathione. It is well known that structurally similar compounds often have antagonistic biological properties (Woolley, 1952), so ophthalmic acid may behave as an anti-coenzyme in those reactions in which glutathione is a coenzyme.

EXPERIMENTAL

Melting points are uncorrected. The analyses and optical rotations were carried out by Weiler and Strauss, Oxford. Details of the paper chromatography are given in Table 2.

L-α-*Amino*-n-*butyric acid.* The preparation by the action of Raney nickel on L-methionine has been described (Fonken & Mozingo, 1947; Vogler, 1947; Berridge, Newton & Abraham, 1952). The use of freshly prepared active catalyst made by the method of Pavlic & Adkins (1946) enabled the reaction to be completed in 16 hr. at room temperature; the yield was 72%. Most of the L-α-amino-*n*-butyric acid used was bought from the California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles, California, U.S.A.

Benzyloxycarbonyl-L-α-*amino*-n-*butyric acid.* Benzyl chloroformate (10·2 ml.) and N-NaOH (10·5 ml.) were added dropwise with stirring and cooling to L-α-amino-*n*-butyric acid (4·3 g.) in 4N-NaOH (10·5 ml.). After 45 min., the mixture was extracted with ether, acidified to pH 2,

Table 2. R_F *values of peptides*

Compounds were chromatographed on Schleicher and Schüll no. 2034*b* paper (Carl Schleicher und Schüll, Dassel, Germany) with *n*-butanol–acetic acid–water (40:9:20, by vol.).

Substance	R_F
Alanine	0·35
α-Amino-*n*-butyric acid	0·44
α-Amino-*n*-butyrylglycine	0·35
γ-L-Glutamyl-α-amino-*n*-butyric acid	0·44
γ-L-Glutamyl-α-amino-*n*-butyrylglycine	0·32

and the product was isolated by extraction with ether. After evaporation of the ether, the residue was dried by addition of acetone–benzene (1:1) and evaporation of the solvent. The syrup was dissolved in dry ether, and light petroleum (b.p. 40–60°) was added; *N-benzyloxycarbonyl-L-α-amino-n-butyric acid* crystallized in acicular plates, m.p. 78–79°, $[\alpha]_D^{16}$ – 32° (*c*, 2·8 in ethanol); yield 5·3 g., 54%; the m.p. was not raised by recrystallization from ether–light petroleum (b.p. 40–60°) (Found: C, 60·6; H, 6·4; N, 5·9. $C_{12}H_{15}O_4N$ requires C, 60·7; H, 6·4; N, 5·9%).

Benzyloxycarbonyl-L-α-amino-n-butyrylglycine ethyl ester (II). Ethyl chloroformate (2·19 ml.) was added to a stirred solution of benzyloxycarbonyl-L-α-amino-*n*-butyric acid (5·46 g.) and tri-*n*-butylamine (distilled over NaOH; 5·48 ml.) in dry toluene (70 ml.) at – 5°. After keeping the solution for 5 min. at this temperature (or for 14 min. at – 15°) glycine ethyl ester, prepared from glycine ethyl ester hydrochloride (3·97 g.) and tri-*n*-butylamine (6·78 ml.) in dry $CHCl_3$ (82 ml.) was added, and the mixture kept for 1 hr. at – 5°, and then for 3 hr. at room temperature. The solution was washed successively with water, 2% (w/v) $NaHCO_3$ and finally water, dried (Na_2SO_4) and concentrated to a volume of 60 ml.; *N-benzyloxycarbonyl-L-α-amino-n-butyrylglycine ethyl ester* separated, and was recrystallized from ethyl acetate–light petroleum (b.p. 60–80°); yield 4·5 g. (61%) of needles, m.p. 114°, $[\alpha]_D^{16}$ – 25° (*c*, 2·7 in ethanol) (Found: C, 60·1; H, 7·0; N, 8·7. $C_{16}H_{22}O_5N_2$ requires C, 59·7; H, 6·9; N, 8·7%).

L-α-Amino-n-butyrylglycine (III). 0·5N-Sodium hydroxide (45·4 ml.) was added to benzyloxycarbonyl-L-α-amino-*n*-butyrylglycine ethyl ester (6·65 g.) in acetone (66 ml.). After the solution had been kept for 2 hr. the acetone was removed at room temperature, and the alkaline solution extracted thrice with ethyl acetate. The ethyl acetate extracts were washed with water and the aqueous layer was added to the aqueous alkaline solution, which was then neutralized with N-HCl (22·7 ml.). The oil was isolated by extraction with ethyl acetate, the ethyl acetate solution freed from Cl^- ions by washing thrice with a little water, and the solvent distilled. The residue was dissolved in acetic acid (20 ml.) and water (10 ml.) and hydrogenated in the presence of palladium black (1 g.); after 4 hr. reduction was complete. The filtered solution was evaporated, the acetic acid removed by adding water and evaporating again, and the residue dried by the addition of acetone (10 ml.) and benzene (10 ml.) and distilling the solvents. Trituration under acetone gave the crystalline *dipeptide* (yield 2·5 g., 76%), m.p. 222° (decomp.), sufficiently pure for the next stage. The analytical specimen, recrystallized from aq. propan-2-ol, had m.p. 225° (decomp.), $[\alpha]_D^{16}$ +72° (*c*, 2·8 in water); (Found: C, 44·7; H, 7·7; N, 17·8. $C_6H_{12}O_3N_2$ requires C, 45·0; H, 7·5; N, 17·5%).

Benzyloxycarbonyl-γ-L-glutamyl-L-α-amino-n-butyrylglycine (IV). Sodium nitrite (1·09 g.) in water (10·9 ml.) was added during 7 min. to a cooled, stirred solution of benzyloxycarbonyl-γ-L-glutamyl hydrazide (LeQuesne & Young, 1950*b*) (3·21 g.) in water (27 ml.) containing 6N-HCl (10·9 ml.) covered with ether (52 ml.). After separation the aqueous layer was extracted with cooled ether, and the combined ethereal layers were washed three times with cooled water and promptly added to L-α-amino-*n*-butyrylglycine (1·75 g.) and $KHCO_3$ (4·36 g.) in water (22 ml.). The mixture was allowed to reach room temperature and stirred for 5 hr. The next day the aqueous layer was acidified, extracted with ethyl acetate (3 × 60 ml.), and the combined extracts were washed thrice with water and then extracted with 2% (w/v) Na_2CO_3 (7 × 10 ml.). The first three extracts were combined, acidified and extracted with ethyl acetate, and the ethyl acetate solution was washed with water, dried (Na_2SO_4) and concentrated to a volume of 15 ml. The *benzyloxycarbonyl tripepetide* (2·4 g.) crystallized, and was united with a further crop (0·3 g.) obtained from the last three fractions of the 2% Na_2CO_3 solution by repeating the fractionation, only the first two fractions obtained by extraction with 2 ml. of 2% (w/v) Na_2CO_3 being used. The product was purified by triturating with ethyl acetate (14 ml.); the residue (1·8 g.; 39%) had m.p. 166–168° (decomp.), not raised by recrystallization from a large volume of ethyl acetate, $[\alpha]_D^{20}$ – 19° (*c*, 3·2 in acetic acid); (Found: C, 53·4; H, 5·85; N, 9·5. $C_{19}H_{25}O_8N_3$ requires C, 53·8; H, 5·95; N, 9·9%).

γ-L-Glutamyl-L-α-amino-n-butyrylglycine (*ophthalmic acid*) (I). Benzyloxycarbonyl-γ-L-glutamyl-L-α-amino-*n*-butyrylglycine (1·7 g.) was suspended in acetic acid (5·8 ml.) and water (40 ml.) and hydrogenated in the presence of palladium black (0·7 g.) for 2·5 hr. The filtered solution was concentrated to a volume of 3 ml., seeded and slowly diluted with acetone (5 ml.); after a short time, the supernatant solution was decanted from the oil, and on standing *ophthalmic acid* separated as an oil which subsequently solidified and was dried *in vacuo* at room temperature (yield 0·8 g., 69%), m.p. 179–180° (decomp.), $[\alpha]_D^{20}$ – 29° (*c*, 2·4 in water); (Found: C, 44·7; H, 6·3; N, 14·5. $C_{11}H_{19}O_6N_3,\frac{1}{2}H_2O$ requires C, 44·3; H, 6·8; N, 14·1%). The water of crystallization was not lost at 100°.

A portion of the tripeptide was hydrolysed with 6N-HCl in a sealed tube at 107° for 16 hr. The hydrolysate was analysed by quantitative paper chromatography (Waley, 1957*b*), 72% (w/w) phenol–aq. 0·2% (w/v) NH_3 soln. being used as solvent; the molar ratios of the amino acids in the hydrolysate were: glycine, 0·98; α-amino-*n*-butyric acid, 0·93; glutamic acid, 1·0.

The tripeptide is readily soluble in water and sparingly soluble in most organic solvents; the partition coefficient (phenol–water) is 0·4. Ophthalmic acid forms a sparingly soluble copper salt from which it may be liberated with 8-hydroxyquinoline (Waley, 1957*b*).

Samples of natural and synthetic ophthalmic acid were submitted to Mrs D. M. Hodgkin, F.R.S., and Dr Beryl Oughton, who wrote as follows: 'Two series of powder photographs were taken, one of the free peptides, the other of the copper salts of the peptides.

'The powder photographs of the natural and synthetic free peptides were identical in line pattern and relative line intensity and clearly indicated the identity of the two preparations. Some difficulties were, however, encountered in the study of the copper salts. The first powder photographs taken of the natural and synthetic compounds were extremely similar and provided strong evidence of the virtual identity of the molecules present in the two crystals. They showed, however, small differences in the wide-angle distribution of a few powder lines. It is difficult to account for these; they suggest the existence of very slightly different crystal modifications, the occurrence of which may be favoured by impurities present or small changes in water content. A later specimen of the natural copper salt, although much discoloured and clearly grossly impure,

gave a pattern closely the same in line distribution as that of the synthetic specimen. This pattern showed much broadened lines, presumably owing to very small crystallite size.'

γ-L-Glutamyl-L-α-amino-n-butyric acid. Sodium nitrite (0·5 g.) in water (5 ml.) was added dropwise to a cooled, stirred solution of benzyloxycarbonyl-γ-L-glutamyl-hydrazide (1·5 g.) in water (12·5 ml.) containing 6N-HCl (5 ml.) covered with ether (25 ml.). After separation, the aqueous layer was extracted with cooled ether, and the combined ethereal layers were washed twice with cooled water and promptly added to L-α-amino-*n*-butyric acid (0·524 g.) and $KHCO_3$ (2 g.) in water (10 ml.). The mixture was stirred for several hours, and allowed to stand overnight. The acidified aqueous layer was extracted with ethyl acetate, and the combined extracts were washed with water and extracted with 2% (w/v) Na_2CO_3 (5 × 5 ml.). The first three extracts were combined, acidified and the oil was isolated by extraction with ethyl acetate. Hydrogenation was carried out in acetic acid (10 ml.) and water (5 ml.) in the presence of palladium black (0·15 g.) for 30 min. The filtered solution was concentrated, and the *dipeptide* precipitated by the addition of 3 vol. of acetone (yield 0·46 g., 38%). The analytical sample, recrystallized twice more from water–acetone, had m.p. 176–178° (decomp.); (Found: C, 45·9; H, 6·5; N, 12·3. $C_9H_{16}O_5N_2$ requires C, 46·5; H, 6·9; N, 12·1%).

In preliminary experiments the cheaper DL-α-amino-*n*-butyric acid was used; the product had the same chromatographic properties as γ-L-glutamyl-L-α-amino-*n*-butyric acid but, being a mixture of diastereoisomers, did not crystallize and was not characterized.

SUMMARY

1. The electrophoretic mobility of ophthalmic acid, a tripeptide isolated from calf lens, shows that the glutamic acid residue is γ-linked, and hence that ophthalmic acid is γ-glutamyl-α-amino-*n*-butyrylglycine.

2. This structure has been confirmed by the synthesis of ophthalmic acid.

3. The structure of opthalmic acid is compared with that of glutathione.

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