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Rat Liver-mediated Degradation of Dibromodulcitol

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Abstract—*The rate and extent of dibromodulcitol (DBD) conversion by* 9000 **g** *rat liver supernatant with an NADPH-generating system (S-9 mix) were studied using* ^{3}H*-labelled drug. Results indicated that S-9 mix seemed to exert an initial protective effect delaying the solvolysis of DBD for about* 30 min *at* 37°C *followed by rapid degradation into exclusively pharmacologically inactive products. Thus S-9 mix contained merely DBD as an effective agent; it amounted to less than* 40% *of the total radioactive compounds by* 120 min. *In the control mixtures the sovolytically produced effective drug content, i.e. the sum of DBD, 1,2-anhydro-6-bromo-6-deoxygalactitol (BrEpG), 1,2-5,6-dianhydrogalactitol (DAG) was* 63%. *Our results suggest the involvement of liver enzymes in the detoxification of DBD into inactive products. Therefore the antitumour effect of DBD cannot be attributed to its active BrEpG and DAG alone. The drug in its unchanged form may contribute to a somewhat greater extent to its cytostatic action than was believed before.*

INTRODUCTION

DIBROMODULCITOL (NSC 104800; DBD; 1,6-dibromo-1,6-dideoxygalactitol) is an alkylating agent which has been used clinically in the treatment of different types of malignancies [1]. During hydrolysis DBD is converted into epoxides and other anhydroderivatives [2] (Fig. 1). In boiling in ion-free water the main product of hydrolysis is 3,6-anhydro-1-bromo-1-deoxygalactitol (BrAG) [3]. Mild alkalinity enhances the formation of the three-membered epoxide ring and results in the production of 1,2-anhydro-6-bromo-6-deoxygalactitol (BrEpG) [4], 1,2-5,6-dianhydrogalctitol (DAG) [5] and 1,2-3,6-dianhydrogalactitol (AEpG) [3]. BrEpG and DAG are more powerful alkylating agents than DBD. Calculated on molecular basis, BrEpG proved to be about 20 times and DAG 30 times more active growth-inhibitory agents with the Walker rat tumour than DBD [4]. The major antitumour activity of the drug has been thought to depend on its conversion into epoxides BrEpG and DAG [4, 5]. The two monofunctional alkylating compounds BrAG and AEpG have no cytostatic activity [6].

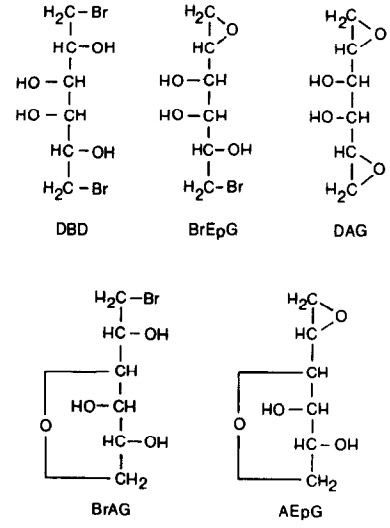

Fig. 1. DBD and its main solvolytic products. Pharmacologically effective compounds: DBD, BrEpG, DAG. Inactive compounds: BrAG, AEpG.

Studies on the transformation of DBD *in vivo* demonstrated the presence of the above solvolytic products in biological fluids. Moreover, six other metabolites were found in humans [7, 8] and two in rats *in vivo* [9].

A previous report demonstrated that DBD exerts a significantly lower toxicity and anti-

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tumour activity in both rats and mice pretreated with phenobarbital [10]. This finding suggests the possibility that microsomal drug metabolizing enzymes may be involved in the biotransformation of DBD, and may influence its antitumour and toxic character.

In the present study the rate and extent of DBD conversion by S-9 mix of rat liver homogenate using ^{3}H-labelled drug is discussed.

MATERIALS AND METHODS

Preparation of [^{3}H]DBD solution in 0.2 M phosphate buffer, pH 7.4

Three milligrams of [^{3}H]DBD, labelled at the C-1 position (obtained from CHINOIN Pharmaceutical Works, Ltd; sp. act. 9.98 MBq/mg, radiochemical purity 98.3%), was dissolved in 20 ml of KH_2PO_4 solution (0.454 mg/ml). It was then mixed with 30 ml of $Na_2HPO_4 \cdot 12H_2O$ solution (31.86 mg/ml) just before use. Thus the spontaneous solvolytic conversion of DBD occurring in a slightly alkaline medium could be initiated from a given point of time.

Preparation of liver S-9 mix

Male Wistar/H-Riop rats (body wt 200 g) were killed and the livers were immediately removed and homogenized in ice cold 0.15 M KCl solution (1:3 w/v) with a Teflon glass homogenizer. The homogenate was centrifuged at 9000 *g* (Beckman Model L ultracentrifuge) for 10 min. The supernatant (S-9 fraction) was then diluted with 0.05 M phosphate buffer, pH 7.4 (1:3 v/v), according to Ames *et al.* [11], then split into 10.0-ml portions. In order to prepare the whole incubation mixture, i.e. the S-9 mix, 1.0 ml of liver S-9 fraction was mixed with the following solutions: 0.2 ml of 0.4 M $MgCl_2$, 0.2 ml of 1.65 M KCl, 0.05 ml of 1 M glucose-6-phosphate, 0.4 ml of 0.1 M $NADP^+$ and 8.15 ml of freshly prepared [^{3}H]DBD solution in phosphate buffer, pH 7.4. The final DBD concentration in 10 ml of S-9 mix portion was 48.9 μg/ml (0.16 mM), which is in the range of the therapeutic level measured in transplantable solid rat tumours [6]. Protein concentration was kept fairly constant at 1.85 ± 0.17 mg/ml [12].

Control mixtures

The following mixtures served as controls: (i) S-9 fraction and the cofactors without $NADP^+$; (ii) $NADP^+$ and cofactors without S-9 fraction; (iii) [^{3}H]DBD in 0.2 M phosphate buffer, pH 7.4.

Estimation of radioactive transformation products

The S-9 mix and the controls were incubated at 37°C for 120 min. The pH values were periodically checked (Universal pH Meter, Type OP-204/1, Radelkis). One-milliliter samples were taken at different time points and added to a suspension of 4 g anhydrous Na_2SO_4 in 5 ml ethyl acetate. Then the solid phase was extracted twice with 2-ml portions of anhydrous ethyl acetate. Each combined extract was evaporated to dryness under an N_2 stream at room temperature. The residue was dissolved in methanol (200 μl), and 100 μl of this solution was applied to a chromatograph. Paper chromatography was carried out on Whatman No. 1 paper and the chromatograms were developed upwards for approximately 30 cm with a solvent system of *n*-butanol–water (86:14). DBD and its transformation products were partly located by cochromatography of the authentic materials synthesized by the method of Vidra *et al.* [3] and Horváth *et al.* [4]. Alkylating substances were detected by the 4-(4-nitrobenzyl)pyridine (NBP) reaction according to Epstein *et al.* [13]. For quantitative determinations the chromatograms were cut into 0.5-cm sections and counted for radioactivity (Beckman LS-9800 using digital integration program) in Aquasol® (New England Nuclear) scintillation cocktail.

RESULTS

DBD and its transformation products

Paper chromatography of the [^{3}H]DBD solution in phosphate buffer, pH 7.4 (Fig. 2A), indicated the presence of the solvolytic products BrAG (R_f 0.72), BrEpG (R_f 0.65), AEpG (R_f 0.55) and DAG (R_f 0.43) simultaneously with the unchanged DBD (R_f 0.80) after 30 min incubation. In the liver S-9 mix, i.e. in the whole incubation mixture, however, no BrEpG and DAG were found; there were only DBD, BrAG and minor quantity of AEpG (Fig. 2B). In addition, a small amount of unidentified material was also detected at R_f 0–0.01. It showed no alkylating ability with the NBP reagent. This material was absent in the buffer solution of [^{3}H]DBD, but it was present in the two other control mixtures.

During incubation up to 120 min, only a negligible decrease of pH from the initial 7.4 to 7.25–7.30 was observed in the various mixtures. Determination of radioactivity in the incubation samples and in the ethyl acetate extracts showed a 83 ± 1.3% recovery of ^{3}H-compounds in the analytical process.

Time course studies

The time course of the transformation of DBD by rat liver S-9 mix and by the three controls can be seen on Fig. 3. In each control mixture the proportion of unchanged DBD declined exponentially, while the amount of the pharmacologically more effective BrEpG and DAG

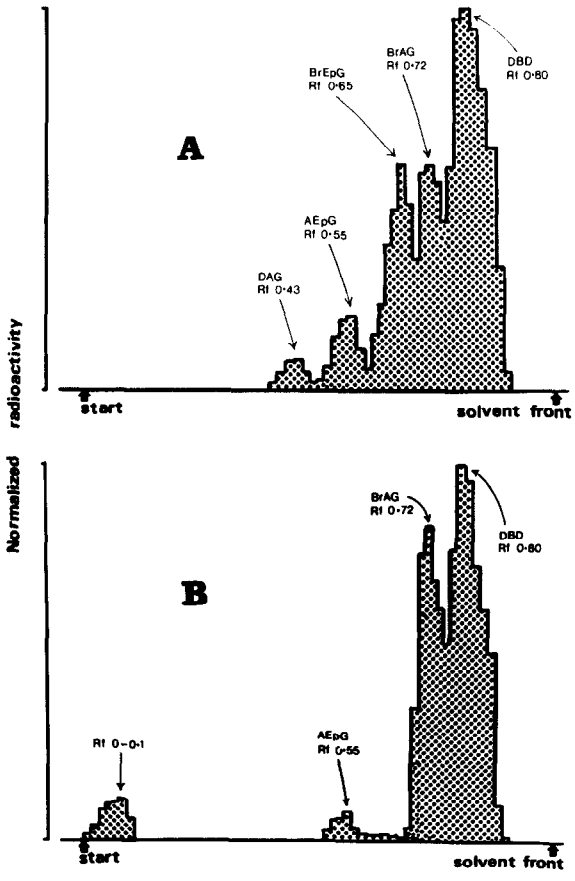

Fig. 2. Distribution of DBD and its transformation products on radiochromatograms of [^{3}H]*DBD in phosphate buffer, pH* 7.4 (*A*) *and in the S-9 mix of rat liver* (*B*), *taken after incubation for* 60 min. *Ascending chromatography on Whatman No.* 1 *paper; solvent system:* n-*butanol-water* (86:14).

increased gradually. The liver S-9 mix seemed to exert an initial protective effect, delaying the solvolysis of DBD for about 30 min. Then the degradation of DBD went on faster than in the controls and resulted only in pharmacologically inactive products, such as BrAG, AEpG and a material with an R_f value of 0–0.1. The main product was BrAG, whose proportion amounted to 50% in the S-9 mix by 120 min. At the same time it reached only 25–30% in the controls.

Without $NADP^+$, the S-9 fraction did not hinder the solvolytic activation of DBD into BrEpG and DAG. Without liver fraction, $NADP^+$ restricted the amount of BrEpG to 15% by 120 min and prevented completely the formation of DAG. The material with an R_f of 0–0.1 seemed to be formed at the expense of AEpG and/or DAG.

The change of the effective drug content, i.e. the sum of DBD, BrEpG and DAG, followed the same time course in the three controls (Fig. 4). Even after 120 min of incubation it amounted to 63% of the total radioactivity, on average. On the other hand, the S-9 mix contained merely DBD as an effective agent, and this was less than 40%.

DISCUSSION

In our present studies the S-9 mix of rat liver proved to modulate the formation of solvolytic products from DBD. It seemed to act protectively against the degradation of DBD during an initial period followed by rapid production of the pharmacologically inactive BrAG. Although the

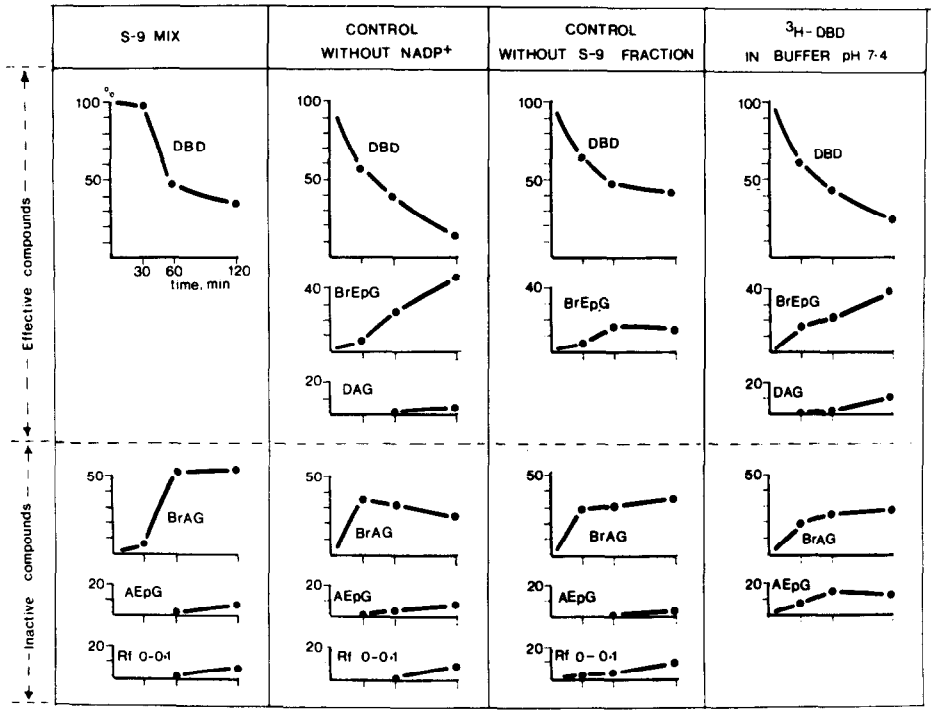

Fig. 3. Time curves for the amount of [^{3}H]*DBD and its transformation products during* 120 min *incubation at* 37°C *in rat liver S-9 mix and in three controls. Values are expressed as a percentage of the total* ^{3}H-*content* (*ordinates*) *measured on the chromatograms. Each point represents a mean of three experiments.*

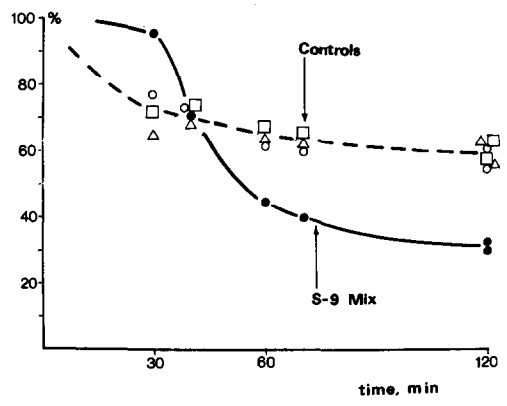

Fig. 4. Time course of the effective drug content, i.e. the sum of DBD, BrEpG, DAG, expressed as a percentage of the total radioactivity found in rat liver S-9 mix (●), and in the three controls: the S-9 fraction and cofactors without NADP$^+$ (△); NADP$^+$ and cofactors without the S-9 fraction (□); [^{3}H]DBD in 0.2 M phosphate buffer, pH 7.4 (○). Each point is the mean of three experiments.

pH remained alkaline, which would be favourable to epoxide formation, the active epoxides, such as BrEpG and DAG, did not appear in this system. NADP$^+$ and cofactors were essential to the modulating action of liver S-9 fraction. The effect was not due to a direct chemical interaction of the drug with NADP$^+$, since without S-9 fraction NADP$^+$ could not prevent completely the appearance of BrEpG, and did not enhance the formation of BrAG.

The material with an R_f of 0–0.1 detected in a minor quantity on the radiochromatograms of the S-9 mix and of the controls without NADP$^+$ and without S-9 fraction (Fig. 3), respectively might consist of galactitol (R_f 0.08) [7, 8] and/or a metabolite (R_f 0.10) found in rats *in vivo* [9].

The present findings are consistent with our previous toxicity studies on rodents pretreated with phenobarbital [10], and suggest the involvement of liver enzymes in the detoxification of DBD. The enzymes may act in two ways: (i) by diverting the solvolytic process towards the formation of the non-toxic BrAG and preventing in this manner the formation of the cytostatically active BrEpG and DAG; and (ii) by enzymatic decomposition of the solvolytically preformed epoxides.

Consequently, the antitumour effect of DBD cannot be attributed alone to the epoxides BrEpG and DAG preformed solvolytically in the plasma. The drug entering the cells, in the form of unchanged DBD, may contribute to a somewhat greater extent to its cytostatic action than was believed before.

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