reported in this study improved peak shape, with the resulting chromatogram resembling the characteristics of partition chromatography; all peaks were well defined and symmetrical (Fig. 1).

The accuracy and precision of the HPLC method were determined by the following experiments. Three weights of a placebo of each pharmaceutical formulation to which known quantities of thiothixene hydrochloride had been added were assayed per day for 3 consecutive days. The average percent recoveries of thiothixene were 97.8, 101.4, and 99.1 for the capsule, injection, and solution, respectively (Table I). The estimates of precision (Table II) were obtained using the analysis of variance statistical technique. Ninety-five percent of the individual results will not vary from each other (*i.e.*, from the mean) by more than ±3.8, ±4.3, and ±2.9% for the capsule, injection, and solution, respectively. The standard errors for the average of two injections per sample were ±1.6, ±1.8, and ±1.1% for the capsule, injection, and solution, respectively.

The proposed HPLC method was compared to the compendial paper chromatographic assays (2), and the results obtained (Table III) were in agreement. All samples were within the requirements of NF XIV for thiothixene formulations by the HPLC methodology (2). Because of the speed, accuracy, and precision of the proposed procedure, it represents a viable alternative to present compendial methodology.

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Structural Features and Protective Activity of Dexamethasone and Pregnenolone-16α-carbonitrile

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Abstract □ Pretreatment with dexamethasone acetate or pregnenolone-16α-carbonitrile markedly diminished the pharmacological effect of zoxazolamine in rats. This prophylactic action was associated with significantly decreased plasma drug levels, which, in turn, were correlated with enhanced hepatic drug biotransformation, induced by the steroids. Dexamethasone proved to be more active than pregnenolone-16α-carbonitrile in this respect. The A-ring conformation as well as the distances of O-3–O-11, O-11–O-17, and the O-3–mean plane C-5–C-17 may be key factors in glucocorticoid activity, and the longer the distances, the greater the potency. These characteristics have no bearing on catatoxic activity for which the 16α-substituent appears to be a structural prerequisite.

Keyphrases □ Dexamethasone acetate—structural and conformational features, effect of pretreatment on pharmacological action, plasma concentrations, and liver homogenate metabolism of zoxazolamine, rats □ Pregnenolone-16α-carbonitrile—structural and conformational features, effect of pretreatment on pharmacological action, plasma concentrations, and liver homogenate metabolism of zoxazolamine, rats □ Structure–activity relationships—dexamethasone acetate and pregnenolone-16α-carbonitrile, structural features, prophylactic activity against zoxazolamine, rats □ Glucocorticoids—dexamethasone acetate and pregnenolone-16α-carbonitrile, structural features, prophylactic activity against zoxazolamine, rats □ Zoxazolamine—effect of pretreatment with dexamethasone acetate and pregnenolone-16α-carbonitrile on pharmacological action, plasma concentrations, and liver homogenate metabolism, rats

Numerous compounds induce liver microsomal enzymes and can be divided into three main classes: barbiturates, polycyclic aromatic hydrocarbons, and steroids (1–3). Pregnenolone-16α-carbonitrile (I), a synthetic "catatoxic" steroid devoid of any other known hormonal or pharmacological activity, and dexamethasone acetate, a synthetic glucocorticoid, are potent microsomal enzyme inducers (3). Pretreatment with I or dexamethasone protects experimental animals against numerous toxic agents, mainly *via* the induction of drug-metabolizing enzymes in liver microsomes (catatoxic mechanism). However, the protective effect of some steroids is attributed to their glucocorticoid activity (3, 4).

Because the mechanism of microsomal enzyme induction at the molecular level is still not known and little accurate structural information is available (5), an investigation was conducted on the structure–activity relationships of these and other similarly acting steroids. In this study, the prophylactic activity of I and dexamethasone against zoxazolamine was correlated with plasma drug concentrations and *in vitro* metabolism by the 9000×*g* liver homogenate fraction. Thus, the protective mechanism was demonstrated and the relative potency of these enzyme inducers was established.

In addition, the steroid structure was determined in an effort to elucidate the common structural and conformational prerequisites for their inductive properties. This information should prove useful in the continuing investigations of the structure–activity relationships of similarly acting steroids. Since information on the structure (6–8) and activity (9, 10) of fludrocortisone, cortisone, and 6α-methylprednisolone was available, it is also discussed.

Table I—Effect of Pregnenolone-16α-carbonitrile and Dexamethasone Acetate on Zoxazolamine Paralysis Time, Plasma Concentrations, and *In Vitro* Metabolism[a]

Pretreatment	Paralysis		Zoxazolamine Concentrations in Plasma, μg/ml	Zoxazolamine Hydroxylation	
	Time, min	Reduction, %		Rate, μmoles/g[b]/hr	Increase, %
Pregnenolone-16α-carbonitrile	52 ± 4***[c] (16)	65	25.8 ± 0.9 (6)	44.7 ± 1.8*** (6)	128
Water	150 ± 8 (10)		36.6 ± 1.2[d]*** (8) 21.2 ± 0.6[e] NS (6)	19.6 ± 1.0 (6)	
Dexamethasone acetate	32 ± 3*** (7)	71	20.4 ± 0.9 (7)	60.8 ± 4.3 (6)	210
Water	110 ± 2 (9)		28.8 ± 1.6[d]*** (9) 17.3 ± 0.6[e]** (10)	19.6 ± 1.0***	

[a] In the 9000×*g* supernatant fraction of the liver. [b] Of liver protein. [c] ** = $p < 0.025$. *** = $p < 0.001$. NS = not significant. Figures in parentheses indicate number of rats used. [d] Unrecovered controls. [e] Recovered controls. (The recovered and unrecovered controls are compared with the steroid-pretreated groups).

EXPERIMENTAL

Female Sprague–Dawley rats[1], averaging 100 g (range 95–105 g) and maintained *ad libitum* on food[2] and tap water, were used for the biochemical study.

Study 1—Compound I[3] or dexamethasone acetate[4] was given orally at a dose level of 20 μmoles in 1 ml of water (micronized with a trace of polysorbate 80) twice daily for 3 days. On the 4th day, 18 hr after the last steroid administration, all rats received 10 mg ip of zoxazolamine[5]. Blood was removed from the pretreated animals when they reacquired the righting reflex; it was also taken from an unrecovered control group killed at that moment as well as from a second control group sacrificed at spontaneous recovery (recovered controls). Drug-free plasma from all rats was used for preparing standards and blanks. The plasma zoxazolamine concentrations were determined by the method of Burns *et al.* (11).

Study 2—The rats were treated as in Study 1 and decapitated 18 hr after the last steroid administration. Their livers were immediately removed, weighed, and washed in an ice-cold 1.15% KCl solution. Liver samples of 3 g were homogenized in 9 ml of isotonic potassium chloride containing 0.02 *M* tromethamine (pH 7.4). The homogenate was centrifuged at 9000×*g* for 20 min, and the supernatant fraction was used for studying zoxazolamine metabolism. The entire procedure was conducted at 0–3°.

Zoxazolamine hydroxylation was determined by the method of Juchau *et al.* (12). The incubation medium contained: tromethamine buffer (pH 7.4), 5 m*M* $MgCl_2$, 5 m*M* glucose 6-phosphate[6], 0.4 m*M* NADP[6], homogenate corresponding to 0.220 g of liver, and 1.2 μmoles of zoxazolamine. The duration of incubation was 30 min. The protein content of the homogenate was determined according to a reported method (13).

Table I shows the effects of I and dexamethasone acetate on: (*a*) zoxazolamine-induced paralysis, (*b*) zoxazolamine concentrations in plasma, and (*c*) zoxazolamine hydroxylation *in vitro*.

The important features of the molecular structures of five steroids were compared. These structures were determined by X-ray analysis from three-dimensional diffraction data, and the nonhydrogen atoms were refined with anisotropic thermal parameters. In all cases, the hydrogen atoms were found by difference Fourier syntheses. The structures compared were: I (R = 3.0%) (14), dexamethasone (R = 2.8%) (14), fludrocortisone (R = 4.6%) (6), cortisone (R = 5.8%) (7), and 6α-methylprednisolone (R = 3.6%) (8). The crystallographic R values ($R = \Sigma||F_o| - |F_c|/F_o|$), which measure the agreement between the observed and calculated structure factors, showed that all these structures are good and that the standard deviations are of the order of 0.005 Å for carbon–carbon bond lengths.

[1] Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec, Canada.
[2] Purina Laboratory Chow.
[3] The Upjohn Co.
[4] Schering Corp.
[5] K. and K. Laboratories.
[6] Sigma Chemical Co.

RESULTS AND DISCUSSION

Compound I or dexamethasone pretreatment significantly reduced zoxazolamine paralysis, which was accompanied by markedly lower plasma drug levels in the pretreated groups as compared with the unrecovered controls. This finding has been attributed to increased drug biotransformation. There were no differences between the plasma zoxazolamine concentrations in the recovered controls and I-pretreated animals, since both groups were in the same clinical state. However, dexamethasone-pretreated rats showed higher zoxazolamine levels than the recovered controls. The reason for this apparent discrepancy was explained earlier (9).

Both steroids greatly enhanced zoxazolamine hydroxylation *in vitro* (Table I). They appeared to protect against this toxicant *via* drug-metabolizing enzyme induction in hepatocytes. Dexamethasone seemed to be more active than I with respect to both *in vivo* prophylaxis and *in vitro* zoxazolamine hydroxylation.

Weeks *et al.* (15) postulated a relationship between glucocorticoid activity and A-ring conformation. They found that the more the ring deviates from the C-5–C-17 mean plane, the greater is the anti-inflammatory activity, for example, of cortisone, 6α-fluorocortisol, 6α-methylprednisolone, and fludrocortisone, the latter having the highest activity. It is known (10) that dexamethasone is considerably more potent than fludrocortisone. As shown in Fig. 1 and Table II, while dexamethasone follows the trend established by Weeks *et al.* (15), the great difference between its activity and that of fludrocortisone cannot be accounted for by the small divergence in the distance of O-3 from the mean plane for these compounds.

Although A-ring conformation may be an important determinant in glucocorticoid activity, other factors such as a C-16 substituent and

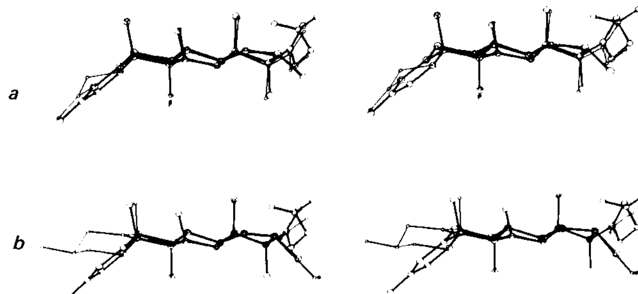

Figure 1—*Projections parallel to the least-squares plane passed through atoms C-5–C-17 inclusive. The molecules of fludrocortisone* (a) *and I* (b) *are successively imposed on those of dexamethasone acetate (large circles).*

Table II—Comparison of Glucocorticoid Activity with Some Features of Steroid Structure (10)

Treatment	Hepatic Glycogen, mg/g	Thymus Weight, g/100 g of Body Weight	O-3—O-11, Å	O-11—O-17, Å	O-3—Mean Plane C-5—C-17, Å
Cortisone	0.67 ± 0.24 NS[a]	0.44 ± 0.003 NS	6.551 (6)[b]	5.195 (6)	1.30
Control water	0.71 ± 0.10	0.48 ± 0.02			
6α-Methylprednisolone	12.30 ± 1.8*	0.24 ± 0.03*	6.593 (4)	5.219 (4)	1.95
Control water	0.71 ± 0.10	0.48 ± 0.02			
Fludrocortisone	11.53 ± 0.33*	0.11 ± 0.01*	6.816 (5)	5.210 (5)	2.43
Control water	0.59 ± 0.10	0.39 ± 0.02			
Dexamethasone acetate	61.99 ± 4.10*	0.08 ± 0.01*	6.822 (5)	5.321 (5)	2.57
Control water	0.59 ± 0.10	0.39 ± 0.02			

[a]NS = not significant. * = $p < 0.005$. [b]Standard deviation.

distances between oxygen functions must also play significant roles. It can be seen in Table II that the oxygen–oxygen distances also follow those of the O-3–mean plane (C-5–C-17). There is actually a better correlation between the O-11–O-17 distances and glucocorticoid activity than with the one just mentioned.

The effect of the oxygen–oxygen distances on the catatoxic activity of I and dexamethasone cannot be very significant since I, which does not have oxygen functions on either C-11 or C-17, is nonetheless a potent catatoxic steroid. Superimposition of the molecule of fludrocortisone on that of dexamethasone (Fig. 1*a*) indicates that C-16 substitution is a decisive factor for strong catatoxic activity. This is further supported by the fact that I (itself a potent agent) and dexamethasone have a C-16 substituent in the α-configuration. Removal of this substituent results in a loss of catatoxic potency.

The present findings established a relationship between A-ring conformation and distances between oxygen functions and glucocorticoid activity. However, such a correlation was not demonstrated for catatoxic activity. Compound I and dexamethasone, both potent catatoxic steroids, have widely differing A-ring conformations (Fig. 1); dexamethasone and fludrocortisone (Table I), with almost identical A-ring conformations, show great differences in catatoxic activity.

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COMMUNICATIONS

Medazepam pKa Determined by Spectrophotometric and Solubility Methods

Keyphrases ◻ Medazepam—pKa determination, spectrophotometric and solubility methods compared ◻ UV spectrophotometry—pKa determination, medazepam, compared to solubility method ◻ Solubility—medazepam, pKa determination, compared to spectrophotometric method ◻ Tranquilizers—medazepam, pKa determination

To the Editor:

The spectral behavior of medazepam shows a marked pH dependence. The peak at 253 nm in acid solutions decreases above pH 3.5 while a new peak appears at 234 nm. The absorptivity and the maximum wavelength of medazepam shift with increasing pH. No variation with pH is observed at the isosbestic point at 243 nm.

All UV absorption spectra were taken at 37° using a spectrophotometer[1] with thermostated cell holders. The pKa was calculated from four series of spectra of pH-varied solutions according to the method of Albert and Serjeant (1) at the wavelengths of 233, 253, and 286 nm (Fig. 1) using:

$$\text{pKa} = \text{pH} - \log\frac{A - B}{B - C} \quad \text{(Eq. 1)}$$

where A is the absorbance of a solution at pH 1.0, B is the absorbance in buffered solution, and C is the absorbance at pH 10.0. The distance $A - B$ is representative for the concentration of the unionized form of the base, whereas $B - C$ is representative for the ionized

[1] Unicam SP 800.