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EFFECTS OF ETHINYL ESTRADIOL ON SUBSTRATE UPTAKE AND EFFLUX BY ISOLATED RAT HEPATOCYTES

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Abstract—Ethinyl estradiol is a well documented, predictable cholestatic agent. The direct effects of this drug on hepatocellular uptake and efflux have been investigated. Uptake of taurocholate and ouabain, which are both actively transported, was inhibited, while uptake of cadmium, which occurs by a combination of facilitated and simple diffusion, was unaffected. The V_{max} for taurocholate uptake was not altered by the presence of ethinyl estradiol, whereas the K_m was increased, suggesting a reduced affinity of receptor for taurocholate. Efflux of taurocholate from pre-loaded cells remained unchanged in the presence of ethinyl estradiol. The data are not entirely consistent with an interference of Na^+, K^+-ATP ase by ethinyl estradiol being an important site of action. The observations may help to explain the mechanism of biliary dysfunction induced by ethinyl estradiol.

Ethinyl estradiol is well documented as an agent that causes cholestasis in susceptible humans and in the laboratory animal [1–3]. It is noted that cholestasis has become such a widely used term that it now suffers from multiple definition [4]. An alternative terminology, bile secretory failure, has been suggested as an appropriate alternative [5]. This then encompasses dysfunction at the hepatocyte level but does not exclude a morphological response. As the present report examines the effects of ethinyl estradiol on the uptake of substrates by liver cells the appreciation of such terminology is important. The role of uptake into the hepatocyte, intracellular movement and the secretory steps at the bile canaliculus have been discussed by others [3, 5, 6]. Interference with such steps in the pathway could account for the interference with biliary function by different chemicals. This has recently been addressed for ethinyl estradiol by Berr *et al.* [7], who showed that pretreatment of rats with ethinyl estradiol resulted in hepatocytes that had reduced capacity to take up taurocholate. In a similar set of experiments efflux of bile acids was also shown to be impaired in hepatocytes isolated from ethinyl estradiol pretreated rats [8, 9]. Neither of these studies examined the effects of ethinyl estradiol added directly to the cell suspension on substrate uptake or efflux. Other studies have shown that other steroids have direct inhibitory effects on hepatocyte uptake [10–12]. However, ethinyl estradiol has been shown to stimulate hexose transport in isolated rat hepatocytes [13]. The present study was therefore undertaken to examine the effects of ethinyl estradiol on uptake and efflux of model substrates by isolated rat hepatocytes, with the aim of determining if such direct effects may play a role in the biliary dysfunction associated with this chemical.

MATERIALS AND METHODS

Tauro [carbonyl-^{14}C] cholic acid, sodium salt (60 mCi/mmol), carrier free ^{109}cadmium chloride (1 mCi/ml, 1 μg/ml) and [G-^{3}H] ouabain (37 Ci/mmol) were purchased from Amersham (Sydney), while taurocholate, ouabain and 17α-ethinyl estradiol (17α-ethinyl-1, 3, 5 (10)-estratriene-3, 17 β diol) were purchased from Sigma (St. Louis). Collagenase was Worthington CLS II. All other chemicals used were reagent grade from local agents.

Sprague–Dawley rats (260–340 g) from the University of Sydney Animal House were used as liver donors. They were allowed free access to food (Allied Stock Feeds, Sydney) and water. Ether was used as the anaesthetic and surgery was performed at about 0930 for each experiment. Hepatocytes were isolated by the method of Berry and Friend [14] with minor modifications as previously described [15]. Hepatocytes were resuspended to 1.3–1.5 × 10^6 cells/ml and initial viability determined by trypan blue exclusion, which was approximately 85%. Cellular potassium ion was also determined as an index of viability and found to be 84.5 ± 7.8 μmoles/g wet wt.

Incubation medium was a Tris-buffered balanced salt solution (131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 1 mM $CaCl_2$, 3 mM Na_2HPO_4, 10 mM Tris (hydroxymethyl) aminomethane, pH 7.4). For determination of effects on uptake, suspension of freshly isolated hepatocytes (2 ml) were preincubated (37°) with shaking at 80 oscillations/min for 15 min with ethinyl estradiol (dissolved in 10 μl of propylene glycol) or propylene glycol control (10 μl). Preliminary studies showed that this amount of vehicle had no effect on uptake of the substrates used. Following preincubation, 80 μl substrate (^{14}C tauro-

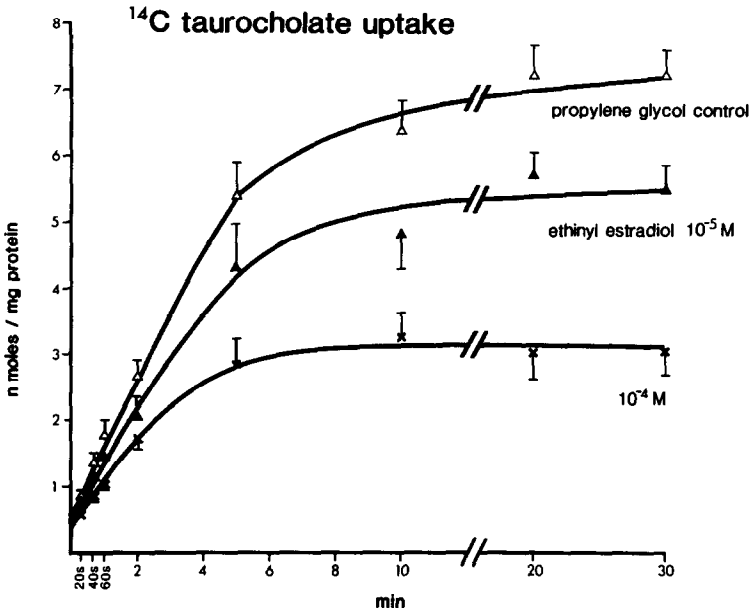

Fig. 1. Effects of ethinyl estradiol on uptake of taurocholate by hepatocytes with time. Symbols represent means and bars the S.E. N = 4 or 5.

cholate, ^{3}H ouabain or ^{109}cadmium each in physiological saline) was added and uptake determined at appropriate times. Each flask contained approximately the same amount of radioactivity for each substrate (taurocholate–80 nCi/ml, ouabain–50 nCi/ml, cadmium–60 nCi/ml). Concentrations of the substrates in the incubation vessels were 25 μM for taurocholate, 125 μM for ouabain and 10 μM for cadmium. Sampling using the silicone oil centrifugation technique was carried out as previously described [16]. The samples containing ^{14}C or ^{3}H were placed in scintillation fluid after the cell pellet was dissolved in the potassium hydroxide and were quantitated in a Packard liquid scintillation counter.

Radiolabeled cadmium containing samples were assayed in a LKB minigamma counter. Determination of the amount of radiolabel and protein concentration [17] allowed calculation of uptake per milligram of protein. Allowance for adherent fluid was made in the calculations as documented by Eaton and Klaassen [11].

In efflux studies, hepatocytes were pre-loaded with ^{14}C-taurocholate by incubation of 2×10^{7} cells/2 ml for 20 min with a final concentration of 50 μM at 160 nCi/ml of suspension. Subsequently an aliquot (100 μl) of this concentrated cell suspension was added to 1.9 ml fresh incubation medium which had been pre-incubated with ethinyl estradiol or vehicle for 15 min. Samples were then taken at appropriate intervals for determination of efflux of taurocholate as described and referenced for the uptake studies. In some experiments ethinyl estradiol was also added to the pre-loading flask at 10 min as well as to the incubation flask for studying efflux.

Statistical analysis was by a two-tailed t-test with a pre-set probability level of $P < 0.05$. Each data point represents a mean value obtained from at least three, but generally four or five, experiments, with each experiment using hepatocytes isolated from a different rat.

RESULTS

The inhibitory action of ethinyl estradiol on uptake of taurocholate by rat hepatocytes over a 30 min incubation period is shown in Fig. 1. This is particularly apparent at the longer incubation times. Figure 2 displays log dose–response curves for the effects of increasing dose of ethinyl estradiol on taurocholate uptake. At 10^{-5} M and higher concentrations the inhibitory action was seen. Concentrations of 10^{-6} and 10^{-7} M were not different to controls. Uptake of ouabain was also found to be suppressed over a 60 min incubation period by ethinyl estradiol (Fig. 3). However, consistent significant inhibition was not seen until 5×10^{-5} M as displayed in the log dose–response curve (Fig. 4). The highest concentration of ethinyl estradiol (10^{-4} M) used in this study showed no effect on the uptake of cadmium by suspensions of rat hepatocytes (Fig. 5).

The effects of ethinyl estradiol on the handling of taurocholate were examined in more detail by investigating uptake over a range of substrate concentrations in the presence and absence of 5×10^{-5} M ethinyl estradiol. Samples were taken at the early incubation times of up to 60 sec. As uptake is linear over this time scale, the data can be used for calculation of kinetic constants such as V_{max} and K_m, which are presented in Table 1. The data show that V_{max} is unaffected by ethinyl estradiol while K_m is significantly increased in its presence.

The efflux of taurocholate from pre-loaded hepatocytes was also examined for the effects of added

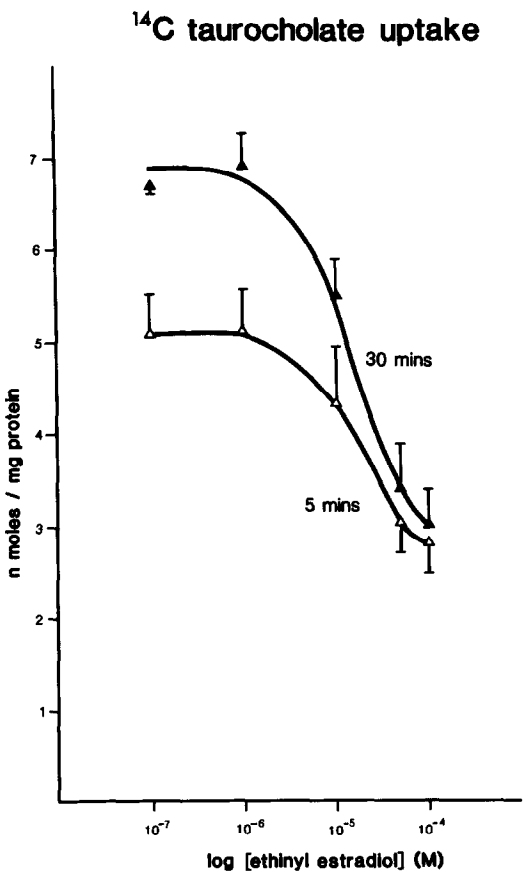

Fig. 2. Log dose–response curve for effects of ethinyl estradiol on taurocholate uptake at 5 and 30 min of incubation. Symbols represent means and bars the S.E. N = 4 or 5.

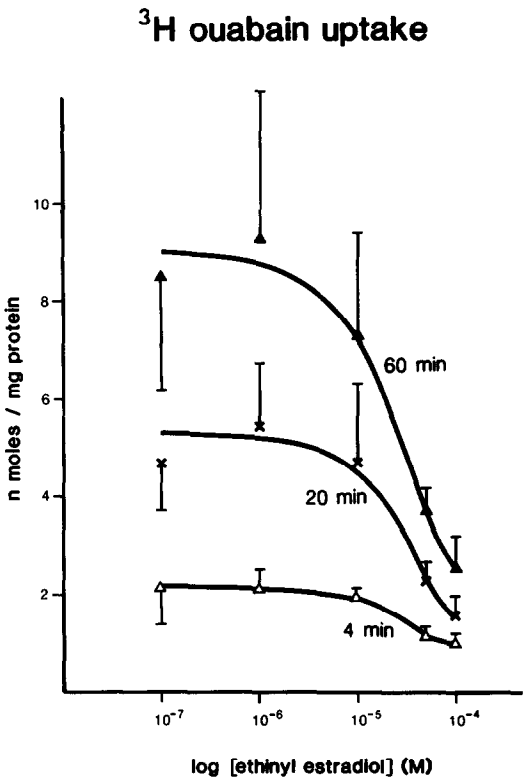

Fig. 4. Log dose–response curve for effects of ethinyl estradiol on ouabain uptake at 4, 20 and 60 min of incubation. Symbols represent means and bars the S.E. N = 4.

ethinyl estradiol. None of the concentrations used in the uptake study had any effect on efflux. The data for the highest concentration only are shown in Fig. 6.

Similar effects of ethinyl estradiol on uptake and efflux of taurocholate were found when hepatocytes isolated from a female rat were used (data not shown). Similarity was both qualitative and quantitative.

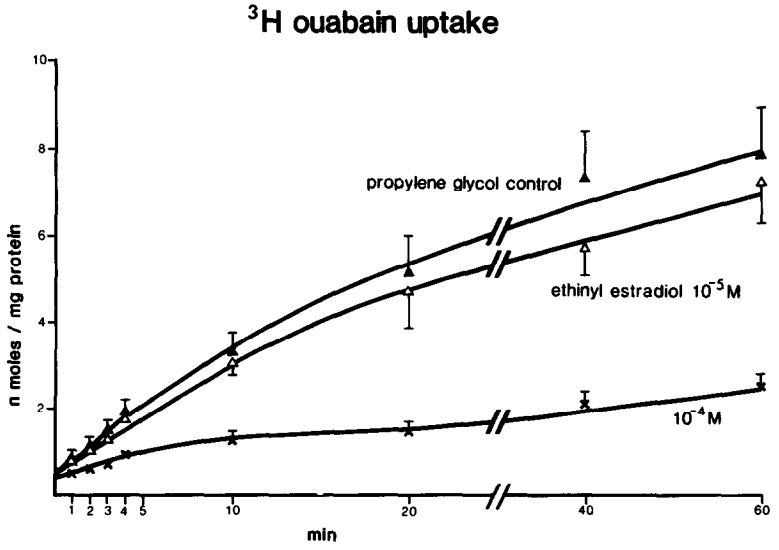

Fig. 3. Effects of ethinyl estradiol on uptake of ouabain by hepatocytes with time. Symbols represent means and bars the S.E. N = 4.

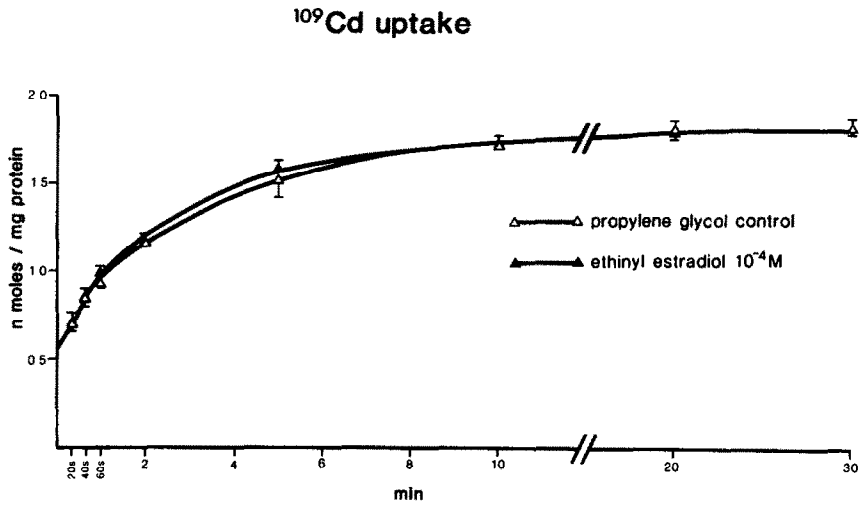

Fig. 5. Effects of ethinyl estradiol on uptake of cadmium by hepatocytes with time. Symbols represent means and bars the S.E. N = 3.

Table 1. Effects of ethinyl estradiol (5×10^{-5} M) on kinetic constants for taurocholate uptake by suspensions of isolated rat hepatocytes

Addition to cells	V_{max}*	K_m†
Propylene glycol control (N = 3)	1.44 ± 0.10	29.6 ± 8.2
ethinyl estradiol (N = 4)	1.64 ± 0.17	93.0 ± 12.3‡

* Values are mean ± S.E. in nmoles/min/mg protein.
† Values are mean ± S.E. in μM.
‡ Statistically different to control.

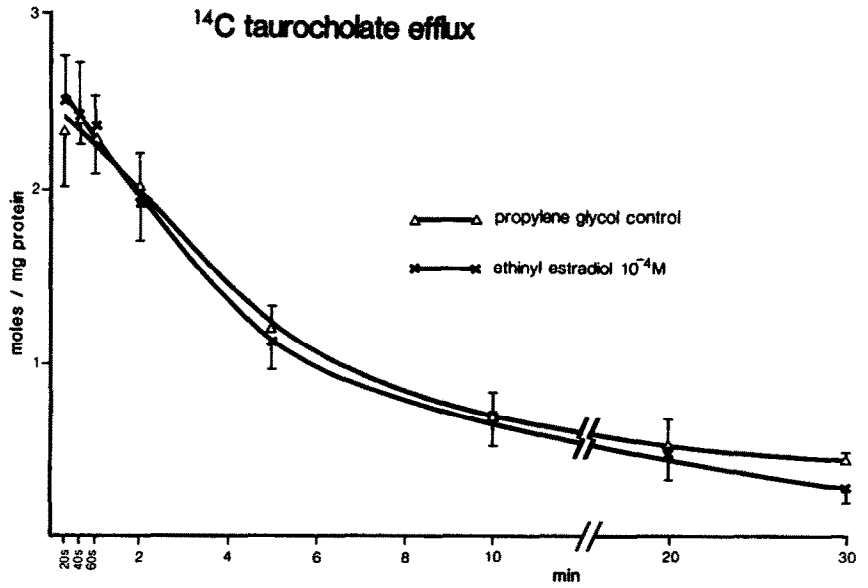

Fig. 6. Effects of ethinyl estradiol on efflux of taurocholate from hepatocytes over 30 min. Symbols represent means and bars the S.E. N = 3.

DISCUSSION

Suspensions of isolated hepatocytes have enjoyed substantial popularity over the last few years as a tool for studying the mechanisms by which various substrates undergo cellular uptake. Some studies have looked at interference with these processes by other chemicals. The advantages of using isolated hepatocytes in such experimental situations have been reviewed [18].

The present study has shown that ethinyl estradiol (or metabolite(s)) can directly inhibit uptake of taurocholate and ouabain by isolated rat hepatocytes, while accumulation of cadmium is unaffected. Further studies using taurocholate showed that V_{max} remains unchanged while K_m is increased in the presence of ethinyl estradiol. Efflux of taurocholate from preloaded hepatocytes was not affected by ethinyl estradiol even at the maximum concentration used.

The effect of ethinyl estradiol on hexose transport was investigated by Madar *et al.* [13], who found that preincubation of hepatocyte suspensions with this drug resulted in a dose dependent increase in uptake of 3–0 methyl-D-glucose. A similar effect was demonstrated in response to 17β-estradiol. It was found that V_{max} increased while K_m remained unchanged. Pretreatment of rats with the estrogen was also found to produce an increase in uptake.

Other steroids have also been investigated for interference with hepatocellular uptake of particular substrates. Schwarz *et al.* [10] found that norethandrolone, 17β-estradiol and progesterone were each able to inhibit taurocholate uptake with 50% inhibition at 18, 48 and 42 μM, respectively. These concentrations are quite similar to those of the present study for ethinyl estradiol. The nature of inhibition, however, was non-competitive, which contrasts with the competitive inhibition displayed by ethinyl estradiol.

Schwarz *et al.* [10] also found little effect on efflux of taurocholate from hepatocytes in response to the above three steroids. Eaton and Klaassen [11] found that estradiol, amongst other steroidal chemicals, inhibited the initial velocity of uptake of ouabain, while mestranol (the 3-methyl ether of ethinyl estradiol) inhibited taurocholate uptake [12]. This again was seen at similar concentrations to those of the present study. Effect on uptake of other substrates under situations that may be associated with biliary dysfunction has been investigated by other authors [19, 20]. Interference with substrate uptake was the predominant finding.

Other studies have utilized hepatocyte suspensions in the study of ethinyl estradiol-induced biliary dysfunction by pretreating the animal with the drug, then isolating the hepatocytes and investigating the handling of substrates by these cells. Tarao and Takamura [8] reported that such treatment resulted in hepatocytes which had a compromised ability to secrete conjugated bile acids. A second report reached similar conclusions [9]. In a study using a similar protocol, Berr *et al.* [7] found that the initial velocity of taurocholate uptake was inhibited by the ethinyl estradiol pretreatment. These authors documented a decrease in V_{max} while K_m remained unchanged. Pretreatment of rats with a different estrogen, estradiol-17β, was not found to cause a decrease in taurocholate uptake but the uptake of estradiol-17β (B-D-glucuronide) was reduced [21].

The mechanism by which ethinyl estradiol causes biliary dysfunction is not clear although changes in membrane Na^+, K^+-ATPase and/or membrane fluidity have been suggested [5, 22, 23]. The results of the present study may well indicate that interference with Na^+, K^+-ATPase is responsible since the uptake of the actively transported substrates, taurocholate and ouabain, was inhibited. Consistent with this, the uptake of cadmium, which moves into the hepatocyte by facilitated and simple diffusion was unaffected. However, the role of Na^+,K^+-ATPase is perhaps questioned on consideration of the observations that the transport of taurocholate is largely sodium dependent [24, 25] while that of ouabain is independent of this cation [11, 26].

The competitive nature of the inhibition found in this study, as demonstrated by similar V_{max} but increased K_m, is consistent with interaction with a common binding site such as the Na^+,K^+-ATPase. However, it is noted that displaying a pattern of competitive inhibition does not rule out a binding at independent sites, if a conformational change has been induced [27]. Thus, the data can also be interpreted as being consistent with a change in membrane fluidity induced by ethinyl estradiol, which would be consistent with the conclusions of Simon *et al.* [28]. It is surprising that the steroids used by Schwarz *et al.* [10] displayed a non-competitive inhibition of uptake which suggests a decrease in binding sites rather than a change in affinity. The indication is therefore that ethinyl estradiol has a different direct mechanism of action to the other steroids.

It is noted that pretreatment of rats with ethinyl estradiol resulted in decreased V_{max} but similar K_m [7], which indicates different effects over longer treatment periods for ethinyl estradiol. This does not preclude a similar direct action for this drug *in vivo*, however. Indeed, there may be a combination of effects via both the longer term mechanism on uptake and efflux and the immediate direct effects in the presence of ethinyl estradiol. This is consistent with the view of Elias and Boyer [29], who suggest that, for a single cholestatic agent, several mechanisms may be operative in the induction of the injury. Investigation of the effects on uptake of ethinyl estradiol added in vitro to hepatocytes isolated from pretreated rats should help to clarify this aspect.

In conclusion, a direct inhibitory action of ethinyl estradiol (or metabolite(s)) on uptake of substrates by hepatocytes has been demonstrated. This in itself may be important in the biliary dysfunction induced by this compound. Taken in conjunction with the previously documented effects by animal pretreatment with ethinyl estradiol, a significant role in bile secretory failure is suggested.

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