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THE EFFECT OF MANNITOL-MYLERAN AND TWO NEW DIBROMO-HEXITOLS ON THE METABOLIC ACTIVITIES OF NUCLEIC ACIDS AND PROTEINS—I.

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Abstract—The effects of dibromo-dulcitol (DBD), dibromo-manitol (DBM) and mannitol-myleran (MM) on the DNA-, RNA and protein synthesis of bone marrow cells *in vitro* and in HeLa cells in tissue culture were studied. The inhibition of DNA synthesis assessed by ^{14}C-formate incorporation displays time and concentration dependence. Forty to fifty μg/ml of DBD were sufficient to produce 50 per cent inhibition in normal rabbit bone marrow cells after 7 hr incubation. Two to two and a half times more of DBM and 4–5 times more of MM are required to produce the same degree of inhibition. Bone marrow cells from rats with Shay leukemia reacted practically to a similar extent. Incorporation of ^{14}C labelled precursors was used to compare the effects exerted on the syntheses of the three biopolymers in bone marrow and HeLa cells. In bone marrow cells DNA synthesis is inhibited to the highest extent, RNA synthesis less and protein synthesis the least. In HeLa cells this order was modified inasmuch as RNA synthesis is inhibited to the same or even to a higher extent than DNA synthesis. The effect exerted on the protein synthesis is 'indirect' since even high concentrations of the compounds failed to inhibit amino acid incorporation. into the cell-free ribosomal system. Nucleic acid synthesis or some metabolic process closely related seems to be the point of attack of these cytotoxic agents.

Degranol®† was synthesized by Vargha when coupling the functional group of nitrogen mustard to mannitol.[1] This was the first hexitol derivative successfully applied in tumour chemotherapy.[2, 3] The tumour growth inhibitory and haematological effects of Degranol were studied by Kellner and Németh[4] and the biochemical studies suggested a strict parallelism between the dynamism of morphological changes and the changes in the metabolic activities of nucleic acids.[5, 6]

To improve the first antiblastic hexitol derivative in clinical practice, Haddow *et al.*[7] and Vargha and Kuszmann[8] attached the functional groups of myleran, the methanesulphonyl (mesyl) group, to mannitol. The pharmacology of the resulting mannitol-myleran‡ (MM) was studied[9] and this compound was successfully applied in the treatment of myeloid leukemias and certain solid tumours.[10]

On basis of theoretical considerations, deviating from the principle of combining hexitols with the well proved functional groups, a series of alpha, omega-substituted poliol derivatives were prepared, containing both lipophilic and hydrophilic molecular moieties. Among these compounds of dual transport characteristics, the halogen

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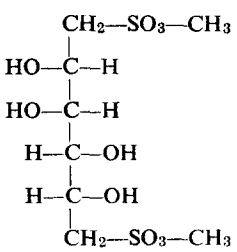
† 1,6-*bis*/β-chloroethylamino/-1,6-dideoxy-D-mannitol = BCM = Degranol®.

‡ 1,6-dimesyl-mannitol = MM = Mannogranol®.

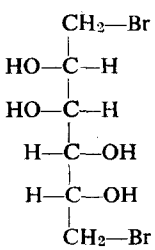
derivatives dibromomannitol* (DBM)[11] and dibromo-dulcitol† (DBD)[12] exerted remarkable cytostatic activity. The pharmacological properties of DBM were described[13] and Sellei and Eckhardt and coworkers successfully applied DBM in clinical practice to treat patients with chronic myeloid leukemia[14–16]

The pharmacology of DBD was studied by Kellner *et al.* recently[17–19] They observed 95 per cent inhibition of tumour growth in sensitive tumours like the Yoshida and Walker tumours and reached even full regression in case of the Shay tumour. In fact, 90 per cent inhibition was observed in semi-resistant Guérin-tumours and inhibition from 90 to 100 per cent in rat rhabdomyosarcomas, known to be resistant practically to all chemotherapeutics. As a characterization of the cytological action of DBD, authors demonstrated the enlargement of the cell, the nucleus and nucleolus and an early and protracted suppression in mitosis.[18]

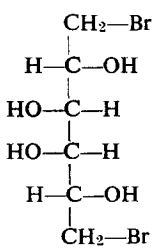
The chemical structures of MM and of the two dibromo-hexitols, DBD and DBM, display a fundamental similarity. All the three of them are alpha, omega-substituted hexitols and the alkylating ability of the two dibromo-hexitols also resembles that of MM.[11] Their pharmacological and clinical properties, however, are different. According to Elson, the effect of MM on the haematopoietic system is similar to the myelotoxicity of mylerans and to the inhibitory action on the lymphoid system[20, 21] of compounds of the nitrogen mustard type (and of epoxides). On the other hand, dibromo-hexitols are selectively myelotoxic, as demonstrated by Kellner *et al.* for DBD[19] and by Institoris *et al.* for DBM.[16]



MM



DBM



DBD

Both clinical and experimental results obtained to date with dibromo-hexitols are very promising. To reveal the mechanism of action of these compounds, we performed biochemical studies. As a first step, we studied the metabolic activities of proteins and nucleic acids of cells treated with these compounds. The data presented here allow the conclusion that all the three compounds appreciably reduce syntheses of nucleic acids and indirectly the synthesis of protein.

MATERIALS AND METHODS

Cell suspension. 'Bone marrow cells' were obtained from the femora of rabbits or rats. Bone marrow cell suspension was prepared according to Antoni *et al.*[22, 23] The cells were suspended and incubated in Tyrode solution, containing 0·1 % glucose and 20 % isologous serum, at 37°, without shaking. The suspension volume was 5 ml

* 1,6 dibromo-1,6-dideoxy-D-mannitol = DBM = Myelobromol®.

† 1,6-dibromo-1,6-dideoxy-dulcitol = DBD.

in 100 ml Erlenmeyer flasks, 15 × 10^6 cells/ml. To produce 'Shay leukemia', solid tumours were treated with trypsin. The cell suspension obtained was injected into the abdominal cavity of adult rats and the resulting ascites cell suspension transplanted. Ascites cells, 1–2 × 10^7, were injected i.v. into 120–150 g rats. Suspension from rat bone marrow cells was prepared on the 7–9th day following transplantation.

'HeLa' cells* were grown in monolayer in Roux flasks at 37°. When adding the compounds, the culture medium was changed. By the end of incubation the cells were quickly collected into cold saline and centrifuged.

The incorporation of ^{14}C precursors into the respective biopolymers of the cell was measured as follows. ^{14}C-formate labelled DNA was isolated from the washed cell suspension practically according to the method of Davidson and Smellie.[24] The labelled DNA was reprecipitated several times[22] and the DNA content was determined by the indole colour reaction.[23] In the case of RNA labelled with ^{14}C-uridine the dry powder obtained by the Davidson-Smellie method was hydrolized in 0·3 N KOH overnight at 37°. Hydrolysis was stopped by acidification with perchloric acid, centrifuged and the supernatant neutralized with potassium hydroxide. Ribonucleotide content was determined by the orcinol colour reaction.[23] The incorporation of ^{14}C-lysine into cellular proteins was measured after precipitation with trichloroacetic acid and purified according to the method of Siekevitz.[26, 23] The residue obtained after washing in alcohol-chloroform was washed once more in alcohol, dissolved in concentrated formic acid, diluted after full dissolution, centrifuged. The sp. act. of this supernatant was determined. The protein content was determined according to the Lowry method[23].

Four parallel samples of the non-treated and two of the treated cell-suspensions were used in each experiment to determine specific radioactivities. Three parallels were used for each colour reaction and two for the determination of radioactivity.

The measurement of ^{14}C*-amino acid incorporation into cell-free ribosomal systems.* Total ribosomal particles were isolated from guinea-pig liver practically according to Munro *et al.*[27] The final volume of 1 ml of the cell-free system consisted of the following components: 2·5 mg ribonucleoprotein, pH 5 fraction (1·5 mg protein) isolated from rat liver according to Hoagland *et al.*[28] 20 μmoles Tris HCl buffer (pH 7·8), 10 μmole $MgCl_2$, 50 μmole KCl, 6 μmole 2-mercaptoethanol, 0·5 μmole ATP, 0·05 μmole GTP, 5 μmole phospho-enol pyruvic acid, 20 μg pyruvate kinase, 0·5 μC ^{14}C-lysine. Incubation lasted for 60 min at 37°, with shaking. The reaction was stopped by trichloroacetic acid. The extraction and determination of labelled proteins were processed as described above. All sp. act. values represent the mean of two samples.

Measurement of radioactivity. A Frieseke–Hoepfner methane gas flow counter was used to measure ^{14}C radioactivity in infinitely thin samples. Specific radioactivity was expressed as cpm/mg of a given biopolymer, i.e. RNA, DNA or protein, respectively.

Isotopes: ^{14}C-formate (Isotope Institute, Budapest) spec. act. 1·54 or 13·26 mC/Mm; ^{14}C-uridine, spec. act. 251 mC/mM; ^{14}C-L-lysine-HCl, spec. act. 4·07 MC/mm were used to label cells and ^{14}C-lysine-HCl, 189 mC/mM, for the labelling of the *in vitro* cell-free system. The three latter compounds were obtained from the Radiochemical Centre, Amersham.

* For the presentation of the HeLa cell culture, our thanks are due to Dr. Éva Csonka.

'Hexitol derivatives' were kindly put at our disposal by "Chinoin" Pharmaceutical Factory, Budapest.

RESULTS

Incorporation of ^{14}C-*formate into the DNA of bone marrow cells.* At first the effect of the compounds exerted on the DNA synthesis in rabbit bone marrow cells was studied *in vitro*. An appreciable period of inhibition could be observed as early as after 2·5 hr of incubation.

TABLE 1. INHIBITION OF ^{14}C-FORMATE INCORPORATION INTO THE DNA OF RABBIT BONE MARROW CELLS *in vitro*, PRODUCED BY 2·5 HR INCUBATION WITH THE COMPOUNDS

Compound	μg/ml	Spec. act cpm/mg/DNA	Inhibition %
—	—	3·480	—
DBD	100	2·270	35
DBD	200	2·070	40
DBM	500	2·170	37
DBM	1000	1·810	48
MM	500	2·740	21

^{14}C-sodium formate, 1 μC/ml (spec. act. 1·54 mC/mM) was added to the cells for the last 90 min of incubation.

As it appears from Table 1, all the three compounds decreased ^{14}C-formate incorporation into the DNA. The higher the concentration, the greater inhibition was found. A high concentration of MM induced slightest inhibition.

The inhibitory effect on the DNA synthesis became even more apparent when the cell suspension was incubated with the compounds for longer times.

As it is evident from Fig. 1, inhibition increased by 2- to 4-fold as compared to the

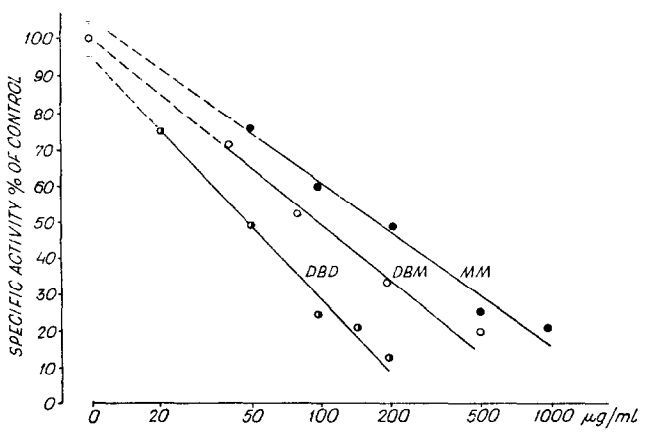


FIG. 1. Concentration dependent inhibitory effect of the compounds on the ^{14}C-formate incorporation into the DNA of rabbit bone marrow cells *in vitro*. Cells were incubated with the compounds for 7 hr. ^{14}C-sodium formate, 1 μC/ml (sp. act. 1·54 mC/mM) was added for the last 90 min of incubation.

values obtained at 2·5 hr. In the experiments with longer incubation period, the differences between the efficiencies of the compounds to decrease the DNA synthesis became more apparent.

In a given concentration of the compound, DNA synthesis was the most inhibited by DBD, the least by MM, while the inhibition degree induced by DBM fell between the two former. For instance, a 2- to 2·5 times higher concentration of the less effective compound was required to produce 50 per cent inhibition.

The effect of the compounds exerted on DNA synthesis in the bone marrow cells of rats injected with Shay myeloid leukemic cells i.v., was also studied.

TABLE 2. INHIBITION OF ^{14}C-FORMATE INCORPORATION INTO THE DNA OF BONE MARROW CELLS FROM RATS WITH SHAY LEUKEMIA *in vitro*, PRODUCED BY 6 HR INCUBATION WITH THE COMPOUNDS

Compound	μg/ml	Spec. act. cpm/mg DNA	Inhibition %
—	—	4·560	—
DBD	40	2·300	51
DBD	80	1·590	65
DBD	200	490	89
DBM	100	3·460	24
DBM	250	1·090	76
MM	200	2·400	47
MM	1000	920	80

^{14}C-sodium formate, 1·0 μC/ml (sp. act. 1·54 mC/mM) was added to the cells for the last 90 min of incubation.

As it appears from Table 2, the inhibitory effect exerted on the DNA synthesis was practically the same as for normal rabbit bone marrow cells. The higher the concentration, the stronger the inhibition. The efficiency of the compounds in comparison to each other was also unchanged.

Incorporation of precursors into RNA *and proteins of the cells.* Rabbit bone marrow cells and HeLa cells were chosen as experimental objects. On the basis of the results of the former bone marrow experiments, two concentrations were chosen which produced about 50 per cent and 75 per cent inhibition in DNA synthesis by the end of 6 hr incubation. As shown in Fig. 2 all the three compounds inhibited both the RNA- and protein syntheses in the cells. For comparison, the inhibition of DNA synthesis measured in this experiment is also indicated. Higher doses produced higher inhibition in the synthesis of all the three biopolymers. Both the order and the degree of inhibition produced by DBD and DBM on any of the three biopolymers were similar: DNA synthesis was inhibited the most, protein synthesis the least, while RNA synthesis inhibition fell between these two biopolymers. MM acted somewhat different as it strongly reduced protein metabolic activity, while incorporation into RNA failed to drop to the same extent as in the case of the two other compounds.

In HeLa cells, with the concentrations applied for rabbit bone narrow cells, no inhibition was found in the metabolic activity of DNA-, RNA or proteins after incubation for 6 hr. The effect, however, developed upon 20 hr incubation. As shown on

Fig. 3 all the three compounds decreased the metabolic activity of DNA-, RNA- and protein synthesis of HeLa cells. Compared to results obtained with rabbit bone marrow cells, some differences could still be observed. In HeLa cells, not the DNA but the RNA-synthesis was the most strongly inhibited. This was even more apparent at low concentrations, enabling a better differentiation of inhibition rates. At high concentrations, DNA synthesis was also strongly inhibited, at least to the same extent as

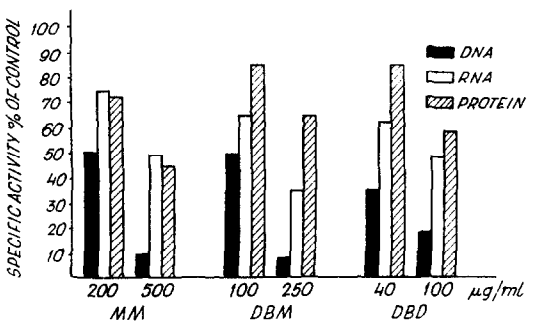


FIG. 2. Inhibition of precursor incorporation into the DNA-, RNA and proteins of bone marrow cells, produced by the compounds *in vitro*. Cells were incubated with the compounds for 6 hr and labelled precursors added for the last 90 min of incubation. ^{14}C-sodium formate, 1·0 µC/ml (sp. act. 13·26 mC/mM), ^{14}C-uridine, 0·12 µC/ml and ^{14}C-lysine, 0·81 µC/ml.

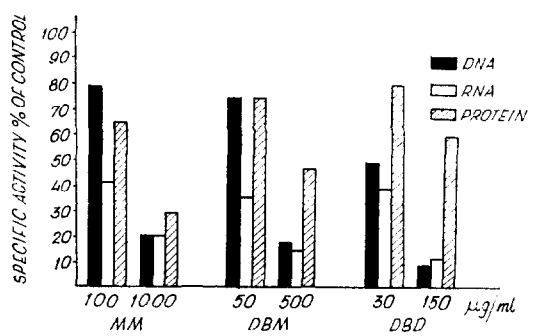


FIG. 3. Inhibition of precursor incorporation into the DNA-, RNA and proteins of HeLa cells, produced by the compounds *in vitro*. Cells were incubated for 20 hr with the compounds and labelled precursors added for the last 90 min of incubation. ^{14}C-sodium formate, 0·18 µC/ml (sp. act. 13·26 mC/mM), ^{14}C-uridine, 0·016 µC/ml and ^{14}C-lysine, 0·025 µC/ml.

RNA synthesis. All the three compounds inhibited the protein synthesis the least in the biopolymers studied. Protein synthesis in HeLa cells, however, was relatively more sensitive to the two mannitol derivatives than to the dulcitol one.

^{14}C-amino acid incorporation into cell-free systems. According to the above results, all the three compounds decreased protein metabolic activity in cells in both experimental objects. This has induced us to reveal whether these compounds act directly or indirectly on the protein synthesizing system.

In these experiments with ribosomes the compound was added to the cell-free system.

As suggested by the data in Table 3 none of the compounds induced any appreciable change in ^{14}C-lysine incorporation.

It should be noted that 2-mercapto-ethanol was one of the components of the *in vitro* amino acid incorporating system. Alkylating agents might be inactivated by mercapto-ethanol. Consequently, the required hexitol concentration would be reduced. Therefore, the experiments were performed also without mercapto-ethanol. Thus, it could

TABLE 3. THE EFFECT OF THE COMPOUNDS ON THE ^{14}C-LYSINE INCORPORATING ABILITY OF THE RIBOSOMAL SYSTEM OF GUINEA-PIGS*

Compound	μg/ml	Sp. act. cpm/mg protein
—	—	1·625
DBD	50	1·610
DBD	100	1·610
DBM	50	1·630
DBM	200	1·850
MM	50	1·600
MM	200	1·640

* For further details see Materials and Methods.

be established that the presence or absence of mercapto-ethanol did not influence the amino acid incorporation *in vitro*. Accordingly, the experimental results given in Table 3 could not be influence by the mercapto-ethanol component.

The conclusion might be drawn that the drop observed in the protein metabolic activity of bone marrow and HeLa cells is the result of some secondary effect, since the compounds failed to act on the protein synthesizing system itself.

DISCUSSION

Alkylating agents acting on the malignant diseases of the blood-forming system were classified into two categories by Elson: lymphotrop drugs acting mainly on the lymphoid system and myelotrop drugs acting mainly on granulopoiesis.[20] The three compounds we studied agreed in their main activity, i.e. all of them are myelotoxic and as such, closely related to the myleran group. Moreover, clinical indications for DBD are about the same as for myleran.[14–16]

According to our experiments, it may be established that all the three compounds inhibited synthesis of the biopolymers DNA, RNA and protein in the bone marrow and HeLa cells. Still, the time course of inhibition displays some differences. The compound acted the most rapidly and most intensively on *in vitro* surviving bone marrow cells. The DNA synthesis was inhibited both in normal and Shay leukemic bone marrow cells to the same extent. As for HeLa cells, a longer, about 20 hr, incubation was required to reach the same inhibition degree as established in bone marrow cells after incubation for 6–7 hr only. Hence, bone marrow cells proved to be more sensitive. The conclusion might be drawn that 'different' incubation times are required for the development of the same inhibition degree in the two different experimental

objects. Furthermore, it might be established that none of the compounds 'acted immediately, only after a certain lapse of time. These facts require further investigations in two different directions. It should be revealed whether the factor responsible for their synthesis inhibition developing during the respective incubation times represents the arrest of a certain phase of the cell cycle and if so, of which phase. Next, since some time is required to inhibit synthesis of biopolymers, it might be assumed that the compounds do not act on the nucleic acids directly but probably on some process involved in nucleic acid synthesis. These two problems will be studied.

The metabolic activity of DNA synthesis does not decrease only as a function of incubation time but depends also on the concentration of the compounds. This appears above all from the experiments with bone marrow cells (Fig. 1.). At the same time, there are differences in the inhibition of DNA synthesis by these compounds. Provided the concentration is identical, the highest inhibition is produced by DBD, less by DBM and the least by MM.

RNA metabolic activity is also appreciably affected by these compounds. Since, however, RNA's of various functions occur in a cell and our results relate to the bulk of cellular RNAs, further investigations are carried on to elucidate which type of RNA is the most affected.*

Though the compounds inhibited also protein synthesis in the cells, experiments with a cell-free system exclude the possibility that the steps of protein synthesis should be their point of attack. The compounds were added to the protein synthesizing ribosomal system in concentrations representing the manifold of the effective concentration of compounds known to inhibit protein synthesis, e.g. chloramphenicol, *p*-fluorophenylalanine, etc.; they failed to induce inhibition. Accordingly, the compounds do not seem to have altered any essential step of protein synthesis, as activation of amino acids, transfer of amino acids to ribosomes or coupling of amino acids. The drop observed in protein synthesis is due to some indirect effect.

Nyhan and Lampert obtained similar results. They observed that a concentration by two orders higher of phenylalanine mustard was required to produce inhibition *in vitro* than that producing inhibition *in vivo*.[29] They emphasized that this suggests the possibility that metabolic alterations in the agent are involved in its action *in vivo*.[29]

For a better approach to the action mechanism of the compounds, their structures should be studied more closely. All the three compounds are alpha, omega-substituted hexitols. When, in course of alkylation, they lose their functional groups (simultaneous to the release of a bromide or methane sulphonate anion), the hexitol skeleton is coupled to the attacked nucleophil centre. Accordingly, DBM and MM introduce 'the same' mannitol radical into the attacked compound and the structure of the radical introduced by DBD differs only in the configuration of two secondary hydroxyl groups. Accordingly, the biological effect of the three compounds should be, of necessity, similar since all the three of them are myelotoxic. However, both the quality of the functional groups and the configuration of the hexitol skeleton do influence the action exerted on cellular metabolism. The DNA synthesis, for instance,

* Labelled RNA, extracted from the treated cells and fractionated by sucrose density gradient centrifugation proved that the labelling of the various RNA fractions was inhibited to different extents. A paper by the authors is in preparation in this series.

is the most inhibited by DBD. However, if bromine is coupled to mannitol, instead of dulcitol, the effect exerted on DNA is reduced. If the bromine on the mannitol skeleton is substituted by a mesyloxy group, inhibition exerted on DNA synthesis is reduced even further.

TABLE 4. CORRELATION BETWEEN CYTOSTATIC ACTIVITY AND CHEMICAL STRUCTURE IN SOME ALPHA–OMEGA DIMESYLOXY AND DIBROMO DERIVATIVES OF HEXITOLS AND ALKANES*

Basic skeleton	Functional group	
	bromide	mesyloxy
Alkane (C = 4 and 6)	—	+ (mylerans)
D-mannitol	+ (DBM)	+ (MM)
Dulcitol	+ (DBD)	—

* + = cytostatically active compound.
— = cytostatically inactive compound.

According to Table 4, it becomes even more apparent that certain structural or configurational changes in the skeleton of the molecule carrying the dibromo- and dimesyl groups might result in the full discontinuance of the cytostatic effect. Thus, e.g. dimesyloxy-alkanes (mylerans and their homologues) are effective cytostatic compounds but dibromo-alkanes of the same alkylating capacity are ineffective cytostatically. According to Timmis and Brown[30] and Vargha *et al.*[31], out of the dimesyl-hexitols, dimesyl-mannitol (MM) alone is cytostatic. Consequently, the configuration of secondary hydroxyl groups is decisive for the cytostatic effect. In the series of dibromo compounds, the configuration change of the hydroxyl groups does not result in the discontinuance of the cytostatic effect since the dulcitol derivative (DBD) is active. As to biological effect, the bromine group is no homologue of the mesyl group. Should it be, the dimesyl-dulcitols and dibromo-alkanes would also be effective cytostatically.

Considering Table 4, it is evident that the way of action of alpha, omega-substituted hexitols cannot be explained by the alkylation reaction 'alone'. The following facts support this statement. The *in vitro* measured alkylation capacity of the three compounds is 'appreciably lower' than that of the nitrogen mustard. This fact alone suggests that alkylation plays a minor and/or secondary role in the effect of these compounds. Studies on the biological transport of DBM and DBD also confirm that the transfer of the alkyl group alone is not exclusively decisive for their effect.[11, 32, 33] The bond between carbon and bromine is more stable in these compounds than the bond of functional groups in the known alkylating agents. The half time of degradation of dibromo-hexitols in the organism is relatively long and among their metabolites there are a fair number of derivatives with bromine in covalent bond.[32–35]

Since these facts and further motives discussed more in detail elsewhere[33, 36] suggest that alkylation is of a relatively minor importance in the effect of these

compounds, reactions of different types potentially responsible for the effect have to be reckoned with.

Alkylating agents generally used in tumour chemotherapy (nitrogen mustards, ethylene-imines, epoxides, mylerans and dibromo hexitol derivatives) do not seem to act in the organism merely by introducing the respective alkyl group into sites important for and sensitive to the biological processes. Namely, in course of alkylation, hydrogen or hydroxyl ions are always 'released'. According to a hypothesis elaborated by two of the authors (I.P.H. and L.I.) it is the nature of the 'reaction accompanying alkylation' that determines the myelotropic or lymphotropic character of a compound.[32, 35] Nitrogen mustards attach a basic side chain to the nucleophil centre. A strong acid might be released owing to the reaction but it is compensated by the basic group. Base is formed simultaneously to the reactions of the ethylene-imines and epoxides. These lymphotoxic compounds are characterized either by the basic group or by the release of basic groups in course of their reaction. The methane sulphonic esters of alkane diols and polyols are belonging to the myleran type. Moreover, the dibromo-hexitols introduce an alkyl- or polyol chain to the point of attack. A strong acid is released owing to the reactions that is not compensated by a basic group as e.g. in case of attack. A strong acid is released owing to the reactions that is not compensated by a basic group as e.g. in case of nitrogen mustards. In view of the low-alkylating reactivity of the myelotropic hexitol derivatives, the effects of an intracellular acidification might play an important part in their cytostatic action.

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