hemoconcentration and death when administered either before or after *placement* of hind leg ligatures, but not after *removal* of these ligatures. Maximal counteraction was secured by administering the drug before or immediately after placement of the ligatures. Chlorpromazine was effective in appreciably counteracting heat trauma induced hemoconcentration and death only if administered prior to traumatization.

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Production of Aldosterone by Rat Adrenal Glands *in vitro*.*† (22635)

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(Introduced by J. S. L. Browne.)
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Rat adrenal glands have been shown to produce corticosteroids *in vitro*(1,2,3) and a stimulation of this production by the addition of ACTH to the incubation medium had been demonstrated(1). Small amounts of aldosterone were found by Wettstein and Anner(4) in the incubation medium of beef and hog adrenals, and by Rosenfeld(5) and associates in the perfusion medium of beef adrenals. The present work shows that the incubation medium from rat adrenals contains a sodium-retaining substance with the properties of aldosterone and describes the results of experiments performed to investigate some of the factors which may affect its secretion.

Methods. In each experiment adrenal glands were obtained from 40 to 90 male rats weighing between 170 to 180 g. These animals were subsequently used for bioassay purposes. The glands were freed of fat and quartered. One quarter of each adrenal was placed in each of 4 flasks containing 1.5 ml per 25 mg of fresh tissue of Krebs-Ringer-bicarbonate solution with added glucose (200 mg%)(6). Thus the 4 flasks contained an equal amount of tissue from each rat in the group. The incubation of the tissue was carried out in a water bath at 38°C. After the first hour the medium was poured off and replaced by an equal quantity of the same solution. The incubation was allowed to continue for an additional 2 hours. A gentle stream of 95% O_2 - 5% CO_2 was passed through the medium continuously. At the end of this period the medium was decanted. A 0.5 ml aliquot was extracted with methylene chloride for determination of the total Δ^4 3-ketosteroid content, according to the method of Saffran and Schally(6). The rest of the medium was extracted with 3.5 times its volume of redistilled chloroform in glass stoppered cylinders. The two phases were separated by centrifugation and the chloroform layer was carefully transferred with a syringe and a long needle into a round bottomed flask and evaporated to dryness *in vacuo* at a water bath temperature of 50°C.

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In earlier experiments this crude extract was assayed directly for sodium-retaining activity. In later studies it was subjected to paper partition chromatography in the benzene-aqueous methanol system of Bush(7), run at room temperature for 4½ hours. The aldosterone band was subsequently bioassayed. Detection and partial characterization of the material after chromatography was done by direct visualization and/or contact photography under ultra violet light; by scanning of the paper strip in a Beckman DU spectrophotometer at 2400 Å; by soda fluorescence, blue tetrazolium and triphenyltetrazolium reactions on the chromatogram; by mixed chromatogram with crystalline compounds and absorption spectrum in sulfuric acid solution. In certain experiments, paper chromatographic separation of aldosterone was followed by spectrophotometric determination of the eluate at 2400 Å. This procedure was made possible without further paper chromatographic purification of aldosterone, since cortisone, which in the benzene aqueous methanol system has a mobility closely related to aldosterone, was not found in the incubation media of rat adrenals. Furthermore after such a chromatographic separation, in 14 experiments with and without ACTH the amounts of aldosterone, as determined both by bioassay and by spectrophotometry, were highly significantly correlated. Sodium-retaining activity was assayed on adrenalectomized rats according to a modification of the method of Singer and Venning(8).

Results. The Na retaining activity of the crude chloroform extracts of rat adrenal incubates varies from 0.27 to 0.73 μg equivalent of aldosterone per 100 mg of tissue per hour of incubation (Mean of 6 experiments, 0.46 ± 0.09).

Paper partition chromatography of these extracts revealed the presence of several ultraviolet-absorbing compounds. Of these, one was tentatively identified as corticosterone by mixed chromatogram, sulfuric acid chromogen, maximum ultraviolet absorption at 2400 Å in methanol and reduction of the tetrazolium reagents. Corticosterone represents about 45 to 50% of the Δ^4 3-ketonic compounds produced *in vitro* by rat adrenals.

A strong sodium-retaining activity was found by biological assay of an ultraviolet absorbing α-ketolic material recovered from the area between the bottom of cortisol and the top of cortisone as defined by a reference strip. This material yielded 0.31 to 0.64 μg equivalent of aldosterone per 100 mg of tissue per hour of incubation (mean of 12 experiments, 0.47 ± 0.03). The sodium-retaining activity separated both from cortisol and cortisone on mixed chromatography and on bioassay showed a dose-response relationship with a slope identical with that of aldosterone. That the sodium-retaining activity was limited to the chromatographic area as defined above, was demonstrated by the fact that eluates of immediately adjacent areas showed no activity on bioassay at level up to 4 times the equivalent dosage. From these findings, it may be concluded with a fair degree of certainty that the ultraviolet-absorbing α-ketolic material under study is aldosterone.

The other compounds present on the chromatograms did not show noticeable sodium-retaining potency in the assay. However, the presence of traces of desoxycorticosterone which, in the chromatographic system used would run close to the solvent front, has not been eliminated.

Action of ACTH. In 4 out of 5 studies the addition of ACTH, 1 to 100 milli-units/100 mg of tissue, significantly increased the

TABLE IA. Sodium Retaining Activity of Incubation Media of Rat Adrenal Glands after Addition of ACTH. (Crude fraction-biological assay.)

ACTH, milliunits per 100 mg tissue	Na retaining activity: Aldosterone eqv., μg/100 mg tissue/hr		Δ^4-3-ketosteroids, μg/100 mg tissue/hr
	Assay 1	Assay 2	
Control	.30	.50	10.3
1	1.25 (<.02)	1.10 (<.01)	10.8
10	1.09 (<.02)	1.00 (=.02)	14.8
100	1.52 (<.01)	1.50 (=.001)	28.2
Control	.14	.21	
100	.61 (<.02)	.40 (>.1)	

Sodium-retaining activity of each medium with added ACTH was compared to that of control medium without ACTH and its degree of significance was calculated by Student's t test. Value of P is shown in parentheses.

TABLE IB. Action of ACTH on Production of Aldosterone by Rat Adrenal Glands Incubated *In Vitro.**

ACTH, milliunits/100 mg of tissue	Aldosterone, μg/100 mg of tissue/hr		Corticosterone, μg/100 mg of tissue/hr	
	Control	ACTH added	Control	ACTH added
100‡	.63	.79	---	5.00
"	.50	.63	2.42	4.60
100§	.54	.71	—	—
"	.52	.59	1.74	5.19
"	.62	.76	2.23	3.55
200‡	.32	.51	2.56	6.94
Mean	.52 ± .05†	.67 ± .04	2.24 ± .18†	5.07 ± .55
	Mean diff. = .14 ± .02 Significant at P <.001		Mean diff. = 2.83 ± .46 Significant at P <.01	

* Quantitative determination of aldosterone in Beckman spectrophotometer at 240 mu.
† Stand. error of mean.
‡ Acton X Nordic Biochemical Labs. Lot 54009.
§ ACTH Connaught Lot 8-3.

sodium-retaining activity of the crude chloroform extract of the incubation media. However, a dose-response relationship was not established between the amount of ACTH and that of the sodium-retaining material (Table 1A). Comparing the action of ACTH on production of the sodium-retaining material and on that of the total Δ^4 3-ketosteroids, it was observed that production of the former was enhanced by a dose of 1 milliunit whereas that of the corticoids increased only at a dose level of 10 and 100 milliunits. Further experiments are needed to establish reproducibility of the response of the sodium-retaining substance to the dose of 1 milliunit of ACTH. To exclude the stimulatory effect of ACTH on other possible Na-retaining substances similar studies were performed with paper chromatographic separation of aldosterone and its quantitative measurement by spectrophotometry (Table IB). They showed that at the dose level of 100 to 200 milliunits/100 mg of tissue, ACTH has a small but consistent effect on the production of aldosterone *in vitro* (mean difference of 6 studies, 0.14 ± 0.02 μg of aldosterone/100 mg of tissue/hr of incubation. Significant at P<.001). The relatively greater increase in sodium-retaining activity of the crude chloroform extracts after ACTH stimulation of the glands is probably due to a sodium-retaining substance or substances other than aldosterone.

Action of growth hormone. Two preparations of growth hormone were tested for their ability to affect the sodium-retaining material released by rat adrenals *in vitro*. The most purified preparation (C. H. Li's Growth Hormone) was without detectable effect at the dose level of 10 and 100 μg/mg of tissue. At the 250 μg dose level, the other preparation (Horner) sensibly increased both the Δ^4 3-ketosteroid content and the biological sodium-retaining potency of the incubation medium, indicating that it was probably contaminated with ACTH (Table II).

Effect of altering Na/K ratio of the incubation medium. Two series of incubations were carried out after alteration of the Krebs-

TABLE II. Sodium Retaining Activity of Incubation Media of Rat Adrenal Glands after Addition of Growth Hormone. (Crude fraction-biological assay.)

Growth hormone, μg/100 mg tissue	Na retaining activity: Aldosterone eqv., μg/100 mg of tissue/hr	Δ^4-3-ketosteroids, μg/100 mg of tissue/hr
Control	.41	10.8
10*	.50	11.3
100*	.45	12.3
Control	.37	
10*	.31	
100*	.28	
Control	.28	10.1
250†	.44‡	18.1

* Growth hormone prepared by Dr. C. H. Li and kindly given to us by Dr. H. Selye.
† Somatrofin, Horner Laboratories, Lot No. P.R. 3.
‡ Sodium retention compared to the control is significant at P <.01.

TABLE III A. Effect of Variation of Na/K Ratio of Incubation Media on Production of Sodium Retaining Material and Aldosterone by Rat Adrenal Glands.

A: Crude fraction biological assay

Na/K*	Na retaining activity: Aldosterone eqv., μg/100 mg of tissue/hr	Δ⁴-3-ketosteroids, μg/100 mg of tissue/hr
24.3	.24	11.8
16.3	.14	14.0
4.0	.32	13.3
17.9	.65 (<.1)	14.5
24.3	.11	9.3
16.3	.46 (<.001)	13.1
4.0	.74 (<.001)	11.4
17.9	.28 (<.01)	12.6

TABLE III B.

B: Paper chromatography

Na/K*	Aldosterone,† μg/100 mg tissue/hr	Corticosterone, μg/100 mg tissue/hr
24.3	.97	4.0
16.3	1.32	5.6
24.3	.69	4.8
4.0	1.02	5.4
24.3	.82	6.3
16.3	1.23	5.7

Mean diff.: .36 ± .025
Significant at P <.01

* The following concentrations of Na and K (in meg/l of medium) have been used: (Na: 143.4, K: 5.9) Na/K = 24.3. (Na: 138.6, K: 8.6) Na/K = 16.1. (Na: 119.8, K: 29.6) Na/K = 4.0. (Na: 96.5, K: 5.38) Na/K = 17.9.

† Quantitative determination of aldosterone in Beckman spectrophotometer at 240 mμ.

Ringer-bicarbonate medium from its original Na/K ratio of 24 to a ratio of 16 and 4 by lowering the Na and increasing the K concentrations. In 2 cases the Na concentration only was decreased, the K concentration remaining about the same as that of the control (Table IIIA). Under these conditions the sodium-retaining potency of the crude fraction of the altered media did not significantly differ from that of the control in the first series of experiments, but did so in the second. However in this second series, the control value of 0.11 μg equivalent of aldosterone is unusually low as compared to the range previously stated for the crude fraction (0.27 to 0.79 μg/100 mg of tissue/hour). More consistent results were obtained after paper chromatographic separation of aldosterone followed by its spectrophotometric determination (Table IIIB). In 3 studies, the production of aldosterone was found to be increased in the media having a Na/K of 16 and 4 respectively. The mean difference of 0.36 ± 0.025 μg of aldosterone 100 mg of tissue/hour is significant at $P<.01$.

Discussion. In the present study bioassays of the crude chloroform extracts of the incubation media were performed only as a guide to production of sodium-retaining substance or substances by the rat adrenals submitted *in vitro* to various stimuli. The inconsistent values sometimes obtained by bioassays of these crude chloroform extracts are not unexpected since such extracts probably represent a mixture of substances which individually might have widely different or even antagonistic effects on sodium retention. Therefore, the biological activity of the crude fractions cannot be compared rigidly with the quantitative measurement of aldosterone separated by paper chromatography and subsequently measured by bioassay or spectrophotometry. Recent reports have shown that aldosterone or a potent electrolyte-regulating substance, in all probability identical with aldosterone, is produced by incubation of beef and hog adrenal homogenates(4) as well as by perfusion of calf adrenal(5). The present data demonstrate that under *in vitro* conditions the yield of aldosterone by rat adrenals is much greater per unit of weight. Studies on hypophysectomized animals have suggested that the pituitary may play a role in the secretion of aldosterone since the amount of the hormone recovered from adrenal vein blood of hypophysectomized rats(9) and dogs(10) was found to be lower than in normal controls. Our findings tend to support this view since a slight but consistent increase in aldosterone production was obtained *in vitro* by addition of 2 different ACTH preparations to the incubation media. However, this effect may be due to contamination of the ACTH preparations with some other pituitary hormone. On the other hand, growth hormone is unlikely to enhance the secretion of aldosterone by a direct effect. Similar conclusions

have been reached by Rosenfeld and collaborators after perfusion of calf adrenals with ACTH and with growth hormone(5). Recent *in vivo* studies have indicated the probability that the main stimulus of aldosterone secretion is directly or indirectly related to certain changes of sodium, potassium, and water metabolism. Although the results presented here show that alteration of the Na/K ratio of the incubation media is accompanied by an increase in aldosterone secretion, the physiological significance of these experiments seems to be limited by the following considerations: 1) They do not disclose whether alteration of Na or K concentrations or both is the effective stimulus for the observed increase of aldosterone secretion. 2) This increase is not very striking, leaving open the possibility that the alteration of Na and K concentrations *in vivo* may exert major effects by an indirect action which would not be demonstrable by the study of isolated glands.

Conclusion. Rat adrenal glands incubated *in vitro* released a sodium-retaining substance with the properties of aldosterone. Purified growth hormone does not seem to increase secretion of sodium-retaining activity. Two ACTH preparations caused a slight but significant increase of aldosterone production *in vitro*. Alteration of the Na/K ratio of the incubation media, by lowering the Na and increasing the K concentration, produces a moderate enhancement of aldosterone secretion.

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Strontium-Calcium Discrimination Factors in the Rat. (22636)

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The behavior of strontium in the animal body has become of particular interest in relation to possible hazards from atomic fission debris. Contamination of the food chain by fallout material may create a situation in which the calcium of the dietary becomes associated with essentially carrier-free radiostrontium. The body burden of radiostrontium, in terms of body calcium, attained after ingestion over long periods of time will be governed by the Sr*/Ca of the intake and by the over-all differentiation between strontium and calcium that may take place in such processes as primary absorption from the tract; incorporation into new bone; incorporation into pre-existing bone; renal excretion; endogenous excretion into the gastrointestinal tract; and reabsorption from the tract.

Comar, Whitney, and Lengemann(1) showed that growing rats utilized dietary calcium in a commercial ration for bone growth by a factor of 3.6 over carrier-free Sr 90 that had been incorporated into the feed, *i.e.,* the Sr 90/Ca ratio of the diet divided by the Sr 90/Ca ratio of the bones equaled 3.6. These rats had been raised for one or 2 generations on a diet in which the Sr 90 to calcium ratio was maintained at a constant level so that their bones were formed entirely from the Ca-Sr 90 of the "spiked" diet. This find-