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THE ACTIVITY OF DIFFERENT STEROIDS IN PRODUCING THYMIC INVOLUTION

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The assay method for corticotrophin, in which the involution of the thymus of the nestling rat is measured, can be applied successfully as a quick and simple screening test for biologically active corticosteroids. The sensitivity of the test is such that injection of a standard dose of cortisone acetate produces different results when made up in different media, or injected by different routes. A standard procedure for dispensing and injecting the steroids has therefore been adopted. Of the steroids investigated, it is noticeable that those possessing marked thymolytic activity are also steroids with therapeutic properties.

An attempt has been made to investigate the thymus-involuting activity of different steroids in relation to their molecular structure, as part of an investigation of the various biochemical properties of steroids, with particular reference to their effects on lymphoid tissue and circulating antibody.

It is generally accepted that the 11-oxysteroid hormones of the adrenal cortex will induce atrophy of lymphoid tissue in rats (White and Dougherty, 1946; Wells and Kendall, 1940; Ingle, 1950). It has been shown (Shewell, 1955; Shewell and Long, 1956) that animal species may be divided into two broad groups by their response to cortisone acetate. Species termed "cortisone-resistant" can withstand prolonged administration of cortisone acetate without losing body weight. Experimental animals, which, like man, are cortisone-resistant, are the guinea-pig and Rhesus monkey. Cortisone-sensitive animals, on the other hand, lose weight and undergo marked muscle wasting when cortisone acetate is administered: the rat, mouse, rabbit and ferret are cortisone-sensitive species. Although a certain degree of atrophy of lymphoid tissue is observed in cortisone-resistant species with cortisone administration, the effect in these species is not nearly as pronounced as that induced in cortisone-sensitive species. It was therefore decided to investigate the activity of steroids related to cortisone in producing involution of lymphoid tissue in the experimental animal found to be most sensitive to cortisone administration, namely, the rat. The thymus gland of the nestling rat was chosen as the most convenient organ to study, as representing mesenchymal tissue in general.

METHODS

Lymphoid tissue involution was measured by studying thymic atrophy. The thymus gland is readily removed by dissection, and consists almost entirely of lymphoid tissue: the sensitivity of its response to the administration of corticosteroids is much greater than that of the spleen (Fig. 1), a difference probably

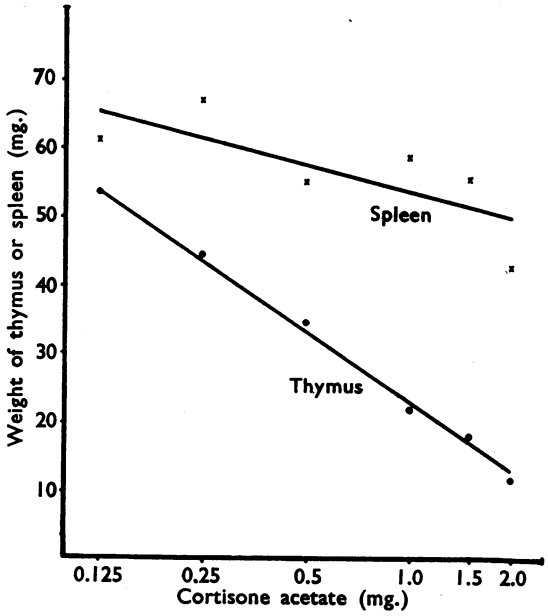


FIG. 1.—Log./dose response of spleen weight and thymus weight to cortisone acetate. Ordinates, weight of spleen and thymus in mg. Abscissae, dose of cortisone acetate on log. scale. Regressions fitted according to the method of least squares. The thymus provides a more accurate and sensitive index of the response of lymphoid tissue to the administration of cortisone acetate than does the spleen.

due to the fact that, among its other functions, the spleen is a blood-depôt. The method used was an adaptation of that developed by Bruce, Parkes, and Perry (1952) for the assay of corticotrophin in nestling rats. Animal variation was reduced by using litter-mate nestling rats of the hooded strain approximately 10 days old, weighing initially 12 to 15 g. Balanced groups of 8 rats were made up from 8 litters, so that one rat in each litter received one of the different treatments available, while at the same time the total weight of nestling rats in each treatment group was the same. The allocation of different treatments within the litter was made at random. One rat served as an untreated control, one as a saline-injected control, and six received doses of the unknown steroid and the laboratory standard steroid, cortisone acetate. The results were analysed as a (3+3) assay, plotting absolute thymus weight (mg.) against log. dose.

The nestling rats were marked with a colour for the litter, and a combination of site of colour and tail-mark for the individual members of the litter. For the next 3 days they were weighed and injected at the same time in each 24 hr. period, and on the fourth day they were weighed, and killed with chloroform. The thymus and spleen from each rat were dissected out and weighed fresh on a torsion balance. Since the administration of certain corticosteroids is known to produce increased glycogen deposition, with liver hypertrophy, the livers were also dissected out and weighed. This was done by clamping off the inferior vena cava with a Spencer-Wells forceps, and freeing the liver from its attachments without tearing the surface. The liver was gently blotted on gauze, and weighed fresh on a torsion balance. By standardizing the dissecting technique it was hoped to minimize errors in liver weight due to bleeding.

All steroid preparations were made up as saline suspensions, and 0.1 ml. of the different dilutions was injected under the loose skin in the rat's neck. A fine gauge (No. 18) needle was always used, to prevent leakage from the injection site. Cortisone acetate (Merck) was used as a laboratory standard. Preliminary studies of the effect of varying injection site and suspending vehicle had emphasized the necessity of comparing steroids under standard conditions. The estimations of comparative potencies in this paper refer to saline suspensions of the acetate injected subcutaneously as described above.

Results

When cortisone (Kendall's Compound E) and hydrocortisone (Compound F) were compared for their effects on thymus and liver weight, both as the alcohols and as acetates, it was found that neither of the alcohols was as effective in inducing either thymus atrophy or liver hypertrophy as was the acetate (Fig. 2). This was surprising, since clinical evidence (Boland, 1952) suggested that F-acetate was less effective in the treatment of rheumatoid arthritis than F-alcohol, and Porter

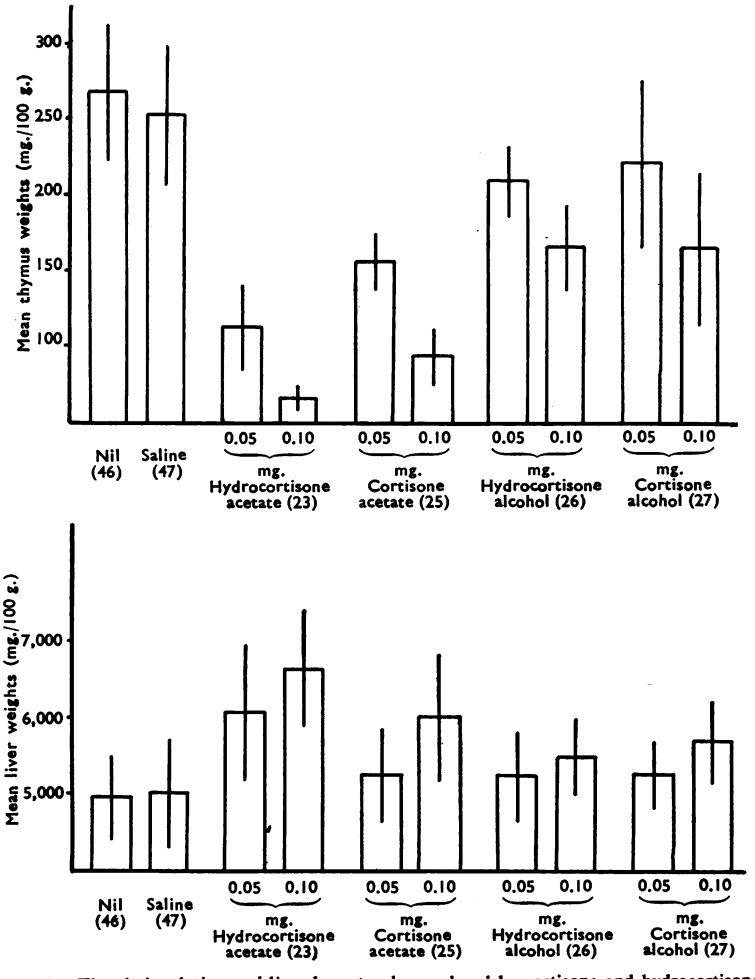


FIG. 2.—Thymic involution and liver hypertrophy produced by cortisone and hydrocortisone as the free alcohols and as acetates. The numerals in brackets give the number of rats in each group. The vertical bars represent the standard deviation from the mean. Both cortisone and hydrocortisone are more effective in inducing thymic atrophy and liver hypertrophy when given as acetates than when given as free alcohols.

and Silber (1953) found the alcohol to be more effective in promoting liver glycogen storage in the rat than hydrocortisone acetate.

It was thought that the relatively poor performance on the part of the alcohols might be due to differential absorption rates: if the alcohols were absorbed and eliminated very rapidly, the lasting concentration of steroids remaining in the blood would be too low to affect the thymus maximally. To delay absorption, therefore, cortisone alcohol and cortisone acetate were ground up in a beeswax-arachis oil mixture (the medium recommended by Bruce *et al.* (1952) to delay the absorption of corticotrophin) and injected subcutaneously into the neck as a 0.1% suspension. When the degrees of thymic involution and liver hypertrophy produced with this suspending agent are compared with those of the saline-suspended alcohol, a marked enhancement of activity occurred. Conversely, cortisone acetate which is absorbed at an optimal rate from a saline suspension is practically ineffective when made up in beeswax-arachis oil (Fig. 3).

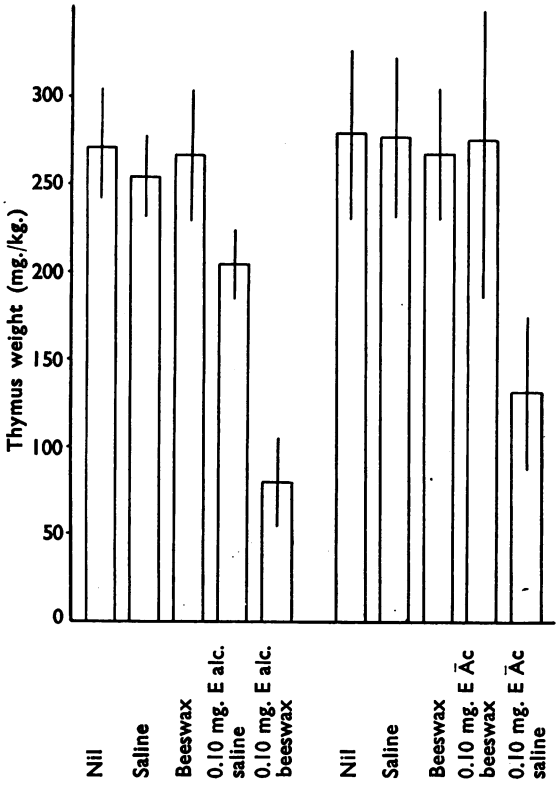


FIG. 3.—The effect of varying the suspending medium on thymic involution. The vertical bars represent the standard deviation from the mean. Cortisone (E) alcohol has only a small effect on thymus weight when made up in saline, while the same dose in beeswax-arachis oil produces much greater involution. On the other hand, cortisone acetate is effective in saline suspension, but has no effect on thymus weight when given in beeswax-arachis oil.

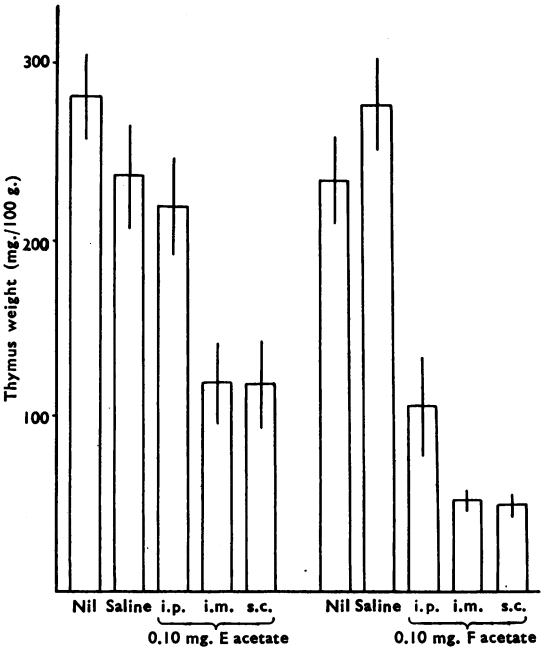


FIG. 4.—The effect of varying the injection route on thymic involution. The vertical bars represent the standard deviation from the mean. Cortisone (E) acetate and hydrocortisone (F) acetate are both less effective in producing thymic involution when injected intraperitoneally than when given either intramuscularly or subcutaneously. i.p., intraperitoneal; i.m., intramuscular; s.c., subcutaneous.

Differences in potency beween saline-suspended preparations when injected into different sites were also demonstrated. Cortisone acetate 0.1% is least effective in inducing thymic atrophy and liver hypertrophy when it is given intraperitoneally: slower absorption from a subcutaneous or intramuscular site results in greater effects on lymphatic tissue (Fig. 4).

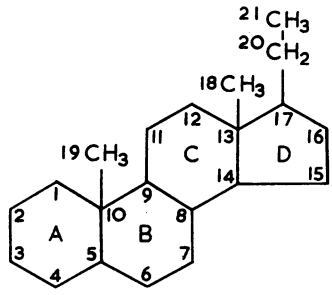
These variations in effect produced by altering either the suspending vehicle or the injection site confirmed the importance of comparing all steroids to be tested under standard conditions. The relative potencies of the different steroids tested in producing thymic atrophy, compared with the standard preparation, cortisone acetate, are set out in Table I.

It will be seen that, as has previously been stated by Dougherty (1952), the criteria for thymolytic activity in the steroid molecule are the same as those for glucocorticoid activity, namely a double

TABLE I

THE RELATIVE POTENCIES OF DIFFERENT STEROIDS IN PRODUCING THYMIC INVOLUTION IN THE NESTLING RAT, AS COMPARED WITH CORTISONE ACETATE

All the steroids were tested as acetate preparations in saline suspension



Pregnane

Trivial Name	Systematic Name	Thymolytic Activity	Potency in Terms of Cortisone	5% Confidence Limits
Cortisone Kendall's Compound E	Pregn-4-ene-17α: 21-diol-3: 11: 20-trione	+	1	—
Hydrocortisone Kendall's Compound F	Pregn-4-11β: 17α: 21-triol-3: 20-dione	+	2·4	1·9– 3·0
9α-Chlorohydrocortisone ..	9α-Chloropregn-4-ene-11β: 17α: 21-triol-3: 20-dione	+	3·4	1·8– 6·3
9α-Fluorohydrocortisone ..	9α-Fluoropregn-4-ene-11β: 17α: 21-triol-3: 20-dione	+	5·3	4·2– 6·5
Prednisone	Pregna-1: 4-diene-17α: 21-diol-3: 11: 20-trione	+	9·9	6·6–14·6
Prednisolone	Pregna-1: 4-diene-11β: 17α: 21-triol-3: 20-dione	+	10·6	Calculated from assay against 9α-fluorohydrocortisone
9α-Fluoroprednisolone ..	9α-Fluoropregna-1: 4-diene-11β: 17α: 21-triol-3: 20-dione	+	13·2	9·8–17·0
*epi*Hydrocortisone	Pregn-4-ene-11α: 17α: 21-triol-3: 20-dione	—	—	—
Dihydro*allo*cortisone ..	*allo*Pregnane-17α: 21-diol-3: 11: 20-trione	—	—	—
Tetrahydrocortisone ..	Pregnane-3α:17α: 21-triol-11: 20-dione	—	—	—
Corticosterone Kendall's Compound B	Pregn-4-ene-11β: 21-diol-3: 20-dione	+	0·2	0·2–0·3
11-Dehydrocorticosterone Kendall's Compound A	Pregn-4-en-21-ol-3: 11: 20-trione	+	0·2	—
Reichstein's Substance S ..	Pregn-4-ene-17α: 21-diol-3: 20-dione	—	—	—
Pregnenetriolone	Pregn-4-ene-17α: 20β: 21-triol-3-one	—	—	—
Reichstein's Substance L ..	*allo*Pregnane-3β: 17α-diol-20-one	—	—	—
DOCA	Pregn-4-en-21-ol-3: 20-dione	—	—	—

bond at $C_{(4)}$, an oxo-group at $C_{(3)}$, and either an oxo-group or a β-hydroxyl group at $C_{(11)}$. Thus "*epi*-hydrocortisone," where the 11-hydroxyl group is in the α- and not in the β-position, has no effect on thymus weight; and both dihydro*allo*cortisone and tetrahydrocortisone, with no double bond at $C_{(4)}$, are inactive.

On the other hand, replacement of the hydrogen atom at $C_{(9)}$ by an α-halogen atom produced a molecule of greater thymolytic activity, together with increased ability to promote liver hypertrophy. 9α-Chlorohydrocortisone acetate is 3.4 times as active as cortisone acetate in causing thymus atrophy (Table I), and 4.8 times as active in promoting liver hypertrophy (Callow, Lloyd, and Long, 1954). An extra double bond in the A ring of the cortisone molecule, as in prednisone or prednisolone, increases thymolytic activity to 10 times that of the cortisone molecule. When the additional 1:2-double bond is combined with an α-fluorine atom at $C_{(9)}$, as in 9α-fluoroprednisolone, a molecule is produced with 13.2 times the thymolytic activity of cortisone acetate. This is not significantly greater, however, than the relative potencies of 10.6 and 9.9 shown by the unhalogenated prednisone and prednisolone molecules, so that there is no suggestion of any synergism between the thymolytic-activity-enhancing effects of the 1:2-double bond and the 9α-halogen atoms.

Some steroid compounds with one less oxygen atom in the molecule also exhibit thymolytic activity, although to a much lesser extent. Thus corticosterone, Kendall's Compound B, has approximately one quarter of the potency of cortisone acetate in producing thymus involution. Kendall's Compound A is indistinguishable from Compound B by this test.

The importance of the $C_{(11)}$ oxo- or β-hydroxyl-group is shown by the fact that neither Reich-

stein's Substances S or L, nor deoxycorticosterone acetate (DOCA), none of which has an oxygen atom at $C_{(11)}$, had any effect upon thymus weight.

Discussion

The thymus is a lymphoid tissue organ particularly susceptible to hormonal-induced atrophy (Weaver, 1955) and was therefore considered a suitably sensitive organ to investigate. A parallel investigation was carried out, through the courtesy of Professor G. R. Cameron, F.R.S., by Dr. V. Udall of the Department of Morbid Anatomy, University College Hospital Medical School, to ensure that it was the lymphoid tissue elements of the thymus that were affected by cortisone acetate, chosen as the standard laboratory steroid. Udall (1955) showed that thymus involution produced by cortisone acetate was apparently due to actual cell damage. Although he found no change in the number of cells in mitosis, the thymocyte count was significantly decreased, and the stem cell count significantly increased; he therefore suggested that, in addition to damaging the thymocytes, cortisone prevented the maturation of the stem cells.

In investigating experimentally-induced thymus atrophy, any possibility that experimental stress, resulting in an increased liberation of adrenocortical hormones, is producing thymic atrophy (Selye, 1950) must, of course, be ruled out. Dougherty and Santisteban overcame this difficulty (Santisteban, 1953; Santisteban and Dougherty, 1954) by studying "lympholytic" agents on the thymus of the adrenalectomized mouse. Their method involves surgery, which has two disadvantages: the number of animals in each experimental group has of necessity to be limited, and surgical procedures themselves stress the animals, so that thymus atrophy is measured after an earlier stress, unknown in extent, has been induced. It was found simpler to adapt the method of measuring thymus involution in the intact nestling rat developed by Bruce *et al.* (1952) for the assay of corticotrophin. This method utilizes Jailer's (1950) observations that the stress mechanism is not completely developed in the young rat, and Bruce *et al.* showed that any stress caused by handling and injecting young rats of up to 10 to 12 days old was insufficient to result in thymus atrophy. Thymic atrophy following injection of any steroid into the intact nestling rat may therefore be regarded as due to that steroid. Stephenson (1954, 1956) has used the thymus involution of 23-day-old weanling rats to compare the relative potencies of some adrenal steroids. While weanling rats have been used successfully in corticotrophin assays, it is felt that for comparing the action of different steroids the younger rat, with an incompletely developed stress mechanism, and with no question of sex difference affecting response, is to be preferred.

The vehicle in which the different steroid preparations are dispensed has been shown to affect the potency of the effect produced on the thymus. Stephenson (1954) considered that the rate of absorption of an 11-oxycorticosteroid from the injection site was inversely proportional to the degree of thymic involution produced, since hydrocortisone acetate given twice daily for three days to weanling rats was more effective in a sesame oil suspending medium than when made up in a 10% ethyl alcohol-saline mixture. This is, however, an over-simplification, since the absorption of cortisone acetate from a beeswax-arachis oil mixture is so slow that not maximal, but negligible, thymic involution takes place.

Similarly, alteration of the injection site, which also alters the rate of absorption of the steroid, is reflected in different potencies. The small degree of thymic atrophy produced by the intraperitoneal injection of cortisone acetate is in accordance with the results obtained by Greenspan, Gifford and Deming (1953), who were unable to influence thymus and adrenal weight in adult rats with intraperitoneal injections of cortisone acetate. They concluded that absorption and excretion had taken place too quickly to allow of any effect. An alternative explanation is that the steroids injected into the peritoneal cavity had been directly absorbed by the liver, and broken down there.

Among the steroids isolated from natural sources, the essential requirements for thymolytic activity are fulfilled only by cortisone, hydrocortisone, corticosterone and 11-dehydrocorticosterone (Kendall's Compounds E, F, B, and A). Of these, marked thymolytic activity is exhibited only by cortisone and hydrocortisone, which also are the only naturally occurring steroids to depress sensitivity to tuberculin in the guinea-pig (Long and Spensley, 1954) and to have any therapeutic effect in man in the treatment of rheumatoid arthritis. Any modification of the structure of the cortisone molecule, other than halogenation at $C_{(9)}$ or the introduction of a 1:2- double bond, results in a loss of thymolytic activity. Thus Kendall's Compounds B and A, with no hydroxyl group at $C_{(17)}$, but still with an oxo group at $C_{(11)}$, retain about one quarter of the thymolytic activity of cortisone. Flux (1954) has claimed thymolytic activity for Reichstein's Substance S. Studying

the effect of corticosteroids on the growth of mammary glands in mice, he weighed the thymuses from ovariectomized mice treated with different steroids and concluded that 2.0 mg. Substance S given daily to an 18 g. CHI mouse produced thymic atrophy. Thymic atrophy in an adult mouse with an intact pituitary-adrenal axis could quite possibly be an effect of stress due to injection technique. Since no involution was observed in the intact "unstressable" nestling rat, it seems most probable that Substance S has no thymolytic activity. No effect on thymus weight was observed with either Reichstein's Substance L or DOCA, although Santisteban and Dougherty (1954) state that deoxycorticosterone increased thymus size in the adrenalectomized mouse, when doses of 0.025 to 0.50 mg. were administered as the glucoside. Under the standardized conditions of the nestling rat experiments just described, doses of up to 0.20 mg./0.1 ml. of DOCA were without effect.

The synthesis of the halogenated cortisones and hydrocortisones by Fried and Sabo (1953) produced the first modification of the cortisone molecule with enhanced biological activity. Since varying only the suspending medium can alter effects in the rat thymus test, it is not surprising that other investigators, using widely differing animal tests, have obtained different relative potencies (Table II). The halogenated cortisone derivatives possess both mineralocorticoid and glucocorticoid activity, so that although clinically more potent than cortisone they are not suitable for use in treating rheumatoid arthritis, as they produce marked salt retention and oedema in man, but can be used to advantage in maintenance therapy after adrenalectomy (Hamwi and Goldberg, 1955).

The introduction of the 1:2- double bond in the A ring, producing prednisone, the unsaturated cortisone derivative, and prednisolone, the hydrocortisone derivative, was reported by Bunim, Pechet and Bollet in 1955. Again, different animal tests give differing values for relative potencies (Table II). The weight of clinical evidence shows that prednisone is approximately 3 to 4 times as effective as cortisone in the treatment of rheumatoid arthritis (Boland, 1956; Bollet, Black and Bunim, 1955; Dordick and Gluck, 1955; Gray and Merrick, 1955; Muller, 1955), about 5 times as effective in allergic disorders and asthma (Arbesman and Ehrenreich, 1955; Feinberg and Feinberg, 1956) and 4 times as effective in treating dermatoses (Robinson, 1955).

From the thymus involution test in the nestling rat, the degree of enhancement of biological activity produced either by halogenation or unsaturation of the cortisone molecule is greater than that borne out by clinical evidence. This is a reflexion of the sensitivity of the rat thymus test. It pro-

TABLE II

VALUES FOR RELATIVE POTENCIES OF THE HALOGENATED HYDROCORTISONES, PREDNISONE AND PREDNISOLONE, OBTAINED WITH DIFFERENT BIOLOGICAL TESTS

The standard steroid used is shown as E (cortisone alcohol), E Ac (cortisone acetate), F (hydrocortisone alcohol) or F Ac (hydrocortisone acetate)

Compound	Test	Potency	Author(s)
HALOGENATED HYDROCORTISONE DERIVATIVES			
9α-Chlorohydrocortisone acetate	Rat liver glycogen deposition	4 × E Ac	Fried and Sabo (1953)
9α-Fluorohydrocortisone ,,	,, ,, ,, ,,	10·7 × E Ac	,, ,, ,, (1954)
,, ,,	,, ,, ,, ,,	12·6 × F Ac	Stafford, Barnes, Bowman, and Meinzinger (1955)
,, ,,	Thymus involution weanling rat	8·8 × F	Stephenson (1956)
,, ,,	"Anti-inflammatory" effect round cotton pellet-rat	7 × F	Dulin (1955)
,, ,,	,, ,, ,, ,,	13·2 × F Ac	Singer and Borman (1956)
,, ,,	Depression eosinophil count in dogs	20 × F Ac	Liddle, Pechet, and Bartter (1954)
,, ,,	Prevention Na^+ loss adrenalectomized dogs	4·7 × DOCA	,, ,, ,, ,, ,,
UNSATURATED CORTISONE AND HYDROCORTISONE DERIVATIVES			
Prednisone	"Anti-inflammatory" effect—rat hind paw	4·5 × E Ac	Ducommun, Ducommun, and Baquiche (1955)
,, ...	Rat liver glycogen deposition	3–4 × E Ac	Bunim, Pechet, and Bollet (1955)
,, ...	Granuloma pouch technique—rat	No different from E Ac	Demartini, Boots, Snyder, Sandson, and Ragan (1955)
,, ...	Thymus involution weanling rat	2·96 × F	Stephenson (1956)
Prednisolone	,, ,, ,, ,,	4·28 × F	,, ,,
,, ...	Rat liver glycogen deposition	2·9 × F Ac	Stafford *et al.* (1955)
,, ...	,, ,, ,, ,,	10·1 × E	Tolksdorf, Battin, Cassidy, MacLeod, Warren, and Perlman (1956)

vides a quick and easily carried out test for biological activity in different steroids; and while steroids with no clinical efficacy, such as corticosterone, produce slight thymic involution, the most effective involuting agents so far tested are those with therapeutic activity.

This work formed part of a thesis accepted by the University of London in part fulfilment of the requirements for the Degree of Doctor of Philosophy. It was carried out under the supervision of Dr. C. H. Andrewes, F.R.S., to whom I am most grateful. The work was done in Dr. D. A. Long's laboratory, and I am greatly indebted to both Dr. Long and to Dr. R. K. Callow for helpful advice. I am grateful to Miss M. V. Mussett, B.Sc., for carrying out the statistical analyses; and would like to thank Mrs. S. Brownstone and Mrs. W. Driver for skilled technical assistance.

The steroids tested came from many sources, and I gratefully acknowledge the following sources: 9α-chlorohydrocortisone acetate and 9α-fluorohydrocortisone acetate, Squibb Institute for Medical Research, New Brunswick, N.J., U.S.A.; prednisone acetate and prednisolone acetate, Schering Corporation, Bloomfield, N.J., U.S.A.; 9α-fluoroprednisolone acetate, Merck & Co. Inc., Rahway, N.J., U.S.A.; *epi*hydrocortisone acetate and Reichstein's Substance L, Syntex S.A., Mexico; dihydro*allo*cortisone acetate, Glaxo Laboratories, Ltd., Greenford, Middlesex; tetrahydrocortisone acetate, Organon Ltd., London; pregnenetriolone, Dr. P. Spensley, Colonial Products Research Council; Kendall's Compound A, Professor J. M. Yoffey, Dept. of Anatomy, Bristol University; DOCA, Ciba Limited, London.

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