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THE FAILURE OF ALDOSE REDUCTASE INHIBITOR 3,3′-TETRAMETHYLENE GLUTARIC ACID TO INHIBIT *IN VIVO* SORBITOL ACCUMULATION IN LENS AND RETINA IN DIABETES

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Abstract—The aldose reductase inhibitor, 3,3′-tetramethylene glutaric acid was investigated as a potential agent to inhibit the accumulation of sorbitol pathway intermediates in various tissues of the rat in diabetes. Two modes of administration were used; the feeding of a 1% (w/v) solution of the disodium salt of the drug; and the injection of the drug three times daily by an intraorbital extra-ocular route. Neither treatment prevented the accumulation of sorbitol and fructose in retina or lens tissue in the diabetic state.

A gas chromatographic method for the analysis of TMG in biological material was developed and applied to the study of the pharmacological properties of the drug. The half-life of the drug in rabbit after a single intravenous injection was 22 min. It was apparent that plasma membranes were impermeable to TMG and it was not possible to achieve significant concentrations of the drug in lens or retinal tissue by its administration orally or intraorbitally. It is concluded that disodium TMG could not be used as an *in vivo* inhibitor of sorbitol pathway activity in retina or lens in diabetes, and that its systemic use to inhibit sorbitol pathway activity in other tissues is not practical.

DIABETES mellitus of long duration is frequently complicated by cataract, peripheral neuropathy and vascular diseases particularly of the retina, kidney and heart. Cataract and peripheral neuropathy have been linked to the accumulation of the polyol sorbitol in lens and peripheral nerve tissue to concentrations sufficiently elevated to cause osmotic damage.[1,2] Retina has also been shown to accumulate sorbitol in diabetes.[3] The retinal sorbitol concentration however is much lower than that in lens or peripheral nerve and cannot presently be invoked as a pathogenic agent in vascular disease of retinal tissue in diabetes.

In retina, lens, and peripheral nerve, sorbitol accumulates in diabetes probably as a result of an increased flux of glucose through the sorbitol pathway of metabolism. The first enzyme of this metabolic pathway, aldose reductase (alditol:NADP oxidoreductase EC 1.1.1.21) has a high Michaelis constant for glucose[4] which, at least in lens, limits the flux of glucose through the pathway under normal conditions.[5] With the elevation of the intracellular glucose concentration following hyperglycaemia, flux through the pathway increases and sorbitol accumulates intracellularly to high concentrations. The application of a drug which could block aldose reductase activity

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in vivo may be considered a potential therapeutic agent of diabetic cataract and peripheral neuropathy,[6] and also as an experimental means to further investigate the relevance of sorbitol metabolism to other diabetic complications.

Inhibition of purified lens aldose reductase by a series of six and eight carbon aliphatic fatty acids, by $NADP^+$, and by a number of dicarboxylic acids has been reported.[4,7] Of the last group of compounds 3,3′- tetramethylene glutaric acid (TMG) has been used extensively for *in vitro* lens and nerve studies of the pathogenesis of cataracts and neuropathies associated with diabetes and galactosaemia.[8,9] The use of TMG to achieve aldose reductase inhibition *in vivo* has not been considered feasible by some authors, since the inhibitory activity of the compound is marginal, and plasma membranes are relatively impermeable to the compound.[10] The substance however, has a low toxicity and large doses may be tolerated by experimental animals. The present communication reports an investigation of the efficacy of TMG administration either locally, or systemically as an inhibitor of sorbitol accumulation in tissues of the diabetic rat. The tissue distribution and biological half-life of the compound are considered in relation to its pharmacological properties *in vivo*.

EXPERIMENTAL

The disodium salt of TMG (R. N. Emanuel & Co., Alperton, Middx., U.K.) was prepared by titration of the acid to pH 7 with sodium hydroxide. The salt was then isolated from solution by precipitation with acetone. The osmolarities of aqueous solutions of this salt determined by freezing point depression (Fiske osmometer) showed that the compound was fully dissociated at concentrations ranging from 0·4 to 40 mM.

Synthesis of the dimethyl ester of TMG. Disodium TMG (2 g) was dissolved in 30 ml of a 5% (v/v) H_2SO_4/methanol solution and heated for 2 hr at 100° under nitrogen in a screw capped culture tube. The solution was then reduced to a volume of approx. 10 ml by evaporation with a Buchi Rotavapor at 40° and then shaken with 10 ml of water and extracted three times with 10 ml of light petroleum (60–80°). The light petroleum extracts were pooled and then successively shaken with 2 ml of 4% (w/v) K_2CO_3 and 20 ml of water before being dried with anhydrous Na_2SO_4. The dried extract was evaporated with a Buchi Rotavapor at 45° to yield an oily residue which could not be reduced in mass.

Samples of this residue were analysed by gas chromatography using the following conditions—Instrument: Packard Series 7300 gas chromatograph equipped with dual flame ionization detectors. Column: 2 m × 4 mm Pyrex glass column packed with Gas Chrom Q (100/120 mesh) coated with 3% (w/w) SE-52 liquid phase. Temperatures: Oven 170°; detector and injection 210°. Flow rates: Carrier gas (N_2) 40 ml/min; hydrogen 20 ml/min; air 400 ml/min.

Two compounds were detected; a minor peak (5 per cent of total peak area; retention time 30 sec) which corresponded in retention time to that of the solvent used in the extraction procedure, and a major peak (95 per cent of total area; retention time 6 min) which was the esterified form of TMG. Microanalysis showed that this liquid contained 62·04% (w/w) carbon and 8·68% (w/w) hydrogen. These values were consistent with the identification of the major component of the liquid as the dimethyl ester of TMG [Fig. 1, 61·68% (w/w) carbon, 8·45% (w/w) hydrogen]. Mass

spectrographic analyses and nuclear magnetic resonance studies were consistent with this identification. The preparative yield of the ester was 84 per cent.

Analysis of TMG in biological material. Tissues (50–500 mg wet wt), and biological fluids (0·1–0·5 ml) were homogenized in 5 ml of 6% (v/v) perchloric acid in an all-glass Potter–Elvehjem homogenizer at 20°. The supernatant fluid (4 ml) which resulted from the centrifugation of each homogenate (2750*g* × 5 min) was neutralized with solid $KHCO_3$ and evaporated to dryness in a Buchi Rotavapor at 40°. The dried extract was vigorously shaken with 5 ml of a 5% (v/v) H_2SO_4/methanol solution and the insoluble material then removed by filtration. The precipitate was washed with 5 ml of 5% (v/v) H_2SO_4/methanol and the washings were pooled with the initial filtrate in a 15 ml screw capped culture tube and heated for 2 hr at 100° under nitrogen. The esterified material in this solution was extracted as described above. The final extract was evaporated using a stream of dry nitrogen, to a volume of approx. 5 μl, then reconstituted to a volume of either 50 or 500 μl with toluene before samples (1–10 μl) were removed for analysis by gas chromatography.

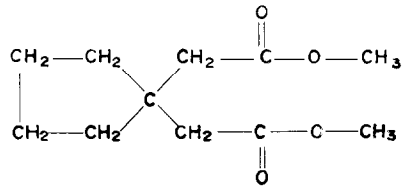

FIG. 1. Proposed structural formula for the dimethyl ester of TMG.

The column specifications, and the operating conditions of the gas chromatograph were as described above. Quantification of each chromatogram was achieved by peak height analysis, using standard solutions of dimethyl TMG.

This method when applied to the analysis of standard solutions of disodium TMG in freshly prepared plasma (rabbit) showed that there was quantitative recovery of the compound over the range 0·8–80 mM. Leaving disodium TMG in solution in plasma for periods of up to one hour before processing did not affect the recovery yield.

Determination of the biological half-life of TMG in the plasma of the rabbit following a single intravenous injection of the drug. Three months old male rabbits were restrained in small portable cages and each was given an intravenous injection (right ear vein) of 5 ml of 30% (w/v) disodium TMG. Blood samples (approx. 2 ml) were removed from the left ear vein of each animal at various intervals before and after the administration of TMG, and were replaced by equivalent volumes of 0·9% (w/v) NaCl. The concentrations of TMG, glucose,[11] and urea[12] were determined in plasma obtained from each blood sample. The microhaematocrit was determined in all blood samples.

Induction of diabetes in rats. Diabetes was induced in 30–40 day old male Norwegian hooded rats (weighing 150–200 g) by the intraperitoneal injection of 100 mg/kg body wt of streptozotocin (Upjohn Pty. Ltd, Kalamazoo, Mich., U.S.A.) in 0·11 M phosphate/0·44 M citrate buffer pH 4·5 (McIlvaine's buffer).[12] The animals had free access to water and a standard rat cube diet (Allied Feeds Pty. Ltd, Sydney, Australia) at all times. Diagnosis of diabetes was established 48 hr after the streptozotocin

injection from the determination of blood glucose concentration[11] and by testing freshly voided urine for the presence of glucose (Labstix: Ames & Co., Mulgrave, Victoria, Australia). Any streptozotocin treated animal which at this time had a blood glucose concentration of less than 300 mg % (w/v) or in which glucosuria was not present, was eliminated from the investigation.

Oral administration of TMG to diabetic rats. Seven days after the induction of diabetes, the drinking water of the animals was replaced by a 1% (w/v) solution of disodium TMG. This solution was consumed at the same rate as the distilled water given to age-matched control animals, and did not result in any apparent side effects. The mean consumption of the drug was 5 g/kg body wt/day in diabetic animals and 1·5 g/kg body wt/day in nondiabetic animals. The drug was fed for 7 days and the animals were then killed.

Intraorbital injection of TMG. Seven days after the induction of diabetes, animals were anaesthetized with ether and 0·1 ml of 30% (w/v) disodium TMG was injected extra-ocularly into both orbits of each animal. Age-matched control normal and control diabetic animals each received intraorbital injections of 0·1 ml of 0·9% (w/v) NaCl. Treatment was given three times daily (9 a.m., 2 p.m., and 6 p.m.) for 5 days and the animals were then killed (approx. 3 hr after the final injection).

The concentrations of free carbohydrates were determined in lenses and retinas of four animals in each treatment group by the gas chromatographic method previously described.[3] Retinas, lenses, aqueous humour, and plasma were also taken from six animals in each treatment group for the analysis of TMG. The free carbohydrate analyses were performed on single retinal or lens samples (i.e. $n = 8$) and the TMG analyses on tissue samples pooled from series of two animals (i.e. $n = 3$).

RESULTS

Figure 2 shows typical gas chromatographic analyses of the methylated and volatile products isolated from the perchloric acid-extracted material from rabbit plasma 20 and 120 min after the intravenous injection of disodium TMG (5 m-moles). A peak with a retention time identical to that of dimethyl TMG is shown in both chromatograms. The concentrations of the other compounds which appeared in these chromatograms were not affected by TMG administration.

The change in the plasma TMG concentration following a single intravenous injection into the rabbit is shown in Fig. 3a. The biological half-life of TMG calculated from a logarithmic plot (Fig. 3b) of the data (Fig. 3a) was 22 min (concentration range 0·1–5·0 m-moles/l.). The logarithmic plot intercepted the vertical axis at a point which corresponded to a plasma TMG concentration of 10 m-moles/l. This value was equivalent to the dose of the drug (approx. 5 m-moles) divided by the extracellular fluid volume (approx. 500 ml for a 2·5 kg rabbit). This result is consistent with TMG being confined to the extracellular space of the animal at all times following injection.

The microhaematocrit (Fig. 3c), the plasma glucose concentration, and the plasma urea concentration were all decreased by approx. 20% immediately following the administration of TMG. The time course of the deviation of the microhaematocrit from its predicted value following TMG injection was correlated to the change in the plasma TMG concentration (Fig. 3a, c). Similar decreases in the microhaematocrit and the plasma urea concentration were observed immediately after the intra-

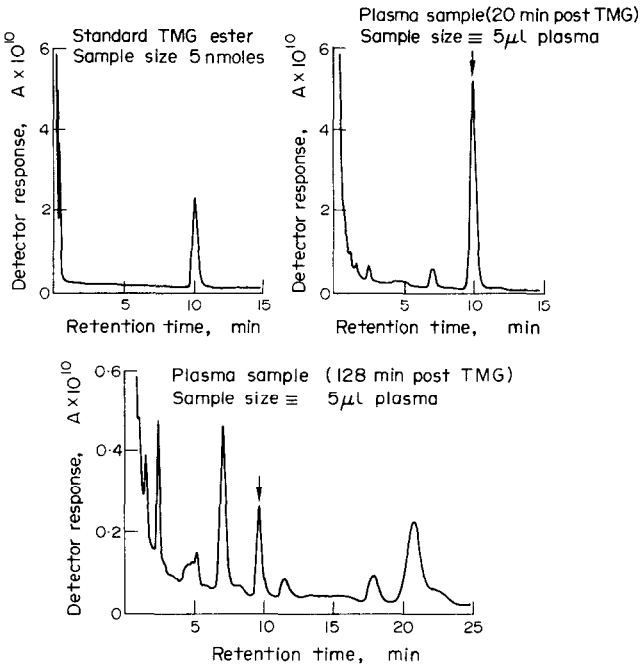

FIG. 2. Gas chromatographic determination of plasma TMG concentration: sample chromatograms.

Experimental. Plasma samples were taken at various intervals from 3 month old rabbits after the animals had received 5 m-moles of disodium TMG by intravenous injection. The TMG in the plasma samples was extracted with perchloric acid and esterified with H_2SO_4/methanol (95:5 v/v). Details are given in experimental section. The methyl esters and other volatile components of the treated plasma samples were analysed by gas chromatography using the following conditions. *Column.* 2 m × 4 mm Pyrex column packed with Gas Chrom Q (100/120 mesh) coated with 3% (w/w) SE52. *Operating conditions.* Temperature: oven 170°, detector and inlet 200°. Flow rate: carrier (Nitrogen) 40 ml/min, air 400 ml/min, hydrogen 40 ml/min.

venous injection of 15 m-moles of mannitol into an age-matched male rabbit. This finding suggested that the changes observed in the microhaematocrit, plasma glucose and urea concentrations following TMG injection were related to an osmotic effect of the drug.

When rats (weighing 150–200 g) were administered by gastric intubation, with two doses each of 5 mls of a 30% aqueous solution of sodium TMG (each dose 6·5 m-moles TMG), the doses being separated by a 2-hr interval and the animals killed 4 hr after the initial dose, the percentage recovery of administered TMG in the urine was 11 per cent, in faeces 22 per cent and in the total eye tissue 0·06 per cent.

The sensitivity of the TMG analyses in rat lens, retina, aqueous humour and plasma, were limited by the presence in the final tissue extracts of a volatile component with a retention time identical to that of dimethyl TMG. The lower limit of sensitivity was: 6 μmoles/l. in plasma, 60 μmoles/l. in aqueous humour, 20 μmoles/kg wet wt in retina and 100 μmoles/kg wet wt in lens. Significant changes in the concentrations of TMG in these tissues were not observed when TMG was administered by either oral or intravenous routes to either diabetic or non-diabetic animals.

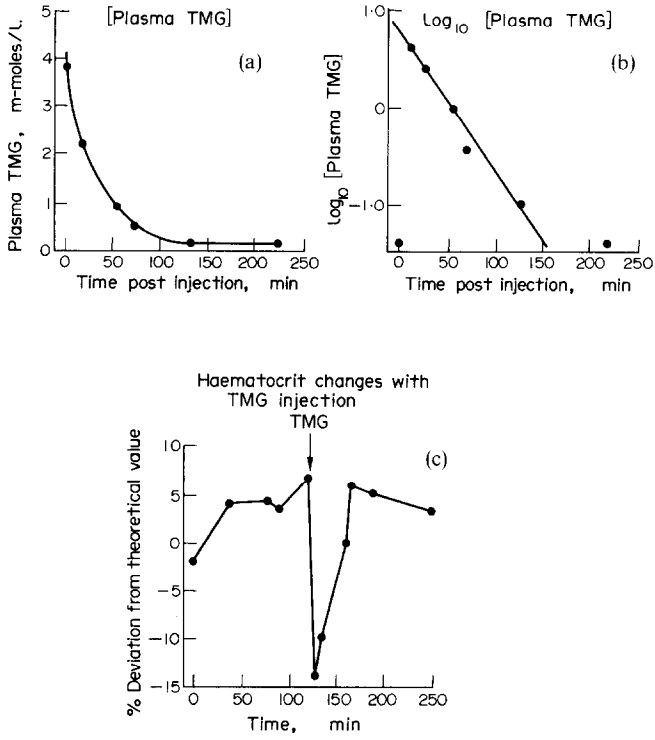

FIG. 3. Plasma TMG concentration and haematocrit changes following a single intravenous injection of TMG into the rabbit.

Experimental. The TMG concentration in the plasma of 3 months-old male rabbits following the intravenous injection of disodium TMG was determined by gas chromatography (Fig. 2). The haematocrit was determined as the microhaematocrit and was corrected for changes due to blood sampling. Each value is the mean of duplicate determinations from two animals.

The effect of TMG administration on the concentrations of free carbohydrates in the lenses and retinas of diabetic and non-diabetic animals is shown in Table 1. The drug, administered either orally or intraorbitally did not prevent the accumulation of sorbitol pathway intermediates in lens or retina in diabetes, nor did it affect the *myo*-inositol concentrations in either tissue. The mean plasma glucose concentration did not vary significantly between TMG-treated and drug control animals in either the normal or the diabetic state.

DISCUSSION

It is improbable that the TMG treatments given in the present experimental series affected the synthesis of sorbitol pathway intermediates in the lens or retina of the diabetic rat. Had the drug inhibited aldose reductase *in vivo* it would be expected that the tissue fructose and sorbitol accumulations would have dissipated as the result of diffusion, or the further metabolism of these intermediates. This conclusion is supported more empirically by the reported finding of Stewart *et al.*,[2] that the sorbitol which accumulated in the sciatic nerves of diabetic rats rapidly disappeared following control of the blood glucose concentration with insulin.

TABLE 1. EFFECT OF TMG ADMINISTRATION ON THE ACCUMULATION OF FREE CARBOHYDRATES BY THE RETINA AND LENS OF THE DIABETIC RAT

(A) Retina

		Retinal free carbohydrates (μmoles/g protein)			
		Fructose	Glucose	Sorbitol	*myo*-Inositol
Treatment					
Diabetic	TMG-injected	4·2 ± 2·5	9·9 ± 5·0	3·1 ± 0·7	10·6 ± 0·6
	TMG-fed	3·1 ± 1·0	11·3 ± 3·8	3·0 ± 0·3	11·2 ± 0·7
	Control	3·7 ± 1·0	9·2 ± 5·0	2·1 ± 0·4	9·6 ± 0·7
Non-diabetic	TMG-injected	<0·2	<0·3	<0·2	14·1 ± 1·7
	Control	<0·2	<0·3	<0·2	10·3 ± 1·0

(B) Lens

		Lens free carbohydrates (μmoles/g protein)			
		Fructose	Glucose	Sorbitol	*myo*-Inositol
Treatment					
Diabetic	TMG-injected	17·2 ± 1·1	5·5 ± 1·1	57·2 ± 18·1	<0·1
	TMG-fed	15·2 ± 0·9	7·6 ± 1.4	73·1 ± 19·2	<0·1
	Control	17·7 ± 0·5	7·4 ± 1·6	57·1 ± 11·2	<0·1
Non-diabetic	TMG-injected	0·3 ± 0·1	0·8 ± 0·1	5·4 ± 1·8	0·5 ± 0·1
	Control	0·2 ± 0·1	0·8 ± 0·2	1·7 ± 0·8	0·7 ± 0·1

Experimental. Animals: Age at killing 42 days. Duration of diabetes 12–14 days. TMG injection: Intraorbital extraocular injections of 30 mg of disodium TMG three times daily for 5 days. TMG feeding: 1% (w/v) disodium TMG solution fed as drinking water for 7 days.

Analysis. Gas liquid chromatography of trimethylsilyl derivatives of free carbohydrates. Values expressed as the mean ± S.E.M. of eight determinations.

The failure of oral or intraorbital administration of TMG to inhibit sorbitol accumulation in lens and retina in diabetic rats was apparently related to a failure to achieve inhibitory concentrations of the drug in these tissues. The observed osmotic effects of TMG on the haematocrit and plasma urea concentrations in the rabbit, and the calculated distribution of TMG in the rabbit, suggest that a factor limiting the tissue concentration of TMG may be the impermeability of plasma membranes to the drug. This property of TMG has been described by other authors[7] and is possibly related to its high aqueous solubility, and to the presence of the two fully dissociated carboxyl groups on the molecule at physiological pH values.

Another factor which may limit the establishment of inhibitory concentrations of TMG in tissues *in vivo* is the rate at which the drug is eliminated or detoxified. From the calculated plasma half-life of the drug in the rabbit, the rate of intravenous infusion of disodium TMG which would be necessary to maintain the plasma TMG concentration between 1–5 mM (an effective inhibitory range) was approx. 10–20 g/kg body wt/day. It was therefore not surprising that in the experiments in which rats were fed approx. 1–5 g disodium TMG/kg body wt/day, that the drug was not detected in plasma, aqueous humour, retina, or lens of the animals.

From these investigations it is concluded that disodium TMG could not be used as an *in vivo* inhibitor of sorbitol pathway activity in retina and lens, and that its systemic use to inhibit sorbitol pathway activity in other tissues is not practical.

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REFERENCES

1. R. VAN HEYNINGEN, *Exptl. Eye Res.* **1,** 396 (1962).
2. M. A. STEWART, W. R. SHERMAN, M. M. KURIEN, G. I. MOONSAMMY and M. WISGERHOF, *J. Neurochem.* **14,** 1057 (1967).
3. J. C. HUTTON, P. J. SCHOFIELD, J. F. WILLIAMS and F. C. HOLLOWS, *Aust. J. exptl. Biol. Med. Sci.* **52,** 361 (1974).
4. S. HAYMAN and J. H. KINOSHITA, *J. biol. Chem.* **240,** 877 (1965).
5. P. I. POTTINGER, *Biochem. J.* **104,** 663 (1967).
6. P. BEAUMONT, F. C. HOLLOWS, P. J. SCHOFIELD, J. F. WILLIAMS and A. W. STEINBECK, *Lancet* **i,** 579 (1971).
7. J. A. JEDZINIAK and J. H. KINOSHITA, *Invest. Ophthal.* **10,** 357 (1971).
8. K. H. GABBAY, *Diabetes* **18,** 336 (1969).
9. J. H. KINOSHITA, D. DVORNIK, M. KRAML and K. H. GABBAY, *Biochim. biophys. Acta* **158,** 472 (1968).
10. K. H. GABBAY and J. H. KINOSHITA, *Lancet* **i,** 913 (1971).
11. A. ST. G. HUGGET and D. A. NIXON, *Biochem. J.* **66,** 12P (1957).
12. R. N. BEALE and N. CROFT, *J. Clin. Path.* **14,** 418 (1961).
13. R. M. C. DAWSON, D. C. ELLIOT, W. H. ELLIOT and K. M. JONES, *Data for Biochemical Research*, 2nd Edn. Oxford University Press, Oxford (1969).