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# INTERACTION OF CYTOTOXIC DIBROMOHEXITOLS WITH ALBUMIN

ETEL INSTITORIS and L. HOLCZINGER

Department of Histo- and Biochemistry,
Research Institute of Oncopathology, Budapest, Hungary

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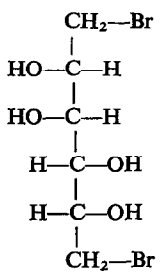
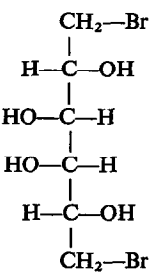
**Abstract**—The binding of [$^{82}$Br]- and [$^{14}$C]-labelled cytotoxic dibromohexitols in bovine serum albumin *in vitro* and to mouse serum proteins *in vivo* was studied by paper electrophoresis. An interaction of dibromohexitols with albumin could be determined. It was found that only molecules containing the $CH_2$—Br molecular segment are capable of interaction with albumin. On the other hand, the binding to albumin seemed to stabilize the C—Br bonds of molecules which, when unbound, have a tendency to lose bromide ion. The rate of bromide loss of DBD under *in vitro* conditions exceeded considerably that of the DBM indicating that DBM is more stable than DBD. Of the serum proteins of treated animals detectable drug adsorption could only be seen in the albumin fraction. The results indicate that serum albumin has a carrier role in the transportation of some part of dibromohexitols introduced into the organism.

THE SYNTHESIS of cytotoxic dibromohexitols afforded an excellent opportunity of study more closely the correlation between chemical structure and biological effect.

DBM and DBD are diastereoisomeric compounds, differing only in the configuration of two secondary hydroxyl groups.



1,6-dibromo-1,6-dideoxy-D-mannitol
(DBM)



1,6-dibromo-1,6-dideoxy-dulticol
(DBD)

The physicochemical properties of the two drugs (e.g. solubility, hydrolysis rate etc.) as well as their biological effect (e.g. dose, cytotoxic effect, selective effect on various tumors, inhibition of DNA synthesis), however, seem to be rather different.[1–6]

On the basis of their chemical structure the dibromohexitols are considered as biological alkylating agents exerting their effect on nucleophilic groups (NH—, SH—, $PO_4$—) of biological systems.

Concerning the mechanism of alkylation *in vivo*, Institoris *et al.*[7,8] suggests that dibromohexitols attack the nucleophil centre directly through their $CH_2$—Br molecular segment capable of electrophilic substitution, while according to Elson *et al.*[9] the

action of alpha-omega-disubstituted hexitols is mediated by diepoxides, which are formed in alkaline solutions by HBr elimination. In both cases the hexitol skeleton is coupled to the attacked nucleophil centre of some biological macromolecule.

A number of experimental data seem to prove, however that beside alkylation the biological transport properties of the substances also play an important role in the biological activity of dibromohexitols[7,10] since the structural properties of the molecule of alkylating agents determining their biotransport—such as the polarity of the molecule its ability for hydrogen-bond formation—could definitively determine the site of biological alkylation i.e. its critical site of action.

The present investigations were performed to compare the stability of the functional groups of DBM and DBD *in vivo* and *in vitro* and to reveal the role of serum proteins in the transportation of these compounds.

## MATERIALS AND METHODS

1,6-dibromo-1,6-dideoxy-1-[$^{14}$C]-D-mannitol (0·2 mc/mg) and 1,6-dibromo-1,6-dideoxy-1-[$^{14}$C]-dulcitol (0·3 mc/mg) were supplied by Chinoin Pharmaceutical and Chemical Works.

### *Preparation of* [$^{82}$*Br*]*-labelled dibromohexitols*

Both 1,6-dibromohexitols were prepared under identical conditions from the corresponding 1,2-5,6-dianhydrohexitols according to the following methods:

0·35 g 1,2-5,6-dianhydrodulcitol[11] was dissolved in 2 ml water and added slowly to 0·8 ml of 50% H Br solution which included 2 mg of 5 mc of [$^{82}$Br] containing NaBr (sp. act. 3·2 mc/mg Br). Following 1 hr stirring at room temperature it was cooled to 0°, whereafter the separated crystals were filtered and eluated bromide free with distilled water.

Yield: 0·6 g 1·6-dibromo-1,6-[$^{82}$Br]-dulcitol.

It was recrystallized in alcohol.

Specific activity: 1·6 mc/mg DBD at the moment of usage. Its radiochemical purity was controlled by thin-layer chromatography in butanol–acetic acid–water (80:20:20, by vol.) mixture, on Kieselgur-G Kieselgel-G (60:40) layer, which was obtained from Merck Co., Darmstadt, Germany. One radioactive spot could be found, $R_f$:0·76. 1,6-dibromo-1,6-[$^{82}$Br]-1,6-dideoxy-D-mannitol was prepared by the same method as described above from 1,2-5,6-dianhydro-D-mannitol.[12] Specific activity: 1·4 mc/mg $R_f$: 0·75.

### In vitro *experiments*

Four mg bovine serum albumin (BSA: Bovine Albumin powder, fract. V. Armour Pharmaceutical Co.) was incubated with 80 m$\mu$ [$^{14}$C]-DBM, [$^{14}$C]-DBD, [$^{82}$Br]-DBM and [$^{82}$Br]-DBD respectively, in 0·8 ml phosphate buffer (M/15) at pH 7·4 for 1 hr at 37°. As control 4 mg of BSA was incubated with 50 $\mu$g [$^{82}$Br]-NaBr under similar conditions.

Aliquots (0·1 ml) of each material were submitted to paper electrophoresis in phosphate buffer (M/30) at pH 7·4 on Schleicher–Schull No. 2043b paper for 3·5 hr. The current applied was 30 mA. The distance between the two electrodes was 10 cm.

In parallel with these procedures the labelled dibromohexitols were dissolved in

phosphate buffer at pH 7·4 and kept in a 37° thermostat for 1 hr and were submitted also to paper electrophoresis to determine the "characteristic site" of compounds.

The papers were cut into strips of 0·5 cm width and eluted with 0·1 N NaOH. The protein content of the eluates was measured by the method of Lowry *et al.*[13] The distribution of [$^{82}$Br] an [$^{14}$C] radioactivity was also determined.

In vivo *experiments*

Male Swiss mice, 22–25 g, were used throughout this work. Twenty animals were divided into four groups of five animals each. A single dose of the labelled compounds was given i.p. to each animal as follows:

| | | |
|---|---|---|
| group 1. | [$^{82}$Br]-DBM | 850 mg/kg |
| group 2. | [$^{82}$Br]-DBD | 850 mg/kg |
| group 3. | [$^{14}$C]-DBM | 210 mg/kg |
| group 4. | [$^{14}$C]-DBD | 210 mg/kg |

The animals were killed 1 hr after the drug administration. The blood was centrifuged at 170 *g* for 15 min at 4°. The serum of each animal was separately submitted to paper electrophoresis as described above.

*Measurement of* [$^{14}$*C*] *radioactivity*

The papers cut into strips of 1 cm (about 50 mg) were burnt to [$^{14}$C]-$CO_2$ in a platinum vessel in Hereus combustion furnace according to Körbl microanalytical method. The [$^{14}$C]-$CO_2$ was absorbed in 1 ml or 2-aminoethanol and its radioactivity was measured by liquid scintillation. Efficiency: 50–60 per cent.[7,14] The measurement was performed with NZ-137 beta radiation counter and NP-241/B single channel amplitude analyser (Gamma Optical Works).

*Measurement of* [$^{82}$*Br*] *radioactivity*

Nk-108 energy selective counter (Gamma Optical Works), SE-2 measuring heads (Electrim NAI/Th Well crystal/Tesla) were used for measuring the radioactivity of the stripes of electrophoretograms.

Efficiency of measurement: 30 per cent.

## RESULTS

In vitro *experiments*

On the electrophoretograms of [$^{14}$C]- and [$^{82}$Br]-labelled dibromohexitols kept in phosphate buffer, a single radioactive peak was found 2–3 cm from the start, representing the "characteristic site" of both drugs. The site of untreated BSA was also detected and was found 7–8 cm from the starting line.

On the electrophoretograms of BSA incubated with labelled DBM two radioactive spots could be detected. One of them corresponded to the "characteristic site" of DBM, the other one coincided with the site of BSA.

Figure 1a shows the amount of free and albumin-bound DBM calculated on the basis of [$^{82}$Br] and [$^{14}$C] radioactivity, respectively. On the "characteristic site" of the drug 6 $\mu$g of [$^{82}$Br]-DBM could be detected which was only about two-thirds of

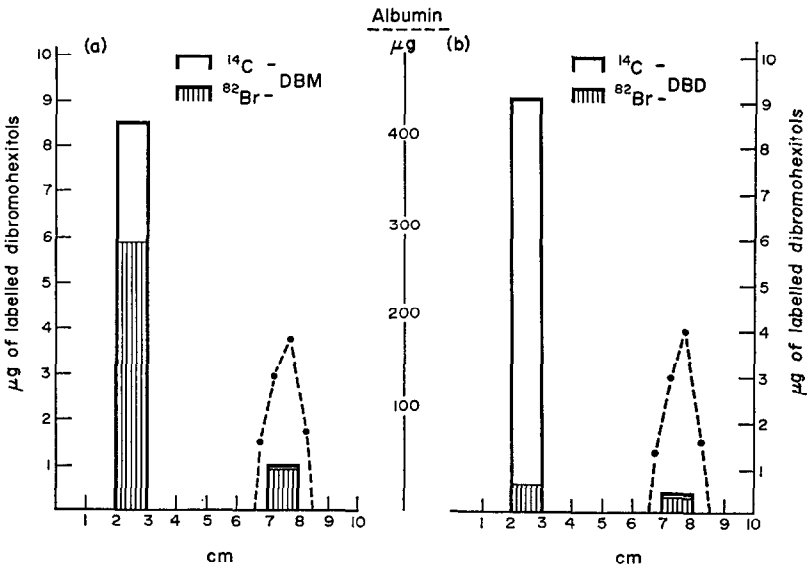



FIG. 1. (a) Paper electrophoresis of 500 μg of BSA incubated with 10 μg of [$^{82}$Br]-DBM and 10 μg of [$^{14}$C]-DBM respectively in phosphate buffer at pH 7·4. (b) Paper electrophoresis of 500 μg BSA incubated with 10 μg of [$^{82}$Br]-DBD and 10 μg of [$^{14}$C]-DBD respectively in phosphate buffer at pH 7·4.

the amount of [$^{14}$C]-DBM (8·5 μg) indicating that during the incubation bromide ions were split off in about one third of the unbound molecules.

By contrast, the calculated amount of [$^{82}$Br]-DBM migrated with the BSA was almost equal to that of the [$^{14}$C]-DBM. This suggests that only molecules containing the intact $CH_2$—Br groups can bind to albumin and the binding to albumin seems to stabilize the reactive groups.

The same phenomenon could be observed in the case of DBD (see Fig. 1b) with the exception that the bromide loss was even more striking and consequently the estimated amount of unbound [$^{82}$Br]-DBD was only one-twentieth that of the [$^{14}$C]-DBD.

Using the same drug/BSA concentration the amount of bound DBM always exceeded that of the DBD.

There was no detectable radioactivity on the electrophoretograms of BSA incubated with [$^{82}$Br]-NaBr indicating that the radioactivity found on the albumin incubated with [$^{82}$Br]-labelled drugs was not due to the adsorption of [$^{82}$Br] ion split off during the incubation.

In vivo *experiments*

The results of electrophoresis carried out on serum of mice treated i.p. with [$^{14}$C] and [$^{82}$Br] labelled dibromohexitols respectively, are shown in Fig. 2. The radioactivity values detected at different sites of the electrophoretograms are expressed in the percentage of overall radioactivity of the entire electrophoretogram. The protein fractions of blood serum are illustrated in Fig. 2c. The diagram of Fig. 2a illustrates the electrophoretograms of sera taken 1 hr after the injection of 210 mg/kg of

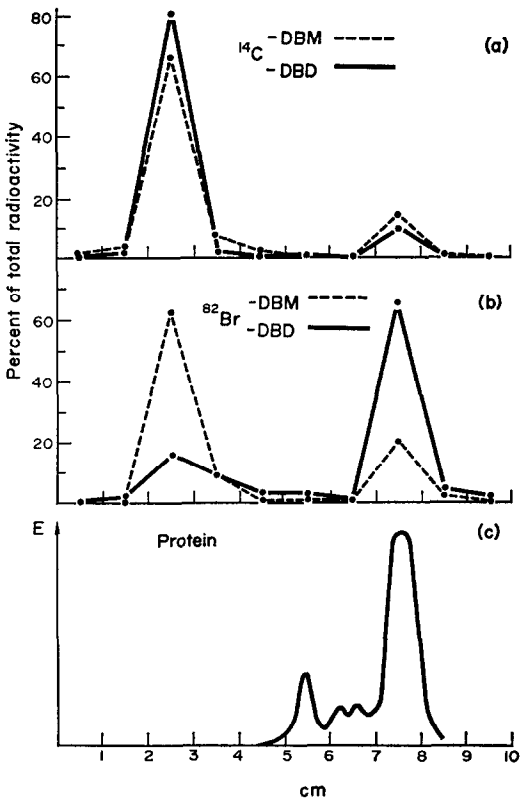



FIG. 2. (a) Paper electrophoresis of mouse serum treated with [$^{14}$C]-DBM and [$^{14}$C]-DBD respectively performed 1 hr after i.p. administration. (b) Paper electrophoresis of mouse serum treated with [$^{82}$Br]-DBM and [$^{82}$Br]-DBD respectively performed 1 hr after i.p. administration. (c) Paper electrophoresis of mouse serum.

[$^{14}$C]-labelled drugs. Similarly to the *in vitro* experiments two radioactive peaks might be detected. Seventy to eighty per cent of the total [$^{14}$C] radioactivity was detected on the "characteristic site" of the free dibromohexitols, whereas 10–14 per cent was found bound to the albumin fraction. The radioactivity measured in the other serum protein fractions was minimal. The electrophoretic patterns of [$^{14}$C]-labelled DBM and DBD are congruent.

Figure 2b shows the electrophoretograms of sera taken 1 hr after i.p. administration of 850 mg/kg [$^{82}$Br]-DBM and [$^{82}$Br]-DBD respectively.

The percentual distribution of DBM-radiobromide is very similar to that of the DBM-radiocarbon (about 15–20 per cent of total activity is bound to albumin and the remaining activity is derived from the free DBM). There is no indication of an *in vivo* split off of [$^{82}$Br] from the DBM molecule bound to albumin and the bromide loss of the unbound compound is also minimal.

The distribution of [$^{82}$Br]-DBD was quite different. The major part of [$^{82}$Br]-DBD (66 per cent of the total [$^{82}$Br] radioactivity) was detected on the site of the albumin fraction while about 26 per cent could be detected on the "characteristic

site". The discrepancy existing between the distribution of [$^{82}$Br] and [$^{14}$C]-DBD refers to an extensive cleavage of $CH_2$—Br bound of the free DBD while no degradation of the DBD molecules bound to albumin could be observed.

## DISCUSSION

When BSA was incubated *in vitro* with radioactive dibromohexitols and the mixtures were subsequently submitted to paper electrophoresis, part of the radioactivity was migrated together with the albumin indicating an interaction of drugs with BSA.

The [$^{82}$Br]-radioactivity of the spots can be taken as being directly proportional to the amount of intact molecules containing the $CH_2$—Br bonds, whereas the [$^{14}$C]-radioactivity represents all the molecules, including also molecules free of bromide. The comparison of [$^{14}$C] and [$^{82}$Br] radioactivity of the unbound molecules *in vitro* revealed a significant bromide loss in the case of both drugs. The rate of degradation of the DBD, however, was always more extensive than that of the DBM indicating that the $CH_2$—Br bonds of DBM are more stable.

On the other hand, the degradation of the albumin-bound molecules was negligible and in this respect there was no difference between DBM and DBD. But, using the same drug/BSA concentration the amount of bound DBM always exceeded that of the DBD.

It is reasonable to postulate that the integrity of molecules i.e. the presence of $CH_2$—Br molecular segment is a precondition of drug–albumin interaction and moreover, the drug–albumin binding enhances the stability of the $CH_2$—Br bonds.

The different rate of *in vitro* absorbtion of the two drugs to albumin may be explained by concentration differences, as the concentration of intact DBD molecules capable of binding, is decreased to a greater extent than that of the DBM, owing to the excessive bromide loss in the medium applied.

The measurements of [$^{14}$C] and [$^{82}$Br] radioactivity of sera taken 1 hr after intraperitoneal administration of the labelled compounds indicated an *in vivo* interaction of the drugs with albumin.

Comparing the percentual distribution of [$^{14}$C] -and [$^{82}$Br]-radioactivity of the electrophoretograms there was no indication of cleavage of radiobromide from the molecules bound to the albumin fraction of the sera, and both DBM and DBD bound to albumin seemed to retain their $CH_2$—Br groups even under *in vivo* conditions.

The unbound molecules, also present in the sera of treated animals did lose a certain amount of bromide ions, but the degradation of DBD molecules always exceeded that of the DBM indicating that the C—Br bounds of DBM are more stable than that of the DBD even *in vivo*.

By evaluating the electrophoretograms of serum proteins of animals treated with dibromohexitols the albumin fraction alone showed a considerably high radioactive peak, while the radioactivity detected in other serum fractions was negligible. According to these findings it may reasonably be assumed that albumin has a carrier role in the transportation of some of dibromo-hexitols introduced into the organism.

It may be also assumed that the albumin–dibromohexitol complex, following the biological pathway of albumin reaches—presumably in intact form—the biological site of action.

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