Table 2. Effect of 6-methylene-4-pregnene,3,20-dione on hepatic bile acid sulfotransferase specific activity

Treatment	N	Bile acid sulfotransferase activity (pmoles/min/mg protein)
Sham-operated		
Vehicle only	6	22.8 ± 2.2
6-Methylene-4-pregnene-3,20-dione	6	56.1 ± 6.5*
5α-Dihydrotestosterone	6	16.7 ± 3.2
5α-Dihydrotestosterone + 6-methylene-4-pregnene-3,20-dione	5	18.5 ± 1.7
Castrated		
Vehicle only	6	60.0 ± 7.8*
6-Methylene-4-pregnene-3,20-dione	7	82.7 ± 3.5*
5α-Dihydrotestosterone	8	12.1 ± 1.2

* Values shown are means of determinations on N animals ± one S.E.M.
* Significantly different, $P < 0.0024$, from sham-operated controls (vehicle only).

The results of present studies indicate that 6-methylene 4-pregnene-3,20-dione treatment can stimulate intrahepatic bile acid sulfotransferase activity without major constitutional effects. Furthermore, the effect of the drug on this metabolic pathway was equivalent to that which results from surgical castration. Since it has been shown that 5α-reductase deficiency can be consistent with normal sexual activity and muscular growth [14], the use of inhibitors of 5α-reductase has been proposed in the treatment of dihydrotestosterone-responsive conditions (e.g. prostatic hypertrophy and prostatic cancer) [11, 15]. The data presented here suggest that a 5α-reductase inhibitor such as 6-methylene-4-pregnene-3,20-dione also may be useful in the study of the treatment of cholestasis.

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Valproic acid teratogenicity in whole embryo culture is not prevented by zinc supplementation

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It has been suggested that some of the toxicities caused by the anticonvulsant valproic acid (2-propylpentanoic acid) may be mediated through an alteration of trace metal status [1, 2]. Many of the side effects of valproate therapy are similar to the symptoms associated with zinc deficiency [3, 4]. Valproate has been linked with an increased incidence of spina bifida in infants born to mothers taking the drug [5, 6] and a connection between zinc deficiency and spina bifida and anencephaly has been suggested [7]. Animal studies show that either zinc deficiency or valproate exposure can induce malformations of the neural tube [8, 9]. Eckhert and Hurley reported a reduction in the incorporation of [^{3}H]thymidine into the DNA of the head region of embryos (embryonic age 13 days) in zinc-deficient

rats which could be reversed by the administration of zinc [10]. Hurd and colleagues have demonstrated that valproate can bind zinc [11] and have reported that valproate depletes plasma zinc in treated rats [2, 12]. As well as being teratogenic in various experimental species *in vivo* [9], valproate has been shown to produce morphological abnormalities in whole mouse and rat conceptuses undergoing organogenesis *in vitro* [9, 13]. The studies reported here were performed in order to examine whether zinc supplementation could ameliorate or prevent the teratogenic effects of valproate on the whole rat conceptus *in vitro*.

Materials and methods

Materials. 2-Propylpentanoic acid (valproic acid, Aldrich Chemical Co., Gillingham, Dorset) and zince acetate (BDH Chemicals, Dagenham, Essex) were neutralized with sodium hydroxide (1N) and diluted in minimum essential medium Eagle (MEM, Gibco, Uxbridge, Middx) before addition to the culture medium.

Methods. The culture system was based on that of New *et al.* [14] and has been described in detail [15]. The conceptuses were explanted from Wistar–Porton rats during the afternoon of Day 10 of gestation (embryonic age 9.5 days). Pairs of conceptuses were cultured for 48 hr in 4 ml of 75% heat-inactivated, immediately-centrifuged rat serum and 25% MEM. Only those conceptuses which were at the mid-headfold stage were used and littermates were distributed between control and treated cultures. At the end of the culture period, yolk-sac diameter, crown–rump and head lengths were measured. Somites were counted and the embryonic morphology was examined [16]. The concentration of zinc in the rat serum was measured using atomic absorption spectrophotometry. The results were analysed using Student's *t*-test.

Results and discussion

Over the 48-hr culture period the early somite stage embryo (embryonic age 9.5 days) developed to the forelimb bud stage, with 28–29 somites. Exposure to 1 mM valproate during the entire culture period resulted in embryos which were significantly reduced in size and somite numbers compared to control embryos, in agreement with previous observations [9]. All the valproate-treated embryos were abnormal, the most frequent defects being retarded otic development, irregular neural suture lines, anomalous segmentation of the somites, and yolk-sac malformation. Unfused brain folds were observed in about 10% of embryos. These observations are also in accord with previous studies [9]. Valproate 1 mM (144 μg/ml) is within the range of maternal serum concentrations observed following a teratogenic dose to mice *in vivo* and serum concentrations of this magnitude have been recorded in patients receiving valproate therapy, although average therapeutic levels are somewhat lower [17].

Three series of experiments were performed: In the first, the concentration of zinc in the culture medium was increased 2- and 4-fold, assuming a mean concentration of 1 μg/ml in the serum (the zinc concentration in rat serum was found to be in the range 0.7–1.3 μg/ml), by the addition of 1 and 3 μg/ml zinc respectively. Embryos developed normally in the presence of these low concentrations of zinc alone and no significant difference was found between the combined effects of zinc and valproate and those of valproate alone (Table 1).

Table 1. Effects of low concentrations of zinc and 1 mM valproate alone and combined on whole rat embryos in culture

Treatment	Total No.	Abnormal embryo no.	Crown–rump (mm) Mean ± SE	Head (mm) Mean ± SE	Somite number Mean ± SE	Abnormal yolk sac No.	Yolk sac diameter (mm) Mean ± SE
Control	7	0	3.6 ± 0.13	1.8 ± 0.07	29.8 ± 0.6	0	4.0 ± 0.09
1 μg/ml zinc	4	0	3.4 ± 0.06	1.9 ± 0.04	28.5 ± 0.3	0	3.8 ± 0.04
3 μg/ml zinc	8	0	3.4 ± 0.13	1.7 ± 0.07	27.9 ± 0.3	0	4.1 ± 0.12
1 mM valproate	10	10	2.7 ± 0.06*	1.2 ± 0.06*	NC	8	3.3 ± 0.04*
1 μg/ml zinc + 1 mM valproate	6	6	2.9 ± 0.08*	1.3 ± 0.04*	NC	6	3.6 ± 0.13*
3 μg/ml zinc + 1 mM valproate	6	6	2.6 ± 0.05*	1.1 ± 0.07*	NC	6	2.9 ± 0.07*†

* Significantly different from control, $P < 0.05$.
† Significantly different from valproate alone, $P < 0.05$.
NC—Not possible to count.

Table 2. The effects of zinc on the development of whole rat embryos in culture

μg/ml zinc treatment	Total No.	Abnormal embryo No.	Crown–rump (mm) Mean ± SE	Head (mm) Mean ± SE	Somite number Mean ± SE	Abnormal yolk sac No.	Yolk sac diameter (mm) Mean ± SE
0	10	0	3.6 ± 0.11	1.8 ± 0.07	28.4 ± 0.7	0	4.1 ± 0.07
10	8	0	3.7 ± 0.13	1.8 ± 0.14	29.1 ± 0.3	0	4.1 ± 0.20
30	8	0	3.7 ± 0.09	1.8 ± 0.03	28.8 ± 0.3	0	4.4 ± 0.03*
50	4	1	3.4 ± 0.31	1.6 ± 0.17	25†	1	3.4 ± 0.20*
100	8	7	3.3 ± 0.07*	1.5 ± 0.06*	26.8 ± 0.3‡	5	3.1 ± 0.17*

* Significantly different to control, $P < 0.05$.
† Three embryos counted.
‡ Five embryos counted.

Table 3. The effects of 1 mm valproate on whole rat embryos pre-incubated for 16 hr in high concentrations of zinc

Treatment	Total No.	Abnormal embryo No.	Crown–rump (mm) Mean ± SE	Head (mm) Mean ± SE	Somite number Mean ± SE	Abnormal yolk sac No.	Yolk sac diameter (mm) Mean ± SE
Control	8	0	3.4 ± 0.06	1.7 ± 0.03	28.0 ± 0.02	0	3.8 ± 0.09
33 μg zinc	7	0	3.1 ± 0.12*	1.6 ± 0.06*	26.0 ± 0.4	0	3.5 ± 0.08*
65 μg zinc	6	0	3.1 ± 0.14	1.6 ± 0.08	26.8 ± 0.04	1	3.5 ± 0.17
1 mM valproate	6	6	2.7 ± 0.12*	1.2 ± 0.11*	21.0†	6	3.2 ± 0.05*
33 μg/ml zinc + 1 mM valproate	8	8	2.9 ± 0.10*	1.2 ± 0.08*	21.6 ± 0.7*‡	8	3.4 ± 0.08*
65 μg/ml zinc + 1 mM valproate	8	8	2.7 ± 0.16*	1.2 ± 0.12*	17.3 ± 1.4*§	8	3.0 ± 0.11*

* Significantly different from control, $P < 0.05$.
† Two embryos counted.
‡ Five embryos counted.
§ Seven embryos counted.

The second series of experiments determined the maximum concentration of zinc which could be added to the culture medium before the embryos showed signs of toxicity. Embryos developed normally in concentrations up to 30 μg/ml but at 50 μg/ml there was a slight reduction in embryonic size and irregular segmentation of the somites was observed (Table 2). At 100 μg/ml the embryos were significantly growth-retarded and the majority had several general, but not severe, anomalies such as incomplete dorsiflexion, retarded cardiac and brain development and disrupted somite segmentation. None of the embryos had open neural tubes or unfused brain folds.

In the final series of experiments embryos were exposed to zinc for the whole 48-hr incubation period, but addition of valproate was delayed until 16 hr of culture. This provided 16 hr of incubation in the presence of zinc followed by 32 hr of concomitant zinc and valproate. Two zinc concentrations were used, 33 and 65 μg/ml, the higher being equimolar to valproate (1 mM). These concentrations of zinc, alone, had a slight growth retarding effect (Table 3), in agreement with findings from experiment 2. Exposure to valproate, alone, for the final 32 hr of culture had similar effects to 48-hr exposure. With this experimental design, there was no significant effect of either concentration of zinc on valproate-induced embryonic effects (Table 3).

None of these experiments provide any support for the suggestion that valproate may exert its teratogenic effects through a depletion of zinc. The first experiment shows that elevation of the medium zinc concentration to physiologically realistic levels has no influence on valproate embryotoxicity. Because of the observed embryonic effects of zinc, alone, in the second experiment it is likely that zinc added to the culture medium does reach the embryonic compartment although an effect mediated through interference with yolk sac function cannot be ruled out. However, little is known of the rate at which embryonic zinc levels equilibrate with exogenous zinc. It was considered possible that concomitant zinc might not protect against valproate if valproate gained rapid access to embryonic compartments which were only slowly repleted with exogenous zinc. We have shown in other studies that valproate equilibrates in this system within less than 4 hr.* For this reason, the 16-hr zinc pre-incubation period was adopted in experiment 3, but again without a protective effect. It was also thought that if valproate were able to bind zinc in a one to one stoichiometry (which seems unlikely) then very high levels of zinc would be required to prevent its action. The results of experiment 3 show that even equimolar quantities of zinc are without effect on valproate teratogenicity.

It remains possible that zinc is compartmentalized within embryonic tissues such that valproate has access to zinc that is not freely exchangeable with zinc from the medium. However, it has been reported that feeding of a zinc-deficient diet to rats over a 3-day period (days 10–12 of gestation) is sufficient to cause a significant increase in abnormal fetuses [18]. If such a transient deficiency in the mother can cause abnormalities in the fetus then it seems unlikely that there are any substantial stores of zinc in the embryo that are not affected by exogenous supplies.

In summary, we have investigated the possibility that valproate teratogenesis is mediated through depletion of zinc. Valproate causes major growth and developmental anomalies in the whole rat embryo in culture. When conceptuses were cultured in the presence of zinc and valproate under a variety of concentrations and conditions it was found that there was no amelioration or prevention of the effects of valproate. Therefore, although valproate may bind zinc, it is unlikely that its primary mechanism of teratogenesis is mediated through a depletion of this trace element.

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Effects of kainic acid analogues on the high affinity uptake of D-[^{3}H] aspartate into rat cerebellar homogenates

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Glutamate and aspartate are considered to serve as excitatory neurotransmitters in the mammalian central nervous system [1]. In common with many transmitters, their physiological actions are thought to be terminated by transport into presynaptic nerve terminals and glia, by a Na^+-dependent high affinity uptake mechanism [2]. Interference with this inactivation process will potentiate the depolarizing actions of these substances [3], and also will reveal their potential excitotoxic properties [4].

A number of closely related excitatory analogues of glutamate and aspartate are effective inhibitors of this uptake system [5]. D-Aspartate for example, is transported equally well as L-glutamate or L-aspartate, and provides a useful non-metabolizable alternative substrate for investigating uptake mechanisms [6].

The availability of glutamate/aspartate uptake inhibitors (lacking overt direct depolarizing actions) is important for investigating the physiological role of excitatory amino acid transport in nervous tissue. The potent excitant and excitotoxin α-kainic acid is a weak inhibitor of high affinity L-glutamate uptake; however, much more potent in this respect is its reduced congener dihydrokainate, which lacks direct excitatory actions at post-synaptic receptors [7].

As part of a study of potential excitatory amino acid receptor antagonists, we have synthesized and examined a number of novel kainic acid analogues [8, 9]. We report here on the ability of these substances to influence the high affinity uptake of D-[^{3}H]aspartate into rat cerebellar crude synaptosomal preparations.

Materials and methods

Preparation of crude cerebellar synaptosomes. Male adult Wistar rats (300 g) were killed by decapitation, and their cerebella excised rapidly. Following removal of the pial meninges, the tissues were homogenized in 10 vol. (w/v) 0.32 M sucrose, with a Teflon-glass homogenizer (0.25 mm clearance), and then diluted to 50 vol. with 0.32 M sucrose, to dilute endogenous amino acids. Following centrifugation at 1000 *g* for 10 min, the supernatant was removed and centrifuged at 17,000 *g* for a further 20 min. The synaptosome-enriched P_2 pellet obtained was resuspended in 10 vol. of 0.32 M sucrose for the assay.

Uptake assay. Assays were performed in 5 ml Teflon centrifuge tubes containing 2 ml Krebs bicarbonate buffer pH 7.4 (NaCl 114 mM; KCl 5 mM; KH_2PO_4 1.2 mM; $MgSO_4$ 1.2 mM; $CaCl_2$ 2.6 mM; $NaHCO_3$ 25 mM; glucose 11.7 mM) together with 20 μl D-[^{3}H]aspartate (10 nM final concentration, sp. act. 22 Ci/mmol, Amersham International, U.K.), gassed with 95% O_2–5% CO_2 and preincubated at 37° for 2 min. Non-specific uptake was defined in a parallel set of tubes where sodium was replaced by an equimolar concentration of choline. The incubations were initiated by addition of 20 μl of the P_2 preparation (equivalent to approximately 50–60 μg protein per assay tube), continued for 3 min at 37° and were terminated by transference onto ice. The tubes were then spun at 16,000 *g* for 10 min, the pellets washed several times with warm buffer (37°) and the tissue was solubilized with 300 μl of 2% SDS overnight. Following the addition of 3 ml scintillant (LKB optiphase 'RIA'), radioactivity was determined by liquid scintillation counting. High affinity uptake was determined by subtraction of the values obtained in the absence of Na^+ from those obtained in the presence of Na^+.

Protein was determined by the method of Lowry *et al.* [10] using bovine serum albumin (Sigma) as standard.

All common analytical reagents were obtained from Sigma UK or from B.D.H. Chemicals, Poole, Dorset, U.K.

Results and discussion

High affinity D-[^{3}H]aspartate uptake into adult rat cerebellar synaptosomes was rapid and showed an absolute dependence on Na^+ (data not shown). Uptake was saturable (Fig. 1a) with a K_m of 10 ± 1.5 μM and a V_{max} of 6.25 ± 0.94 nmol/mg protein/3 min (Fig 1b).

A number of kainic acid analogues were tested for their ability to influence this high affinity uptake (Fig. 2). α-Dihydrokainate was the most potent of the analogues tested with an IC_{50} of approximately 110 μM, whilst α-kainate was slightly weaker with an IC_{50} of about 350 μM (Table 1). The other kainate analogues tested were substantially less potent, and several, *viz.* α-homokainate, β-homokainate, α-alloketokainate, β-alloketokainate, α-ketokainate, β-ketokainate, α-kainyl glycine, β-kainyl glycine, α-kainyl-aminomethylphosphonate, β-kainyl aminomethylphosphonate and α-carboxykainate were all totally ineffective in influencing high affinity D-[^{3}H]aspartate uptake into adult rat cerebellar synaptosomes at concentrations up to 1.5 mM.

As reported previously [7], both α-kainate and, more potently, α-dihydrokainate were found to inhibit the high affinity uptake of D-[^{3}H]aspartate into rat brain tissues.