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AN *IN VIVO* STUDY OF THE ACTION OF ANTIGLUCOCORTICOIDS ON THYMUS WEIGHT RATIO, ANTIBODY TITRE AND THE ADRENAL-PITUITARY-HYPOTHALAMUS AXIS

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SUMMARY

Forty-one steroids were assayed for their ability to act as antiglucocorticoids *in vivo* in intact rats using thymus weight, antibody titre and plasma ACTH and corticosterone levels to assess such activity. Progesterone, an antiglucocorticoid extensively studied *in vitro*, demonstrated significant *in vivo* antagonism of endogenous corticosterone by increasing all the measured parameters except thymus weight. Competition studies against exogenous corticosterone using adrenalectomized rats indicated that the increased corticosterone blood levels mask progesterone antagonism on the thymus. Like progesterone, several other compounds including cortexolone, 17α-hydroxyprogesterone, 16α-methylprogesterone and 16α-ethynylprogesterone demonstrated multi-site antiglucocorticoid activity *in vivo*. One previously untested compound, 17α-methylprogesterone demonstrated *in vivo* an ability to significantly elevate antibody titres and increase thymus weight while simultaneously having no effect on ACTH and corticosterone blood levels. The possible therapeutic use of such selective antiglucocorticoids as immunopotentiators is discussed.

INTRODUCTION

Since the discovery of the antiglucocorticoid properties of progesterone on the cortisol mediated inhibition of uridine incorporation into RNA in isolated rat thymocyte suspensions [1, 2] many steroidal compounds have been reported to antagonize glucocorticoid activity in a wide range of *in vitro* test systems [3–6]. Likewise, the mechanism of action of antiglucocorticoids at the cellular, subcellular, and receptor levels has been extensively studied *in vitro* [7–11]. However, attempts to demonstrate antiglucocorticoid activity *in vivo* have been scant and unsuccessful [9, 12, 13].

In this report antiglucocorticoid activity *in vivo* is studied by administering multiple daily doses of antiglucocorticoid compounds to intact prepubertal male Wistar rats for periods of 8 or 16 days. The parameters measured are: aspects of the immune system, thymus weight/body weight ratios and antibody titres raised in response to egg albumin, as well as aspects of the adrenal-pituitary-hypothalamus axis, i.e., plasma ACTH and corticosterone concentrations.

EXPERIMENTAL

Steroids. Testosterone, 17α-ethynylestradiol-17β, 11β-hydroxyprogesterone, 3β-hydroxy-5β-pregnan-20-one and 3β-hydroxy-5α-pregnan-3-one were obtained from Sigma (Si). Estradiol-17β, cortisol, corticosterone, 17α-hydroxyprogesterone, 5α-dihydrocortisol, 5β-dihydrocortisol, 5α-dihydroprogesterone and 5β-dihydroprogesterone were from Schwarz-Mann (S-M), while progesterone was from ICN. Other steroids were from Ikafarm (Ik) or gifts from Ciba-Geigy (Canada) Ltd. (C-G) including 17α-methyltestosterone and Upjohn (Canada) Ltd. (Up) as listed

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Trivial nomenclature: *cortexolone* 17,21-dihydroxy-4-pregnene-3,20-dione (S-M); *cortexone* 21-hydroxy-4-pregnene-3,20-dione (S-M); *dehydrospirocortisol* 11β-hydroxy-4-androstene-3-one-17(R)-spiro-2'-(2',3'-dihydroxol-3'-one); *5β-dihydrocortexolone* 17,21-dihydroxy-5β-pregnane-3,20-dione(Ik); *20α-dihydroprogesterone* 20(S)-hydroxy-4-pregnene-3-one(Si); *20β-dihydroprogesterone* 20(R)-hydroxy-4-pregnene-3-one(Si); *ethisterone* 17α-ethynyl-17β-hydroxy-4-androstene-3-one(C-G); *fluoxymesterone* 9α-fluoro-17α-methyl-11β,17β-dihydroxy-4-androstene-3-one (Up); *21-hydroxymethylprednisolone acetonide* 21-homo-11β,17-dihydroxy-21,22-isopropylidenedioxy-1,4-pregnadiene-3,20-dione; *medroxyprogesterone acetate* 6α-methyl-17-hydroxy-4-pregnene-3,20-dione (17-acetate) (Up); *mestranol* 17α-ethynyl-3-methoxy-17β-hydroxy-1,3,5(10)-estratriene(Si); *methandrostenolone* 17α-methyl-17β-hydroxy-1,4-androstadien-3-one(C-G); *norethindrone* 17α-ethynyl-17β-hydroxy-19-nor-4-androsten-3-one(Si); *norethynodrel* 17α-ethynyl-17β-hydroxy-19-nor-5-(10)-androsten-3-one(Si); *19-nortestosterone* 17β-hydroxy-19-nor-4-androsten-3-one(Si); *17-O-methylethisterone* 17α-ethynyl-17β-methoxy-4-androsten-3-one; *17-O-methylnorethindrone* 17α-ethynyl-17β-methoxy-19-nor-4-androsten-3-one; *sodium cortexolone hemisuccinate* sodium (17,21-dihydroxy-4-pregnene-3,20-dione) 21-succinate; *sodium cortexone hemisuccinate* sodium (21-hydroxy-4-pregnene-3,20-dione) 21-succinate; *sodium corticosterone hemisuccinate* sodium (11β,21-dihydroxy-4-pregnene-3,20-dione) 21-succinate; *sodium cortisol hemisuccinate* sodium (11β,17,21-trihydroxy-4-pregnene-3,20-dione) 21-succinate (S-M); *tetrahydrocortexolone* 3α,17,21-trihydroxy-5β-pregnan-20-one(Ik); *tetrahydrocortisol* 3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one(Si); *tetrahydroprogesterone* 3α-hydroxy-5β-pregnan-20-one(Si).

in the general footnote by the abbreviations bracketed above. All steroids were tested without further purification. Dehydrospirocortisol was prepared in this laboratory by Dr. S. Wang. The following steroids were synthesized by literature methods: 17-O-methylethisterone and 17-O-methylnorethindrone [16], 16α-ethynylprogesterone [17], 17α-methylprogesterone and 17α-ethylprogesterone [18], 21-hydroxymethylprednisolone-21,22-acetonide [19], 4,4-dimethyl-5-pregnene-3,20-dione and 4,4-dimethyl-11β,17,21-trihydroxy-5-pregnene-3,20-dione [20], and 21-hemisuccinates [21].

The structures of the synthesized compounds were confirmed by I.R. and N.M.R. spectroscopy. Steroids for injection were prepared as solutions or fine suspensions in isotonic phosphate buffered saline pH 7.2, containing 6% polyethylene glycol 400 and 3% tween 80. During treatment periods fresh injection preparations were made every 4 days.

Treatment of animals. Prepubertal male Wistar rats weighing 65–70 grams were obtained from Canadian Breeding Laboratories (St. Constant, Quebec) and housed in conditions of regulated light (light 7 a.m.–7 p.m.), with free access to Purina rat chow and water. Adrenalectomized rats were obtained from the same source and maintained on 0.9% saline. After 3 days of acclimatization rats were sorted into groups of equal weight containing 6–10 rats.

Intact studies. Rats were injected subcutaneously on the back with 0.2 ml of injection preparation every 4 h 5 times daily between 7 a.m. and 11 p.m. for either 8 or 16 days, as well as at 3 a.m. on the last day of treatment. In 16 day studies rats were immunized with 1 mg of egg albumin (Sigma: grade V) in 1 ml of pertussis vaccine (Connaught Laboratories: 15,000 million killed bacilli per ml) *via* a single intraperitoneal injection on day 5 of treatment, similar to Crunkhorn [22]. Controls were treated identically except they received the steroid injection vehicle minus the steroid. At the termination of 16 days of treatment, rats were anaesthetized 1.5 h after the last steroid injection by a subcutaneous injection of sodium barbitone (90 mg/rat) and stresslessly killed one at a time 1–2 h later by carbon dioxide asphyxiation. Immediately after death rats were weighed and the thymus dissected free of contaminating tissue, including extrathymic lymph nodes, and weighed wet on a Mettler analytical balance to the nearest mg. Blood was sampled from the right superior vena cava. Equal volumes (1.5–2 ml) from each rat were pooled in heparinized tubes (33 units/ml) for plasma and in nonheparinized tubes for serum, centrifuged at once and refrigerated. The 8 day studies were terminated in an identical manner except that 970 units of sodium heparin was injected along with the barbitone since only plasma was required, and the rats were killed in groups. All rats were killed between 8:30 a.m.–1:00 p.m. when corticosterone [23] and ACTH [24] levels are low and stable. Average thymus weight/body weight ratios and standard deviations were determined for each test group and expressed as a percentage of the control group.

Competition studies. Competition studies were performed over an 8 day period in an identical manner to the intact studies, but using adrenalectomized rats. Here the rats were injected with a preparation that contained the antiglucocorticoid plus sodium corticosterone hemisuccinate in a dose of 7.5 μmol/kg/day. Controls received only the corticosterone. An adrenalectomized group not injected with corticosterone was also run for comparison. Other competition studies were done in an analogous manner using progesterone (60 μmol/kg/day) as the competitor.

Doses. Doses are reported as μmol/kg/day for comparative purposes. In more conventional terms the dose in μg/ml equals μg/rat/day which is the dose (μmol/kg/day) × average rat weight over an eight day study × molecular weight. The average rat weight is necessary as the rats grow from 80–135 gm over 8 days. In 16 day studies it is necessary to increase the number of μg/ml by 50% over days 9–16, as the rats increase in weight to 190 gm.

Antibody titre. The antibody titres of the pooled serum samples were determined by the Farr technique [25]. Ovalbumin was labelled using the iodine monochloride method. The reported antibody binding capacity (ABC) is the number of μg of I^{125}-labelled ovalbumin bound per ml of undiluted serum as calculated from the ABC-33. The ABC was determined for each pool in duplicate and expressed as a percentage of the control group.

ACTH assay. ACTH plasma concentrations of pooled samples were determined using a commercial radioimmunoassay kit (Amersham-ACTH RIA kit). Briefly, the kit measures ACTH extracted from plasma by glass beads versus human ACTH standards using rabbit anti-hACTH serum and I^{125}-hACTHα^{1-24}. Separation of free from bound ACTH was done using dextran coated charcoal. Maximum antigen binding capacity was 35–40% and the standards generated a 6 point saturation type curve between 0–800 pg/ml. Manipulation of volumes allows for a sensitivity as low as 10 pg/ml and an inter-assay standard deviation of 16% over 60 assays is reported by the suppliers. Concentrations were determined in duplicate, reported as pg/ml, and expressed as a percentage of the control group.

Corticosterone assay. Pooled plasma corticosterone concentrations were measured by radioimmunoassay using a commercial rabbit raised antisera (AS-B-159; Bio-RIA, Division of the Institute of Bioendocrinology, Montreal, Quebec) and [1,2-^{3}H]-corticosterone (Amersham-41 Ci/mmol). The corticosterone used for standards was Schwarz-Mann purified by silica gel chromatography and double crystalization. All solvents used were redistilled reagent grades.

Briefly, 2 ml of sample plasma, to which a small amount of [^{3}H]-corticosterone tracer (less than 1%) was added to determine efficiency of extraction, is extracted with 10 ml of methylene dichloride. The

Table 1. Accumulated values for control animals injected with only vehicle

Study type	Number of rats	Body weight (g)	Thymus weight Body weight	Antibody titre (μg/ml)	ACTH (pg/ml)	Corticosterone (μg/dl)
8 Day intact	120	136.3 ± 5%	3.93 ± 5%		1753 ± 23%	0.521 ± 26%
16 Day intact	90	196.2 ± 4%	3.26 ± 5%	3.14 ± 16%	1074 ± 23%	0.608 ± 22%
8 Day adrenalectomized	55	135.2 ± 12%	4.58 ± 5%		11,119 ± 28%	
16 Day adrenalectomized	10	190.1 ± 4%	4.42 ± 9%	15.0 ± 28%	9420 ± 16%	

Values are the mean ± percentage standard deviation. Body weight and thymus weight ratios are the means of the number of rats listed, while the others are the means of several pools of serum or plasma. All of the values from adrenalectomized animals except body weight are significantly different from values from intact animals of the same study length ($P < 0.05$ by two tailed Student's *t*-test).

extract is evaporated under nitrogen and the residue spotted on self-made silica gel PF_{254} (EM Reagents) plates and run in benzene–ethyl acetate (1:3, v/v) or chloroform–acetone (7:3, v/v) to separate corticosterone from larger concentrations of injected steroid. The spot corresponding to corticosterone is extracted with acetone and 50 μl samples evaporated for assay. The assay is done in 250 μl of RIA solution composed of: 1 vial antisera, 60 μl of 2.5% human gamma globulin solution in 0.1 M phosphate buffer pH 7.4, 200,000 c.p.m. [^{3}H]-corticosterone in 100 μl methanol and 10 ml of 0.05 M borate buffer pH 8.0. After a 2 h incubation at room temperature 100 μl of 6% dextran coated charcoal (Amersham) is added, incubated for 10 min at 4°C and centrifuged. 300 μl of the supernate was then counted in a modified Bray's solution. Maximum antigen binding was 70–80% and the standards generated a straight line between 0 and 2000 pg. The results are reported in μg/dl after correction for extraction efficiency and manipulative loss. Each pool was done in duplicate and expressed as a percentage of the control group.

RESULTS

General

The results reported in the figures and tables are presented as a percentage of simultaneously run controls so that results may be easily compared from group to group. The accumulated averages for controls are presented in Table 1. By comparing the adrenalectomized to the intact controls we may clearly set our positive controls: 8 day studies: thymus weight/body weight + 17%, ACTH + 543%, and 16 day studies: thymus weight/body weight + 36%, antibody titre + 378%, and ACTH + 777%. All the adrenalectomized values are significantly different from the intact values. In one experiment (data not shown) sham adrenalectomized animals were compared to intact animals and revealed no significant difference in the measured parameters.

The corticosterone values for the intact animals agree well with those reported by others [26, 27]. However, the ACTH values are much greater than those reported previously using a radioimmunoassay [24]. The higher ACTH values cannot be explained by stress as untreated and unimmunized rats housed for 16 days and killed by decapitation gave ACTH (−19% + 6%), corticosterone (−2% + 2%), and thymus weight/body weight (+7% + 10%) values no different from normally treated and immunized controls. The false high ACTH readings are most likely a fault of the assay method; measuring rat ACTH with a human ACTH immunoassay. However, while the absolute ACTH values may well be incorrect the relative values are still comparable as adrenalectomy increases and corticosterone administration proportionally decreases ACTH values, as seen in Fig. 3.

Studies with progesterone and cortexolone

Since progesterone and cortexolone have been the most often studied compounds *in vitro* [1–15] we have studied them in the most detail *in vivo*. The results of 16 day treatment of intact rats is presented in Fig. 1. At lower doses (less than 200 μmol/kg/day) progesterone shows good *in vivo* antiglucocorticoid activity (to endogenous corticosterone) since antibody titre, ACTH and corticosterone levels are all significantly increased. However the thymus weight ratio was unchanged. Progesterone antiglucocorticoid activity was maximum at 60 μmol/kg/day and decreased with increasing dose giving glucocorticoid activity at 900 μmol/kg/day, when it caused a significant depression of all parameters. Cortexolone was tested as the sodium hemisuccinate and results (Fig. 2) are similar to progesterone except that antiglucocorticoid activity is expressed over a smaller concentration range peaking at 15 μmol/kg/day, while having significant glucocorticoid action at only 600 μmol/kg/day. Similar results were recorded in a more detailed 8 day study (data not shown) for both progesterone and cortexolone. In contrast, adrenalectomized rats studied for 8 days gave no antiglucocorticoid responses but gave a magnified glucocorticoid response at the higher doses (data not shown).

Since progesterone and cortexolone show antiglucocorticoid action in isolated rat thymocyte suspensions *in vitro* [1–3], their failure to increase thymus weight ratios is of special interest. Postulating that the increased corticosterone levels may be blocking

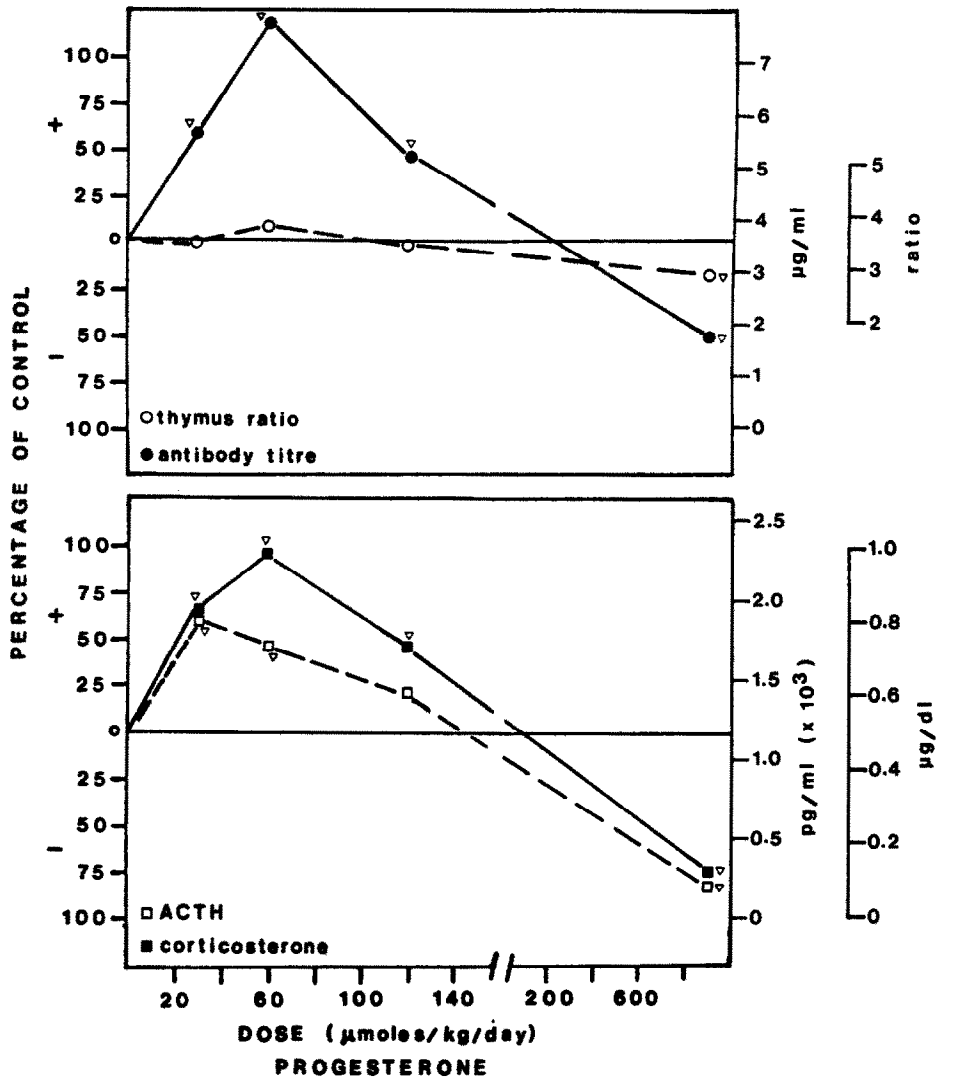

Fig. 1. Effects of progesterone on intact rats over a 16 day period. Points are the means plotted in both absolute and relative terms. Relative values are expressed as a percentage of simultaneously run controls. Thymus ratio is the thymus weight/body weight ratio and is the mean of 6–10 rats, while antibody titre (μg/ml), ACTH (pg/ml), and corticosterone (μg/dl) are the means of duplicate assays of pooled serum or plasma from the 6–10 rats. Standard deviations are not shown but are similar to those listed in Tables 2–8. Points marked with (▽) are significantly different from control ($P < 0.05$ by the two tailed Student's t-test).

significant antiglucocorticoid activity at the thymus, competition studies in physiologically reconstituted adrenalectomized rats were performed. To determine the dose of sodium corticosterone hemisuccinate which would give physiological levels of corticosterone and reduce thymus weight ratios and ACTH to intact values adrenalectomized rats were given increasing doses of sodium corticosterone hemisuccinate for 8 days (Fig. 3) with the result being that 7.5 μmol/kg/day was chosen as the physiological dose. The competition studies run with progesterone and cortexolone versus sodium corticosterone hemisuccinate over an 8 day period are plotted in Figs 4 and 5. The results show that low doses of the antagonists inhibit the physiological dose of corticosterone at both the thymus and pituitary-hypothalamus, as they increased both thymus weight ratios and ACTH. At high doses both compounds again show glucocorticoid activity by depressing thymus weight and ACTH levels.

Studies with other antiglucocorticoids

Numerous other compounds besides progesterone and cortexolone have been shown to be antiglucocorticoids in various *in vitro* systems [1–6]. These compounds as well as other structurally related steroids

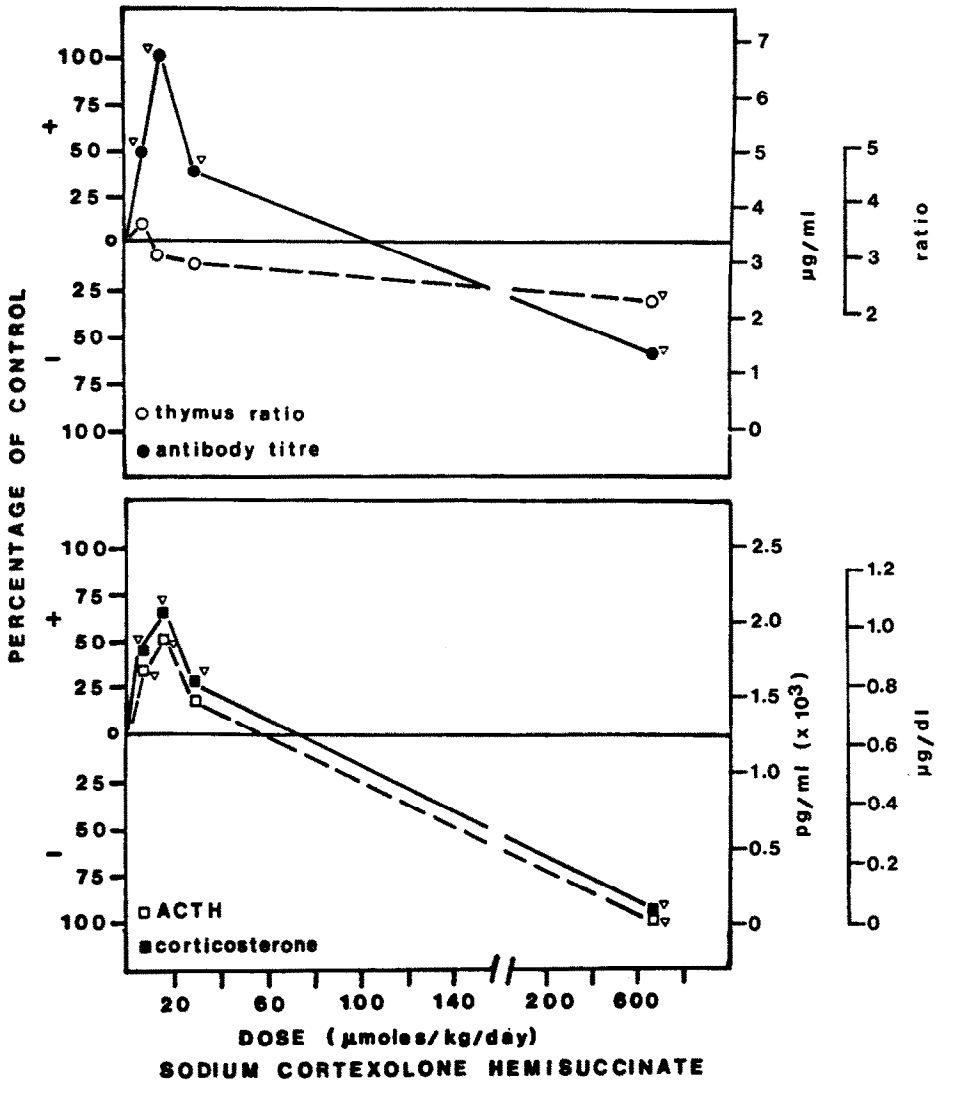

Fig. 2. Effects of sodium cortexolone hemisuccinate on intact rats over a 16 day period. Details as in Fig. 1.

were tested *in vivo* for antiglucocorticoid activity in intact rats for both 8 and 16 days. The results obtained have been tabulated in Tables 2–7 and have been classified into groups according to the activities observed.

Nonthymotrophic antiglucocorticoids (Table 2) show activity similar to progesterone and cortexolone being antiglucocorticoid without causing thymus hypertrophy at low dose, although not always glucocorticoids at high dose. *Glucocorticoids* (Table 3). These are so classified since they cause significant decreases in the measured parameters at low doses. *Inactive* compounds (Table 4) have no significant effect on the measured parameters at the doses tested. *Estrogens and androgens* (Table 5) cause a nonglucocorticoid thymus involution and affect ACTH and corticosterone through an enzymic mechanism. 19-nortestosterone is an exception to this and gives a true antiglucocorticoid effect on the pituitary-hypothalamus. *Synthetic progestins* (Table 6) cause a similar thymus involution but depress ACTH and corticosterone *via* a true glucocorticoid effect at the pituitary-hypothalamus. *Immunopotentiating antiglucocorticoids* (Table 7) are not antiglucocorticoids at the pituitary-hypothalamus, as they do not effect ACTH and corticosterone levels but do cause an increase in antibody titre and thymus weight ratios at low doses.

Antiglucocorticoid competition studies with progesterone

A number of compounds showed unexpected glucocorticoid activity when tested in intact animals. To investigate this further, 8 day competition studies were performed in adrenalectomized rats using the

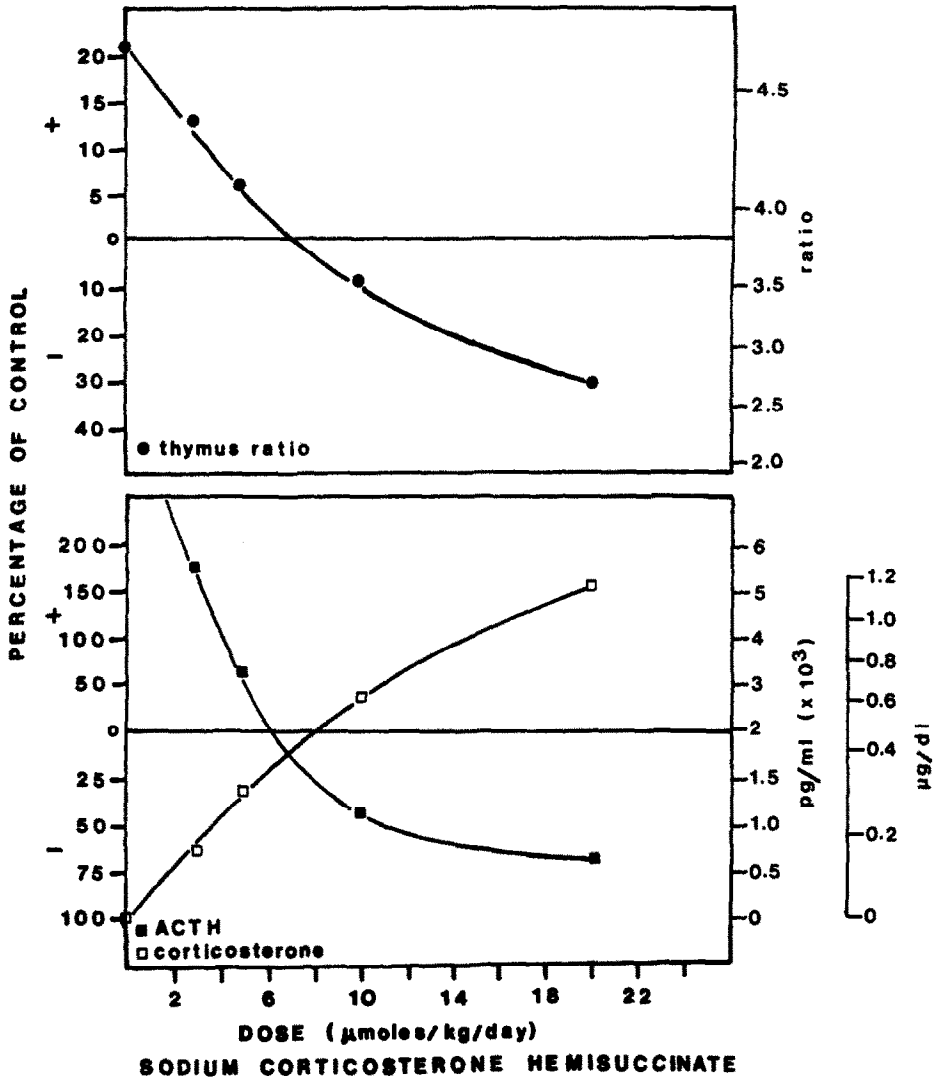

Fig. 3. Administration of sodium corticosterone hemisuccinate to determine its physiological dose. The determination was an 8 day study performed by administering sodium corticosterone hemisuccinate to adrenalectomized rats. Relative results are expressed as a percentage of intact control. Other details as in Fig. 1.

antiglucocorticoid progesterone (60 µmol/kg/day) to antagonize the glucocorticoid effect. In Table 8 progesterone significantly reduced all the glucocorticoid effects of medroxyprogesterone acetate and the glucocorticoid effect of the synthetic progestins on ACTH but did not prevent their thymus involution nor that of estradiol-17β or testosterone.

DISCUSSION

Glucocorticoids can suppress both T-dependent humoral and cellular immune responses in many intact experimental animals, such as mouse, rat and rabbit, as shown by their ability to suppress antibody levels [35], decrease homograft rejection [36] and decrease responses to bacterial infection [37]. Similarly, in certain cases, growth of tumors is facilitated by glucocorticoids [38]. This immunosuppressive effect is believed to be the result of cytolytic [39] or redistributive [40] action on the cells of the lymphoid system, as glucocorticoid administration causes a lymphopenia and a decrease in the size of the lymphoid organs, including the thymus [32, 45].

In contrast to immunosuppression following glucocorticoid treatment, removal of the glucocorticoid producing organs, the adrenal glands, causes a general immunopotentiation. Adrenalectomy substantially shortens the time for skin graft rejection in male mice [41], increases the number of plaque forming cells in the mouse spleen [42], and increases the anti-

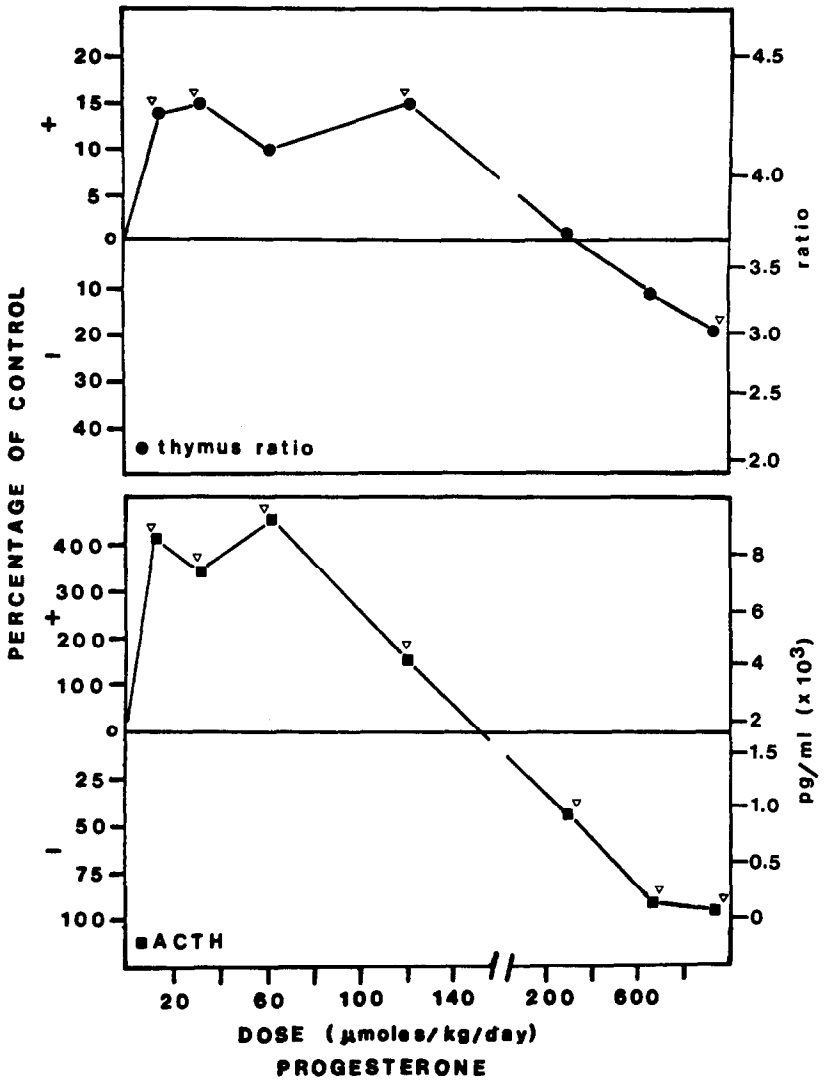

Fig. 4. Effects of progesterone on adrenalectomized rats dosed with sodium corticosterone hemisuccinate (7.5 µmol/kg/day): 8 day competition study. Controls were adrenalectomized rats dosed with sodium corticosterone hemisuccinate (7.5 µmol/kg/day) only. Other details as in Methods and Fig. 1.

body levels in rats [22]. In certain cases adrenalectomy also prevents tumor induction in rats, although the tumor may have some hormone dependence [43]. This immunopotentiation is a result of lymphatic tissue hypertrophy, including the thymus, caused by the removal of endogenous glucocorticoid [44, 45].

Recently a number of steroids relatively devoid of glucocorticoid activity themselves have been shown to be antagonistic to glucocorticoids in a number of *in vitro* test systems which measure both biological activity and receptor affinity [1–11]. One logical clinical use for these agents could be as immunopotentiators which duplicate the effects of adrenalectomy without requiring irreversible surgery. Such an immunopotentiator could have possible therapeutic value in the treatment of malignant and infectious disease. However, antiglucocorticoids have seldom been tested *in vivo* and then only in single dose competition studies. In these earlier studies, adrenalectomized rats were injected intraperitoneally with progesterone dissolved in corn oil in competition with cortisol [13]. This is unfortunate since progesterone is known to be rapidly absorbed from injection sites when administered in oil solutions [29, 30], while cortisol is much more slowly absorbed from aqueous-ethanol solutions [31] which give comparable effects to an oil solution [32]. Furthermore, progesterone is more rapidly removed from the blood (human half-life 15 minutes) than cortisol (human half-life 1.4–3 h) [33], making it unlikely that the progesterone would be present in competitive concentrations at the site of action (thymus or liver) at the same time the

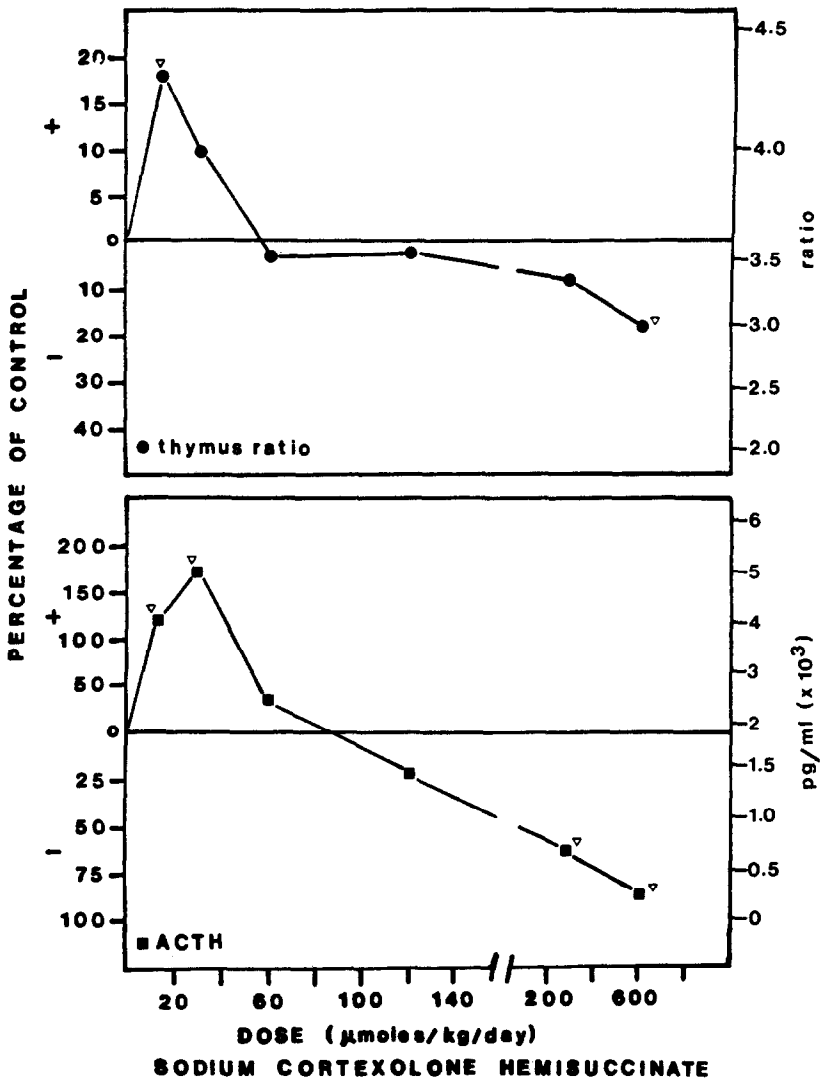

Fig. 5. Effects of sodium cortexolone hemisuccinate on adrenalectomized rats dosed with sodium corticosterone hemisuccinate (7.5 μmol/kg/day): 8 day competition study. Controls were adrenalectomized rats dosed with sodium corticosterone hemisuccinate (7.5 μmol/kg/day) only. Other details as in Methods and Fig. 1.

cortisol was present. Likewise, the dose of progesterone employed was in excess of the antiglucocorticoid range we found for progesterone.

In view of these facts, and in view of possible therapeutic relevance, this study was performed using intact rats in an attempt to antagonize the endogenous corticosterone which is always present at constant (within diurnal rhythm) and known levels. We administered the antiglucocorticoids as an aqueous suspension to decrease the absorption rate and 5 times daily every 4 h in an attempt to maintain fairly constant antiglucocorticoid blood levels. While we did not measure the blood levels obtained, the results seem to indicate that this has at least been partially achieved. Competition studies were done using sodium corticosterone hemisuccinate, since cortisol hemisuccinate has been shown to be more rapidly absorbed from intramuscular depots [34] than the free alcohol [31], making it more likely that the antiglucocorticoid and the corticosterone would be present at the same time at the target organs. The dose of sodium corticosterone hemisuccinate used was such as to give physiological levels of corticosterone, thus making the competition studies more comparable to the intact studies and to possible therapeutic situations. Because of the small glucocorticoid effect caused by single physiological doses of sodium corticosterone hemisuccinate, the competition studies were done with multiple doses as well.

We found that a number of compounds showing antiglucocorticoid activity *in vitro* have antiglucocorticoid activity *in vivo* as well, when tested using the

Table 2. Steroids demonstrating nonthymotrophic antiglucocorticoid activity *in vivo* in intact rats

Compound	Dose (μmol/kg/day)	Thymus weight Body weight (% control)	Antibody titre (% control)	ACTH (% control)	Corticosterone (% control)
17α-Hydroxyprogesterone	25	−1 ± 14	—	+20 ± 20	+15 ± 10
	25	−7 ± 15	+25 ± 14	+35 ± 15*	+28 ± 6*
	50	+2 ± 5	—	+15 ± 6	+39 ± 5*
	600	0 ± 10	—	+10 ± 20	+15 ± 5
16α-Methylprogesterone	25	0 ± 19	+61 ± 14*	+48 ± 2*	+32 ± 6*
	50	+2 ± 12	+107 ± 4*	+59 ± 2*	+50 ± 4*
	100	+6 ± 11	+23 ± 9	+36 ± 19	+39 ± 5*
	800	+12 ± 10	+30 ± 12	−45 ± 10*	−59 ± 16*
16α-Ethynylprogesterone	50	−2 ± 9	+121 ± 6*	+69 ± 8*	+65 ± 4*
4,4-Dimethyl-5-pregnene-3,20-dione	2.5	+4 ± 9	—	+23 ± 3	+23 ± 7*
	6.25	+7 ± 7	—	+58 ± 5*	+68 ± 13*
	12.5	+2 ± 9	—	−26 ± 4*	+9 ± 6
	25	−6 ± 9	—	−26 ± 13*	−17 ± 1*
5α-Dihydrocortisol	12.5	−3 ± 7	—	+19 ± 11	+45 ± 7*
	25	+8 ± 9	—	+10 ± 15	+38 ± 7*
	50	+1 ± 5	—	+26 ± 10*	+59 ± 16*
5β-Dihydrocorticol	25	−4 ± 6	—	+46 ± 13*	+95 ± 2*

Values shown are the means expressed as a percentage of simultaneously run controls ± percentage standard deviation. Thymus weight ratios are the mean of 6–10 rats while the others are the means of duplicate assays of pooled serum or plasma from the 6–10 rats. Those listed with an antibody titre are for 16 day studies, while unimmunized 8 day studies are listed with a (—). Values marked by (*) are significantly different from control ($P < 0.05$ by two tailed Student's *t*-test).

proper protocol. Progesterone, a model *in vitro* antiglucocorticoid, shows good but nonthymotrophic antiglucocorticoid activity *in vivo* in intact animals causing an increase in antibody, ACTH, and corticosterone levels by approximately 100% with maximum activity demonstrated at a dose of 60 μmol/kg/day. In competition studies (Fig. 4) progesterone also increased thymus weight ratios (+15%) indicating that progesterone is an antiglucocorticoid at the thymus *in vivo* but that the increased corticosterone levels caused by antagonism at the pituitary-hypothalamus reduces antiglucocorticoid activity at the thymus to insignificant levels in intact animals. Why antibody titres are not also reduced to insignificant levels may

Table 3. Steroids demonstrating glucocorticoid activity *in vivo* in intact rats

Compound	Dose (μmol/kg/day)	Thymus weight Body weight (% control)	Antibody titre (% control)	ACTH (% control)	Corticosterone (% control)
Sodium corticosterone hemisuccinate	25	−28 ± 13*	—	−73 ± 8*	+180 ± 13*
	25	−41 ± 14*	−48 ± 6*	−87 ± 12*	+160 ± 8*
Sodium Cortisol hemisuccinate	25	−46 ± 13*	—	−71 ± 9*	−67 ± 4*
Sodium cortexone hemisuccinate	6.25	+3 ± 7	—	−16 ± 12	0 ± 1
	12.5	−3 ± 7	−49 ± 19*	−9 ± 15	−16 ± 18
	12.5	+4 ± 8	—	−20 ± 18	−49 ± 18*
	25	+5 ± 11	—	−23 ± 6*	−28 ± 9*
	50	−2 ± 7	—	−50 ± 2*	−43 ± 22*
	300	−23 ± 14*	—	−95 ± 4*	−86 ± 10*
11β-Hydroxyprogesterone	12.5	+3 ± 11	—	−1 ± 13	+15 ± 15
	25	−3 ± 11	—	−45 ± 15*	−36 ± 4*
21-Hydroxymethylprednisolone acetonide	1.25	−1 ± 9	—	−9 ± 7	−33 ± 17
	2.5	+9 ± 11	—	+16 ± 14	−27 ± 11
	2.5	−3 ± 16	−18 ± 22	−26 ± 6	−39 ± 4*
	6.25	+8 ± 6	—	−9 ± 7	−78 ± 3*
	12.5	−6 ± 5	—	−20 ± 19	−61 ± 10*
	25	−15 ± 12*	—	−31 ± 5*	−65 ± 7*
Medroxyprogesterone acetate	0.625	0 ± 11	—	+10 ± 15	+26 ± 20
	25	−29 ± 12*	—	−81 ± 3*	−72 ± 7*
	600†	−93 ± 11*	—	−90 ± 25*	−83 ± 11*
Dehydrospirocortisol	25	−5 ± 14	−39 ± 13*	+2 ± 9	−25 ± 16
	25	0 ± 11	—	+3 ± 4	+17 ± 14

For details see Table 2. † Caused a significant body weight loss and testis atrophy at this dose.

Table 4. Steroids inactive *in vivo* in intact rats

Compound	Dose (μmol/kg/day)	Thymus weight / Body weight (% control)	Antibody titre (% control)	ACTH (% control)	Corticosterone (% control)
5α-Dihydroprogesterone	25	+2 ± 12	—	+25 ± 8	+28 ± 9
5β-Dihydroprogesterone	12.5	+6 ± 13	—	+5 ± 10	+8 ± 11
	25	+8 ± 12	—	+14 ± 14	+4 ± 9
	50	+5 ± 9	—	−18 ± 2	+12 ± 2
	600	−5 ± 5	—	−24 ± 14	−29 ± 18
5β-Dihydrocortexolone	25	+2 ± 4	—	+23 ± 15	+31 ± 15
Tetrahydrocortisol	25	+1 ± 18	−4 ± 22	+5 ± 9	−18 ± 13
Tetrahydrocortexolone	25	0 ± 10	−21 ± 35	+23 ± 14	+19 ± 18
Tetrahydroprogesterone	25	−2 ± 9	+28 ± 16	+2 ± 3	−17 ± 7
3β-Hydroxy-5β-pregnan-20-one	25	0 ± 6	—	+7 ± 4	+21 ± 8
3β-Hydroxy-5α-pregnan-20-one	25	+1 ± 7	—	+17 ± 14	+36 ± 14
	50	−4 ± 17	—	+12 ± 4	+9 ± 4
20α-Dihydroprogesterone	25	+7 ± 18	−11 ± 7	+20 ± 13	−13 ± 15
20β-Dihydroprogesterone	25	+5 ± 8	−13 ± 11	+19 ± 17	−9 ± 5
Sodium (4,4-dimethyl-11β,17,21-trihydroxy-5-pregnene-3,20-dione)-21-succinate	12.5	+8 ± 9	—	+4 ± 5	−7 ± 15
	12.5	−7 ± 16	−19 ± 15	−28 ± 19	−12 ± 10
	25	+6 ± 15	—	+16 ± 3	−9 ± 5
	600†	−9 ± 4	—	−42 ± 17*	−37 ± 5*

For details see Table 2. † Shows glucocorticoid activity on ACTH and corticosterone at this dose.

involve differences in the distribution of progesterone/corticosterone between the thymus and the lymph nodes/spleen (the sites of antibody synthesis) or differences in numbers or types of glucocorticoid receptors in each tissue. The further increases in ACTH (+460%) found in adrenalectomized competition studies in comparison to the intact studies (+60%), also occurs because of the absence of corticosterone feedback.

The maximum antagonistic dose found for progesterone is approximately ten times the dose of sodium corticosterone hemisuccinate needed to restore physiological levels of corticosterone in adrenalectomized rats. These relative doses agree well with those found effective in thymocyte suspensions [1, 2], and in hypothalamic fast feedback antagonism studies done *in vitro* [14, 15]. At higher doses progesterone shows glucocorticoid activity, a fact also supported

Table 5. Estrogens and androgens tested for antiglucocorticoid activity *in vivo* in intact rats

Compound	Dose (μmol/kg/day)	Thymus weight / Body weight (% control)	Antibody titre (% control)	ACTH (% control)	Corticosterone (% control)
Estradiol-17β†	0.0625	−7 ± 8	—	+16 ± 14	+8 ± 2
	0.125	−16 ± 16*	—	+4 ± 25	+7 ± 5
	0.25	−31 ± 15*	—	+8 ± 2	+18 ± 14
	1.25	−26 ± 11*	—	−6 ± 4	+9 ± 6
	2.5	−37 ± 8*	—	−38 ± 4*	+37 ± 4*
	2.5	−45 ± 12*	−50 ± 18*	−30 ± 15*	+29 ± 13*
17α-Ethynylestradiol-17β†	2.5	−45 ± 12*	—	−21 ± 1*	+38 ± 16*
Mestranol†	2.5	−36 ± 8*	—	−14 ± 1*	+38 ± 9*
Testosterone	0.625	−5 ± 13	—	+20 ± 10	+4 ± 10
	1.25	−2 ± 11	—	−6 ± 4	−8 ± 10
	2.5	−3 ± 11	—	+17 ± 11	+3 ± 16
	12.5	−21 ± 15*	—	−12 ± 9	+13 ± 6
	25	−23 ± 19*	—	−49 ± 9*	+24 ± 7*
	25	−30 ± 10*	−45 ± 13*	−33 ± 12*	+18 ± 9*
17α-Methyltestosterone	25	−38 ± 4*	—	−22 ± 10*	+17 ± 10*
Fluoxymesterone	25	−42 ± 10*	—	−45 ± 12*	+20 ± 1*
Methandrostenolone	25	−24 ± 7*	—	−16 ± 6*	+29 ± 10*
	25	−19 ± 12*	+16 ± 17	−30 ± 8*	+65 ± 15*
19-Nortestosterone	2.5	−7 ± 4	—	+20 ± 1*	+12 ± 7
	6.25	−15 ± 4*	—	+55 ± 8*	+65 ± 3*
	12.5	−18 ± 14*	—	+62 ± 3*	+29 ± 1*
	25	−26 ± 10*	—	+74 ± 20*	+35 ± 6*

For details see Table 2. † Caused a significant body weight loss and testis atrophy at these doses.

Table 6. Synthetic progestins tested for antiglucocorticoid activity *in vivo* in intact rats

Compound	Dose (μmol/kg/day)	Thymus weight / Body weight (% control)	Antibody titre (% control)	ACTH (% control)	Corticosterone (% control)
Norethindrone	0.156	+1 ± 7	—	−4 ± 8	+2 ± 10
	0.313	0 ± 4	—	+7 ± 3	+12 ± 10
	0.625	+7 ± 7	—	+25 ± 13	+20 ± 9
	1.25	−12 ± 19	—	−9 ± 1	−10 ± 16
	2.5	−4 ± 14	—	+3 ± 1	+5 ± 3
	12.5	−20 ± 12*	—	−25 ± 9*	−22 ± 11*
	25†	−47 ± 7*	—	−42 ± 22*	−54 ± 6*
	25†	−51 ± 20*	−3 ± 9	−28 ± 11*	−37 ± 9*
Norethynodrel	0.625	+2 ± 9	—	+13 ± 8	+24 ± 11
	25†	−47 ± 13*	—	−65 ± 12*	−50 ± 9*
Ethisterone	0.625	+3 ± 6	—	+11 ± 1	+23 ± 14
	25†	−16 ± 12*	—	−55 ± 10*	−52 ± 8*
	50†	−19 ± 12*	+7 ± 13	−32 ± 15*	−42 ± 16*
	600†	−50 ± 13*	—	−67 ± 13*	−68 ± 18*
17-O-Methylethisterone	25	+7 ± 13	−4 ± 2	−16 ± 24	−8 ± 10
	50	+4 ± 14	−12 ± 8	+31 ± 15*	+45 ± 3*
	100	−9 ± 14	+14 ± 20	+56 ± 5*	+84 ± 6*
17-O-Methylnorethindrone	25	−44 ± 19*	−3 ± 9	+72 ± 3*	+47 ± 8*

For details see Table 2. † Caused a significant body weight loss and testis atrophy at this dose.

Table 7. Steroids demonstrating immunopotentiating antiglucocorticoid activity *in vivo* in intact rats

Compound	Dose (μmol/kg/day)	Thymus weight / Body weight (% control)	Antibody titre (% control)	ACTH (% control)	Corticosterone (% control)
17α-Methylprogesterone	25	+5 ± 8	+66 ± 6*	−26 ± 18	−24 ± 9
	50	+1 ± 8	+114 ± 7*	−29 ± 16	−24 ± 14
	100	+18 ± 11*	+185 ± 10*	−12 ± 8	−25 ± 9
	600	+6 ± 16	+35 ± 6*	−35 ± 2*	−37 ± 9*
17α-Ethylprogesterone	25	−3 ± 15	+43 ± 2*	−14 ± 10	+27 ± 18
	50	+11 ± 18	+50 ± 10*	+4 ± 18	−10 ± 11
	100	−1 ± 11	+48 ± 5*	−6 ± 10	+9 ± 15

For details see Table 2.

by *in vitro* evidence [28, 53, 54], indicating that progesterone is only a partial agonist or a sub-optimal inducer in the nomenclature of Tomkins[4], who offers one possible explanation at the receptor level for the activity of such compounds. This biphasic action of progesterone has also been confirmed by further *in vitro* studies using a glucocorticoid induced thymocite cytolysis assay [46].

The antiglucocorticoid/glucocorticoid biphasic action of progesterone may be a natural physiological action in pregnant animals. In pregnancy peripheral blood levels of progesterone correlate well with our antiglucocorticoid doses, while concentrations in the placental tissue correlate with our higher glucocorticoid doses [47]. This could partially explain the higher ACTH and cortisol levels found in pregnancy [48]. Likewise, it has also been suggested that progesterone may be a physiological immunosuppressant at the placental barrier [53, 54].

Cortexolone gave *in vivo* antiglucocorticoid activity similar to progesterone but over a smaller dose range. This may be because cortexolone was tested as the sodium hemisuccinate which would result in more transient blood levels.

Although progesterone showed good antiglucocorticoid activity *in vivo* it is far from being an ideal immunopotentiating agent since it only increased antibody titre to 33% of adrenalectomized controls and was not at all thymotrophic. Thus other *in vitro* antiglucocorticoids [2–4, 13] and related compounds were tested to investigate structure activity relationships in the hope of finding an ideal immunopotentiator. Compounds listed in Table 2 also have nonthymotrophic antiglucocorticoid activity *in vivo*. The addition of a 17α hydroxy group lowered the antiglucocorticoid responses, while addition of a nonpolar methyl or ethynyl 16α group did not change the antiglucocorticoid activity. 4,4-Dimethylation simply shifts the range of potency. The antiglucocorticoid activity of the dihydrocortisols, as contrasted to progesterone and cortexolone dihydro compounds, may be due to additional receptor binding points at the 11β hydroxyl. A number of compounds showed only glucocorticoid action *in vivo* (Table 3). The glucocorti-

coid effect of compounds similar to medroxyprogesterone actetate have been noted before [13]. The failure of cortexone to show antiglucorticoid activity may be due to the fact that it was tested as the hemisuccinate. Dehydrospirocortisol is interesting since it showed glucocorticoid activity on antibody titre only. Some compounds, chiefly metabolites of cortexolone, progesterone and cortisol showed neither glucocorticoid or antiglucocorticoid activity *in vivo* (Table 4).

Almost all the steroids listed in Tables 5 and 6 cause significant thymus involution at low doses. However, since all of these compounds are estrogen or androgen in structure it seems likely that the thymus involution is a sex hormone response rather than a glucocorticoid action. Although the sex steroids and the glucocorticoids produce the same end result (thymus involution) it is likely that they act through a different mechanism. Glucocorticoids have been shown (by *in vitro* testing) to act directly on the thymus lymphocytes [2, 3, 39], while estradiol-17β and testosterone exert no biological activity on, or binding to receptors from these thymocytes [2, 3, 39]. Similar to their action on isolated rat thymocytes, the sex steroids demonstrated no activity (depression of phytohaemagglutinin response) on, or binding to isolated human peripheral blood lymphocytes, while glucocorticoids do [53–55]. The same occurs when the lymphocytes are separated into T-cells, B-cells, and monocytes [56]. *In vitro* work using isolated rat thymocytes performed in this laboratory [46] has confirmed that estradiol-17β and testosterone have no biological activity (cell lysis) at 10^{-6}–10^{-5} M. Likewise all the other compounds in Tables 5 and 6 were found to be inactive as well. Similarly in this study an antiglucocorticoid dose of progesterone could not prevent the thymus involution caused by estradiol, testosterone, ethisterone, norethindrone, or 19-nortestosterone, but did reverse the involution caused by medroxyprogesterone acetate as detailed in Table 8. Thus, although these compounds cause thymus involution, they do not act directly on the thymus lymphocytes at these doses as do the glucocorticoids. It has been suggested that the sex hormones may act on the epithelial cells of the thymus [49–51] and regulate the synthesis or release of a thymic hormone such as thymosin [52]. These thymic hormones act on thymus lymphocytes as well as peripheral lymphocytes in the lymph nodes, spleen, and blood.

Estradiol-17β and testosterone also affect antibody synthesis *in vivo* as indicated by their lowering of serum antibody titres and the depression of the number of plaque forming cells in the spleen [57–59]. In our study estradiol-17β and testosterone again caused a decrease in antibody titre but neither methandrostenolone nor ethisterone and norethindrone or their 17-O-methylethers did.

While these compounds all cause thymus involution, they differ in their effects on ACTH and corticosterone levels. The estrogen-androgen compounds (Table 5) cause a small increase in corticosterone and a similar decrease in ACTH, an effect which has also been observed by others [26]. This too is not a glucocorticoid action since testosterone and estradiol do not bind to glucocorticoid receptors in the pituitary or hypothalamus [60–61]. The observed values most likely occur because estradiol and testosterone are inhibitors of the adrenal enzyme responsible for the 5α-reduction of corticosterone [62, 63]. This results in increased corticosterone levels and thus depressed ACTH levels *via* feedback on the pituitary-hypothalamus. The synthetic progestins (Table 6) appear to have a true glucocorticoid action at the pituitary-hypothalamus and cause a decrease in ACTH and

Table 8. Progesterone antagonism of some glucocorticoid effects *in vivo*: 8 day competition studies

Compound	Dose (μmol/kg/day)	Thymus weight / Body weight (% adrenalectomized) (control)	ACTH (% adrenalectomized) (control)
Estradiol-17β	2.5	−34 ± 18	−12 ± 16
+ progesterone	60	−27 ± 10	−16 ± 6
Testosterone	25	−25 ± 3	−7 ± 12
+ progesterone	60	−25 ± 15	−20 ± 15
Norethindrone	25	−48 ± 16	−60 ± 10
+ progesterone	60	−53 ± 13	−30 ± 9*
Ethisterone	25	−15 ± 10	−65 ± 12
+ progesterone	60	−13 ± 14	−32 ± 8*
Medroxyprogesterone acetate	25	−40 ± 7	−96 ± 10
+ progesterone	60	−18 ± 10*	−42 ± 12*
19-Nortestosterone	25	−25 ± 9	−8 ± 10
+ progesterone	60	−27 ± 11	−20 ± 12
Sodium cortexolone hemisuccinate	650	−32 ± 6	−95 ± 7
+ progesterone	60	−35 ± 7	−96 ± 20
Progesterone	900	−26 ± 9	−90 ± 9
+ sodium cortexolone hemisuccinate	15	−28 ± 6	−95 ± 12

Data was determined as in Table 2. (*) indicates a significant difference between progesterone treated and corresponding non-progesterone treated control ($P < 0.05$ by two tailed Student's t-test).

corticosterone which can be blocked by progesterone (Table 8). 19-nortestosterone is in a class by itself being antiglucocorticoid at the pituitary-hypothalamus.

One interesting structural feature is observed in the compounds listed in Tables 5 and 6. The addition of a 17α-ethynyl group to both testosterone (to give ethisterone) and to 19-nortestosterone (to give norethindrone) changes the pituitary-hypothalamus action of these compounds without affecting their action on the thymus. In the former, the action is changed from inactive to glucocorticoid and in the latter, from antiglucocorticoid to glucocorticoid. This lead us to speculate that if progesterone could be 17α-ethynylated it might lose its antiglucocorticoid activity at the pituitary-hypothalamus and reveal its latent thymotrophic activity, creating a more effective *in vivo* immunopotentiator.

In view of difficulty in synthesizing 17α-ethynylprogesterone the simple 17-O-methylethers of ethisterone and norethindrone were prepared. While these two compounds showed some antiglucocorticoid activity *in vitro* in isolated thymocytes [46], they retained their sex hormone thymus involution properties while being antiglucocorticoid at the pituitary-hypothalamus and having no effect on antibody titre *in vivo* (Table 6). Finally, 17α-methyl and ethyl progesterone were prepared and tested. The results show that 17α-methylprogesterone has good immunopotentiating antiglucocorticoid activity *in vivo* by significantly increasing both thymus weight ratios and antibody titres to 50% of adrenalectomized controls without affecting ACTH and corticosterone levels at a dose of 100 μmol/kg/day (Table 7). The 17α-ethyl derivative was not as potent as the methyl derivative which is parallel to their activity on thymocytes *in vitro* [46]. At higher doses (800 μmol/kg/day) 17α-methylprogesterone has an increasingly glucocorticoid action causing a significant reduction in ACTH and corticosterone and much smaller increases in thymus weight ratios and antibody titre. With regard to possible clinical applications a dose of 100 μmol/kg/day in rats represents approximately 400–500 mg daily in humans, a large although not unreasonable dose.

Our results also give support to the theory that not all glucocorticoid receptors are identical, but show tissue differences. These tissue differences are inherent in the activity of a number of tested compounds which show different activities on our three test tissues; thymus, lymph nodes/spleen, and pituitary-hypothalamus. Taking into consideration *in vitro* studies as well, the synthetic progestins have respectively: antiglucocorticoid activity, no activity, and glucocorticoid activity. 19-nortestosterone, 17-O-methyl ethisterone, and 17-O-methylnorethindrone respectively: antiglucocorticoid activity, no activity, and antiglucocorticoid activity. 17α-methyl and ethyl progesterone have respectively: antiglucocorticoid, antiglucocorticoid, and no activity. With the exception of 19-nortestosterone all these compounds have nonpolar 17α ethynyl, methyl or ethyl substituents. Such substituents most likely modify the accessibility of the important 17β polar substituents (hydroxyl, O-methyl or acetyl) to receptor binding points. This may also occur with dehydrospirocortisol where the 20-ketone is held in a rigid β-side position by the spiro ring. Dehydrospirocortisol is inactive on the thymus and pituitary-hypothalamus and only causes depression of antibody titres. Steric hindrance of the 17β substituent function by nonpolar 17α groups is supported by evidence that the formation and hydrolysis of the 20-ethylene ketal of corticosterone is greatly impeded by a 17α-methyl group [64]. The different activities of these compounds at the three receptor sites indicates that certain receptors tolerate steric hindrance of the 17β substituents while others do not, revealing at least one characteristic difference between the receptors of the different tissues.

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