microsomal lipid peroxidation (E. G. Mimnaugh, personal communication). Increasing the concentration of VP-16 above 50 μM had no additional inhibitory effect on ascorbate-promoted peroxidation. These preliminary observations suggest that the antioxidant effects of VP-16 may involve either (a) scavenging of lipid or lipid peroxy radicals, and/or (b) chelation of metal ions by the drug or by a metabolite formed by microsomal oxidation. Currently, we are attempting to synthesize the *o*-dihydroxy derivative and evaluate its biological properties.

In summary, incubation of VP-16 with mouse liver microsomes in the presence of NADPH resulted in O-demethylation which was enhanced by phenobarbital-induced microsomes. This O-demethylation may be significant since it would result in the formation of the *o*-dihydroxy derivative of VP-16 which may then be subsequently oxidized to the respective quinone. Such a quinone derivative of VP-16 could bind to critical cellular macromolecules causing cytotoxicity. VP-16 was also a potent dose-dependent inhibitor of both the basal and daunomycin-promoted microsomal lipid peroxidation. By contrast, podophyllotoxin, the parent compound, had very little effect on lipid peroxidation, suggesting that the 4′-OH group of VP-16 was necessary for this activity.

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# Hydrogen bond structure in the glucocorticoid agonist–receptor complex

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The interaction between various steroids and the glucocorticoid receptor is largely determined by van der Waals interactions [1]. In addition, 11β hydroxysteroids form a hydrogen bond with the receptor as we have shown recently [2]. This hydrogen bond appears to have considerable functional significance: its formation may render a conformation of the complex preferable, starting from which it may easily undergo activation. This is probably why most of the optimal glucocorticoids have an 11β-hydroxyl group [3].

The oxygen atom of that hydroxyl may in principle, donate as well as accept, a hydrogen atom when participating in a hydrogen bond. To find out how the hydrogen bond is directed we used in this study two steroids that have 11β-chloro- rather than 11β-hydroxy-substituents and can thus be acceptors, but not donors, in hydrogen bonds.

## *Materials and methods*

*Chemicals, media and buffers.* [1,2$^3$H]Triamcinolone acetonide (26 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, U.K. Dichlorisone and 16α-methyl dichlorisone were donated by the Steroid Reference Collection, Hampstead, London (Curator: Prof. D. N.

Kirk). Tissue culture medium and fetal calf serum were from Gibco, Uxbridge, U.K. All other chemicals were from Reanal (Budapest, Hungary).

The following buffers were used. Buffer H: 0.01 M Tris/HCl, 1.5 mM EDTA, 2 mM dithiothreitol. Buffer D: same as buffer H except that solvent was 99% $^2H_2O$. Activity of buffers H and D was adjusted to pL 7.4 using the correction formulae pL = pH meter reading + 0.3314 $n$ + 0.0766 $n^2$ where $n$ is the $^2H_2O$ fraction of the solvent [4].

*Animals, cell lines and assays.* 'Hunnia hybrid' roosters and male Wistar rats (150–200 g body weight and 6–9 weeks old) were used. Rats were bilaterally adrenalectromized 2–5 days prior to the experiment. Chick thymus and rat liver cytosols were prepared in buffer H or buffer D as described ealier [2]. Methods of determination of the rate constants are published in ref. [5]. In these conditions, about 90% of the high affinity glucocorticoid binding sites was available in chick thymus cytosol.

Fao cell clone [6] was derived from the H4IIEC3 Reuber rat hepatoma cell line and was kindly provided by Dr. M. C. Weiss (Centre de Génétique Moléculaire du C.N.R.S., Gif-sur-Yvette, France). Tyrosine aminotransferase enzyme (TAT: E.C.2.6.1.5.) is inducible in Fao cells by glucocorticoids [6]. Culture conditions have been described previously [6, 7]. Tyrosine aminotransferase, enzyme activity and protein were assayed according to the method of Diamondstone [8] and Lowry *et al.* [9]. One unit of TAT catalyses the formation of 1 μmole of parahydroxyphenylpyruvate/min at 37°.

*Results and discussion*

Hydrogen bond rearrangements can be visualized by use of $^2H_2O$ as a solvent, since the reaction whose rate-limiting step involves formation or decomposition of such a bond would proceed considerably slower in heavy than in ordinary water. We have shown earlier by this method that hydrogen bond rearrangement was rate limiting for decomposition but not for formation of 11β-hydroxysteroid-glucocorticoid receptor complexes [2]. We have also found that neither the 3,20-oxo- nor the 17α,21-hydroxy-groups participated in kinetically relevant hydrogen bonds. Therefore we reasoned that should deuterium isotope effect occur during dissociation of the glucocorticoid receptor-dichlorisone or -16α-methyl dichlorisone complex it would indicate that the 11β-chloro atom of the above steroids participates in a hydrogen bond accepting the hydrogen atom donated by some suitable side chain at the steroid binding site of the receptor. The results are summarized in Table 1. As can be seen, a statistically significant kinetic deuterium isotope effect was observed for both test compounds. Ratios of dissociation rate constants in different solvents were similar for the two steroids and were not very different from that observed in the case of corticosterone ($k_H/k_D$ = 1.82 ref. [2]).

These steroids were not only able to form a kinetically relevant hydrogen bond with the receptor but also behaved like optimal glucocorticoid agonists on the basis of TAT induction using a clonal line of Reuber rat hepatoma cells. Their effect was almost as large as that of dexamethasone, with half maximal doses of about 1 nM for both steroids (Fig. 1). Moreover, none of them could antagonize, in 10-fold excess, the effect of dexamethasone (data not shown). The values of half maximal doses compared well with the dissociation equilibrium constants determined in a cell-free system, using rat liver cytosol (Table 2). Deuterium isotope effect was not studied in this system, since the dissociation rate constant was too small in ordinary water to permit us to measure its decrease in heavy water with the required precision.

In conclusion, we found that dichlorisone and 16α-methyl dichlorisone, two steroids that have 11β-chloro-substituents, formed a kinetically relevant hydrogen bond with the receptor similar to the natural and synthetic glucocorticoid agonists that have 11β hydroxyls. This finding suggests that a 11β substituent of high enough electronegativity is required on the steroid molecule to accept the hydrogen atom donated by a corresponding side chain of the receptor. The hydrogen bond formed between the receptor and dichlorisone or 16α-methyl dichlorisone was functioning properly, since both compounds were optimal glucocorticoids.

Table 1. Deuterium isotope effect on steroid-receptor dissociation*

| Steroid | $k_{diss}$ (per min) × $10^3$ In buffer H | In buffer D | $k_H/k_D$ |
|---|---|---|---|
| Dichlorisone | 2.49 ± 0.25 | 1.47 ± 0.08 | 1.54† |
| 16α-Methyl dichlorisone | 2.50 ± 1.00 | 1.87 ± 0.94 | 1.45‡ |

* In each case, thymus of a single rooster was halved, one half was homogenized in buffer H, the other in buffer D. $k_H$, $k_D$ dissociation rate constants in buffer H, buffer D. Three independent experiments were performed.

† Different from 1.00 by the $t$-test at $p < 0.01$ level.

‡ Different from 1.00 by the $t$-test at $p < 0.10$ level.

Table 2. Kinetic parameters of the steroid–receptor complex*

| Steroid | $k_{ass}$ (per mole/min) × $10^{-5}$ | $k_{dis}$ (per min) × $10^4$ | $K_D$ (nM) |
|---|---|---|---|
| Dichlorisone | 6.9 ± 2.4 | 9.5 ± 1.3 | 1.40 |
| 16α-Methyl dichlorisone | 9.7 ± 1.3 | 6.4 ± 1.6 | 0.66 |

* Rat liver cytosol was used. Three independent experiments were performed for both steroids.

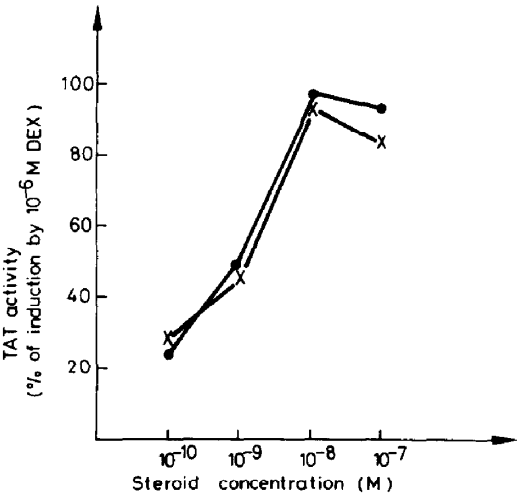


Fig. 1. Induction of TAT by dichlorison (X——X) and 16α-methyl dichlorison (○——○) in Fao cells. Monolayer cultures were exposed to varying concentrations of steroids for 18 hr and harvested for determination of TAT specific activity as previously described [7, 8]. The increase in TAT activity above the basal level was plotted as per cent of the TAT activity induced by $10^{-6}$ M dexamethasone. Sp. act.: 20.65 (basal), 189.75 (induced by $10^{-6}$ M DEX). Concentration of DEX required for half-maximum induction of TAT is: 1 nM. Each point represent the average of 2–4 determinations.

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# Reduced cyclic-AMP responsiveness in the colliculus inferior of audiogenic seizure-prone rats

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A relationship between cyclic AMP (cAMP) and epileptic seizure activity has been demonstrated in a large number of experiments. Accumulation of cAMP has been observed in the brain electric-shock treatment [1, 2] or following administration of several convulsant drugs [3–7]. The concentration of cAMP was also increased in epileptic foci [8]. In addition, it has been found that dibutyryl cAMP injected intracerebrally alters seizure threshold, however, data on this point are contradictory [9–11].

Although it is generally accepted that genetic factors are involved in the development of human epilepsy, the possible role of cAMP in epileptic mechanisms has not been studied in genetically epilepsy-prone animals. Although many neurochemical parameters involved in seizure-prone animals have been extensively investigated [12–14], no data is available on the function of cAMP in this regard. Therefore the aim of this paper was to clarify whether there is any difference between epilepsy-prone and normal animals in their basal and stimulated brain cAMP content. Since stimulation of the cAMP system of the brain during pentylenetetrazol (PTZ)-induced seizures is well described [3, 4], PTZ was used to test the capacity of the cAMP apparatus for increased synthesis.

According to large number of publications audiogenic seizure-prone rats (ASPR) are good objects for examining a genetic disposition to epilepsy [12–14]. The relative neutrality of the cortex temporalis (CT) and the important role of the colliculus inferior (CI) in the development of audiogenic convulsions have also been shown [15, 16]. Therefore basal and stimulated cAMP levels in CT and CI of ASPR and normal rats were determined in these experiments.

## *Methods*

Male CFY rats (180–240 g) were used in the experiments. Separation of ASPR from controls was made in a special