Biochemical Pharmacology, 1964, Vol. 13, pp. 963–968. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF GLUCOSE PRETREATMENT ON THE ANTI-TUMOUR ACTION OF MANNITOL MYLERAN

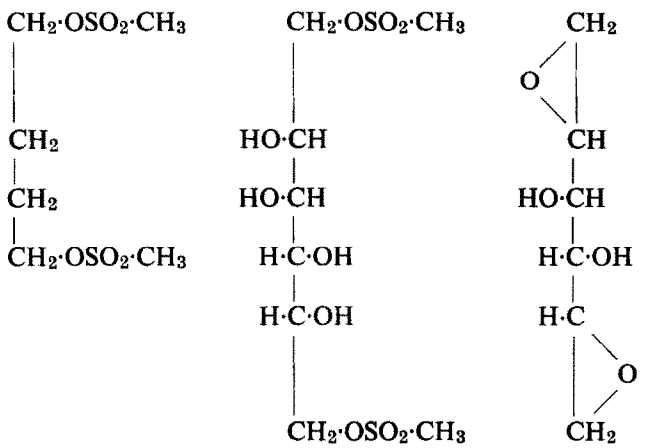
T. A. CONNORS, L. A. ELSON and C. L. LEESE

Chester Beatty Research Institute,
Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3

(*Received* 3 *March* 1964; *accepted* 24 *March* 1964)

Abstract—Up to 70% of Mannitol Myleran is excreted as unchanged drug in the urine of rats within 5 hr after i.p. administration. Glucose pretreatment greatly potentiates both lethality and anti-tumour action. This enhancement is due to an induced anuria which prevents excretion of unchanged drug. Sorbitol, and to a lesser extent, anti-diuretic hormone, cause a similar potentiation consistent with their restrictive effects on urine production.

BIS-METHANE sulphonoxy alkanes (e.g. Myleran I) may be distinguished from other cytotoxic bifunctional alkylating agents on the basis of their toxic and haematological effects in growing rats.[1] As an example of a water soluble derivative of Myleran, Timmis and Brown[2] prepared 1,6-bis mesyloxy-1,6-dideoxy-D-mannitol (Mannitol Myleran: II). This compound has been shown to possess biological characteristics of an epoxide-like nature[3] unlike those of Myleran and it is suggested that Mannitol Myleran is converted to a large extent *in vivo* to 1,2-5,6-dianhydromannitol III.



I II III

Ross and Davis[4] have shown that the conversion of Mannitol Myleran to a *bis*-epoxide occurs *in vitro* under physiological conditions. It has been suggested by Ross[5] that the selectivity of certain epoxides as anti-tumour agents may be increased by pretreating tumour bearing rats with glucose. Large doses of glucose have been shown

to lower selectively the pH of tumours.[6] A lower pH would increase the reactivity of some epoxides and enhance their cytotoxic action. Since Mannitol Myleran probably acts to a large extent through an intermediate *bis*-epoxide its anti-tumour action was investigated in rats pretreated with glucose. It was found that both the toxicity and anti-tumour action were increased approximately 8-fold, with no evidence for improvement in selectivity.[7] No other alkylating agents tested in glucose pretreated rats exhibited such a large enhancement of lethality. In most cases the potency of the drug remained unaltered or, at the most, was increased by a factor of two. The mechanism by which large doses of glucose potentiate the action of Mannitol Myleran has now been investigated.

MATERIALS AND METHODS

Female Wistar rats from an inbred colony, and 200–250 g in weight were used. They were allowed water and rat cake *ad libitum*, except when in metabolism cages when water only was given. All compounds were injected intraperitoneally in aqueous solution, except for anti-diuretic hormone which was administered s.c. Glucose treatment consisted of three i.p. doses of 5 g/kg of a 50% aqueous solution administered 1 hr before, at the time of, and 1 hr following, injection of the drug. No deaths have ever resulted from the glucose treatment alone, although at the dose level employed it was irritant at the site of injection and caused malaise and anorexia for up to 24 hr after administration. Glucose pretreatment could not be used if the drug was administered in arachis oil, since arachis oil and glucose alone caused severe peritonitis and death in 50% of the animals treated.

Estimation of Mannitol Myleran in urine and blood

Mannitol Myleran in the urine and blood of treated animals was estimated by a modification of methods previously employed for the determination of alkylating agents using γ-(4-nitrobenzyl) pyridine as the colourimetric reagent.[8] It was found advantageous in the present work to replace methyl ethyl ketone, used in previous investigations as a solvent for the chromogenic reagent, with ethylene glycol, so reducing the volatility and obviating the biphasic reaction mixture obtained when the ketone is employed. As Mannitol Myleran is a relatively inert alkylating agent a temperature of 90° was employed for the incubation with ethylene glycol as solvent. Under these conditions maximum colour development was obtained in 90 min. At optimum conditions it was found that Mannitol Myleran exhibited a linear relationship with optical density over the concentration range 0–100 μg.

Reagents

γ-(4-nitrobenzyl) pyridine (prepared by the method of Bryans and Pyman[9]), 2% w/v in ethylene glycol,

0·1 M Potassium hydrogen phthalate buffer, pH 6·0.

Triethylamine (redistilled) 50% w/v in dry acetone.

Isotonic sucrose 0·25 M.

Urine

The urine sample was diluted to give a concentration of Mannitol Myleran in the range 0–100 μg. A 1-ml aliquot of the diluted urine was mixed with 2 ml of the nitrobenzyl pyridine solution and 0·1 ml of buffer in a stoppered tube. The mixture was

heated at 90° for 90 min, ice cooled and treated with 2 ml of the triethylamine solution. After mixing, the optical density was determined immediately at 565 mμ.

Blood

A suitable volume of blood (up to 1 ml) was mixed with 3 ml of isotonic sucrose. After centrifugation 1 ml of the supernatant was mixed with 2 ml of the nitrobenzylpyridine and 0·1 ml of buffer. The mixture was incubated as described above, centrifuged, and 2 ml of the supernatant treated with 2 ml of triethylamine and the optical density measured.

Chromatographic separation of Mannitol Myleran

Mannitol Myleran was separated chromatographically on Whatman No. 1 paper using *iso*-propanol–water (4 : 1) as the mobile phase. The compound was detected by dipping the paper sheets through a 0·5% solution of γ-(4-nitrobenzyl) pyridine in acetone, and drying at 80–90° for 5 min. The dried papers were then dipped through a 0·5% solution of sodium hydroxide in ethanol and dried for 5 min at 80–90°. The position of the substance was shown by the development of a fugitive blue colour. Ten μg of Mannitol Myleran was readily detectable after development and reaction as above. The R_f and R_g values in the above solvent were 0·69 and 1·86 respectively.

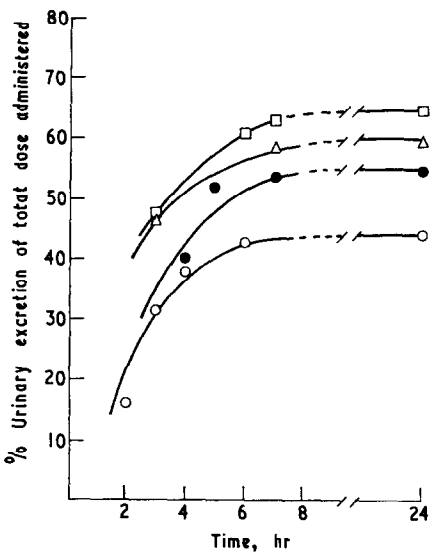


FIG. 1. Urinary excretion of Mannitol Myleran (2 g/kg, i.p.) in rats
Each curve represents an individual rat

RESULTS

The excretion of Mannitol Myleran in the urine was first studied in rats given an i.p. dose of 2 g/kg. Thirty min after the injection an alkylating agent was detectable in the urine and paper chromatography confirmed that this was unchanged Mannitol Myleran. Estimation of the drug in the urine showed that between 40% and 70% of the total dose administered was excreted within 5 hr (Fig. 1).

In the glucose-treated rats receiving Mannitol Myleran (2 g/kg, i.p.) there was a prolonged anuria lasting about 12 hr. This delays the excretion of the drug and might be expected to lead to higher or more prolonged blood levels of the compound, hence extending the time available for alkylation by the drug. This phenomenon could account for the general enhancement of t he activity of the drug in glucose-treated animals. Confirmation of raised and prolonged levels of the drug *in vivo* was obtained by comparing its concentration in the blood of rats given 2 g/kg of Mannitol Myleran alone and also with glucose pretreatment. In animals receiving the drug alone there was a rapid rise in the blood concentration followed by a sharp fall as the drug is distributed intracellularly and then excreted or inactivated by hydrolysis and alkylation. (Fig. 2). In glucose-treated rats there was a slower rise in the blood concentration of the drug, due possibly to the presence of the hypertonic glucose solution in the peritoneum restricting absorption. The blood concentration subsequently reached higher levels than those of the control animals and was maintained at these high levels for a prolonged time (Fig. 2).

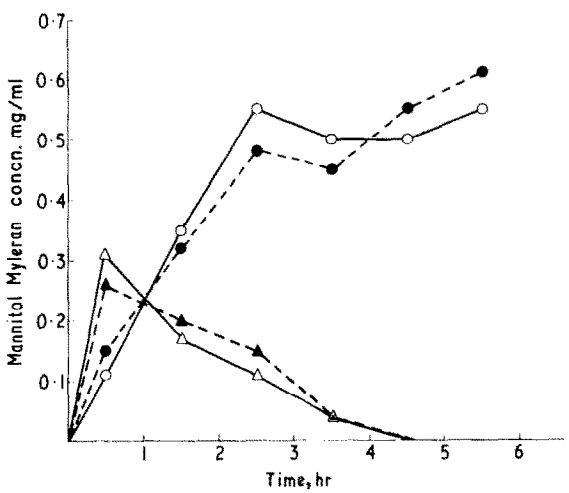


FIG. 2. Blood concentration of Mannitol Myleran (2 g/kg) following i.p. administration
△——△ ▲——▲ —Control animals (Mannitol Myleran alone) ●——● ○——○ —Glucose-treated animals

If glucose enhances the potency of Mannitol Myleran by inducing an anuria, then it would be expected that other compounds causing powerful anti-diuresis would have a similar effect. From the results shown in Table 1 it can be seen that sorbitol (5 g/kg of a 50% solution, 3 doses) causes an anuria for at least 3 hr and enhances the lethality of Mannitol Myleran similarly to that produced by glucose. Anti-diuretic hormone ('Pitressin') 0·33 I.U. administered s.c. 1 hr before Mannitol Myleran injection, only caused a slight antidiuresis, but, nevertheless, produced a moderate increase in lethality.

Sodium chloride (0·9 g/kg, 9% solution, 3 doses) had a powerful diuretic effect and whilst it did not significantly affect the LD_{50} of Mannitol Myleran it did delay the time of death.

DISCUSSION

Since Mannitol Myleran has been demonstrated to be excreted to a large extent as unchanged drug in rats, the anti-diuretic effect of hypertonic glucose solutions could account for its increased potency in glucose pretreated animals. The toxicity, haemotoxicity and tumour inhibitory properties of Mannitol Myleran remain unaltered in pattern after glucose pretreatment, and it does not appear likely that any selective pH fall in tissues which might occur following glucose administration, is selectively affecting the distribution or reactivity of this drug.

TABLE 1

Pre-treatment	Urine production* (ml)	LD_{50} Mannitol Myleran (mg/kg)
None	2·2	2,103
Glucose 5 g/kg × 3	0	312
Sorbitol 5 g/kg × 3	0	407
Sodium Chloride 0·9 g/kg × 3	9·0	2,205
A.D.H. 0·33 I.U. × 1	0·6	1,594

* Expressed as vol. of urine excreted per rat, during the 3 hr following Mannitol Myleran injection. Mean of 10 animals per group.

Connors *et al.*[10] have shown that TEM has a better therapeutic index in rats bearing the Walter 256 tumour when pretreated with glucose. It is suggested that this improvement of anti-tumour action over lethality is due to the selective pH fall in tumour tissue increasing the cytotoxicity of TEM at this site. However, in view of the recent report by Jackson and James[11] on the powerful diuretic effect of ethylene-imine and derivatives, it is possible that TEM may also induce a diuresis and its own rapid excretion, so precluding its optimum distribution to the relatively avascular tumour. After induction of an anuria with glucose, the TEM might now stay in the body for a longer period and obtain an optimum penetration of tumour tissue.

It should be possible to potentiate the action of other drugs excreted in unchanged form by inducing an anuria. Both Methotrexate and ThioTEPA have been demonstrated to be excreted in a large proportion as unchanged drug.[12] In preliminary experiments we have shown that glucose pretreatment increases the lethality of these compounds by approximately four-fold. It is not thought that glucose pretreatment will necessarily improve the therapeutic index of epoxides and ethylene-imines but where these compounds are being excreted unchanged, glucose at high doses will exert a sparing action, reducing the amount of drug required for any effect. For such drugs the rate of urine formation of treated animals will presumably be an important factor in determining their toxicities. It would seem possible that in patients with impaired kidney function, Mannitol Myleran, by being less rapidly excreted, might well show a greatly increased toxicity.

Acknowledgements—The authors wish to thank Mr. M. Jones and Mr. M. Tanner for technical assistance. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the

British Empire Cancer Campaign, the Tobacco Research Council and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

1. L. A. ELSON, *Radiation and Radiomimetic Chemicals*, p. 16. Butterworths, 1963.
2. S. S. BROWN and G. M. TIMMIS, *J. chem. Soc.* 3656 (1961).
3. T. A. CONNORS and L. A. ELSON, unpublished work.
4. W. DAVIS and W. C. J. ROSS, *Biochem. Pharmacol.* 915 (1963).
5. W. C. J. ROSS, *Biochem. Pharmacol.* **8**, 235 (1961).
6. H. KAHLER and W. B. ROBERTSON, *J. Nat. Cancer Inst.* **3**, 495 (1943).
7. T. A. CONNORS, L. A. ELSON and V. M. ROSENOER. Unpublished work.
8. J. EPSTEIN, R. W. ROSENTHAL and R. J. ESS, *Analyt. Chem.* **27**, 1435 (1955).
9. F. BRYANS and F. L. PYMAN, *J. chem. Soc.* 549 (1929).
10. T. A. CONNORS, B. C. MITCHLEY, V. M. ROSENOER and W. C. J. ROSS, *Biochem. Pharmacol.* **13**, 395 (1964).
11. H. JACKSON and R. M. V. JAMES, *Brit. J. Pharmacol. Chemother.* **21**, 581 (1963).
12. H. G. MANDEL, *Pharmacol. Rev.* **11**, 743 (1959).