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The Relationship between Glucocorticoid Structure and Effects upon Thymocytes

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SUMMARY

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Glucocorticoid hormones inhibit uridine incorporation into ribonucleic acid of thymocytes. The relationship between this effect and the binding to glucocorticoid receptors in mouse thymocytes was studied in parallel. The 27 different steroids examined could be classified as either agonists (e.g., dexamethasone, hydrocortisone, corticosterone), which bind to thymocytes and have an inhibitory effect on uridine incorporation; antagonists (e.g., 6α, 16α-dimethylprogesterone, progesterone), which bind to receptors without significant effect on uridine incorporation and which inhibit the biochemical effect of agonists; or inactive steroids (e.g., estradiol, cortisone), which neither bind nor have any effect on uridine incorporation. Combined evaluation in mouse thymocytes thus appears to be a useful tool in screening of steroids for glucocorticoid activity.

INTRODUCTION

Glucocorticoids exert a number of inhibitory effects in lymphoid cells from rat and mouse. Such inhibitory effects include a decrease in glucose uptake (1) and a decrease in the uptake of both nucleic acid precursors and amino acids (2), resulting in inhibition of nucleic acid and protein synthesis (3-7).

The currently accepted mechanism of glucocorticoid action follows a pattern which applies to all steroid hormones and includes the following steps: (a) binding of the hormone to high-affinity stereospecific proteins in the cytoplasm of target tissue cells; (b) transfer of the steroid-protein complex to the nucleus, where it binds to chromatin; (c) initiation of DNA-dependent, RNA-mediated protein synthesis; and (d) mediation of the physiological effect by the induced protein (8).

The purpose of the present study was to establish whether, in view of this mechanism of action, it is possible to deduce a correlation between glucocorticoid binding and activity in thymocytes; to determine whether isolated thymocytes constitute a convenient model for the study of structure-activity relationships and for screening of the glucocorticoid activity of steroid hormones; and to define the structural requirements involved in the binding process.

MATERIALS AND METHODS

Materials

[^{3}H]Dexamethasone (28 Ci/mmole) was

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purchased from the Radiochemical Centre, and [^{3}H]uridine (25 Ci/mmole), from C. E. N., Saclay, France. Nonradioactive dexamethasone was obtained from Sigma Chemical Company, and other steroids, from Roussel-Uclaf. The structures of the steroids are shown in Fig. 1. Stock solutions of steroids (3 mM) were prepared in absolute ethanol and stored at 4°. Tris buffer solution (5 mM Tris-HCl, pH 7.4, 133 mM NaCl, 6 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose) was used for binding studies. Protein-free medium used for [^{3}H]uridine incorporation studies was Eagle's minimal essential medium containing Earle's salts (Gibco), supplemented with 2 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Female C57 Bl_6 mice, 7–8 weeks old (15–25 g), were adrenalectomized under pentobarbital anesthesia 4–6 days before use. Thereafter the animals were maintained on normal chow, but with 0.9% NaCl solution to drink in place of tap water.

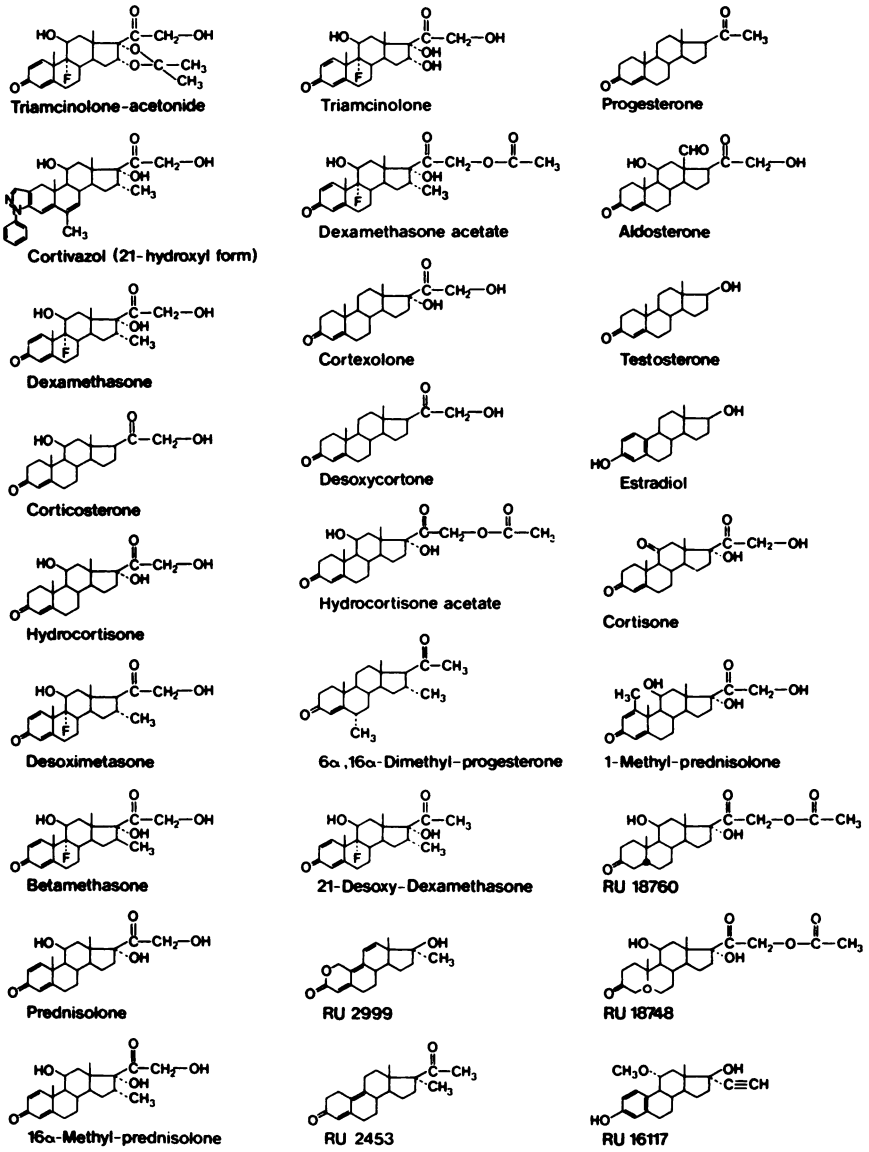

FIG. 1. *Structures of all steroids tested*

Methods

Thymus glands were removed from two to four mice, pooled, minced in Tris buffer solution, and gently homogenized using a Teflon-glass homogenizer. The homogenate was filtered through nylon gauze and subsequently centrifuged at 800 × *g* for 10 min. The pellet was resuspended in 0.84% NH_4Cl in order to disrupt red blood cells. The cell suspension was again centrifuged at 800 × *g* for 10 min, and the pellet was washed once in Tris buffer. Cells were resuspended by vigorous shaking, filtered through nylon gauze, and counted using a Malassez chamber. Cells were prepared at 0–4°, and the initial viability of these thymocytes, estimated by dye exclusion, was over 97%.

Binding studies. Aliquots (600 μl) of the cell suspension, adjusted to 6 × 10^6 cells/ml, were incubated in ice for 2 hr with continuous shaking in the presence of [^{3}H]dexamethasone (final concentration of labeled steroid ranged from 1 to 200 nM).

At the end of incubation, the residual free dexamethasone was separated from bound dexamethasone by centrifugation for 15 sec in an Eppendorf 3200 centrifuge. Supernatants were discarded, and cells were resuspended in 300 μl of steroid-free medium. Aliquots (200 μl) of this suspension were filtered through Whatman GF/A filters and washed three times with 10 ml of cold Tris buffer solution. The radioactivity retained on the filters was counted by liquid scintillation. Each experiment was performed in the absence and presence of 50 μM unlabeled dexamethasone. The residual binding in the presence of unlabeled dexamethasone was considered to be nonspecific binding.

To compare the ability of various steroids to compete with [^{3}H]dexamethasone for binding sites in thymocytes, cells were incubated for 2 hr at 0–4° with a single concentration of [^{3}H]dexamethasone (50 nM, which is slightly higher than the K_d value), alone or in the presence of a 1-, 10-, or 100-fold excess of competing unlabeled steroid. The specific binding of [^{3}H]dexamethasone to thymocytes in the presence of the various competitors was expressed as a percentage of the binding recorded in the absence of competitor.

[^{3}H]Uridine incorporation studies. Inhibition of labeled uridine incorporation into RNA was used as a measure of the effect of glucocorticoids in thymocytes incubated at 37° (9). Cells prepared as described above were adjusted to 6 × 10^6 cells/ml in minimal essential medium. Aliquots (600 μl) of the cell suspension were incubated at 37° for 3 hr in the presence of various steroids. [^{3}H]Uridine (1 μCi) was then added to each sample, which was incubated at 37° for another hour. The viability of these thymocytes, incubated in the presence and absence of dexamethasone, was estimated by dye exclusion to be more than 97%. Samples were chilled and centrifuged (10 min at 3000 × *g*), and the pellets were resuspended in tracer-free medium and precipitated with cold 5% (w/v) trichloracetic acid. Precipitates were collected on Whatman GF/A filters and washed three times with 15 ml of cold trichloracetic acid. Radioactivity was measured by liquid scintillation spectrometry.

RESULTS

Relationship between dexamethasone binding and inhibition of uridine incorporation. In preliminary experiments (not shown), using a single high dose of [^{3}H]dexamethasone (50 nM), we observed that the specific binding of the steroid in mouse thymocytes reached a plateau after 90 min of incubation at 0–4° and after 15–20 min of incubation at 37°. The binding of [^{3}H]dexamethasone to mouse thymocytes after 2 hr of incubation at 0–4°, over a range of [^{3}H]dexamethasone concentrations, is shown in Fig. 2. At 200 nM the concentration of specific binding sites for [^{3}H]dexamethasone was 9.8 ± 0.75 fmoles/10^5 cells (n = 10). [^{3}H]Dexamethasone bound to a single class of sites with a dissociation constant (K_d), calculated from a Scatchard plot (10), of 40 nM. The concentration (R_n) of these glucocorticoids receptors was 10 fmoles/10^6 cells, which corresponds to about 6000 sites/cell. K_d and R_n values measured after 15–20 min of incubation at 37° were not significantly different from those determined at 0–4°.

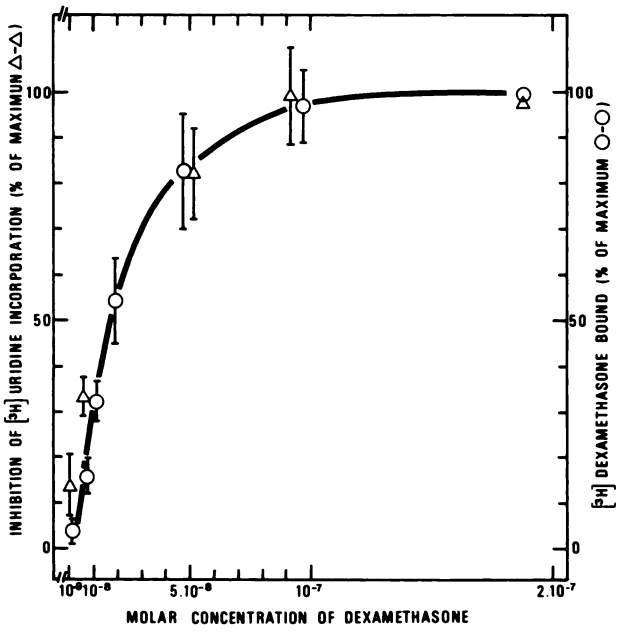

FIG. 2. *Relationship between dexamethasone binding and inhibition of uridine incorporation*

Specific [^{3}H]dexamethasone binding (n = 10) at 0–4° (○——○) was determined as described in *Methods*. At 200 nM [^{3}H]dexamethasone, the specific binding was 9.8 ± 0.75 fmoles bound per 10^6 cells. This value was taken as 100%, and values at other concentrations were expressed as a percentage thereof ± standard error of the mean. The effect of 200 nM unlabeled dexamethasone (n = 9) on [^{3}H]uridine incorporation (▲——▲) was taken as 100% (29.9 ± 3.2% of the incorporation in control samples; 0.78 ± 0.09 pmole of [^{3}H]uridine incorporated per 10^6 cells), and results at other concentrations were expressed as a percentage thereof ± standard error of the mean.

Using a single high dose of dexamethasone (200 nM), we studied the time course of the effect of steroid *in vitro* on incorporation of [^{3}H]uridine into the acid-insoluble fraction of thymocytes (not shown). After a lag period of about 1–1.5 hr after addition of dexamethasone, there was progressive inhibition of [^{3}H]uridine incorporation. In the fourth hour of incubation with steroid, [^{3}H]uridine incorporation into RNA in thymocytes was reduced to 29.9 ± 3.2% (n = 9) of the incorporation in control samples. The data in Fig. 2 show that during the 4-hr incubation, increasing concentrations of dexamethasone produced increasing inhibition of [^{3}H]uridine incorporation, which reached a plateau at 100 nM.

These data establish a relationship between occupancy of binding sites by dexamethasone and inhibition of uridine incorporation. Both binding and inhibition of uridine incorporation increased with increasing concentrations of dexamethasone. The concentration of dexamethasone producing a half-maximal effect on uridine incorporation (ED_{50}) was identical with that necessary for half-maximal binding.

Relationship between binding of other steroids and inhibition of uridine incorporation. The inhibitory effects of various concentrations of dexamethasone, triamcinolone acetonide, hydrocortisone, progesterone, and estradiol on the binding of 50 nM [^{3}H]dexamethasone to mouse thymocytes at 0–4° is shown in Fig. 3. The I_{50} of dexamethasone, i.e., the concentration of unlabeled compound that depressed [^{3}H]dexamethasone binding by 50%, was 67 ± 32 nM. The I_{50} of each steroid was similarly determined, and the potency relative to dexamethasone was calculated from the ratio of the I_{50} values. Relative potencies are indicated in Table 1.

The effects of dexamethasone, triamcinolone acetonide, hydrocortisone, progesterone, and estradiol on [^{3}H]uridine incor-

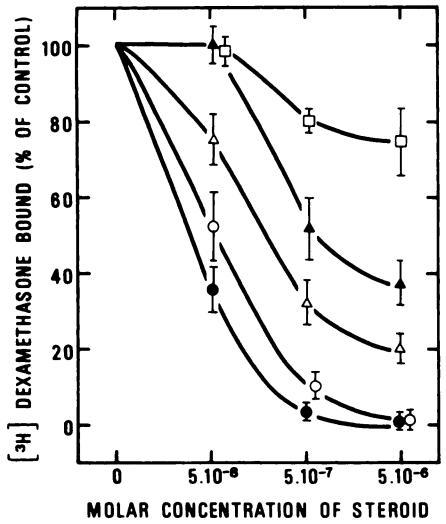

FIG. 3. *Effects of various steroids on specific binding of dexamethasone*

Specific binding of 50 nM [^{3}H]dexamethasone at 0–4° in mouse thymocytes: competition by unlabeled dexamethasone (○——○), triamcinolone acetonide (●——●), hydrocortisone (△——△), progesterone (▲——▲), and estradiol (□——□). The specific binding of [^{3}H]dexamethasone alone (mean value for seven experiments, 6.9 ± 0.5 fmoles bound per 10^6 cells) is plotted as 100%, and the binding of [^{3}H]dexamethasone in the presence of the competitors is shown as a percentage thereof ± standard error of the mean (n = 7).

poration into RNA in thymocytes are shown in Fig. 4. Inhibition of [^{3}H]uridine incorporation increased with increasing concentrations of dexamethasone or triamcinolone acetonide, and reached a plateau at 100 nM. This concentration of both steroids reduced incorporation to 30% of that in control samples. With hydrocortisone, the plateau was obtained 1 μM, and incorporation was also reduced to 30–35% of that in control samples. The concentration producing a half-maximal effect (ED_{50}) on uridine incorporation was determined for each steroid. Relative potencies were 3.06 ± 0.39, 1.00, and 0.33 ± 0.04 for triamcinolone acetonide, dexamethasone, and hydrocortisone, respectively. Thus all three steroids exhibited a good correlation between their relative potencies in inhibiting [^{3}H]dexamethasone binding and [^{3}H]-uridine incorporation into RNA in thymocytes. On the other hand, progesterone and estradiol had a weak effect on [^{3}H]uridine incorporation into RNA, which corresponded to their low affinity for binding sites.

The relative binding affinities of 27 steroids for dexamethasone binding sites and their relative biological effects are given in Table 1. The compounds could be classified in the three categories: (a) agonists, which bind to dexamethasone binding sites and inhibit [^{3}H]uridine incorporation, with a good correlation between binding affinity and relative potency; (b) antagonists, which are able to bind to glucocorticoid binding sites but have no effect on [^{3}H]uridine incorporation, and, in addition (Table 2), can reverse the inhibitory effect of dexamethasone; and (c) inactive steroids, which do not bind to glucocorticoid binding sites and are without effect on [^{3}H]uridine incorporation.

DISCUSSION

The data obtained in the present investigation indicate that mouse thymocytes are target cells for a variety of structurally related steroids, since the high-affinity specific binding of the steroids to these cells may be correlated with a biological response, i.e., [^{3}H]uridine incorporation into RNA.

In the present study dexamethasone was shown to bind to thymocytes with high affinity; K_d and R_n values measured at 0–4° were not significantly different from those determined at 37° (11). However, experiments were conducted at 0–4° in order to measure total, essentially cytoplasmic, binding, since nuclear transfer of the cytoplasmic steroid-receptor complexes takes place mainly above 20–25°.

An extremely good correlation was found between affinity for dexamethasone binding sites and inhibitory effect on [^{3}H]uridine incorporation into RNA for a variety of steroids. These steroids may be classified as agonists. Other steroids, such as 6α,16α-dimethylprogesterone, RU 2999, RU 2453, a highly potent progestin (12), cortexolone, and progesterone, had pronounced affinity for the dexamethasone binding sites, but little or no inhibitory effect on [^{3}H]uridine incorporation. They

TABLE 1

Relative inhibition by various steroids of dexamethasone binding and uridine incorporation in mouse thymocytes

The inhibition of specific [^{3}H]dexamethasone binding by each steroid was determined as described in Fig. 3, and the affinity relative to dexamethasone was calculated from the ratio of the I_{50} values. The effects of the steroids on [^{3}H]uridine incorporation were studied as described in Fig. 4, and the potency relative to dexamethasone was calculated from the ratio of ED_{50} values. Results are the means ± standard errors of seven experiments. For agonists, correlation between affinity for binding sites and potency of biological effect is significant at $p < 0.001$.

Steroid	Relative affinity for binding (dexamethasone = 1)	Relative potency (dexamethasone = 1)
Agonists		
Triamcinolone acetonide	2.53 ± 0.14	3.06 ± 0.39
Cortivazol (21-hydroxyl form)	1.62 ± 0.08	2.15 ± 0.30
Dexamethasone	1.00	1.00
Corticosterone	1.01 ± 0.07	0.92 ± 0.07
Hydrocortisone	0.32 ± 0.04	0.33 ± 0.04
Betamethasone	0.29 ± 0.03	0.32 ± 0.07
Desoximetasone	0.20 ± 0.07	0.30 ± 0.06
21-Desoxydexamethasone	0.14 ± 0.02	0.12 ± 0.02
16α-Methylprednisolone	0.11 ± 0.02	0.32 ± 0.07
Triamcinolone	0.10 ± 0.03	0.17 ± 0.07
Prednisolone	0.08 ± 0.02	0.30 ± 0.05
Dexamethasone acetate	0.08 ± 0.01	0.09 ± 0.02
Desoxycortone	0.08 ± 0.01	0.09 ± 0.03
Antagonists		
6α,16α-Dimethylprogesterone	2.91 ± 0.14	<0.01
RU 2999	0.38 ± 0.07	<0.01
RU 2453	0.25 ± 0.04	<0.01
Cortexolone	0.14 ± 0.02	<0.02
Progesterone	0.12 ± 0.02	<0.01
Inactive		
Aldosterone	0.01	0.02
Testosterone	0.01	0.01
Hydrocortisone acetate	0.01	<0.01
Cortisone	<0.01	<0.01
Estradiol	<0.01	<0.01
1-Methylprednisolone	<0.01	<0.01
RU 18760	<0.01	<0.01
RU 18748	<0.01	<0.01
RU 16117	<0.01	<0.01

have been demonstrated to be antiglucocorticoid hormones. Progesterone has been reported to display antiglucocorticoid activity toward tyrosine aminotransferase in rat hepatoma tissue culture cells (13) and rat thymus cells *in vitro* (14), and cortexolone, in rat thymus cells *in vitro* (1). Moreover, it has been shown that potent androgens (15) such as testosterone and 17α-methyltestosterone are anti-inducers of tyrosine aminotransferase. In our studies the highly potent anabolic androgen RU 2999 (16) was classified with antiglucocorticoid hormones, whereas testosterone was considered inactive, since its binding affinity was considerably lower. The low competitive effect of testosterone is in agreement with the value recorded in rat skeletal muscle (17).

The structural requirements for optimal glucocorticoid binding and inhibition of [^{3}H]uridine incorporation into RNA in mouse thymocytes are in agreement with the characteristics of glucocorticoids: an unsaturated A ring, a ketone in position 3, a hydroxyl group in position 11β, and a 2-carbon side chain in the 17β-position with a keto and a hydroxyl group in positions 20

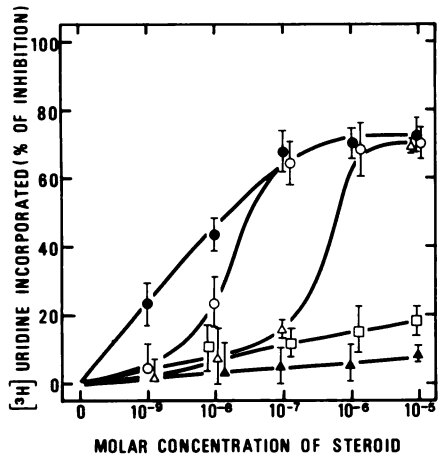

FIG. 4. *Effects of various steroids on uridine incorporation*

Effects of increasing concentrations of dexamethasone (○——○), triamcinolone acetonide (●——●), hydrocortisone (△——△), progesterone (▲——▲), and estradiol (□——□) on [^{3}H]uridine incorporation into RNA in thymocytes at 37°. In the absence of steroid, incorporation was 0.64 ± 0.06 pmole of [^{3}H]uridine incorporated per 10^6 cells. Results are expressed as percentage inhibition of this control value. All points are the means and standard errors of seven experiments.

and 21, respectively. The addition of either a 9α-fluoro group or a 1,2-double bond is known to result in compounds which are even more potent agonists (18). The absence of the 21-hydroxyl group (e.g., 6α,16α-dimethylprogesterone, RU 2453, and progesterone) was found to result in compounds with antiglucocorticoid activity. However, the addition of a 9α-fluoro group and a 1,2-double bond abolished this antagonistic activity and enhanced potency as an agonist, as in 21-desoxymethasone. It was previously reported (19) that cortivazol and other heterocyclic corticosteroids, which have no keto group in position 3, exhibit potent glucocorticoid and anti-inflammatory activities *in vivo*. Our results in mouse thymocytes *in vitro* substantiate these observations and refute the absolute necessity of a ketone in this position for glucocorticoid activity. The caveat to this statement is, of course, that these compounds are not metabolized under the experimental conditions. Acetylation in position 21 led to a decrease in relative

TABLE 2

Effects of antagonists on inhibition of [^{3}H]uridine incorporation into thymocytes by dexamethasone

Mouse thymocytes were incubated with steroids as described in *Methods*. Values are expressed relative to a control incubated without dexamethasone (mean of five experiments, 0.77 ± 0.07 pmoles of [^{3}H]uridine incorporated per 10^6 cells) and are the means and standards errors of five experiments.[a]

Dexamethasone	Inhibition of [^{3}H]uridine incorporation
	%
Alone (50 nM)	40.4 ± 1.7
+6α,16α-Dimethylprogesterone (1 μM)	1.2 ± 2.0
+Progesterone (1 μM)	21.7 ± 2.0
+RU 2453 (1 μM)	23.5 ± 2.2
+RU 2999 (1 μM)	25.4 ± 1.8
+Cortexolone (1 μm)	27.8 ± 1.7

[a] $p < 0.001$ compared with value for 50 nM dexamethasone.

binding affinity and in the inhibition of [^{3}H]uridine incorporation, whether in the case of dexamethasone or hydrocortisone. These acetates, however, are metabolized *in vivo* and exert an activity comparable to that of the nonacetylated derivatives. Moreover, it is well known that cortisone (which has a keto group in position 11) has to be converted to cortisol (which has a hydroxyl group in position 11) in the organism in order to be active (20).

Thus, within the limits of a technique *in vitro*, which cannot take metabolism into account, isolated mouse thymocytes appear to be a useful tool for the rapid screening of steroid glucocorticoid activity.

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