NOTES

The *in vivo* Synthesis of Diethylriboflavin Phosphate[1,2]

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Diethylriboflavin has been shown to be a very potent antagonist of riboflavin in the rat.[3] Since part of the biological activity of diethylriboflavin appears to be due to its ability to displace riboflavin from tissues, it was felt that a study of the tissues of animals fed this riboflavin homolog should be undertaken. It was believed that to be an effective displacing agent the diethylriboflavin must be converted to some form resembling those products which are normally derived from riboflavin. For this purpose the livers of rats fed diethylriboflavin were examined for diethylriboflavin and its derivatives. Rats of Group 1 (seven animals) received 2 mg. of diethylriboflavin each day until they died or for a maximum of 36 days, and those of Group II (six animals) received a flavin-free diet for 36 days. The diet and the mode of administration of the diethylriboflavin have been described.[3]

After death or termination of the experiment, the livers were removed, frozen, lyophilized and ground to a powder in a porcelain mortar. The liver powders of animals of Group I were combined, as were those of Group II, and the flavin concentration per gram of each group was determined fluorometrically.[4] Protein-free and salt-free extracts, containing 44.4 μg. of flavin, were prepared by the method of Yagi[5] and aliquots, containing equal amounts of flavin (4.4–8.8 μg.), of each of the extracts were placed on Whatman No. 1 paper and chromatographed using the upper phase of a water–*n*-butanol–acetic acid (5:4:1) solvent system.

Riboflavin, riboflavin phosphate and flavin adenine dinucleotide (R_f 0.30, 0.10 and 0.05, respectively) were detected in the chromatograms of Groups I and II. Two spots of R_f 0.19 and 0.54 were noted in the Group I chromatograms and were not present in those of Group II. The two spots had a yellow–green fluorescence when viewed under ultraviolet light as is characteristic of flavin compounds. The two new spots had R_f values identical with synthetic diethylriboflavin 5′-phosphate (0.19) prepared by the use of the Flexser and Farkas method[6] for the synthesis of riboflavin 5′-phosphate, and synthetic diethylriboflavin (0.54) prepared by the method of Lambooy.[7] Furthermore, when two-dimensional paper chromatography was employed, the two new spots behaved identically with diethylriboflavin 5′-phosphate and diethylriboflavin. Solvent 1 was used for the first dimension and solvents 2, 3 or 4 for the second dimension (Table I).

Table I

R_f Values (Ascending)

Solvent[a]	1	2	3	4
Riboflavin	0.30	0.34	0.77	0.66
Diethylriboflavin	.54	.34	.84	.77
Riboflavin 5′-phosphate	.10	.50	.18	.06
Diethylriboflavin 5′-phosphate	.19	.53	.21	.11
Flavin adenine dinucleotide	.05	.40	.22	.17
FAD-X[8]	.05	.40	.47	.30
Riboflavin phosphate-X[8]	.13	.54	.50	.15
Lumichrome[8]	.68	.07	.88	.72
Lumiflavin[8]	.48	.18	.94	.68
Riboflavinyl glucoside[8]	.22	.40	.60	.50

[a] Solvent (1) water:*n*-butanol:acetic acid 5:4:1 (top phase); (2) 5% Na_2HPO_4 in water and isoamyl alcohol[9]; (3) 160 g. phenol:30 ml. *n*-butanol:100 ml. water (lower phase)[10]; (4) collidine saturated with water.

The failure to detect the diethylriboflavin homolog of flavin adenine dinucleotide in the tissues of animals fed diethylriboflavin may be due to its having an R_f value similar to FAD in the solvents used or because the FAD homolog was not formed.

(1) This study was supported in part by research grant No. G-3326 from the National Institutes of Health, United States Public Health Service.

(2) Reference was made to this study in a paper presented at The National Vitamin Foundation Symposium on Antimetabolites, New York City, March 1, 1955.

(3) J. P. Lambooy and H. V. Aposhian, *J. Nutrition*, **47**, 539 (1952).

(4) Association of Vitamin Chemists, "Methods of Vitamin Assay," Interscience Publ. Co., Inc., New York, N. Y., 1951.

(5) K. Yagi, *Journal of Biochem. (Japan)*, **38**, 161 (1951).

(6) L. A. Flexser and W. G. Farkas, U. S. Patent 2,610,177 (September 9, 1952).

(7) J. P. Lambooy, This Journal, **72**, 5225 (1950).

(8) These R_f values taken from reference 8.

(9) C. E. Carter, This Journal, **72**, 1466 (1950).

(10) F. M. Huennekens, D. R. Sanadi, E. Dimant and A. I. Scheppartz, *ibid.*, **75**, 3611 (1953).

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The Structure of Macrocyclic Glycine Peptides

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The synthesis of two macrocyclic glycine peptides, "cyclo-(triglycyl)"[1] and cyclo-(hexaglycyl)[2] have been reported recently. Comparison of these materials in this laboratory has shown them to be identical and to possess the cyclo-(hexaglycyl) structure.

The assignment of the cyclo-(triglycyl) structure to the compound prepared by the cyclization of diglycylglycine azide[1] appears to have been made on the basis of the method of preparation only, no confirmatory molecular weight evidence being described. The cyclo-(hexaglycyl) structure proposed for our material (prepared from N-carboxyglycine anhydride) was established by many reproducible molecular weight determinations by the vapor pressure technique of Menzies,[3] adapted to

(1) J. C. Sheehan and W. L. Richardson, This Journal, **76**, 6329 (1954).

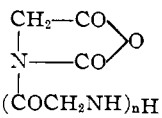
(2) D. G. H. Ballard, C. H. Bamford and F. J. Weymouth, *Proc. Roy. Soc. (London)*, **A227**, 155 (1955).

(3) A. W. C. Menzies, This Journal, **32**, 1615 (1910).

a semi-micro scale, and by partial alkaline hydrolysis.[2] We have now obtained further confirmation of the cyclo-(hexaglycyl) structure by application of the cyclization procedure of Sheehan and Richardson[1] to pentaglycylglycine azide. The latter was prepared from pentaglycylglycine methyl ester[4] which was converted to pentaglycylglycine hydrazide by treatment with hydrazine using a 30% solution of lithium chloride in methanol as solvent. Treatment with nitrous acid and cyclization again yielded cyclo-(hexaglycyl) (23% yield from the hydrazide, 8% over-all from pentaglycylglycine methyl ester). The identity of the materials has been established by comparison of the infrared spectra and of powder and single crystal X-ray photographs of cyclo-(hexaglycyl) with the compound prepared by us from diglycylglycine azide.

The analytical and recrystallization data recorded for cyclo-(hexaglycyl) and the "cyclo-(triglycyl)" of Sheehan and Richardson show certain discrepancies. "Cyclo-(triglycyl)" is described as forming a prismatic hemihydrate on crystallization from water which, if allowed to remain in contact with water, changes to fine needles of an anhydrous form. Cyclo-(hexaglycyl) prepared in these laboratories normally crystallizes from water as a prismatic hemihydrate showing inclined extinction; the formulation of this compound is supported by X-ray unit cell and density measurements. The material appears to be completely stable in contact with its aqueous mother liquor. The other principal forms observed by us are a monohydrate (analysis equivalent to the cyclo-tripeptide hemihydrate), and the anhydrous compound. The monohydrate, obtained by slow crystallization from water at low temperatures, rarely forms crystals of well defined prismatic habit and also appears to be stable in its aqueous mother-liquor. The only metastable modification encountered may be obtained by rapid cooling of hot aqueous solutions and forms fine needles showing parallel extinction. This form is converted to a mixture of the monohydrate and hemihydrate on standing in water at 25°.

The observation that cyclization of diglycylglycine azide yields the cyclic hexapeptide, and that the same material is the major cyclic product formed from N-carboxyglycine anhydride polymerization intermediates such as I, from which the



I, n = 1, 2, 3, etc.

formation of any cyclic peptide (excluding 2,5-diketopiperazine) is theoretically possible, indicates that, for this amino acid, the formation of the 18-membered ring is particularly favored. The other cyclic peptides isolated in lower yields from the N-carboxyglycine anhydride polymerization appear from molecular weight studies to be mixtures of the cyclic penta-, hepta- and possible octa-peptides which are not readily separable. These results indicate that the ease of cyclization of bifunctional peptide derivatives is not a simple function of chain length only, as has been suggested, for example, by Boissonas and Schumann.[5] Such would be the case only if the chains were perfectly flexible and if steric complications were absent. Clearly interaction between the CO and NH groups of the polypeptide chains could result in a very considerable loss of flexibility.

The great stability of cyclo-(hexaglycyl) is shown by its resistance to acid and alkaline hydrolysis, this compound being relatively unaffected by conditions causing a rapid breakdown of 2,5-diketopiperazine. Paper chromatographic studies of partial hydrolysates show the cyclic hexapeptide to be considerably more stable than any of its linear degradation products, glycine and glycylglycine being the only fragments readily observable during partial acid and alkaline hydrolysis.

(4) E. Pacsu and E. J. Wilson, *J. Org. Chem.*, **7**, 117 (1942).

(5) R. A. Boissonas and I. Schumann, *Helv. Chim. Acta*, **35**, 2229 (1952).

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Soluble Ester Derivatives of Pentaerythritol Trinitrate

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The use of polynitrate esters as medicinal agents for the treatment of hypertension and as vasodilators has been practiced for many years. Materials such as mannitol hexanitrate, glyceryl trinitrate and pentaerythritol tetranitrate are examples of such compounds. Although these materials have proven to be valuable therapeutic agents, they are characterized by an extremely low degree of solubility in water and other body fluids. The recent availability of the polynitrato alcohol, pentaerythritol trinitrate,[1] has prompted work in this Laboratory toward the synthesis of polynitrate esters containing functional groups which were amenable to water solubilization.

Several monoacid esters of pentaerythritol trinitrate have been prepared by the esterification of this nitrato alcohol with dibasic acid anhydrides. In this manner pentaerythritol trinitrate was esterified with succinic, phthalic, glutaric and β,β-dimethylglutaric anhydrides. The reaction of pentaerythritol trinitrate with methylsuccinic anhydride gave a mixture of the α- and β-methyl hydrogen succinate esters which could not be separated. The monoacid esters thus obtained were then converted to their sodium salts for the purpose of providing water solubility. The pharmacological evaluation of these compounds is in progress and will be reported elsewhere. As a preliminary report, it has been shown that all of the compounds have a very pronounced hypotensive effect when given intravenously to anesthetized dogs.

Experimental[2]

Materials.—Pentaerythritol trinitrate was prepared by the method described by Marans, Elrick and Preckel.[1] Glutaric anhydride was prepared by the reaction of glutaric

(1) N. S. Marans, D. E. Elrick and R. F. Preckel, THIS JOURNAL, **76**, 1304 (1954).

(2) Melting points were taken on a Fisher–Johns melting point apparatus and are uncorrected. The microanalyses were performed by H. L. Hunter, G. Maciak and Gloria Beckmann.