BAA may be an explanation for the findings of Greenbaum and Fruton.

Hydrolysis of GTAA by the mitochondrial fraction of liver homogenates suggests that cathepsin C is associated with this same particulate. Cathepsin C has an optimal hydrolytic activity at pH 5.2. From pH 6.6 to 7.8 extensive transamidation has been observed with progressively less hydrolysis(10). Using the preparations described in this article one will be able to test cytoplasmic particles for transamidation and other transfer reactions ascribed to the cathepsins. Their role in the nitrogen metabolism of normal cells may well be larger than the peptidase activity demonstrated by the results presented.

*Summary.* The mitochondria of rat liver homogenates contain proteinases similar to cathepsins B and C. An inhibitor for the hydrolytic activity of cathepsin B is present in the supernatant fluid of the same homogenates.

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## *In vitro* Response of Functional Experimental Adrenal Tumors to Corticotropin (ACTH).* (23202)

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Two secretory adrenocortical tumors are being reported; one originated in an irradiated mouse and the other in a radiothyroidectomized rat(1). The animals bearing the tumor transplants secreted steroids with glucocorticoid and mineralocorticoid but no gonadal activities. The urine of tumor-bearing mice contained an increased amount of 17-ketosteroids, particularly the 11-oxygenated 17-ketosteroids (Bloch and Cohen, unpublished data). These tumors grew in hypophysectomized animals. The adrenals of unoperated hosts were markedly atrophic indicating inhibition of ACTH discharge. In the following experiments the *in vitro* responsiveness of mouse and rat adrenal tumors to ACTH was studied in comparison with normal adrenal tissues and the nature of the steroidal secretions was investigated.

*Materials and methods. Adrenal tissues.* The previously described mouse strain 2 and rat strain 1 adrenal tumors were used(1). Normal adrenals were obtained from 3-month-old animals of the same strains ($LAF_1$ mice and WR rats). All animals were killed by decapitation. Tumors measuring 2-4 cm in diameter were excised and placed in cold Krebs-Ringer bicarbonate buffer, pH 7.4, fortified with 200 mg % glucose(2). Tumor slices were cut free hand with a razor and placed in a Petri dish containing cold buffer.

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TABLE I. *In Vitro* Response of Male $LAF_1$ Mouse and WR Rat Adrenal Tumor Slices to ACTH.

| Tumor size* (cm) | Treatment | mU ACTH per 100 mg | μg equiv. cortisol/100 mg/2 hr: No ACTH | μg equiv. cortisol/100 mg/2 hr: ACTH added | Stimulation ratio† |
|---|---|---|---|---|---|
| Mouse | | | | | |
| 4 | None | 200 | 1.8 | 12.6 | 7.0 |
| 2.5 | " | 100 | 3.3 | 15.2 | 4.7 |
| 3.5 | " | 50 | 2.6 | 15.4 | 5.9 |
| 2.5 | " | 60 | 2.4 | 15.2 | 6.4 |
| 3 | " | 45 | 1.7 | 12.0 | 7.1 |
| 3 | " | 25 | 1.4 | 11.0 | 8.1 |
| 3 | " | 25 | 5.3 | 13.9 | 2.6 |
| | Avg | | 2.6 ± 1.3‡ | 13.6 ± 1.8 | 6.0 |
| Rat | | | | | |
| 2 | None | 150 | 3.4 | 11.3 | 3.3 |
| 3 | " | 130 | 3.3 | 17.8 | 5.4 |
| 5 | " | 130 | .9 | 17.9 | 19.9 |
| 2.5 | " | 130 | 1.7 | 17.9 | 10.5 |
| 3 | " | 130 | 1.8 | 23.6 | 13.1 |
| 2.5 | Adrenalectomy | 140 | .6 | 16.5 | 27.6 |
| 2 | Gonadectomy | 140 | 1.5 | 17.0 | 11.3 |
| | Avg | | 1.9 ± 1.1 | 17.4 ± 3.6 | 13.0 |

* Estimated avg tumor diameter.

† Stimulation ratio $= \frac{\mu g \text{ equiv. cortisol secreted with ACTH}}{\mu g \text{ equiv. cortisol secreted without ACTH}}$.

‡ Mean ± stand. dev.

The normal adrenals were dissected, carefully cleaned and cut in halves on buffer moistened filter paper. *Tissue incubation and steroid extraction.* The *in vitro* method of Saffran and Schally for bioassay of corticotropin was modified to measure the steroid production by tumor slices and normal adrenal segments(3). Approximately 30-50 mg of normal adrenals (about 10 mice or 1 rat were necessary) or 100 mg of tumor slices were blotted on filter paper, weighed accurately on a Roller-Smith torsion balance and transferred to a 10 ml Erlenmeyer flask containing 3 ml of buffer. The flasks were flushed with a 95% $O_2$-5% $CO_2$ gas mixture, stoppered and shaken in a water bath at 38°C for 1-1½ hours. At the end of this preincubation period, the buffer was removed and 3 ml of fresh buffer were added to rewash the slices in order to eliminate all traces of any interfering substances. The flasks received 2.9 ml of buffer and various concentrations of U.S.P. Reference Standard Corticotropin dissolved in 0.9% saline, the final volume being 3.0 ml. The samples were again flushed with the gas mixture and incubated for 2 hours in a shaking water bath at 38°C. At the end of the incubation period, 2.5 ml of buffer were transferred from each flask to a glass stoppered 15 ml centrifuge tube containing 2.5 ml of purified methylene chloride. The tubes were well shaken and centrifuged at 2500 rpm to separate the 2 phases. After removal of the lower phase, this extraction procedure was repeated with 2.5 ml of methylene chloride to insure complete removal of the steroids from the buffer. The combined extracts were evaporated to dryness and the residue redissolved in 1 ml or 2 ml of purified 95% ethanol. All determinations were done in duplicate or triplicate. *Estimation of* $\Delta^4$*-3-ketosteroids.* The optical densities of aliquots of the ethanolic extracts were measured at 242 mμ in the Beckman model DU ultraviolet spectrophotometer against an ethanolic blank of a methylene chloride extract of the buffer. The $\Delta^4$-3-ketosteroid concentrations were obtained from a standard cortisol curve and are expressed as μg equivalents of cortisol/100 mg tissue/2 hr of incubation. Recovery tests were run with 2 and 10 μg quantities of standard cortisol in 2.5 ml of buffer to check ef-

TABLE II. *In Vitro* Response of $LAF_1$ Mouse and WR Rat Adrenal Tumors to Low Dosages of ACTH.

| | μg equiv. cortisol/100 mg/2 hr | | | |
|---|---|---|---|---|
| mU ACTH per 100 mg | $LAF_1$ 3773 ♂ | $LAF_1$ 3680 ♂ | $LAF_1$ 3672 ♂ | WR 274 ♀ |
| .0 | 2.3 | 2.9 | 2.3 | .7 |
| .5 | 6.7 | 10.6 | | 2.2 |
| 1.0 | 8.7 | 12.6 | 11.7 | 3.0 |
| 2.0 | 10.7 | 15.6 | 13.4 | |
| 2.5 | | | | 10.0 |
| 3.0 | | | 14.4 | |
| 4.9 | | | | 9.9 |
| 7.0 | | | | 9.6 |
| 12.6 | | | | 11.5 |
| 24.6 | | | | 12.2 |

ficiency and precision of the extraction procedures. In 5 experiments at each concentration, the mean recoveries were 2.1 ± 0.2 μg and 9.8 ± 0.3 μg, respectively. When no peaks in absorption were observed at 242 mμ, the data were not included in the calculations. The steroidal composition of the ethanolic extracts was determined by paper chromatographic methods(4,5).

*Results. Incubation studies.* The mouse and rat adrenal tumor $\Delta^4$-3-ketosteroid production with and without ACTH is recorded in Table I. The mouse adrenal tumors increased their secretion of steroids about 6 fold following addition of ACTH to the incubation medium. With the rat adrenal tumors, the increase was greater due to lower basal secretory capacity of the tumor slices in the absence of ACTH and higher secretory rates in the presence of ACTH. The steroid secretory rates of the tumor slices seemed to fall in the same range despite variations in tumor latency periods in the rat(1) following different types of operative treatment(Table I).

The sensitivity of these experimental adrenal tumors to ACTH was very high. In Table II, maximal steroid secretion of both rat and mouse adrenal tumor slices was obtained between 2 and 3 mU of ACTH per 100 mg tissue. These peak steroid values are similar to those in Table I where higher dosages of ACTH were given.

Incubation time studies of the total $\Delta^4$-3-ketosteroid secretion of mouse and rat adrenal tumor slices are shown in Fig. 1.

The basal secretory rates of the normal

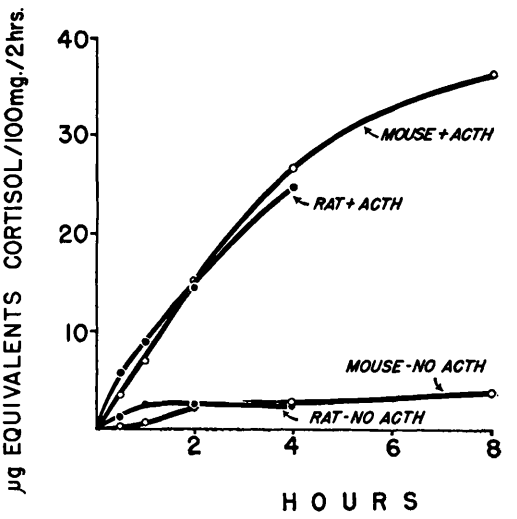


FIG. 1. Rates of $\Delta^4$-3-ketosteroid secretion by mouse and rat adrenal tumor slices with maximal amounts of ACTH (60 mU for the mouse, 115 mU for the rat) and control slices.

mouse and rat adrenal segments (medulla intact) were higher per unit weight than those exhibited by the respective adrenal tumor slices (Tables III and IV). In turn, the maximal responses to ACTH by the normal tissues were also higher, resulting in activity ratios approximating that of the mouse adrenal tumor slices. Minimal stimulatory concentrations of ACTH for the normal mouse and rat adrenals were not determined. In testings with our group of normal rat adrenals, ACTH concentrations of 75-100 mU were maximal; with the normal mouse adrenals,

TABLE III. Response of Normal Male $LAF_1$ Mouse Adrenal Halves to ACTH.

| | mU ACTH per 100 mg | μg equiv. cortisol/100 mg/2 hr* | Stimulation ratio† |
|---|---|---|---|
| | 0 | 6.4 | 10.4 |
| | 336 | 66.5 | |
| | 0 | 14.4 | 4.1 |
| | 210 | 59.5 | |
| | 0 | 6.3 | 5.6 |
| | 640 | 35.3 | |
| | 0 | 6.9 | |
| | 630 | 70.0 | 10.1 |
| | 560 | 54.7 | 7.9 |
| Avg | 0 | 8.5 | 7.6 |
| | 210-640 | 57.2 | |

* One flask containing 10-11 adrenal pairs was used for each determination.
† See footnote Table I.

TABLE IV. Response of Normal Male WR Rat Adrenal Halves to ACTH.

| | mU ACTH per 100 mg | No. of* determinations | μg equiv. cortisol/100 mg/2 hr | Stimulation ratio† |
|---|---|---|---|---|
| | 0 | 2 | 18.4 (17.5–19.2)‡ | 4.9 |
| | 100 | 2 | 90.5 (89.5–91.5) | |
| | 0 | 2 | 18.3 (18.2–18.3) | 3.9 |
| | 400 | 5 | 72.1 (62.5–87.5) | |
| | 0 | 3 | 9.1 ( 7.1–10.4) | 5.6 |
| | 600 | 3 | 50.5 (46.7–55.7) | |
| Avg | 0 | | 15.3 | 4.6 |
| | 100–600 | | 71.0 | |

* Each flask contained 1 pair of adrenals.
† See footnote Table I.
‡ Avg and range.

the levels of ACTH used were assumed to be maximal.

In Fig. 2, the absorption curves of standard cortisol solutions are compared to experimental curves of adrenal tumor ethanolic extracts. Since the curves appeared to be identical in shape at similar peak readings, the presence of an interfering substance seems unlikely.

*Chromatography.* Under ACTH stimulation, the major steroid synthesized by normal mouse adrenal incubates was corticosterone

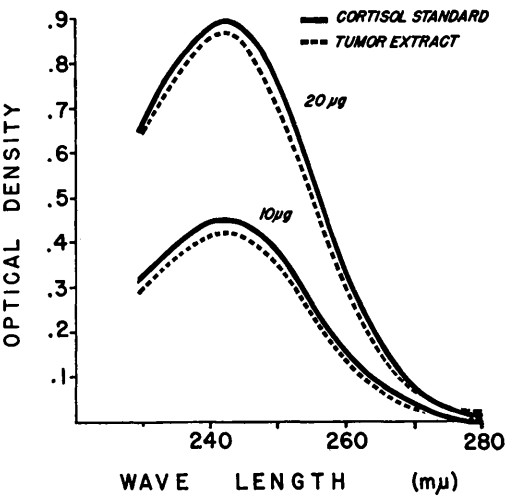


FIG. 2. Comparison of absorption spectra of standard cortisol in 95% ethanol and of methylene chloride extracts of adrenal tumor incubates redissolved in 95% ethanol.

(72% of the total $\Delta^4$-3-ketosteroids), 11β-hydroxy-$\Delta^4$-androstene-3,17-dione being present in small quantities (Table V). Adrenal tumor tissue, when incubated with ACTH, produced approximately equal amounts of these 2 steroids. However, 25-65% of the total $\Delta^4$-3-ketosteroids in the tumor incubates

TABLE V. Corticosterone and 11β-Hydroxy-$\Delta^4$-androstene-3,17-dione Content of $\Delta^4$-3-Ketosteroids Extracted from Incubates of Normal Adrenals and Adrenal Tumor Tissue from $LAF_1$ Mice.

| Exp. | mU ACTH per 100 mg | Condition of incubation | mg tissue | $\Delta^4$-3-ketosteroids, μg/100 mg‡ | Corticosterone μg/100 mg | % | 11β-Hydroxy-$\Delta^4$-androstene-3,17-dione μg/100 mg | % |
|---|---|---|---|---|---|---|---|---|
| | | | Normal mouse adrenals | | | | | |
| 32, 38 | 0 | Standard† | 90 | 10.4 | × | | × | |
| " | 275 | " | 80 | 63.0 | 45.0 | 72 | 8.7 | 14 |
| | | | Mouse adrenal tumor | | | | | |
| 22–24 | 0 | Standard | 1000 | 3.3 | × | | × | |
| 22 | 1.5* | " | 1100 | 13.2 | 2.6 | 20 | 2.0 | 15 |
| 23 | 1.5* | " | 1200 | 8.6 | 1.6 | 19 | 2.3 | 27 |
| 24 | 2.0* | " | 900 | 14.1 | 3.2 | 23 | 3.4 | 24 |
| 28 | 0 | " | 1100 | 2.5 | × | | .5 | 20 |
| " | 60 | ½ & 1 hr incub. | 600 | 5.5 | 1.0 | 18 | 3.2 | 58 |
| " | " | 2 hr incub. | 300 | 15.7 | 9.5 | 60 | 2.9 | 18 |
| " | " | 4 " " | 200 | 27.2 | 12.1 | 45 | 7.1 | 26 |
| " | " | 8 " " | 300 | 34.3 | 4.7 | 14 | 3.0 | 9 |
| 36, 37 | 25 | No glucose in buffer | 600 | 8.0 | × | | 3.4 | 43 |
| " | " | Standard | 600 | 11.5 | 4.9 | 43 | 1.9 | 17 |

* Avg ACTH conc. in 3 flasks. Individual flasks contained .5, 1 and 2 mU ACTH (Exp. 22 and 23), and 1, 2 and 3 mU ACTH (Exp. 24).
† Standard incubation conditions as described in text.
‡ Determined as "μg cortisol" by measuring O.D. at 242 mμ in Beckman model DU Spectrophotometer.
× = Not detected on paper chromatograms.

consisted of unidentified compounds. Analysis of 2 normal rat adrenal incubates for steroid content yielded 63% (11.4 μg/100 mg tissue without ACTH) and 39% (20.0 μg/100 mg tissue with ACTH) corticosterone in the total $\Delta^4$-3-ketosteroid fraction. In 2 rat adrenal tumors incubated with ACTH, 5.7 μg and 2.3 μg of corticosterone and 2.0 μg and 2.6 μg per 100 mg tissue of a compound with chromatographic behavior of cortisone, respectively, were found. The sums of the 2 steroids represented 54% and 35%, respectively, of the total $\Delta^4$-3-ketosteroids present in the tumor slice incubates, the remainder consisting of compounds yet to be identified.

The influence of glucose on steroid synthesis by the mouse adrenal tumor slices was investigated. The absence of glucose from the incubation medium had no effect on basal secretory rate, but the steroid secretion at maximal levels of ACTH with glucose was increased about 40% in 2 experiments as was in experiments of Schönbaum *et al.* using rat adrenal segments *in vitro*(6). The presence of glucose, however, effected a change in concentration of corticosterone and 11β-hydroxy-$\Delta^4$-androstene-3,17-dione in a reciprocal manner (Table V, Exp. 36, 37).

Three adrenal tumors‡ originating in castrated ce strain mice and transplanted in the strain of origin were investigated for steroid secretion. At autopsy the tumor-bearing hosts (2 male and 1 female) showed signs of stimulation by gonadal hormones. Tumors 4-6 g in weight from the 3 strains were incubated with and without ACTH. No extractable $\Delta^4$-3-ketosteroids were detected.

*Discussion.* The secretory response of the mouse and rat adrenal tumor slices to a low and narrow range of ACTH concentrations indicated a much greater sensitivity of adrenal tumor than normal adrenal tissue to the hormonal stimulation. Maximal stimulation of the normal rat adrenals (per unit weight) required about 30 times more ACTH than rat adrenal tumor. If log-dose response curves are drawn for the mouse values in Table II and the linear curves extrapolated to the base secretory levels, these tumor slices may well respond to 0.1 or 0.2 mU ACTH.

These adrenal tumors were able to secrete adrenocorticoids in the absence of ACTH. A theoretical comparison of the secretory potency of a 5 g tumor versus that of intact adrenals in the mouse and rat reveals some very striking results. A 5 g mouse adrenal tumor could theoretically secrete 130 μg of corticoids in 2 hours in the absence of ACTH and about 680 μg in 2 hours in the presence of maximal quantities of ACTH. On the other hand, a pair of normal mouse adrenals weighing about 4 mg would secrete less than 0.4 μg of corticoids in 2 hours in the absence of ACTH and about 2.3 μg in 2 hours in the presence of ACTH. Similar calculations comparing a 5 g rat adrenal tumor with a pair of rat adrenals (about 33 mg/pair) would show a secretion of 95 μg and 5 μg corticoids in the absence of ACTH and 870 μg and 25 μg corticoids per 2 hours with excess of ACTH, respectively. Thus, no endogenous ACTH would be necessary for the maintenance of hypercorticoidism in the tumor-bearing mouse or rat. Blood corticoid assays are being carried out to determine if the corticoid level is really as high as indicated by the tumor secretory potential.

The presence of corticosterone, 11-dehydrocorticosterone, cortisone and possibly 11β-hydroxy-$\Delta^4$-androstene-3,17-dione, among other steroids, in rat adrenal effluents has been demonstrated(7,8,9). Corticosterone and 20α-dihydrocorticosterone have been identified in mouse serum(10). Under the experimental conditions reported here, the primary adrenal steroids synthesized by mice of the $LAF_1$ strain appear to be corticosterone and 11β-hydroxy-$\Delta^4$-androstene-3,17-dione. In addition to these compounds, adrenal tumor incubates yielded substances suggestive in their chromatographic behavior of progesterone, 11β-hydroxyprogesterone, 11-dehydrocorticosterone and $\Delta^4$-3-keto-20, 21-dihydroxy steroids. The quantitative (and possibly qualitative) differences in steroid production between normal mouse adrenals and adrenal tumor suggest different enzyme patterns and/or reaction kinetics and equilibria in the 2 types of tissues. The marked decrease in

‡ Kindly given to us by Dr. Arthur Kirschbaum.

corticosterone accumulation in tumor tissue may be explained by a combination of reduced synthesis and increased adrenal metabolism of corticosterone to other steroids. The finding of $\Delta^4$-3-ketosteroid with 21-desoxy or 20,21-dihydroxy side chains, and of 11-dehydrocorticosterone lend support to this hypothesis.

The absence of any detectable amounts of 17$\alpha$-hydroxylated corticosteroids in the mouse adrenal tumor or normal adrenal tissues confirms the *in vivo* study of Southcott(10) and the *in vitro* study of Hoffman(11).

*Summary.* 1) Slices from transplants of 2 secretory adrenocortical tumors were incubated with and without ACTH. Incubation mixtures were extracted and the total $\Delta^4$-3-ketosteroids quantitatively determined from peak optical densities at 242 m$\mu$. Basal steroid secretion of the mouse and rat tumor slices averaged 2.6 and 1.9 $\mu$g equiv. cortisol/100 mg/2 hours respectively; with maximum stimulating amounts of ACTH, the average rates were 13.6 and 17.4 $\mu$g, respectively. Both tumors responded maximally to 2-3 mU of the hormone. 2) Under ACTH stimulation, corticosterone and 11$\beta$-hydroxy-$\Delta^4$-androstene-3,17-dione were produced in approximately equal amounts by the mouse adrenal tumors. However, 25-65% of the total $\Delta^4$-3-ketosteroids consisted of compounds still to be identified. The secretion of the rat adrenal tumor slices consisted mainly of corticosterone. 3) Glucose in the buffer stimulated the total steroid secretion of the mouse adrenal tumor slices about 40%. 4) An adrenal tumor that secreted gonadal hormones was incubated with and without ACTH. No measurable $\Delta^4$-3-ketosteroids were detected in the incubates.

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## Effect of Varying Levels of DL-Tryptophan in Diet on Dental Caries in Rats.* (23203)

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The chemistry of the salivary glands of the rat has recently received new interest due to experimental evidence indicating a correlation between activity of the thyroid gland, histologic picture of the salivary glands, and incidence of dental caries(1,2). Moreover, protein metabolism in the salivary glands as it relates to caries experience deserves special consideration, since proteolytic activity of rat salivary glands has been shown to be dependent upon normal thyroid function(3). Tryptophan is of particular interest in this relationship since "significantly greater amounts of tryptophan or tryptophan-like compounds in caries-free (human) salivas compared to caries-susceptible" has been reported(4).

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