Steroids in Adrenal Venous Blood of the Dog.*† (20549)

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Adrenal venous blood of the dog has been quantitatively analyzed for steroid components. Fourteen fractions have been isolated, 10 of which appear to be steroids. Three have been identified as 17-hydroxycorticosterone, corticosterone, and 11-desoxy-17-hydroxycorticosterone. Certain chemical characteristics of the remaining fractions have been determined.

Methods. Male dogs were anesthetized with sodium pentobarbital. Adrenal venous blood was collected from a cannula placed in the left lumboadrenal vein. All collateral branches of the lumboadrenal vein were ligated and the adrenal vein ligated between the gland and the inferior vena cava. Thirty to 50 mg heparin were injected in divided doses to prevent clotting. Blood was collected in lots of approximately 250 ml. Immediately after collection, each lot was diluted 1:1 with water and extracted twice with a volume of chloroform equal to that of blood plus water. Centrifugation was necessary to break the emulsion formed during extraction. The pooled chloroform extracts from blood of a single dog were evaporated to dryness immediately or kept overnight at —10°C and evaporated the next day. A total of 3500 ml of adrenal venous blood was obtained from 4 dogs; the dried extracts were pooled and partitioned between hexane and 70% ethanol. The 70% ethanol fraction was chromatographed on paper by the method of Burton *et al.*(1) using the solvent system propylene glycol-toluene. An outline of the design of chromatography is presented in Table I. The developed chromatograms were examined for steroids as follows: 1) the entire strip was divided at right angles to its length into a series of ½- to 1-inch cuts which were eluted in methanol in individual test tubes and subsequently analyzed, or 2) color tests (reaction with ammoniacal $AgNO_3$, triphenyltetrazolium, or concentrated sulfuric acid streaked on a glass plate) were applied to narrow strips cut the full length of the chromatogram and those portions of the chromatogram corresponding to the indicated locations of steroids were cut out and eluted in methanol for further analysis. The first method was used in screening preliminary chromatograms; the second, in isolating individual fractions. The eluted substances were quantitatively analyzed by 4 methods: 1) ultraviolet absorption spectrum, 2) reaction with phenylhydrazine, 3) reaction with concentrated sulfuric acid, and 4) reaction with m-dinitrobenzene.

Results. Fourteen fractions were isolated. (Tables I and II). *Fraction 6* chromatographed in the same area as 17-hydroxycorticosterone. Ultraviolet absorption, 240 mμ; reaction with sulfuric acid, orange color with strong fluorescence; absorption in phenylhydrazine reagent to give the 400 mμ peak characteristic of 17,21-dihydroxy-20-ketosteroids; absorption maxima at 240, 280, 390, and 470 mμ in concentrated sulfuric acid. On the basis of these findings, Fraction 6 was identified as 17-hydroxycorticosterone. Its concentrations, 147 μg/100 ml adrenal venous blood, was the highest of the various fractions isolated.

Fractions 1 through 5 were more polar than 17-hydroxycorticosterone. A narrow strip cut from Chromatogram 3 and placed on a glass plate streaked with concentrated sulfuric acid exhibited the following bands of color starting at the origin and progressing toward the front: 1) green fluorescence, 2) pink, 3)

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TABLE I. Paper Chromatography of Extract of Adrenal Venous Blood (Propylene Glycol Stationary Phase, Toluene Mobile Phase).

3500 ml adrenal venous blood

Combined $CHCl_3$ extracts partitioned, 70% ethanol : hexane

Chromatogram 1—72 hr, HSPG*: toluene, 70% ethanol fraction. The first inch, heavily pigmented, was discarded; the remainder was eluted and rechromatographed.

Chromatogram 2—72 hr, HSPG : toluene, eluate of Chromatogram 1. Components incompletely resolved. Chromatogram divided.

Chromatogram 3—240 hr, HSPG : toluene, first 2 inches of Chromatogram 2. Fractions 1 through 6 resolved.

Chromatogram 6—15 hr, HSPG : toluene, overflow of Chromatogram 1. Components incompletely resolved.

Chromatogram 7—22½ hr, HSPG : toluene, eluate of Chromatogram 6. Incomplete resolution.

Chromatogram 8—23½ hr, FSPG*: toluene, eluate of Chromatogram 7. Fractions 9 and 10 incompletely resolved. Fractions 11 and 12 resolved.

Chromatogram 9—48 hr, HSPG : toluene, fractions 9 and 10 from Chromatogram 8. Fractions 9 and 10 resolved.

Chromatogram 4—144 hr, HSPG : toluene, remainder of Chromatogram 2. Fractions 6 and 7 resolved.

Chromatogram 5—96 hr, HSPG : toluene, overflow of Chromatogram 4. Fraction 8 isolated.

Chromatogram 10—5½ hr, FSPG : toluene, combined overflows from Chromatograms 6, 7 and 8. Fractions 12 and 13 incompletely resolved. Fraction 14 isolated.

Chromatogram 11—24 hr, FSPG : toluene, fractions 12 and 13 of Chromatogram 10. Fractions 12 and 13 resolved.

* HSPG = half-strength propylene glycol. Propylene glycol is diluted 1:1 with methanol before application to the paper and the methanol allowed to evaporate.
FSPG = full-strength propylene glycol.

green fluorescence, 4) green fluorescence and 5) pinkish brown. Immediately below Fraction 5 was the brilliant green fluorescence of Fraction 6 (17-hydroxycorticosterone). Fractions 1, 3, and 4 reduced ammoniacal $AgNO_3$ and reacted with phenylhydrazine; they exhibited no definite maxima at 240 mμ. Fractions 2 and 5 absorbed in the ultraviolet but did not react with phenylhydrazine. Fraction 2 absorbed maximally at 255 mμ, and 5, at 285 mμ. Concentrations were estimated on the basis of the phenylhydrazine reaction: Fraction 1, 1.25; Fraction 3, 3.8; Fraction 4, 1.0 μg/100 ml. Fractions 2 and 5 could not be quantitated.

Fraction 7. About 5 μg/100 ml of this unidentified steroid were present. It reduced ammoniacal $AgNO_3$, absorbed at 230 mμ and reacted with phenylhydrazine reagent to give the 340 mμ maximum characteristic of Δ^4-3-ketosteroids without the 17,21-dihydroxy-20-ketone grouping. It gave an orange solution without fluorescence which absorbed maximally at 240, 285 and 370 mμ in concentrated sulfuric acid. Ultraviolet absorption, phenylhydrazine reaction and $AgNO_3$ reduc-

TABLE II. Substances Found in Adrenal Venous Blood of the Dog.

Fractions in order of polarity	Ammoniacal $AgNO_3$ reduction	Ultraviolet absorption (max. mμ)	Absorption in phenylhydrazine reagent (max. mμ)	Concentration (μg/100 ml)	Identification
1	+	—	400	1.25	Not identified
2	—	255	0	†	" "
3	+	—	400	3.80	" "
4	+	—	400	1.0	" "
5	—	285	0	†	" "
6	+	240	400	147.0	17-hydroxycorticosterone
7	+	230	340	5.0	Not identified
8	+	230–240*	400	3.0	" "
9	—	0	0	†	" "
10	—	215, 265	0	†	" "
11	+	240	400	15.0	11-desoxy-17-hydroxycorticosterone
12	+	240	340	47.0	Corticosterone
13	+	235	340	16.0	Not identified
14	?	230–235	330–340*	18.0	" "

* Inflection but no definite maximum.
† Quantitation not possible.

tion suggest the presence of a Δ^4-3-ketone configuration and a α-ketol side chain. However, the presence of 2 more atoms of oxygen on the steroid nucleus is suggested by the position of this steroid on the chromatogram.

Fraction 8 was present in a concentration of about 3 μg/100 ml and chromatographed in the same region as cortisone. It reduced $AgNO_3$ and exhibited a maximum at 400 mμ with phenylhydrazine. The ultraviolet absorption curve was suggestive of absorption in the region 230 to 240 mμ. In concentrated sulfuric acid, the substance exhibited maxima at 285 and 390 mμ (cortisone exhibits maxima at 280, 340 and 400 mμ). The steroid did not react with iodine reagent (cortisone gives a brilliant blue color when treated with this reagent).

Fractions 9 and 10. Two unidentified substances were found whose polarity was slightly less than that of Fraction 8. The more polar of these, Fraction 9, became distinctly pink when treated with sulfuric acid, and exhibited maxima at 270, 410 and 485 mμ with this reagent; it did not absorb in the ultraviolet or react with phenylhydrazine. $AgNO_3$ reduction was questionable. Fraction 9 is probably not a steroid. Fraction 10 was slightly less polar than Fraction 9. It was seen as an absorbing band when the chromatogram was viewed under ultraviolet light. The material did not reduce $AgNO_3$, but rather it retarded the usual reduction which occurs when the paper strip, freshly dipped in ammoniacal $AgNO_3$, is exposed to light. It absorbed at 215 and 265 mμ and exhibited maxima at 210, 270 and 550 mμ with sulfuric acid. It did not react with the phenylhydrazine reagent. Fraction 10 is probably not a steroid.

Fraction 11, present in a concentration of about 15 μg/100 ml, was tentatively identified as 11-desoxy-17-hydroxycorticosterone. It reduced $AgNO_3$, absorbed at 240 mμ and gave a 400 mμ peak with phenylhydrazine. It became pink when treated with sulfuric acid, and the sulfuric acid chromogen was identical with that of 11-desoxy-17-hydroxycorticosterone. A mixture of 11-desoxy-17-hydroxycorticosterone and Fraction 11 chromatographed as a single spot.

Fraction 12 found on chromatograms 8, 10 and 11 was identified as corticosterone. It reduced $AgNO_3$, absorbed at 240 mμ, gave a brilliant green fluorescence with sulfuric acid and reacted with phenylhydrazine to give a maximum at 340 mμ. The sulfuric acid chromogen was identical with that of corticosterone. It was present in a quantity of 47 μg/100 ml, the second highest concentration of the fractions isolated.

Fraction 13 has not been identified. It re-

duced $AgNO_3$, absorbed at 235 mμ, gave a 340 mμ maximum with phenylhydrazine reagent and exhibited maxima at 280 and 370 with sulfuric acid. It was less polar than corticosterone, but considerably more polar than desoxycorticosterone. These data suggest a C_{21} O_4 compound with Δ^4-3-ketone and α-ketol groupings. The sulfuric acid chromogen was not the same as that of 11-dehydrocorticosterone (maxima at 280, 350 and 410 mμ). On the basis of the phenylhydrazine reaction, 16.0 μg/100 ml were estimated to be present.

Fraction 14 chromatographed close to the solvent front. The high mobility of this material in the solvent system precluded adequate resolution. It had a weak reducing effect on ammoniacal $AgNO_3$, absorbed strongly in the ultraviolet at 230 to 235 mμ and exhibited a maximum at 290 mμ in sulfuric acid. The absorption curve in the phenylhydrazine reagent exhibited an inflection but not a definite maximum in the region 330 to 340 mμ. Fraction 14 did not give a reaction with m-dinitrobenzene characteristic of 17-ketosteroids. If the ultraviolet extinction coefficient is of the same order of magnitude as that of the cortical steroids, Fraction 14 is present in a concentration of 18 μg/100 ml.

An eluate of that portion of Chromatogram 10 which would have contained substances less polar than Fraction 14 and the overflow of Chromatogram 10 were analyzed in the ultraviolet region of the spectrum and tested with m-dinitrobenzene. There was no evidence for the presence of Δ^4-3-ketones or 17-ketosteroids.

Discussion. Reich *et al.*(2) demonstrated the presence of 17-hydroxycorticosterone and corticosterone in dog adrenal venous blood. Bush(3) found 17-hydroxycorticosterone, corticosterone and 2 substances more polar than 17-hydroxycorticosterone. He depended largely upon position on the paper chromatogram for identification. Zaffaroni and Burton(4) reported the presence of 9 $AgNO_3$-reducing substances in dog adrenal venous blood. Two of these were identified as 17-hydroxycorticosterone and corticosterone; the other 7 were characteristized as α-ketols. The results presented in this paper confirm that 17-hydroxycorticosterone and corticosterone are the 2 major components present in dog adrenal venous blood. 11-Desoxy-17-hydroxycorticosterone has been identified as a component of adrenal venous blood for the first time. Eleven other fractions have been isolated and partially characterized and it appears that 7 of these are steroids. It is not certain that all these substances are produced by the adrenal. A definitive answer must await analyses of the concentrations of these substances in both arterial and venous blood of the adrenal.

The physiological significance of the complex pattern of steroids in adrenal venous blood cannot be evaluated at this stage of development of the problem. Each fraction must be assayed for biological activity regardless of its concentration as determined by chemical methods. Furthermore, the fractions must be assayed alone and in combination to test the possibility that the steroids in adrenal venous blood act synergistically or antagonistically. The biological activities of 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone have been described. Fraction 8, present in relatively small concentration, has been demonstrated recently to exert a very potent sodium-retaining effect in adrenalectomized rats(5). Fraction 8 appears to be approximately 25 times as potent as DOCA and may be identical with the sodium-retaining factor recently described by Simpson *et al.*(6). This fraction is an example of a substance, present in very small amount in adrenal venous blood, which nevertheless exerts a very marked biological effect.

The adrenal cortex may exert a variety of metabolic effects by secreting a variety of patterns of adrenocorticosteroids. An aberration in electrolyte metabolism may be associated with the secretion of a pattern with a higher than normal concentration of those steroids whose predominant influence is on electrolyte metabolism. On the other hand, an aberration in carbohydrate metabolism may be associated with the secretion of a pattern with a higher than normal concentration of those steroids whose predominant in-

fluence is on carbohydrate metabolism. An approach to this problem is made possible by the development of technics for the quantitative analysis of steroids in adrenal venous blood.

Summary. Adrenal venous blood of the dog has been quantitatively analyzed by paper chromatography for steroid components. Fourteen fractions have been isolated; 3 have been identified as 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone; 7 of the unidentified fractions exhibit chemical reactions characteristic of adrenocorticosteroids.

ADDENDUM. In a subsequent experiment conducted in collaboraton with Dr. Hans Hirschmann a sample containing Fractions 7 and 8 was acetylated and chromatographed on paper 90½ hours, employing the system propylene glycol-hexane described by Savard. Five distinct zones were found by examining the strip under ultraviolet light. These subfractions (numbered 1-5 in order of their position on the chromatogram, starting at the origin) were eluted with methanol and were examined spectroscopically in this solvent and after reaction with phenylhydrazine-sulfuric acid reagent. Subfractions 1, 2, and 3 absorbed maximally near 240 mμ; subfraction 4, near 235 mμ, while subfraction 5 showed only a shoulder in this region. With phenylhydrazine reagent subfractions 1 and 5 absorbed maximally at 390-400 mμ, whereas subfractions 2, 3, and 4 gave products which did not exhibit absorption peaks in this region. Each subfraction was treated with acetylcholinesterase in glycylglycine buffer at pH 7.5 for 4 hours at 30°C, and assayed for sodium-retaining activity by the method described in an earlier communication(5). All subfractions were inactive except subfraction 4 which possessed high sodium-retaining activity. No claim of homogeneity is made for any of these subfractions, but it would appear that the sodium-retaining activity and the chromogenicity in the phenylhydrazine reagent shown by our previously described Fraction 8 are not due to the same substance.

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Increased Excretion of Dehydroascorbic and Diketogulonic Acids in Urine of Rats after Standardized Temperature Shock. (20550)

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Increased urinary excretion of 2 oxidation products of ascorbic acid (AsA), namely dehydroascorbic acid (DHA) and diketogulonic acid (DKA) has been observed in rats during exposure to an environmental temperature of 0°C(1) and after exposure to high doses of X-ray(2), as compared to control excretions on comparable rats under normal conditions. A third type of stress, namely standardized temperature shock(3), has been tested and the results observed are reported here.

Experimental. The experimental animals were adult albino rats of both sexes, belonging to the same strain as those employed in the previous work(1,2), which were housed in individual metabolism cages as previously described. At the beginning of the control period, each animal was anesthetized with nembutal, and immersed up to the neck in a water-bath maintained at 37°C for 15 seconds.