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Structure of alkyl radicals upon alkylation by thiotepa

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Summary. *Aim*. To determine conditions for reactions of alkylation by thiotepa (as exemplified by adenine) to obtain most efficient antitumoral drugs and identify their construction. For this two monofunctional analogs of thiotepa, ethyleneimine and monoaziridindiethylphosphate were used. *Methods*. Reversed-phase HPLC, acid hydrolysis, UV-spectroscopy, paper and thin-layer chromatography. *Results*. At one and the same site of alkylation can be generated up to 4 products with differing degree of thiotepa aziridine cycle opening and, accordingly, various antitumoral activities. In the alkylation by thiotepa depending on conditions four different alkyl radicals can be formed. *Conclusions*. Reactions of alkylating by thiotepa should be carried out in neutral and alkalescent media.

Keywords: alkylation, thiotepa, DNA, nucleic acids bases.

1. Introduction.

Thiotepa (N,N',N''-triethylenethiophosphoramide). In the 1960s and 70s threefunctional alkylating agent thiotepa (N,N',N''-triethylenethiophosphoramide) was successfully employed in chemotherapy of malignant neoplasm as an antitumoral drug. However, it generally exerts a pronounced toxic effect [1-8]. Minor doses of thiotepa were found to have cytostatic and cytotoxic effects, while higher ones exhibited mutagenic effect [9, 10]. The most effective antitumoral drugs have proved to be products of the alkylating with thiotepa of some biologically active substances such as alkaloids, particularly the celandine (Chelidonium majus) alkaloids [11-13], and nucleic acids. Therefore, in the experimental models of the animal transplantable tumors, thiotepa-modified DNA and RNA inhibited tumor growth by 90-100 %, while the efficacy of the thiotepa action *per se* usually does not exceed 50-55 % and nucleic acids themselves possess no pronounced antitumoral effect. It was also shown that both embryonic and thiotepa-modified human DNA have weak mutagenic effects with almost equal activities. They increase the frequency of spontaneous mutations only three times, whereas the mutagenic effect of thiotepa is 20 times as high [14].

Pioneering studies on alkylation of nucleic acids and their components, followed by isolation and identification of modification products have been started some 50 years ago. Besides the common alkylating agents (diazomethane, dialkyl sulfates, alkyl methane sulfonates and others), mustard gas and its nitrogen-containing analogs, N-2-chloroethylmorpholine and N,N-diethyl-2-chloroethylamine were used. Similar to ethyleneimine and its derivatives, only two latter named compounds form during alkylation an active intermediate, immonium cation [15-17].

The early works on thiotepa alkylation appeared later on and dealt primarily with pharmacokinetic studies [18], mutagenic and toxic effects of this alkylating agent itself in comparison with the same effects of intact and thiotepa-modified nucleic acids [14]. The fact that these was interaction between thiotepa and nucleic acids showed by the changes in their melting temperature curves or DNA lumines-

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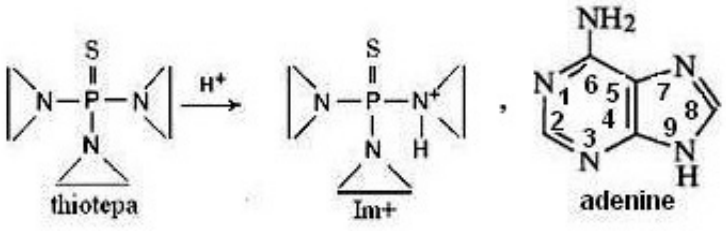
Scheme 1



cence that indicates DNA alkylation at the position 7 of guanine [19, 20]. For the first time we isolated products of heterocyclic bases alkylated by thiotepa and proposed the construction of the alkyl radicals.

Having a high pKa (7.8), thiotepa is able to alkylate the nucleophilic centers even in neutral media, however the efficiency of alkylation is rather low [21]. The alkylation rate substantially increases in the presence of a proton donor, which facilitates the formation of active alkylating particle, the immonium cation (Im+), generated by protonation of the ethyleneimine (aziridine) ring [22] (see scheme 1).

But thiotepa may directly alkylate the acids themselves, which are proton donors. The stability of the immonium cation and therefore the efficiency of alkylation reaction are determined by the nature of proton donor, i.e. the nucleophility of its anion.

For alkylation reactions performed at pH 4.5-7.0 we choose as the most preferable proton donor perchloric acid ($HClO_4$). Its anion occupies the last place in the row of anion nucleophility, and therefore the product of its interaction with thiotepa displays the ionic structure, while HCl is covalently linked to thiotepa and the chloride-anion competes with nucleophils in alkylation reaction, including the nucleic acid bases. We found that if the reaction is performed in the presence of immonium cation the increase in temperature not only accelerates the hydrolysis of the P—N bonds but also provokes a sulfur release as a hydrogen sulfide. This results in a complex mixture of thiotepa degradation products including various phosphoric acid derivatives.

Taking into account all above mentioned the alkylation reactions in the presence of $HClO_4$ were performed at temperature not higher than 37 ˚C. When the reactions were conducted under the neutral conditions the yield of products was increased either by heating up to 100 ˚C or by raising the alkylating agent concentration (adenine-thiotepa, 1:5, Scheme 1). Since in great excess of thiotepa the medium became weakly alkalized, it was neutralized by adding diluted $HClO_4$.

To identify the resultant products alkylation by thiotepa in parallel was carried out alkylation by its monofunctional analogs, ethyleneimine (EI) and monoaziridinediethylphosphate (MAEP), and also UV characteristics of adenines alkylated by different alkylating agents were used [23]. The data obtained provide evidence for alkylation sites and the structures of alkyl radicals.

Materials and methods. All chemicals and solvents were of analytical or HPLC grade. In the work were used: acetonitrile (Lichrosolv), sodium dihydrogen phosphate and adenine from Serva (Germany), Filtrak FN-12 paper (Germany) and Silufol^R UV-254 plates (Czech Republic). The alkylating agents (thiotepa, ethyleneimine and monoaziridinediethylphosphate) were synthesized as described previously [24, 25].

Equipment, separation conditions and identification of alkylated bases. UV spectra were recorded with a Specord UV-VIS spectrophotometer (Karl Zeiss, Germany). The alkylated mixtures were separated by using a Bio-Rad HPLC system (USA) with a flow-through UV detector of the LTV monitor type, model 1306, of the same firm. The chromatography of the alkylated bases was carried out under the conditions of reversed-phase HPLC on a Bio-Sil ODS-5S column (4x150 mm) at a rate of elution of 0.7 ml/min in a 0-20 % concentration gradient of acetonitrile in 0.05 M sodium phosphate buffer, pH 7.0.

Alkylation of the nitrogenous bases.

In acidulous medium. 13-15 mg (≈0.1 mmol) of adenine was dissolved upon heating in 3-5 ml of water and then added to freshly prepared solu-

Table 1

UV spectral characteristics and dissociation constants of adenines alkylated by thiotepa and isolated by reversed-phase HPLC. A — our data, B — data from B. Singer review [23]

Sites of alkylation	pH	λ_{max} - λ_{min}, nm		pKa [23]
		A	B	
Adenine	1	262.5–228	–	4.15
	12	269.5–237	–	
N1	1	263–235	262–233	7.2
	12	272–246	271–242	
N3	1	276–233	275–236	6.0–6.5
	12	274–245	274–245	
N^6	1	272–233	272–234	4.2
	12	273–244	273–236	
N9	1	259–228	258–227	4.0
	12	261–233	261–229	
(N7)**	1	272–235	273–237	3.6
	12	270–234	270–230	

** — *minor product obtained by means of PC and TLC chromatography (from Fig. 4).*

tion of Im+ obtained by mixing of 19 mg (0.1 mmol) thiotepa and 17.4 mg (0.1 mmol) $HClO_4$ both dissolved in 1 ml of water. The mixture was stirred at 20 ˚C for 3 h and kept at 37 ˚C for 18 h. Non-reacting thiotepa was extracted with ether, the rest of solution evaporated at 35-40 ˚C in the vacuum of the water-jet pump, the 0.2-0.4 mg base aliquot was chromatographed on a column.

In neutral medium. 0.1 mmol of bases were dissolved in 4-6 ml of water and mixed with 0.5 mmol of alkylating agent. To neutralize the weak alkaline medium the diluted (1:200) 5.74 N $HClO_4$ was added. The mixture was incubated at 37 ˚C for 24 h, and then analyzed as described above.

In the absence of $HClO_4$ (water solutions of thiotepa have pH≈8, those of EI have pH≈10). A mixture containing 100 mg of adenine and 300 mg of thiotepa in 10 ml of water was heated at 100 ˚C under reflux for 8-10 h. After cooling, filtration, and extraction of thiotepa with ether the mixture was analyzed as described above.

Results and discussion. To identify the alkylation sites and structures of alkyl radicals of modified bases the reactions of adenine alkylation by thiotepa under different conditions (pH and temperature) were studied in details. As seen from Figures 1a and 2a, alkylated adenines are eluted both before and after adenine fraction.

By HPLC method the reaction mixture containing products of adenine alkylation in the presence of $HClO_4$ was separated into four fractions. Three fractions are eluted far before the adenine fraction (Fig. 1a). The UV spectra of these fractions were completely identical to those of the products of adenine substitution in the positions N1, N3 and N9 [23]. It can be seen from the review Singer and also Table 1 that the spectral characteristics of the products of substitution at one and the same position of the heterocycle do not depend on the structure of the alkylating agent (the deviations do not exceed 1-2 nm), while for different alkylation centers such a dependence was observed, and the deviations could amount to 10 nm and more. Remarkably, the isolated products were eluted from a column in the order correlating with their pKa values (Table 1).

To explain the different chromatographic mobility of adenines alkylated by thiotepa and to determine the structures of alkyl radicals, we carried out the alkylation reactions with EI and MAEP. EI presents the single aziridinic cycle, while the MAEP like the thiotepa has one aziridinic cycle, one phosphorus and one amide bond which can be hydrolyzed.

Fig. 1 demonstrates that the chromatogram of the mixture of adenines alkylated with thiotepa in the presence of $HClO_4$ is similar to that as in the case of ethyleneimine alkylation in the absence of $HClO_4$.

Conditions for alkylation reaction and mixture separation are presented in section «Materials and methods». N1, N3, N9 — sites of adenine alkylation. Ade — peak of adenine.

Under the alkylation with MAEP two types of products were separated (Fig. 2a).

Both types have the same spectral characteristics corresponding to alkylation at the N1 and

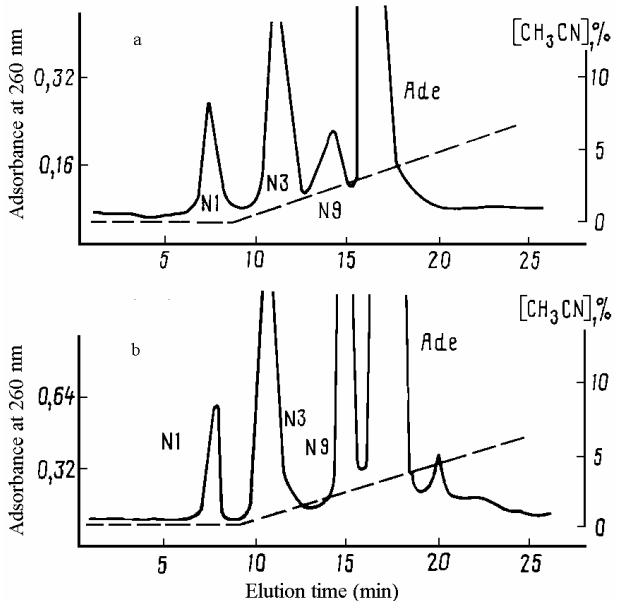


Fig. 1. HPLC of adenine alkylation products: a) by thiotepa in the presence of the perchloric acid (starting pH 4.5) and b) by ethyleneimine in the absence of proton donor (pH 8.0).

N3 positions, but having different elution times (before and after adenine). Contrary to alkylation of adenine by ethyleneimine and thiotepa, the N9-substituted adenine was not detected before adenine, but it appeared after the acid hydrolysis of the mixture (Fig. 3b).

Based on the results obtained for adenine alkylation with MAEP at neutral pH one may suppose that alkylation could lead to two types of the alkyl radicals: phosphoraminodiethyl one — R*, resulting from opening of the protonized aziridine cycle, and the aminoethyl radical — R, resulting from the hydrolysis of the amide (P-N) bond of radical R*. The following structures are proposed for these MAEP radicals (Scheme 2).

Amide bond may be cleaved both during the alkylation reactions and during the storage of alkylated mixtures. R* radical seems to impart

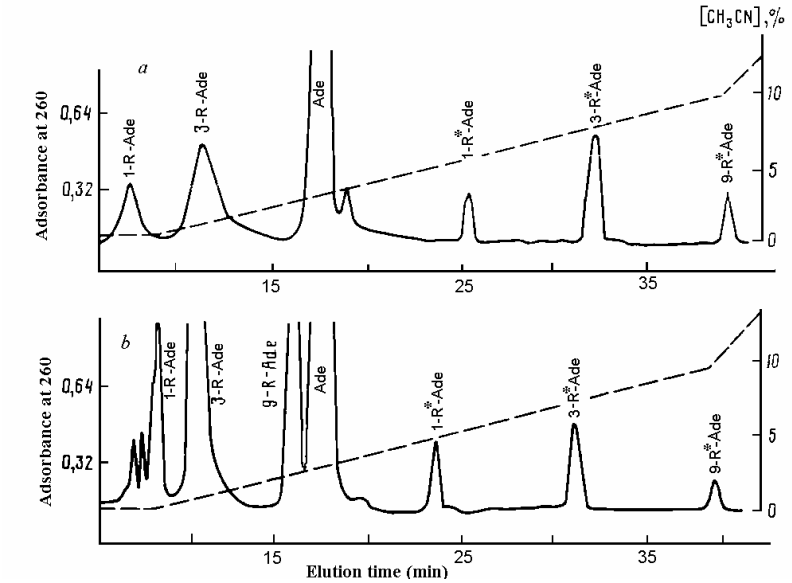


Fig. 2. HPLC on the column (4x150 mm) Bio-Sil ODS-5S for mixture of adenines alkylated by monoaziridinediethylphosphate at 37 ˚C during a day and in adenine-MAEP-$HClO_4$ 1:1:0.1 ratio (a), the same mixture after acid (0.5 N HCl, 100 ˚C, 1 h) hydrolysis (b). R — radical with amide bond cleavage, R— radical without such cleavage.*

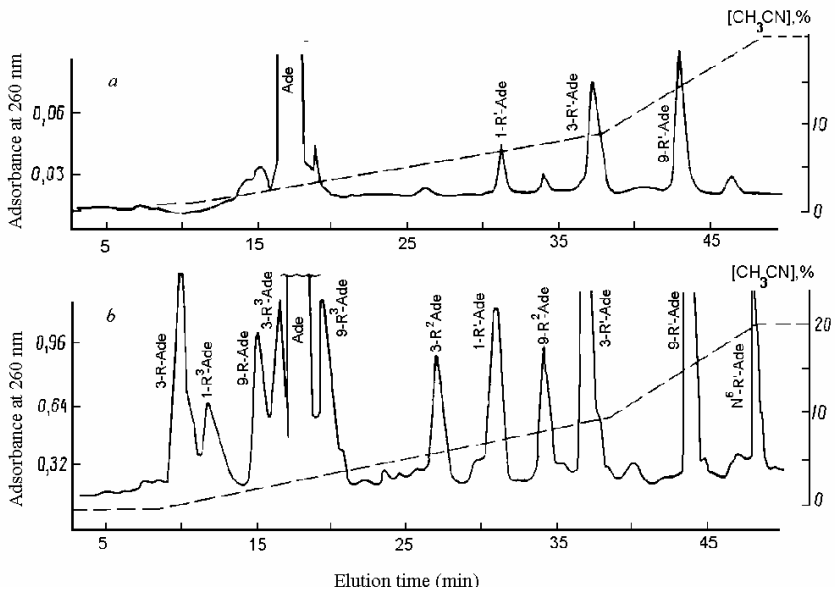


Fig. 3. HPLC on the column (4x150 mm) Bio-Sil ODS-5S for the mixture of adenines alkylation by thiotepa during a day in neutral conditions at room temperature and in adenine-thiotepa-$HClO_4$ 3:15:0.1 ratio (a) and after 8 h of heating at 100 °C and in adenine-thiotepa 1:2 ratio (b). 1, 3, and 9 — sites of adenine alkylation. The structures of R, R^1, R^2 and R^3 radicals are presented in scheme 3.

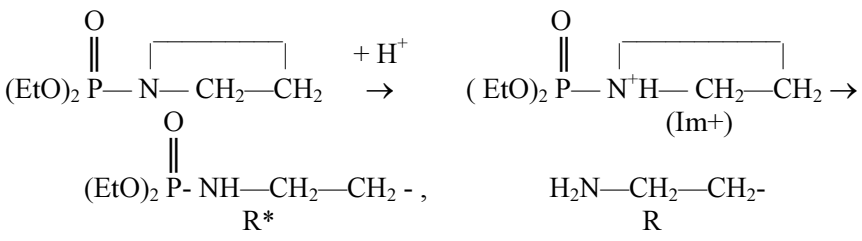
hydrophobic properties to the product, while R - hydrophilic ones, which explains their emergence after and before adenine, respectively. This also provides an explanation for different chromatographic mobility of the modified products under various alkylation conditions. Upon alkylation in weak acid media (with Im^+ involved) the aminoethylation products (with R radical) are produced as a result of the amide bond hydrolysis, whereas in neutral media the phosphoraminoethylation products (with R* radical) are produced and eluted after adenine (Figs 2 and 3).

By analogy, having three phosphor amide bonds thiotepa is suggested to form four types of alkyl radicals at the each position and therefore four types of alkylation products are produced by the consecutive cleavage of three amide bonds. In common case, four structures of thiotepa radicals are depicted in scheme 3.

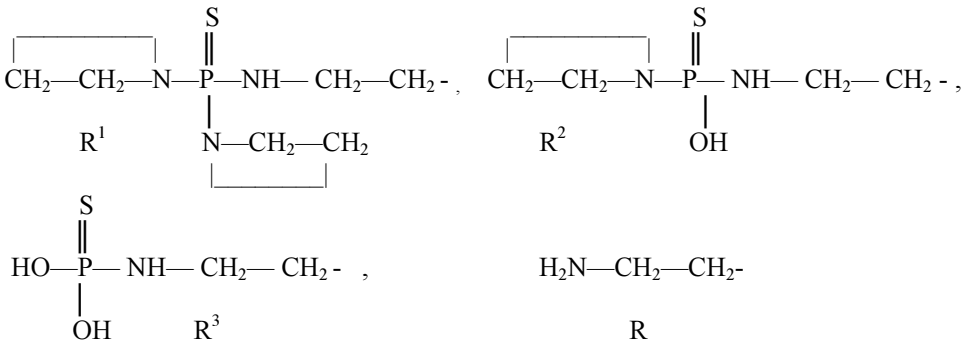
Products with these radicals were isolated from the mixture of alkylation products upon boiling of adenine with thiotepa. As seen from Fig. 3b the mixture contains four products with spectral characteristics of 3-substituted adenines and as many products alkylated at N9 position of the heterocycle. Identification of the fractions on the chromatogram was accomplished by comparison with the appropriate peaks in Figs 1b and 2a, where the radicals were determined as R (this radical is the only one for EI alkylation) and R* (R^1 for thiotepa). Decrease of product hydrophobity with the hydrolysis of its amide bonds (R^2 and R^3) was also considered to identify fractions.

The last product eluted from the column was adenine substituted at the exocyclic nitrogen in the N^6 position. Direct alkylation at this position proceeds extremely rarely and with low effi-

Scheme 2



Scheme 3



ciency. Adenine N^6-derivatives are generally formed from 1-substituted products as the result of a Dimroth regrouping at relatively low alkalinity (pH 8-9) and even in neutral media [19]. Such transformation into N^6-derivative may be a reason for the absence of 1-aminoethyladenine (1-R-Ade) fraction in Fig. 3b. N^6-R-Ade is eluted together with adenine (their pKa values are very close, Table 1), while are separated from each other on the column with sephadex G10. In acidulous media with conditions lacking for such regrouping, 1-substituted adenine can amount to 20 % of the total of alkylated products (Fig. 1a).

Spectral characteristics of adenine alkylated at the exocyclic amino group and isolated both by thin layer (TLC) and paper (PC) chromatography (Table 2, Fig. 4) slightly differ from those of N^6-alkyl adenine isolated by HPLC. At the same time, both products have analogs reported in literature [23].

Thus, UV absorption spectra of N^6-(2-hydroxyethyl)adenine were analogous to those of adenine alkylated by thiotepa without cleaving P-N bond (N^6-R1-Ade), while UV spectra of N^6-Me- (or Et-) adenines correspond to that of the N^6-aminoethyladenine isolated in Silufol plates. Apparently, the N6 of adenine is the only position for which UV spectra are determined not only by the alkylation site but also by the structure of the alkylating particle and possibly by conditions of product isolation (neutral medium in case of HPLC and alkalescent in case of PC and TLC).

The values in parentheses are presumably attributed to N1-substituted adenines, which

Table 2

UV spectral characteristics of alkylated adenines isolated by TLC method. Solvent systems: A — methanol-ammonia (25 %) — water (5:1:7), B — isopropanol-ammonia (25 %) — water (7:1:2)

Sites of alkylation	pH	$\lambda_{max} - \lambda_{min}$, nm	R_f(against Ade), in system		A_{280}/A_{260} (isolated in system B)
			A	B	
N3(R^1)	1	275-236	0.6-0.7	0.08	1,3
	12	273-244			1.3
N^6	1	267-233	(0.9)	(0.47) **	(0.9)
	12	273(280)-244 -2222224-224245			(1.1)
N3(R)	1	275-238	1.45	0.7	0.87
	12	273-247			0.85
N9	1	258-231	1.16	0.85	0.23
	12	261-233			0.16
Adenine	1	262.5-228	1.0	1.0	-
	12	269.5-237			
N7	1	272-235	-	1.15	-
	12	270-234			

Table 3

UV spectral characteristics of alkylated guanines, cytosines and uracil isolated by reversed-phase HPLC. A — our data, B — data from B. Singer review [23]

Sites of alkylation	pH	λ_{max} - λ_{min}, nm	
		A	B
		Guanine	
N1	1	253(272)–233	251(274)–229
	12	277(261)–246	278(260)–243
N7	1	252(272)–232	249(272)–233
	12	283–257	280–258
N7*	1	262–230	262–221
	12	260–248	261–243
N9	1	253(277)–231	251(276)–230
	12	256(269)–241	(258)268–238
		Cytosine	
N3	1	277–246	275–242
	12	297–258	294–254
N^4	1	280–248	277–244
	12	289–259	284–253
		Uracil	
N3	1	262–235	259–230
	12	281,5–248	218, 283–245

* *with cleavage of the imidazole ring leading to 5 — R and R'-pyrimidines.*

were converted into N6-derivatives under separation in alkaline medium.

Besides the products isolated by HPLC, we isolated the minor product by means of PC and TLC. When bulk of the products of alkylation remained close to the start in a neutral medium (Fig. 4a, R_f 0-0.3), we managed to isolate the minor product with R_f 1.36 in sufficient amount. This product displays UV spectral characteristics of N7-alkyladenine. It goes before the adenine in chromatogram, since its pKa is to be much more lower than that of adenine. This is the second supporting evidence that this product is N7-substituted adenine (Table 1).

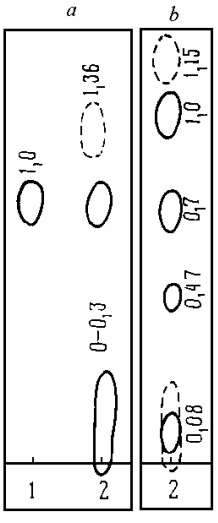


Fig. 4. Separation in the system i-propanol - ammonia (25 %) — water 7:1:2 ratio of adenine products alkylated by thiotepa (37 °C, 24 h) on the paper Filtrak FN-12 (a) and plates Silufol UV-254 (b): 1 — adenine, 2 — reaction mixture. Numerals near the spots — their R_f comparative to adenine. Starting pH - 7 (a) and 4.5 (b). Operating conditions for experiment are presented in section «Materials and methods».

The analogous product was detected on the Silufol UV-254 (R_f 1.15, Fig. 4b) plates. It is noteworthy, that reaction mixture after alkylation in acidulous medium was divided into three spots apart from adenine. Apparently, these are the products of substitution with various alkyl radicals (R^1, R^2 and R^3, in the order of their hydrophobity decrease). The data obtained in different conditions of alkylation and separation showed that the main products of alkylation in neutral medium are formed without the cleavage of the P—N bonds of thiotepa, while the more mobile ones are formed through aminoalkylation and their yield decreased with pH raising (Fig. 4b).

Thus, alkylation of adenine by thiotepa proceeds similarly to alkylation by other electrophilic agents at the N9, N3 and N1 positions with decreasing efficiency consistent with their pKa (Table 1) and partial regrouping of the N1 into N^6-derivative in alkalescent media. The least effective alkylation may proceed at the N7 position of heterocycle. Unlike the reactions with monofunctional alkylating agents (dialkyl sulfates, alkyl halides, alkylene oxides etc.), the reactions with thiotepa are rather dependent on pH, temperature and duration of reaction. Under reaction performed within the pH range 4.5-5.0 and at room temperature, the products with R

radical were obtained. In neutral and alkalescent media (without proton donor) alkylation is preferentially realized by the uncleaved molecule of alkylating agent (R^1 radical). In acidulous media, where the alkylation reactions are generally performed, radicals of various types can appear, since the starting pH 4.5 can rise as the reaction proceeds. Such radicals can also be produced upon heating (up to 100 ˚C and more) and long-term storage (about a year) of alkylated mixtures. Upon separation by reversed-phase HPLC, the modified products with R, R^3, R^2 and R^1 radicals were sequentially eluted from the column. Remarkably, the products with R1 radical exhibit a higher antitumoral effect than those with other radicals [26].

The thiotepa-alkylated products of other heterocyclic bases were also obtained and then identified (Table 3). Guanine is preferably alkylated at the N9 and N7 positions and to a lesser extent at the N1 position. As in the case of adenine, UV absorption spectra for alkyl derivatives of guanine, cytosine and uracil appeared to be practically identical for all the alkylating agents used (EI, MAEP, thiotepa) and the data obtained are consistent with the data of B. Singer review.

Pyrimidine bases of nucleic acids are alkylated much less effectively than purinic ones and with very small output (to 1-2 %), preferably at the N3 position, while cytosine is also alkylated at N^4.

In conclusion, thiotepa as a polyfunctional alkylating agent should cross-link the DNA double strands. However, there is no evidence in literature on the chemical nature of cross-linking induced by thiotepa. In our study, we failed to establish cross-linking between two molecules of any base under alkylation by thiotepa. And also, we did not find bases disubstituted by thiotepa. Most likely, thiotepa induces cross-linking only in DNA, when one molecule of thiotepa alkylates two guanines at the N7 position in double strands, as shown in case of «nitrous mustard gas» [27].

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Структура алкільних радикалів при алкілуванні тіотефом

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Резюме. *Мета.* Визначити оптимальні умови реакцій алкілування (на прикладі аденіну) для отримання ефективних протипухлинних препаратів і встановити їх будову. Для цього використані 2 монофункціональних аналога тіотефа — етиленімін і моноазиридиндиетилфосфат. *Методи.* ВЕРХ, кислотний гідроліз, УФ-спектроскопія, паперова та тонкошарова хроматографія. *Результати.* При алкілуванні тіотефом по одному і тому сайту можуть утворюватися до 4 продуктів алкілування з різним ступенем розкриття азиридинових циклів тіотефа і, відповідно, різною протипухлинною активністю. *Висновок.* Реакції алкілування тіотефом варто проводити в нейтральних або слабколужних середовищах.

Ключові слова: алкілування, тіотефа, ДНК, основи нуклеїнових кислот.

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