Growth-Survival and Sodium Retaining Activity of 9 α-Halo Derivatives of Hydrocortisone.* (21168)

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(Introduced by O. Wintersteiner.)
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Fried and Sabo(1,2) have recently reported a series of 9 α-halo substituted derivatives of hydrocortisone and cortisone possessing unusual glucocorticoid-like activity in the rat. We have examined these compounds for growth and survival as well as for sodium retention in the adrenalectomized rat. The results obtained with 9 α-fluoro, 9 α-chloro-, and 9 α-bromo-hydrocortisone acetate are here reported.

Material and methods. The growth-survival technic of Gaunt *et al.*(3) was used to study the effects of these compounds in maintaining life. Immature male rats, weighing 60 to 70 g, were bilaterally adrenalectomized and immediately injected subcutaneously with an aqueous suspension† of the steroid being tested. The animals used for each test dose were kept as a group in a cage and allowed our stock diet‡ and water *ad libitum*. The cages were inspected daily for dead animals; the rats were weighed twice each week. A survival period of 28 days was used. Animals surviving beyond this period were taken, for purposes of calculation of the data found in Table I, as surviving only 28 days. Data obtained from animals dying within 3 days after adrenalectomy were discarded. The average maximum weight gain, listed in Table I, was determined by summing the maximum gains achieved by each animal in the test group, using a figure of zero for those animals that did not gain or that even lost weight, and dividing by the total number of animals in the group. Daily gain was calculated by dividing the sum of the maximum gains, just described, by the sum of the number of days required by each animal to reach this maximum weight. When the animals either gained no weight or lost weight, their total days of survival were used in obtaining the latter sum. The sodium excretion test used is a modification of the method reported by Dorfman, Potts, and Feil(4). Male rats, weighing 140 to 160 g, were bilaterally adrenalectomized and maintained for 48 hours on our stock diet with tap water containing 1% saline and 5% glucose. This saline-glucose drinking water was then replaced with a 5% glucose solution for an additional 24 hours. Two hours prior to the start of the test all food and water was withdrawn. A minimum of 5 animals per dose level of steroid and 10 animals for untreated controls were used in each assay. Each steroid was tested simultaneously against desoxycorticosterone acetate (DCA) as well as against the untreated controls. Steroids tested for sodium retention were administered by subcutaneous injection of 0.5 ml of the compound dissolved in 20% alcohol. This was followed 1 hour later by subcutaneous injection of 0.5 ml of an aqueous solution containing 35 μg of sodium chloride per g of body weight and 2 μc of Na^{22}. The animals were placed in individual 2-liter beakers containing a raised wire screen for 6 hours. The screen served as a urine-feces separator. After this 6-hour test period, the animals were made to urinate by allowing them to inhale ether and then applying suprapubic pressure. The beakers and screens were thoroughly

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† Aqueous suspensions are prepared by grinding crystalline steroids in mortar and passing through a 200-mesh screen. The finely divided material is suspended in a menstruum consisting of 0.04% Tween 80, 0.7% NaCl, 0.9% benzyl alcohol, and 0.5% carboxymethylcellulose.

‡ The stock diet consists of the following ingredients: yellow corn, 42.5%; linseed meal, 10.5%; casein, 7.5%; alfalfa, 3.0%; skimmed milk powder, 26.0%; yeast, 2.0%; corn oil, 6.3%; cod liver oil, 1.0%; bone ash, 0.7%; and NaCl (fortified with KI), 0.5%.

TABLE I. Growth-Survival of Immature Bilaterally Adrenalectomized Male Rats Receiving Single Injections of Steroids.

Compound	Test dose (μg)	No. of animals	Avg survival time (days)	% surviving 28 days	Avg wt gain Max (g)	Avg wt gain g/day
Aqueous menstruum (controls)	—	105	6.8	2	3.6	.60
E acetate	5000	10	14.1	0	9.5	.87
	2500	10	10.9	10	9.5	1.13
	2000	20	13.4	10	14.6	1.08
	1600	20	10.5	0	3.6	.47
	800	20	9.2	5	9.8	1.40
F "	1600	20	13.5	10	12.4	1.37
	800	9	11.6	0	1.7	.16
DCA	2000	10	16.6	10	27.7	2.40
	1600	10	15.9	0	27.4	2.26
	1000	10	12.0	0	15.8	2.11
	800	39	11.0	5	18.1	2.64
	400	27	10.6	4	11.4	1.93
Bromo F acetate	1600	20	21.2	15	53.0	3.35
	800	19	20.5	31	50.1	3.33
	400	10	9.1	0	12.1	2.14
	200	9	10.1	11	22.3	2.80
Chloro F "	2000	10	28.0	100	72.3	2.96
	1600	19	24.4	74	77.4	3.69
	800	10	17.1	30	51.5	3.75
	400	20	22.9	35	58.3	3.03
	200	9	18.2	11	31.9	2.84
	100	20	13.8	0	27.1	3.00
Fluoro F "	3200	10	8.5	0	.3	.04
	1600	29	12.3	0	6.9	.78
	400	10	8.7	0	5.6	1.24
	100	10	8.7	0	3.1	.49

rinsed with tap water into 250 ml beakers. The combined urine and wash was then evaporated to dryness overnight on a steam bath. Four ml of water were then added to the solids and as uniform a sample as possible was made by scraping the sides and bottom of the beaker with a rubber policeman. Two ml of this suspension were then transferred to one-inch cupped planchets and the amount of radio-activity determined with a scintillation counter.

Results and discussion. From the data in Table I it can be seen that control animals receiving single injections of the aqueous menstruum have an average survival time of 6.8 days. These results are in accord with the results reported by other workers(3). In the groups receiving DCA a survival time of 10.6 to 16.6 days is noted. Very few animals survive the 28-day period. Using DCA as a standard of growth-survival, it can be readily seen in Table I that cortisone acetate (E acetate), hydrocortisone acetate (F acetate), and 9 α-fluoro-hydrocortisone acetate (Fluoro F acetate) elicit much smaller growth responses and somewhat lower survival times. Of these compounds, Fluoro F acetate appears to be the least effective. With 9 α-bromo-hydrocortisone acetate (Bromo F acetate), significant increases were noted in survival time (9.1-21.2 days) and in growth. The data indicate that Bromo F acetate is more active than DCA, since 200 to 400 μg of the bromo compound are about as effective in both growth and survival as 800 to 1000 μg of DCA.

The administration of 9 α-chloro-hydrocortisone acetate (Chloro F acetate) gave the best results in growth survival. All of the animals receiving the 2000 μg dose survived the 28-day period. At the 1600 and 800 μg levels, 74% and 30%, respectively, of the animals survived. When the dose levels were lowered to 100 to 200 μg, the incidence of survival dropped. Even at these dose levels a marked

TABLE II. Comparison of Fluoro F Acetate and DCA in Sodium Retention. (Excretion in Na^{22} expressed as % of controls.)

Dose, μg	Fl.F	DCA	Fl.F	DCA	Fl.F	DCA	Fl.F	DCA	Fl.F	DCA
2- 3	40	82	72	60	33	36	37	35	41	90
8-10	20	52	61	21	27		37	37	47	
22.5							68	33	44	37

effect on survival time (13.8 to 18.2 days), average maximum gain, and daily gain was noted. From the data in Table I chloro F acetate appears to be 10 to 20 times as effective as DCA in stimulating growth and survival.

The sodium-retaining activity of Bromo F acetate was examined over a range of doses from 0.03 μg to 200 μg. At levels of 2.3 μg and 7 μg, responses of 100% and 80% Na^{22} excretion, respectively, were obtained when compared with controls. At a level of 200 μg the sodium excretion was only 60% that of the controls, indicating that Bromo F acetate is considerably less active than DCA. Application of the F-test in a comparison of dose-response curves between DCA and Bromo F acetate indicates that the curves are not parallel.

Early experiments with Fluoro F acetate in sodium retention showed a significant dose-response curve that was parallel to that obtained with DCA. However, later experiments failed to confirm the earlier observations although analysis of variance(5) of the response showed a highly significant difference between the response for the controls and the treated animals. The data are summarized in Table II and indicate that at dose levels of 2 to 3 μg, Fluoro F acetate is somewhat more effective than DCA in the retention of sodium. At higher dose levels the increased retention of sodium is not always seen with this fluoro derivative.

The possibility that a different mechanism of action exists for the retention of sodium by the fluoro and bromo derivatives than by DCA must be considered.

In contrast, the dose-response curve with Chloro F acetate is parallel to that with DCA (Fig. 1), indicating a similar mode of action for these 2 steroids. The results of 4 independent assays of the chloro derivative are shown in Table III. Using the statistical methods of Bliss(5), the sodium-retaining activity of chloro F acetate, obtained from the

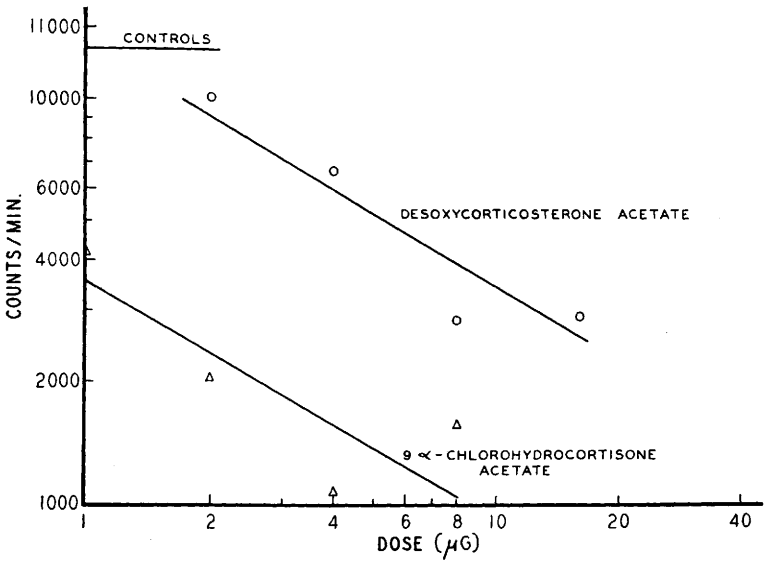

FIG. 1. Comparison of effect of Chloro F acetate and DCA on urinary Na^{22} excretion of adrenalectomized rats.

TABLE III. Assays of Chloro F Acetate for Sodium-Retaining Activity, 4 Experiments.

No. of animals	Potency (DCA = 1)
40	9.5
50	12.3
36	16.9
36	10.2

pooled data listed in Table III, is 10.8 times that of DCA with 95% confidence limits of 6.8 to 17.1. The analysis of variance showed that the departure from parallelism and curvature are not significant. This indicates that the requirements for a valid assay have been met.

The mineralo-corticoid-like activity of Chloro F acetate is of considerable interest in view of the previously reported unusually high glucocorticoid-like action of this compound(1,6). Thus, in the rat this compound is unique in that it manifests both gluco- and mineralo-corticoid-like properties to such a high degree.

It is also of interest to note that growth and survival can be markedly enhanced by a substance, such as Bromo F acetate, which has little sodium-retaining activity. Conversely, a compound with sodium-retaining activity, such as Fluoro F acetate, is not always effective in stimulating growth and survival.

Summary. 1. Growth and survival studies on adrenalectomized immature rats following single injections of aqueous suspensions of steroids indicate that Chloro F acetate is 10 to 20 times and Bromo F acetate 2 to 5 times as active as DCA. E acetate, F acetate, and Fluoro F acetate are less effective than DCA. 2. Sodium retention tests with Na^{22} indicate that Chloro F acetate is 10.8 times as potent as DCA, and that Fluoro F acetate is somewhat more effective and Bromo F acetate considerably less effective than DCA. The dose-response curves suggest that the steroids cause sodium retention by more than one mechanism. 3. The data indicate that, with the steroids studied, there is not always a direct correlation between sodium-retaining activity and growth-survival activity.

1. Fried, J., and Sabo, E., *J. Am. Chem. Soc.*, 1953, v75, 2273.

2. ———, *ibid.*, 1954, v76, 1455.

3. Gaunt, R., Leathem, J. H., Howell, C., and Antonchik, N., *Endocrinology*, 1952, v50, 521.

4. Dorfman, R. I., Potts, A. M., and Feil, M. L., *ibid.*, 1947, v41, 464.

5. Bliss, C. I., *The Statistics of Bioassay*, New York, Academic Press, 1952.

6. Borman, A., and Singer, F. M., *Fed. Proc.*, 1954, v13, 185.

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Effects of an Antihyaluronidase Substance and of Hyaluronidase on Growth of Virus-Induced Fibromas. (21169)

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The spreading effect of hyaluronidase has been amply demonstrated by Duran-Reynals (1) and Sprunt(2,3) for India ink, vaccinia virus, fibroma virus, and virus III. We have obtained results similar to theirs with the fibroma virus but have not published these previously since augmentation of the lesion in the rabbit skin brought about by the simultaneous injection of hyaluronidase and virus seemed such an obvious and predictable phenomenon. However, the recent *in vitro* development of an "antihyaluronidase substance" by Hadidian and Pirie(4) stimulated the experiments here described. These indicate that an antihyaluronidase prepared outside of the animal body has an effect exactly the reverse of that of hyaluronidase on the formation of virus-induced fibromas in that it can prevent the formation of the fibroma or markedly diminish its size.