SYNTHESIS OF {[1-^{14}C]HYDANTOIC ACID, 5-VALINE}ANGIOTENSIN (II) AND A COPOLYMER OF POLYSUCCINYLLYSINE C WITH [1-GLYCINE, 5-VALINE]ANGIOTENSIN (II)

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In order to investigate the inactivation and metabolism of angiotensin* in living organisms, and also to develop a variant of the radioimmunobiological determination of this hormone, it was necessary to synthesize the compound labeled with a radioisotope. The main requirements for a labeled preparation and for the method of its production are as follows: in connection with the specific nature of the study of the metabolism of angiotensin, the label must be introduced into the N end of the molecule; the method of preparing the labeled compound must be simple and economical and guarantee the maximum yield of compound containing a radioisotope of high activity; and the product must not differ substantially in its biological action from the natural hormone.

The processes of the tritiation [1] and iodination (^{131}I) [2, 3] of peptides which are widely used for the introduction of radioisotopes clearly do not satisfy these requirements. On tritiation, if the tritium is not introduced by a special method [4], the radioactivity is uniformly distributed along the polypeptide chain and there is no possibility of selective labeling. In addition, the rapid exchange of tritium with the protons of the surrounding medium complicates the evaluation of the results obtained [5, 6]. Iodination mainly affects the tyrosine residues, but the properties and biological activities of the labeled polypeptides differ considerably from those of the natural compounds [7]. This also applies to the introduction of a radioactive label by thiocarbamoylation (^{35}S) with phenyl isothiocyanate [8]. The requirements presented are most nearly satisfied by the carbon isotope ^{14}C, which permits a radioactive center to be formed in a particular part of the molecule with a relatively high activity [5, 9, 10].

We have shown previously that [1-hydantoic acid]angiotensin (II) sterically corresponding to the natural hormone (its β-amide) but differing from the structure of the latter by the absence of a free N-terminal amino group, which has shifted along the skeleton of the molecule, possesses a pressor activity of 150% (of the natural hormone) [11]. The present paper describes a method for the production of (II) and also its radioactive analog (III) by the selective carbamoylation of the amino group of [1-glycine]angiotensin (I) with sodium cyanate according to the scheme

$$H_2N-CH_2-CO-Arg-Val-Tyr-Val-His-Pro-Phe-OH \quad (I)$$

$$\downarrow \text{NaCNO}$$

$$H_2N-CO-NH-CH_2-CO-Arg-Val-Tyr-Val-His-Pro-Phe-OH \quad (II)$$

Conditions for the carbamoylation of amino and other functional groups have been described in detail in the literature [12-15]. We synthesized substance (II) in a phosphate buffer (pH 6) with a twofold excess of sodium cyanate at room temperature. It was assumed that under these conditions the cyanic acid would

*Here and below, [5-valine]angiotensin (II).

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react selectively only with the N-terminal amino group of (I), since the nucleophilicity of the guanidine group of arginine at pH 6 is completely suppressed by protonation [16]; according to Stark [13], the carbamoyl derivative of the imidazole ring of histidine is extremely unstable in aqueous solution, and in the pH range from 6 to 12 it dissociates in a few minutes with the formation of a histidine residue; the carbamoylation of the hydroxy group of tyrosine at pH 6 takes place far more slowly than the carbamoylation of a N-terminal amino group, whose rate of reaction is determined by the effective concentration of its nonprotonated form and by the concentration of undissociated cyanic acid [15].

After the completion of the reaction, which took 16 h, the phosphate ions and the excess of cyanate were eliminated by means of ÉDÉ-10p ion-exchange resin (in the acetate form): to split off the carbamoyl groups from the tyrosine residues, the product obtained was kept at pH 9 and 30°C for 3 h [15]. The residual desalting was performed on Amberlite IRC-50 [17]. The unchanged substance (I) was separated by ion-exchange chromatography on CM-cellulose. On paper chromatography in two systems, and also on paper electrophoresis, compound (II) was revealed with Pauli's [18] and Ehrlich's [19] reagents and with the phenanthrenequinone reagent [20] in the form of a single spot, and was not revealed with ninhydrin, which shows the presence in it of free functional groups of arginine and histidine and the absence of an O-carbamoyl group in tyrosine [15]. From its electrophoretic and chromatographic characteristics, (II) was identical with [1-hydantoic acid]angiotensin [11]. The pressor activity of (II) on nephrectomized rats [21] was 150% (of that of the natural hormone).

The radioactive {[1-^{14}C]hydantoic acid}angiotensin (III) was synthesized by the following route:

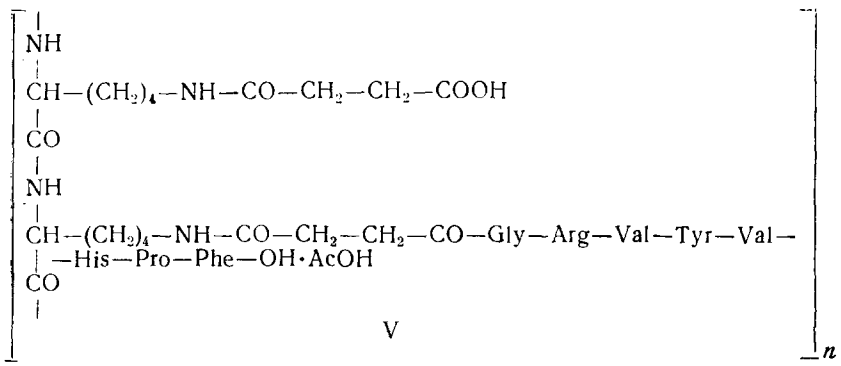
$$H_2N-{}^{14}CO-NH_2 \rightarrow Na^{14}CNO \rightarrow [H-N={}^{14}C=O]$$

$$[H-N={}^{14}C=O]+I \rightarrow H_2N-{}^{14}CO-NH-CH_2-CO-Arg-Val-Tyr-Val-His-Pro-Phe-OH \quad (III)$$

By isomerizing [^{14}C]urea in n-butanolic solution in the presence of an equimolecular amount of sodium butoxide [22], we obtained sodium [^{14}C]cyanate with a yield of 99%; the carbamoylation of (I) with the [^{14}C]cyanate according to the procedure that we have proposed gave (III) with a yield of 41.5%. The synthetic product (III) was homogeneous in two chromatographic systems on paper and in paper electrophoresis. The radiochemical purity of the (III) was shown by scanning a paper chromatogram and an electrophoregram of 0.0675 nmole of the labeled preparation. Scanning showed only one radioactive spot corresponding to the labeled compound (III) (Figs. 1 and 2). The radioactivity of compound (III) was determined by the activity of the initial ^{14}C-labeled urea. We used urea with a specific activity of 110 mCi/g (6.6 mCi/mmole). Product (III), after purification and freeze-drying, possessed a specific activity of 5.04 μCi/mg, which corresponded to a content of peptide material in the sample of 77% [23].

To develop a variant of the radioimmunological determination of the angiotensin [24] we also synthesized a copolymer of polysuccinyllysine and (I) – (V). For this purpose, a solution of polylysine hydrobromide in borate buffer was acylated with succinic anhydride by a modification of the method of Stason et al. [25], and then in 50% aqueous dioxane solution it was condensed with substance (I) by means of N-cyclohexyl-N'-[β-(4-methylmorpholino)ethyl]carbodiimide p-toluenesulfonate.

The amino-acid analysis and the calculated percentage content of the elements of (V) based on it, which agreed well with the results of the elementary analysis, show that one molecule of (I) was attached on an average to every second succinylated lysine residue. Consequently, 68% of the mass of the copolymer (V) consisted of [1-glycine]angiotensin connected by a covalent bond with the succinylated polylysine, the most probable structure of the polymer chain being as follows:



V

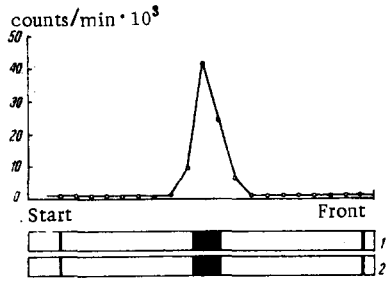


Fig. 1

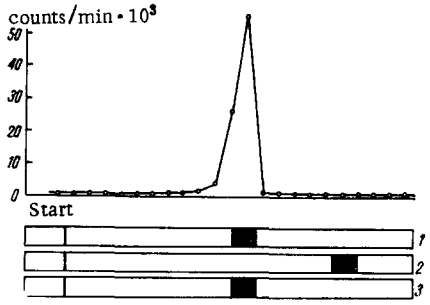


Fig. 2

Fig. 1. Distribution of radioactivity when 0.0675 nmole of {[1-^{14}C]hydantoic acid}angiotensin was subjected to chromatography (system 1). The lower part of the figure shows the position of the spot giving a positive reaction with Pauli's (1) and Ehrlich's (2) reagents.

Fig. 2. Distribution of the radioactivity when 0.0675 nmole of {[1-^{14}C]-hydantoic acid}angiotensin was subjected to paper electrophoresis (pH 2.4). The lower part of the figure shows the positions of the spots with a positive reaction to Pauli's (1) and Ehrlich's (2) reagents relative to that of histidine (II).

A sample of [1-glycine]angiotensin was given to us by A. P. Pavars the pressor activity was determined by Z. P. Aune, the amino acid analysis was performed by R. F. Platnietse, and the radioactivity measurements were performed by V. M. Berzin'sh.

EXPERIMENTAL

The work was carried out with [1-glycine]angiotensin [26], ^{14}C-labeled urea with a specific activity of 110 mCi/g (supplied by the All-Union combine "Izotop"), polylysine hydrobromide (mol. wt. 40,000-100,000) (Schuchardt, German Federal Republic), and N-cyclohexyl-N'-[β-(4-methylmorpholino)ethyl] carbodiimide p-toluenesulfonate (Fluka, Switzerland). The n-butanol was purified as described in the literature [23], with additional drying over potassium hydride. The melting (decomposition) points were determined in open capillaries (without correction) and the angles of rotation on a Perkin-Elmer model 141 polarimeter. Descending chromatography was performed on Filtrak FN-16 paper in the systems 1) butan-1-ol–acetic acid–water (4:1:5) and 2) sec-butanol–3% ammonia (3:1). Electrophoresis on Filtrak FN-16 paper was performed in 1 M acetic acid (pH 2.4), 15.5 V/cm, 1.5 h. The electrophoretic mobilities of the substances were characterized as the ratios of the distances traveled by the substances to the distance traveled by histidine. For elementary analysis, the substances were dried over P_2O_5. The elementary analyses of the compounds synthesized corresponded to the calculated figures.

In the determination of amino acid compositions (Biocal BC-200 amino acid analyzer), the substances were hydrolyzed with 6 N hydrochloric acid at 105-108°C in tubes sealed under vacuum for 21 h. In column chromatography, the peptides were detected by scanning the fractions in an SF-4 spectrophotometer at 275 nm, and also with Pauli's reagent on paper.

To determine specific radioactivity, 100 μl of the substance under investigation was dissolved in 5 ml of Bray's solution [27], and the ^{14}C activity was measured in a Packard Tri Carb model 3380 scintillation spectrometer with a counting efficiency of 68%. The distribution of the ^{14}C activity on paper chromatograms and electrophoregrams was determined by scanning fragments 1 × 1.5 cm of the corresponding chromatograms and electrophoregrams in 5 ml of Bray's solution on the above-mentioned spectrometer.

The pressor activity of the compound was determined by a published method [21] on nephrectomized male rats weighing 150-200 g under urethane narcosis (1.25-1.75 g/kg).

The standard used was [1-asparagine, 5-valine]angiotensin (II), whose activity was taken as 100%.

[1-Hydantoic acid]angiotensin (II). To a solution of 0.0307 g (26 μmoles) of (I) in 2 ml of water were added 0.004 g (60 μmoles) of sodium cyanate and 0.2 ml of a 3 M solution of monosodium phosphate (pH 6). The course of the reaction was monitored by paper electrophoresis. After 16 h, substance (I) had been con-

verted almost completely into the carbamoylated product (E_{His} 0.60, Pauli- and Ehrlich-positive, ninhydrin-negative, spot). Then 25 ml of water was added, and the mixture was stirred for 45 min with the moderately basic ÉDÉ-10p ion-exchange resin (in the acetate form). The mixture was filtered, and the resin was washed on the filter with 10 ml of water. The filtrate was treated with 1 M sodium acetate solution to pH 9 (0.6 ml) and was kept at 30°C for 3 h. Then it was passed through a column (0.9 × 8.5 cm) containing Amberlite IRC-50 (in the H^+ form). The column was washed with 300 ml of 0.25% acetic acid, and the peptide was eluted with 50% acetic acid. The fractions containing peptide material were evaporated, and the residue was dissolved in 10 ml of 0.25% acetic acid and deposited on a column (1.2 × 6 cm) of CM-cellulose (in the H^+ form). The column was washed with water and eluted with solutions of ammonium acetate with a linearly increasing concentration. (The mixing vessels contained 300 ml of water and 300 ml of 0.1 N ammonium acetate.) The rate of elution was 1.5 ml/min, the fraction volume being 7.4 ml. The fraction containing the main peak was evaporated and freeze-dried once from acetic acid and twice from water. Yield 0.010 g (32%), mp 229-232°C, R_f 0.48 (system 1), 0.27 (system 2), E_{His} 0.62, $[\alpha]_D^{22} -57.2°$ (c 0.36; 20% acetic acid). $C_{48}H_{68}N_{14}O_{11}$. On the basis of the nitrogen analysis, the calculated content of peptide material was 82% [23].

In its chromatographic and electrophoretic characteristics the substance was identical with [1-hydantoic acid]angiotensin [11].

Sodium [^{14}C]Cyanate. To a solution obtained by dissolving 0.035 g (1.52 mmole) of metallic sodium in 2 ml of n-butanol at 90°C was added 0.091 g (1.52 mmole) of [^{14}C]urea, and the mixture was kept at 140°C for 20 h. The crystals that deposited were filtered off and were washed on the filter twice with 2 ml of n-butanol, twice with 1.5 ml of ethanol, and with 15 ml of ether. Yield 0.098 g (99%).

{[1-^{14}C]Hydantoic acid}angiotensin (III). To a solution of 0.303 g (0.26 mmole) of (I) in 11 ml of water were added 0.1 ml of a 3 M solution of monosodium phosphate and a solution of 0.0381 g (0.586 mmole) of sodium [^{14}C]cyanate in 8 ml of water; the pH was kept at 6 ± 0.5 by the addition of 3 M monosodium phosphate (a total of 1.1 ml). After 23 h, 170 ml of water and 25 ml of ÉDÉ-10p ion-exchange resin (in the acetate form) were added and the mixture was stirred for 0.5 h. The solution was passed through a column containing 20 ml of ÉDÉ-10p ion-exchange resin (in the acetate form). The column was eluted with water, and the fractions containing the peptide were combined (180 ml), the pH was brought to 9 by the addition of 3 ml of a 1 M solution of sodium acetate, and the solution was left at 30°C for 3 h. Then it was deposited on a column (1.6 × 18 cm) containing 23 ml of IRC-50 ion-exchange resin (in the H^+ form). The column was eluted with 500 ml of 0.25% acetic acid and then with 50% acetic acid. The fractions containing the peptide were combined and evaporated. The residue was dissolved in 5 ml of 0.25% acetic acid and deposited on a column (2.2 × 16 cm) containing 61 ml of CM-cellulose (in the H^+ form). The column was eluted with 120 ml of water and with ammonium acetate solution having a linearly rising concentration. (The mixing vessels contained 500 ml of water and 500 ml of 0.1 N ammonium acetate.) The rate of elution was 1.2 ml/min, and the fraction volume 6 ml. The fractions including the diffuse main peak were treated with acetic acid, evaporated to small volume, and freeze-dried. The resulting product was twice freeze-dried from water. Yield 0.139 g (41.5%). The product possessed a specific activity of 5.04 μCi/mg, which corresponded to a 77% content of peptide material [23] in the sample (at a radioactivity of the initial compound of 6.6 mCi/mole). In its chromatographic and electrophoretic characteristics, preparation (III) was identical with [1-hydantoic acid]angiotensin [11]. The investigation of the distribution of radioactivity on paper chromatograms and electrophoregrams showed only one radioactive spot corresponding to labeled compound (III).

Washing the column with 0.1 N ammonium acetate (after similar treatment) gave another 0.029 g (9.6%) of a substance corresponding in electrophoretic mobility (E_{His} 0.85) to unchanged (I).

Polysuccinyllysine (IV). A suspension of 0.163 g (0.78 mmole of lysine residues) of carefully comminuted polylysine hydrobromide in 21 ml of borate buffer (pH 9.5) was stirred until dissolution was complete (45 min). With constant stirring, 0.72 g (7.2 mmoles) of succinic anhydride was added in separate portions to the solution over 2 h. The pH of the solution was kept between 9 and 10 by the addition of a 1 N solution of sodium hydroxide. After the end of the reaction (judging from the disappearance of the ninhydrin-positive reaction), the solution was acidified with 5 N hydrochloric acid to pH 3. The solution together with the precipitate that had deposited was dialyzed against distilled water for 40 h. The precipitate was centrifuged off, washed with water, and recentrifuged. The resulting polysuccinyllysine was dried in vacuum over P_2O_5/KOH. Yield 0.146 g (82%). $(C_{10}H_{16}N_2O_4)_n$.

Polysuccinyllysine-[1-glycine]angiotensin (V). A suspension of 0.045 g (0.195 mmole of succinyllysine residues) of (IV) in 25 ml of 50% dioxane was treated with 0.021 ml (0.15 mmole) of triethylamine. The poly-

(amino acid) swelled strongly and partially dissolved. To this suspension was added 0.15 g (0.128 mmole) of (I) and 0.194 g (0.45 mmole) of N-cyclohexyl-N'-[β-(4-methylmorpholino)ethyl]carbodiimide p-toluenesulfonate, and the mixture was stirred vigorously for 24 h. The precipitate that had deposited was centrifuged off and washed with water, and then it was recentrifuged and dried in vacuum over P_2O_5/KOH. Yield 0.144 g. Amino acid composition: Arg 1.00, Gly 0.71, His 1.02, Lys 2.01, Phe 1.03, Pro 0.99, Tyr 0.83, Val 2.07. $(C_{67}H_{98}N_{17}O_{17} \cdot AcOH)_n$.

SUMMARY

1. The direct carbamoylation of [1-glycine]angiotensin has given a highly active analog of the natural hormone – [1-hydantoic acid]angiotensin.

2. The synthesis of [1-hydantoic acid]angiotensin labeled with the ^{14}C isotope in position 1 has been effected by the proposed method.

3. A copolymer of polysuccinyllysine and [1-glycine]angiotensin has been synthesized.

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