INVESTIGATION OF THE PATHWAYS OF BIOTRANSFORMATION OF SPIROBROMINE

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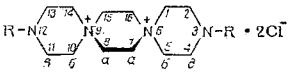
In the plan of development of studies of the molecular mechanism of the biological action and pathways of biotransformation of antitumor drugs from the group of dispirotripiperazinium derivatives, we made an investigation of the conversions of spirobromine — 3,12-bis(3'-bromopropionyl)-3,12-diaza-6,9-diazoniadispiro [5,2,5,2]hexadecane dichloride (I) — in aqueous media at 37°C and pH values close to physiological ($5.0 < pH < 8.0$). Is is a new, original antitumor drug, highly effective in the treatment of acute leukemia and a number of forms of malignant neoplasms [3, 7].



I: R = $-COCH_2CH_2Br$; II: R = $-COCH=CH_2$; III: R = $-COCH_2CH_2Cl$; IV: R = —H

We used PMR and ^{13}C NMR spectroscopy as the main method of investigation. The assignment of the signals (Table 1) was based on the nature of the multiplicity, the values of the chemical shifts δ, and the spin-spin coupling constants (SSCC) of the signals, as well as on a comparison of the spectral parameters with those described previously for a number of related compounds [1, 4, 8].

Our investigation showed that the main process of conversion of I in the range of pH from 5.0 to 8.0 is the dehydrobromination of the bromopropionyl groups, with the formation of 3,12-bisacrylyl-3,12-diaza-6,9-diazoniadispiro[5,2,5,2]hexadecane dichloride (II). The spiroheterosystem does not undergo any change under these conditions (Fig. 1).

$$\rangle N{-}COCH_2CH_2Br\ (I) \xrightarrow[-HBr]{5.0 < pH < 8.0} \rangle N{-}COCH{=}CH_2 \cdot (II)$$

The structure of the reaction product II was determined on the basis of an analysis of the PMR and ^{13}C NMR spectra. Thus, signals at 130.1 and 126.2 ppm are observed in the spectrum of ^{13}C (see Fig. 1a). The values of the chemical shifts, multiplicity, and SSCC $^1J(^{13}C^1H)$ indicate that these signals belong to the ethylene group $CH{=}CH_2$. In the PMR spectrum, during the reaction the appearance of signals in the olefin region is observed (Fig. 2a, b, c), the multiplicity of which and the values of the chemical shifts (see Table 1) are also characteristic of the $CH{=}CH_2$ group. The parameters of the PMR spectra of compound II coincide with the analogous values for this compound synthesized by an independent method [8].

Under conditions close to physiological (pH = 7.0, 37°C), the dehydrobromination reaction proceeds at a substantial rate: at an initial concentration of the preparation 0.1 M, its half-conversion time is ~50 min.

The reaction rate increases with increasing pH of the medium. Measurement of the dependence of the rate constant of dehydrobromination, calculated according to a first-order kinetic equation, on the pH (Fig. 3) showed that in the range $5.0 < pH < 8.0$ it is satisfactorily described by a linear equation $\log k = 0.4\ pH - 6.4$.

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TABLE 1. Parameters of the PMR and ^{13}C NMR Spectra of Compounds I-IV, VII

Compound	Chemical shifts, δ (^1H), ppm					Chemical shifts, δ (^{13}C), ppm						SSCC ^1J ($^{13}C^1H$), Hz				
	H_a†	H_b	H_c	H_β	H_γ	C_a†	C_b	C_c	C_α	C_β	C_γ	C_a†	C_b	C_c	C_β	C_γ
I	4,22	3,93	4,06	3,15	3,67	51,77	58,77	38,96* 35,24	171,28	35,11	27,37	149	150	143* 145	128	157
II	4,24	3,85	4,12	6,73	6,25 5,91	51,78	58,70	39,27 35,36	167,80	126,23	130,05	149	150	143 145	166	164
III	4,22	3,93	4,06	3,03	3,88	51,77	58,77	38,96 35,24	171,07	35,02	39,82	149	150	143 145	130	155
IV IV+2	4,11 4,41	3,74 4,28	3,20 3,88	— —	— —	52,9 52,6	60,9 56,9	39,3 38,0	— —	— —	— —	148 150	146 150	139 148	— —	— —
VII	4,23	4,05	3,95	2,85	2,85	51,62	58,59	38,83 35,10	172,01	26,81	32,44	149	150	144 146	143	142

*Doubling of the signals of C_c in compounds I-III, VII as a result of amide isomerization (the indicated values correspond to the spectrum measured at 35°C).

†For the notation of a, b, and c, see the formula in the text; here and in Table 3: the letters α, β, and γ denote the carbon atoms in the side chains of I-III, respectively.

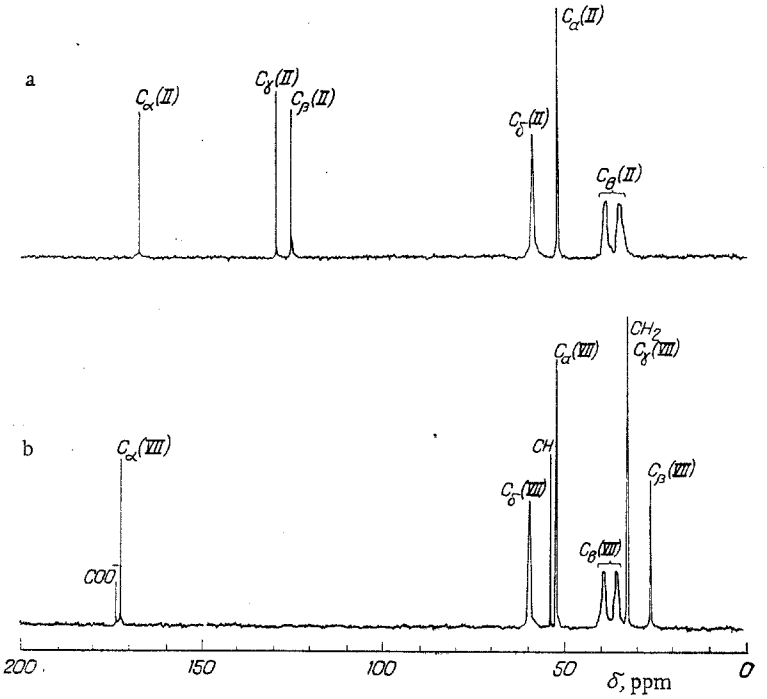


Fig. 1. ^{13}C NMR spectra. a) solution of II before addition of an equimolar amount of VI; b) after addition of VI.

Since acid is liberated in the course of the reaction, the reaction is autoinhibited in the absence of buffering capacity of the solution. Thus, to lower the pH of a 0.1 M solution of I in water to a value of 4.0, at which the rate of dehydrobromination is negligible, only 0.05% I must be converted. This explains the cause of the low pH values of aqueous solutions of I, whereas impurities of II in them cannot be detected by the method of NMR spectroscopy. However, in buffer solutions the amount of acrylate II formed increases substantially, and when the buffering capacity of the solution is sufficient, virtually complete conversion I → II is possible (see Fig. 2c).

We should mention that a study of the hydrobromination reaction in phosphate buffer became possible on account of the absence of alkylating ability of compounds I and II with respect to phosphate ions, in contrast, for example, to prospidin [5].

The only side process of the conversion of I in the range of $5.0 < pH < 8.0$ at 37°C is the exchange of halogen in the γ-position of the side chain of I, leading to the formation of compound III. However, under the indicated conditions the rate of this reaction proves substantially lower than the rate of the dehydrobromination reaction.

A study of the conversions of I in solutions of organic phosphates (potassium D-glucose-1-phosphate, sodium D-ribose-5-phosphate), nucleotides (sodium guanosine-5'-monophosphate, potassium inosine-5'-monophosphate), and amino acids (arginine, lysine) showed that under these conditions a dehydrobromination reaction is observed.

The process I → II is virtually irreversible. Thus, conducting it in solutions of D_2O does not lead to the formation of products of deuterosubstitution in the β position of the side chain of I or II, which should have been observed in the reverse reaction. Heating of II in weakly acid medium (pH > 1.0) in the presence of an excess of Cl^- or Br^- ions does not lead to the formation of products of addition of HCl (HBr) at the double bond. When the HCl concentration is increased to 1 M, and in the case of heating to 70°C, hydrolysis of II is observed at the N-C bond with the formation of 3,6,9,12-tetraazoniadispiro[5,2,5,2]hexadecane tetrachloride (IV^{2+}) and acrylic acid (V).

$$\rangle N{-}COCH{=}CH_2\ (II) \xrightarrow[70\ ^\circ C,\ 1\ M\ HCl]{H_2O,\ H^+} \rangle NH_2\ (IV) + HO{-}COCH{=}CH_2\ (V)\ .$$

The structure of the hydrolysis products was established on the basis of an analysis of the PMR spectra (see Fig. 2d). The multiplicity, the values of the chemical shifts and SSCC

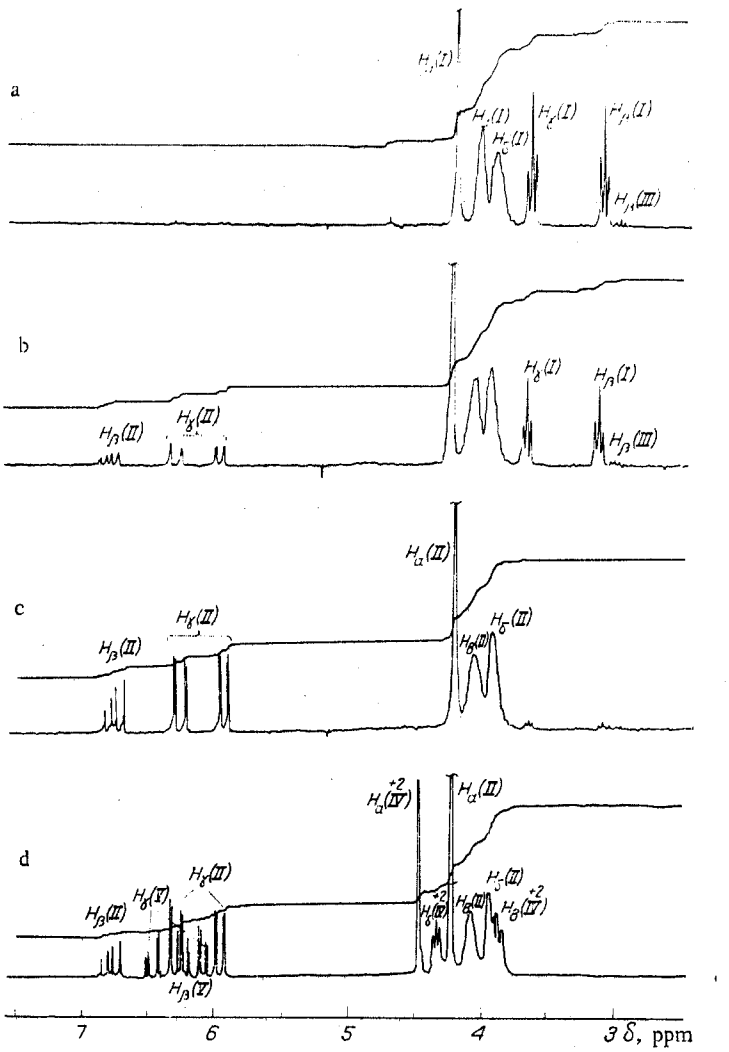


Fig. 2. PMR spectra. Solution of I in phosphate buffer at pH 7.0: *a*) before heating; b) after 1 h of heating at 37°C; c) after 7 h (under the same conditions as in b); d) solution of II in 1 M HCl after 30 min of heating at 70°C:

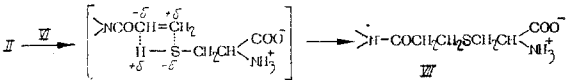
TABLE 2. Parameters of the PMR Spectra of Unsaturated Compounds II and V

Compound	Chemical shifts, δ (^{1}H), ppm			SSCC, Hz			Spin-lattice relaxation time, T_1, sec		
	H_β	H_γ^{cis}	H_γ^{trans}	$^3J(H_\beta H_\gamma^{cis})$	$^3J(H_\beta H_\gamma^{trans})$	$^2J(H_\gamma^{cis} H_\gamma^{trans})$	H_β	H_γ^{cis}	H_γ^{trans}
II	6,73	6,25	5,91	16,9	10,8	1,4	0,7	0,9	0,7
V	6,21	6,46	6,01	17,0	10,3	1,6	6,0	4,0	3,5

of the signals of the reaction product, observed in the olefin region, are characteristic of the CH=CH$_2$ group, just as in the case of II. However, in contrast to II, these signals belong to a low-molecular-weight compound, as evidenced by the substantially longer spin-lattice relaxation times T_1 (Table 2). The parameters of the PMR spectrum of compound V are close to those cited in the literature [13].

The ease of occurrence of the reaction I → II under conditions close to physiological suggests that it may play a substantial role in processes of biotransformation of I. It is known that compounds containing an ethylene bond conjugated with the carbonyl are highly reactive electrophilic agents, which is responsible for the presence of antiblastic properties in certain compounds of this series [6, 10]. It has been established that compounds with an activated double bond interact especially readily with thiols, being added to the sulfhydryl groups [6]. The presence of an activated ethylene bond in II suggests an increased reactivity of this compound in the alkylation of the SH groups, which play an important role in the functioning of protein systems [9]. Considering that these groups are contained in proteins in the form of amino acid residues of cysteine (VI), it seemed advisable to study the interaction of II with VI.

It was found that II interacts readily with cysteine, forming the thioester VII.



The structure of the reaction products was confirmed by the PMR and ^{13}C NMR spectra. Thus, in the ^{13}C spectra the signals characteristic of the olefin group disappear, and signals appear in the aliphatic region (see Fig. 1) belonging to the CH_2CH_2 group of compound IV (see Table 1).

The rate of interaction of II and VI is high: in the PMR spectra of the reaction mixture, measured directly after the beginning of the reaction at 25°C, no signals of the initial compounds can be detected. The pH of the medium has no appreciable influence on the reaction rate in the range $4.0 < pH < 9.0$. This is evidence of the participation of nonionized sulfhydryl groups in the reaction (since for the SH groups of VI, $pK_a = 10.0$ [11]).

At pH > 5.0, I also interacts with VI, forming VII; however, in this case the reaction procees through an acrylyl derivative II. This is confirmed by the fact that under conditions when the rate of conversion I → II is negligible (pH < 5.0), no interaction of spirobromine with VI is observed.

Thus, under conditions close to physiological, I is converted to the compound II, which possesses greater reactivity in comparison with I, at least with respect to sulfhydryl groups. This suggests that the acrylate II may play an important role in the biological action of I if it is formed as a product of the biotransformation of I.

To resolve the question of whether the process I → II occurs *in vivo*, using PMR spectroscopy we studied the structure and quantitative composition of the products of the metabolism of I after intravenous injection of the drug. The object of investigation was the urine of the experimental animals, since it is known that more than 98% of the drug and/or its metabolic products are eliminated in this way [2]. The method that we used for isolating biotransformation products does not exclude a loss of products of profound destruction of the drug, which are not quaternary ammonium bases; however, the dispirotripiperazinium derivatives are isolated quantitatively (within the limits of error of the measurement by the PMR method).

Our investigation showed that the main products of the isolation of I are the drug itself, the acrylyl derivative II, and the product of halogen exchange III. Moreover, the content of I in the total of excretory products in the first 7 h came to 75-85%, II 10-15%, and III 5-10%. The content of II in the urine may be below the amount of the substance actually formed in the organism as a result of the possibility of its bonding to the blood and tissue proteins, which contain free SH groups.

A determination of the relative formation of I, II, and III in the urine showed that the amount of II increases, while that of I decreases with time, beginning with the moment of administration of the drug (Table 3). A certain increase in the content of III is also observed.

As shown by a study of the dynamics of the excretion of the drug and its metabolites, about 85% of the amount excreted in 7 h is excreted in the first 3 h; moreover, about 70% corresponds to the period from 1 to 2 h after administration of I. This agrees with the results of a study of the pharmacokinetics of [^{14}C]spirobromine [2].

The results obtained are evidence that the dehydrobromination reaction occurs *in vivo*. Thus, the compound II formed may play an important role in the molecular mechanism of the biological action of I, acting at the stage of binding of the preparation to the biological systems of the cell.

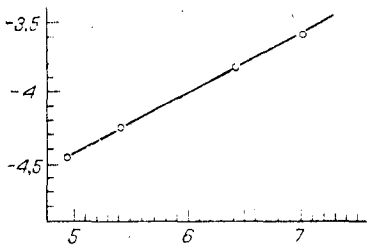


Fig. 3. Dependence of the logarithm of the rate constant of the dehydrobromination of I on the pH of the medium. Along x-axis: pH; along y-axis: log k.

TABLE 3. Relative Content (in %) of Metabolites of Spirobromine (I) after Intravenous Injection

Compound	Time after administration, h								Total in 7 h
	0.5	1	2	3	4	5	6	7	
I	93	85	82	79	74	66	65	58	81
II	5	10	11	12	16	24	25	32	13
III	2	5	7	9	10	10*	10*	10*	6

Note. The sum of I, II, and III was taken as 100% in each time interval.
*The content of III was arbitrarily taken as 10%, since its precise amount cannot be measured as a result of the overlapping of the signals of III with the signals of endogenous compounds in the PMR spectra.

EXPERIMENTAL

Compunds I and II were synthesized as described earlier [7, 8].

The ^{13}C NMR spectra were recorded on a Varian XL-100-12/A instrument (Switzerland) with working frequency 25.2 MHz. The internal standard was acetone, the chemical shift of which with respect to TMS was assumed equal to 29.4 ppm. The PMR spectra were measured on an XL-200 instrument from Varian (Switzerland) with working frequency 200.06 MHz. Internal standard: DSS. The spectra of all the compounds were recorded in D_2O solutions. To suppress the signal of residual protons of the solvent we used the method of saturating it during the period of lag between data collections. The relaxation time $T_1(H)$ was measured on nondegasified samples using a pulse sequence of inversion–reduction.

The pH values of the solutions were measured on a pH-Meter-27 instrument from Radiometer (Denmark). The values of pD in the case of measurement in D_2O were converted to the corresponding pH values by means of the function pH = pD − 0.4 [12].

The kinetics of the conversion I → II was studied according to the PMR spectra of 0.1 M solutions of I in 1 M KH_2PO_4–NaOH. The pH values of the buffer solutions were varied by varying the ratio of the salt and alkali.

Experiments on the isolation of products of biotransformation of I were conducted on noninbred male rats weighing 120 g. Feeding of the animals was stopped 24 h before the beginning of the experiment, giving only water. The urine was collected from the rats before and at periods of 30 min, 1, 2, 3, 4, 5, 6, and 7 h after intravenous injection of I (50 mg per rat). After concentration of the sample under vacuum to one fifth of the original volume (at 30-35°C), the residue was treated with acetone to remove the bulk of the endogenous organic compounds. Derivatives of dispirotripiperazinium, insoluble in acetone, were isolated in the form of a precipitate, which was then centrifuged, dried under vacuum, and dissolved in D_2O for the measurement of the PMR spectra. The concentration of biotransformation products in solution was judged according to the ratio of the integral intensities of their signals to the signal of DSS, added to D_2O as a standard.

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THE ROLE OF THE SORBITOL PATHWAY OF GLUCOSE METABOLISM IN THE MECHANISMS OF THE THERAPEUTIC ACTION OF PANTETHINE IN EXPERIMENTAL DIABETES

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Pantethine — D-bis(N-pantothenyl-β-aminoethyl)disulfide (I) — represents the disulfide form of pantethein, the main biologically active form of pantothenic acid (II). The latter, as is well known [4], participates in the biosynthesis of the coenzyme of acetylation (CoA) by means of a series of successive reactions of transformation of the vitamin II to 4'-phospho-II (III), 4'-phosphopantothenoylcystein, 4'-phosphopantethein, and dephospho-CoA. Preparations of I have been proposed in Japan and Italy, chiefly as agents for the prophylaxis and therapy of atherosclerosis. The normalizing influence of I on the ratio of lipoproteins of the atherogenic classes and high-density lipoproteins [14, 15] was confirmed in the course of a clinical study of the drug, including studies in diabetes [19].

In recent years drugs containing sulfhydryl and disulfide groups have been used successfully in the treatment of chronic diabetic lesions: unithiol, sodium thiosulfate, lipoic acid, and lipamide [2, 6, 7], which, in particular, improve the objective symptomatics of diabetic polyneuropathy, increasing the rate of conduction of excitation along the peripheral nerves.

Despite the prophylactic effect of I in diabetic polyneuropathy [16], its influence on the carbohydrate metabolism in diabetes has practically not been investigated. And yet, it has been established that the disruption of the regulation of the sorbitol pathway of glucose metabolism in insulin-insensitive tissues plays a substantial role in the pathogenesis of chronic diabetic lesions [3, 8, 9, 18].

The purpose of the present work was to study the infleunce of I in comparison with other derivatives of II — calcium salts of II and III — on the sorbitol content in the sorbitol pathway of glucose metabolism in the tissues of rats with chronic streptozotocin diabetes.

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