804. *Amino-acids and Peptides. Part III.* The Constitution of Hypoglycin B.*

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The natural product hypoglycin B is shown to be γ-L-glutamyl-α-amino-β-2-methylenecyclopropylpropionic acid on the basis of both degradative and synthetic evidence.

In 1955, Hassall and Reyle [1] reported the isolation, from the fruit of *Blighia sapida*, of two compounds that had the unusual property of lowering blood-sugar levels in test animals. One of these compounds, hypoglycin A, had approximately double the hypoglycæmic activity of the other, hypoglycin B. The structure of hypoglycin A has been established as α-amino-β-2-methylenecyclopropylpropionic acid by independent investigations in several laboratories.[2,3] In a preliminary communication [4] we proposed that hypoglycin B is the dipeptide γ-L-glutamyl-α-amino-β-2-methylenecyclopropylpropionic acid (I). In what follows, a detailed account is given of the evidence supporting this proposal.

Analytical data for hypoglycin B indicate the formula $C_{12}H_{18}N_2O_5$. Hydrolysis with 50% formic acid in a sealed tube has been found [5] by paper chromatography to give hypoglycin A and glutamic acid in excellent yield. Thus it only remains to define the amino- and carboxyl functions of hypoglycin A and glutamic acid which are involved in the peptide link.

Hydrolysis of 2,4-dinitrophenylhypoglycin B gave a mixture containing 2,4-dinitrophenyl-L-glutamic acid as a major component. This showed that the free amino-group in the dipeptide is in the glutamic acid residue. van Slyke estimation of amino-nitrogen in hypoglycin B indicated two atoms per mole. As Sachs and Brand [6] have shown that α-glutamyl-peptides react normally with nitrous acid to give one atom of amino-nitrogen per mole whereas γ-glutamyl-peptides give approximately two atoms, the result suggested that the γ- rather than the α-carboxyl group of glutamic acid was involved in the amide link. The reaction with phenyl isothiocyanate confirmed this. Treatment under the conditions of the Edman technique [7] for *N*-terminal amino-acid determinations led to the formation of the phenylthiohydantoin derivative (II) of hypoglycin B. The structure of this compound was indicated by the characteristic ultraviolet absorption maximum [8] at 269 mμ and the infrared bands at 3320, 1758, 1600, 1400 cm.$^{-1}$. The derivative behaves on titration as a monocarboxylic acid. There was no indication of a phenylthiocarbamyl derivative ($\lambda_{max.}$ 240 mμ) which is to be expected when phenyl isothiocyanate reacts under similar conditions with an α-glutamyl-peptide.

The structure of hypoglycin B has been confirmed by synthesis.† The methyl ester of (+)-hypoglycin A was condensed with the triethylamine salt of *N*-trityl-L-glutamic acid in the presence of dicyclohexylcarbodi-imide. The product was saponified with sodium hydroxide under mild conditions and then hydrolysed with aqueous acetic acid to a dipeptide shown to be identical with hypoglycin B by comparison of melting points,

* Part II, *J.*, 1959, 80.

† With the definition of the structure of hypoglycin B as γ-L-glutamylhypoglycin A we propose to simplify the trivial nomenclature in future publications by referring to hypoglycin A as hypoglycin and to hypoglycin B as γ-L-glutamylhypoglycin.

[1] Hassall and Reyle, *Biochem. J.*, 1955, **60**, 334.

[2] Ellington, Hassall, Plimmer, and Seaforth, *J.*, 1959, 80.

[3] (*a*) Anderson, Johnson, Nelson, Olson, Speeter, and Vavra, *Chem. and Ind.*, 1958, 330; (*b*) Wilkinson, *ibid.*, p. 17; von Holt and Leppla, *Angew. Chem.*, 1958, **70**, 25; Renner, Jöhl and Stoll, *Helv. Chim. Acta*, 1958, **41**, 589; (*c*) Carbon, Martin, and Swett, *J. Amer. Chem. Soc.*, 1958, **80**, 1002; (*d*) de Ropp, Van Meter, De Renzo, McKerus, Pidacks, Bell, Ullman, Safir, Fanshawe, and Davis, *ibid.*, p. 1004.

[4] Hassall and John, *Tetrahedron Letters*, 1959, No. 3, p. 7.

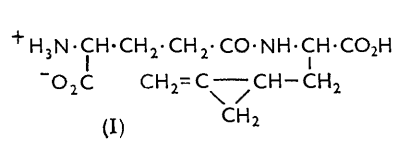
[5] von Holt, Leppla, Kröner, and von Holt, *Naturwiss.*, 1956, **12**, 279.

[6] Sachs and Brand, *J. Amer. Chem. Soc.*, 1954, **76**, 3601.

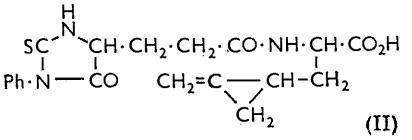
[7] Edman, *Acta Chem. Scand.*, 1955, **4**, 277, 283.

[8] Ramachandran, Epp, and McConnell, *Analyt. Chem.*, 1955, **27**, 1734.

infrared spectra, optical rotations, and crystalline 2,4-dinitrophenyl derivatives. Experiments have shown that the use of *N*-tritylglutamic acid leads to γ- rather than α-peptides.[9] Results reported by Jöhl and Stoll [10] after the completion of this work provide independent



(I)



(II)

support for this structure of hypoglycin B. Hydrolytic studies of hypoglycin B have also been reported by von Holt and Leppla,[11] who suggested that hypoglycin B is an α-glutamyl derivative of hypoglycin A but their evidence left open the alternative.

Experimental

M. p.s were determined by means of a Kofler block. Paper chromatography employed Whatman No. 1 paper. Ultraviolet spectra were determined on a Unicam spectrophotometer. Infrared spectra were measured with potassium bromide discs unless otherwise stated. We are grateful to Dr. H. E. Hallam, University College, Swansea, for determinations of infrared spectra.

Purification of Hypoglycin B.—Hypoglycin B was prepared by Hassall and Reyle's procedure.[1] The crude material, after four crystallisations from acetone–water, gave a product giving a single spot (R_F 0·56) on a paper chromatogram in the system butan-1-ol–acetic acid–water (4 : 1 : 5) and having $[\alpha]_D^{32}$ +9·7° ± 2° (*c* 1·12 in H_2O) [Found: C, 52·9; H, 6·9; N, 10·6; N(van Slyke), 9·8. Calc. for $C_{12}H_{18}N_2O_5$: C, 53·3; H, 6·7; N, 10·4%], $\nu_{max.}$ 3320, 1640, 1530 (amide), 1725, 1218 (CO_2H), 1410 (CO_2^-), and 889 cm.$^{-1}$ ($>C=CH_2$); pK_2' and pK_3' values, 3·73 and 9·36 (these are in the range of a γ-glutamyl peptide).[12]

Hydrolysis of Hypoglycin B with 50% Formic Acid.—Hypoglycin B (12 mg.) was heated with 50% formic acid (1·5 c.c.) at 110° in a sealed tube for 25 hr. The residue obtained when the mixture was evaporated to dryness, was dissolved in a small quantity of water and applied to Whatman No. 1 paper. Paper chromatography [with butan-1-ol–acetic acid–water (4 : 1 : 5)] indicated the major products as two ninhydrin-positive compounds, R_F 0·19 and 0·60 respectively. These correspond to L-glutamic acid and (+)-hypoglycin, respectively. Very faint spots were obtained at R_F 0·36 (purple), 0·44 (purple), 0·46 (yellow), and 0·50 (purple). These correspond to minor hydrolysis products of hypoglycin.[2]

N-2,4-*Dinitrophenylhypoglycin B.*—Hypoglycin B (150 mg.) in water (10 c.c.) was titrated potentiometrically with 1·1N-sodium hydroxide to pH 9·0. 1-Fluoro-2,4-dinitrobenzene (100 mg.) was added, with stirring, to the solution at 40°. The reaction was allowed to proceed with pH maintained at 9·0 by the addition of alkali. The end of the reaction was indicated by the fact that no further alkali was required. Excess of fluorodinitrobenzene was extracted with ether (2 × 5 c.c.), and the aqueous layer was then acidified with concentrated hydrochloric acid (0·1 c.c.). The *dinitrophenyl derivative*, which was precipitated as an oil, was extracted with ether (7 × 15 c.c.). The extract was dried (Na_2SO_4) and evaporated to dryness. The residue (240 mg., 98%) crystallised from methanol–water as needles (175 mg.), m. p. 166·5—169°, R_F 0·46 [phenol–t-butanol–phosphate buffer of pH 5·91 (1 : 1 : 1 v/v) in 2% ammonia], $[\alpha]_D^{19}$ −84° (*c* 0·6 in 4% aqueous sodium hydrogen carbonate), $\lambda_{max.}$ 359 mμ (ε 16,600) [Found: C, 49·9; H, 4·7; N, 13·1%; *M* (Battersby and Craig [13]), 446. $C_{18}H_{20}N_4O_9$ requires C, 49·5; H, 4·6; N, 12·8%; *M*, 436].

Hydrolysis of N-2,4-*Dinitrophenylhypoglycin B.*—*N*-2,4-Dinitrophenylhypoglycin B (162 mg.) was refluxed in the dark with 5·7N-hydrochloric acid (40 c.c.) for 26 hr. The mixture was extracted with ether (7 × 15 c.c.) and both the aqueous and the ether solution were evaporated to dryness. The ether extract yielded a yellow gum (103 mg.) which crystallised from ethyl

[9] Amiard, Heymès, and Velluz, *Bull. Soc. chim. France*, 1956, 698.
[10] Jöhl and Stoll, (*a*) *Helv. Chim. Acta*, 1959, **42**, 156; (*b*) *ibid.*, p. 716.
[11] von Holt and Leppla, *Z. physiol. Chem.*, 1958, **313**, 276.
[12] Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Co., New York, 1943.
[13] Battersby and Craig, *J. Amer. Chem. Soc.*, 1951, **73**, 1887.

acetate–light petroleum (b. p. 60—80°), then having m. p. 165—171° (11 mg.) undepressed on admixture with authentic DL-2,4-dinitrophenylglutamic acid (m. p. 170—172°), and having the correct infrared spectrum for this product. Further purification of the mother-liquor on a Hyflo-Supercel column gave a product which again did not crystallise.

A second hydrolysis of 2,4-dinitrophenylhypoglycin B (115 mg.) was carried out for a shorter time (2 hr.), to avoid racemisation. Working-up in a similar way gave a yellow oil (74 mg.) (from the ether extract), $[\alpha]_D^{24}$ $-79° \pm 2°$ (*c* 2·5 in AcOH). L-2,4-Dinitrophenylglutamic acid [14] has $[\alpha]_D$ $-81°$

Paper chromatography of the ether extract [using the system phenol–t-butanol–phosphate buffer of pH 5·91 (1 : 1 : 1 v/v) in 2% ammonia] gave R_F 0·23, which corresponded to that of authentic L- and DL-2,4-dinitrophenylglutamic acid.

Chromatography of the aqueous solution [with butan-1-ol–acetic acid–water (4 : 1 : 5)] gave a pattern of ninhydrin-positive spots (see Table) which was very similar to that obtained from an identical hydrolysis of hypoglycin (10·3 mg. with 5·7N-hydrochloric acid at 110° for 26 hr.).

Paper chromatography of 26 hr. hydrolysis mixtures.

Hypoglycin A hydrolysate			Dinitrophenylhypoglycin B hydrolysate		
R_F	Intensity *	Colour with ninhydrin	R_F	Intensity *	Colour with ninhydrin
0·15	II	Purple	0·15	IV	Purple
			0·19	III	Purple
0·25	I	Purple	0·25	I	Purple
0·29	II	Purple	0·29	II	Purple
0·33	II	Yellow	0·33	II	Yellow
0·45	V	Yellow	0·45	V	Yellow
0·60	I	Purple			
0·70	I	Purple	0·70	I	Purple

* Intensity increases from I to V.

Phenylthiohydantoin Derivative of Hypoglycin B.—Hypoglycin B (103 mg.) in water–dioxan (10 c.c.; 1 : 1) was adjusted to pH 9·0 by addition of N-sodium hydroxide. Phenyl isothiocyanate (0·3 c.c.) was added with stirring at 40°, and the pH kept at 9·0 by frequent addition of N-sodium hydroxide. The reaction was allowed to proceed for 100 min. The solution was extracted with benzene (7 × 10 c.c.), acidified to pH 3·0 with N-hydrochloric acid, left overnight at 0°, and then extracted with ether. The dried extract was evaporated to dryness, to yield a white residue (129 mg.). Recrystallisation from ethanol–water gave the *phenylthiohydantoin derivative* as needles, m. p. 186—188° [Found: C, 58·7; H, 5·5; N, 10·6%; equiv. (by potentiometric titration), 408. $C_{19}H_{21}NO_4S$ requires C, 58·9; H, 5·4; N, 10·8%; *M*, 387], $\lambda_{max.}$ 268—269 mμ (ε 16,000), $\nu_{max.}$ 3320 (NH), 1758 (ring CO), 1713 (CO_2H), 1621 (CO·NH), 1600 (Ph), 1506 (NH), 1400 (CS), 893 cm.$^{-1}$ ($>C=CH_2$). The principal maxima correspond to those assigned to a phenylthiohydantoin ring.[8]

Paper chromatography on starch-impregnated Whatman No. 1 paper [in butan-1-ol–heptane–90% formic acid (4 : 4 : 2) and made visible with iodine–azide [15] reagent] gave R_F 0·85.

Attempted Cleavage of Product from Phenyl Isothiocyanate Reaction.—The derivative described above (56·8 mg.) in anhydrous nitromethane (3 c.c.), saturated with hydrogen chloride, was stirred at 40° for 15 min. The nitromethane solution, when evaporated to dryness, gave unchanged starting material. These conditions have been shown to lead to cleavage of a phenylthiocarbamyl derivative, which would be formed by the action of phenyl isothiocyanate on α-glutamylhypoglycin.[7]

Hypoglycin Methyl Ester Hydrochloride.—(+)-Hypoglycin (1·51 g.) was added to anhydrous methanol (15 c.c.) containing thionyl chloride [16] (0·93 c.c.) and kept at −10° until all had dissolved. After refluxing for 2 hr., the solution was evaporated to dryness in a vacuum. The residue crystallised from methanol–ether to give *hypoglycin methyl ester hydrochloride*, needles, m. p. 151—152°, $[\alpha]_D^{22}$ $+36° \pm 1·5°$ (*c* 2·0 in H_2O) (Found: C, 49·8; H, 7·3; N, 7·5; O, 17·1; Cl, 18·9. $C_8H_{14}ClNO_2$ requires C, 50·1; H, 7·4; N, 7·3; O, 16·7; Cl, 18·5%).

Triethylammonium Trityl-L-glutamate.—L-Glutamic acid (5·8 g.) was shaken with water (120 c.c.), ether (120 c.c.), and diethylamine (8 c.c.), until all the glutamic acid was in solution.

[14] Rao and Sobers, *J. Amer. Chem. Soc.*, 1954, **76**, 1328.
[15] Sjøquist, *Acta Chem. Scand.*, 1953, **7**, 447.
[16] Boissonas, Guttmann, Jacquenaud, and Waller, *Helv. Chim. Acta*, 1956, **39**, 1421.

Triphenylmethyl chloride (14·4 g.) was added, with vigorous stirring, to the mixture at −10° under nitrogen. Stirring was continued for 3 hr. at approximately −10° and the mixture kept at 0° overnight. The ether was removed in a vacuum. The aqueous mixture which remained gave, on acidification, a precipitate of tritylglutamic acid. The mixture was extracted with ethyl acetate (2 × 100 c.c.). The dried extract was treated with excess of triethylamine and concentrated to a small volume. Addition of ether precipitated triethylammoniun trityl-L-glutamate (4·28 g.). Recrystallisation from ethyl acetate–ether yielded colourless needles (3·87 g.), m. p. 97—100°, $[\alpha]_D^{16}$ −36° ± 2·5° (*c* 2·0 in $CHCl_3$) (Found: N, 5·8. Calc. for $C_{30}H_{38}N_2O_4$: N, 5·7%). Amiard, Heymès, and Velluz [9] give m. p. "approx. 100°," $[\alpha]_D^{20}$ −32° ± 2° (*c* 2·0 in $CHCl_3$).

N-*Trityl Derivative of γ-L-Glutamyl-(+)-hypoglycin Methyl Ester.*—Hypoglycin methyl ester hydrochloride (500 mg.) in methylene chloride (4 c.c.) was shaken with a slight excess of aqueous N-ammonia (3·6 c.c.). Dicyclohexylcarbodi-imide (670 mg.) and the triethylammonium trityl-L-glutamate (1·37 g.) were added to the methylene chloride solution with shaking. Soon after, a uniform solution was obtained, and precipitation of dicyclohexylurea commenced. The reaction was allowed to proceed at room temperature overnight. Excess of dicyclohexylcarbodi-imide was destroyed by addition of a few drops of acetic acid. The filtrate obtained on removal of dicyclohexylurea was washed with 3N-hydrochloric acid and water, dried (Na_2SO_4) and evaporated to give the ester as a white solid (1·26 g., 92%). No attempt was made to crystallise this product.

Hypoglycin B.—The *N*-trityl derivative of γ-L-glutamyl-(+)-hypoglycin methyl ester from the previous preparation was hydrolysed. Trials led to choice of following conditions. The crude ester (1·04 g.) in ethanol (8 c.c.) was treated with N-sodium hydroxide (4·5 c.c.) and left at 20° for 1 hr. Water (60 c.c.) was added. The solution was extracted with chloroform, and the extract rejected. The aqueous layer was acidified with 3N-hydrochloric acid and extracted with chloroform. The residue (631 mg.) obtained by evaporating the dried, chloroform extract was heated with 50% acetic acid (5 c.c.) on a boiling-water bath for 3 min. Dilution with water precipitated triphenylmethanol, which was removed. The filtrate was extracted with ethyl acetate. Evaporation of the aqueous layer gave a residue (123 mg.) which, after four recrystallisations from acetone–water, yielded needles having, before and after admixture with hypoglycin B, double m. p. 189—191°, 200—204°, R_F 0·56 [butan-1-ol–acetic acid–water (4 : 1 : 5)] $[\alpha]_D^{19}$ +9° ± 2·5° (*c* 1·4 in H_2O) [Found: N, 10·3; N(van Slyke), 9·8. Calc. for $C_{12}H_{18}N_2O_5$: N, 10·4%]. The infrared spectrum was identical with that of natural hypoglycin B.

The 2,4-dinitrophenyl derivative of the synthetic product was prepared and shown by mixed m. p. determination and comparison of infrared spectra and paper chromatograms to be identical with the corresponding derivative from natural hypoglycin B.

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