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Isolation of Aldosterone (Electrocortin)

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Isolation of crystalline aldosterone[3] from beef adrenal glands by partition chromatography is described. A series of three partitions of defatted adrenal extract, the first between benzene and 50% aqueous methanol, the next two between 10% ethyl acetate in benzene and 50% aqueous methanol, provided 10–25 mg. of the crystalline hormone per 500 kg. of glands. Some physical properties of the hormone are recorded.

The isolation from extracts of beef adrenal glands of a new crystalline hormone with high activity in the control of mineral metabolism has been reported briefly by laboratories abroad[1] and in this country.[2] The new crystalline hormone was originally named electrocortin by the English–Swiss group. With establishment of its chemical structure as 11β,21-dihydroxy-3,20-diketo-4-pregnene-18-al, these investigators assigned to the hormone the new designation aldosterone.[3]

Non-crystalline concentrates of presumably the same hormone have been prepared from hog adrenal gland extracts,[4] dog adrenal venous blood[5,6] and human urine,[7] and by perfusion of monkey adrenal glands.[6] In addition it has been implied that the same mineral-active substance is present in placental extract.[8]

We wish to describe here the details of our own experience in isolation and characterization of crystalline aldosterone from beef adrenal glands. A comparison of sources, methods of isolation and biological and physical properties leaves no doubt that the hormone isolated here is the same as that reported by Simpson, *et al.*,[1] and by Mattox and co-workers.[2]

Experimental

Assay.—The bioassay was a modification of the urinary sodium retention assay described by Dorfman, *et al.*[9] Test samples and desoxycorticosterone acetate for comparison were injected subcutaneously in 0.25 ml. of 30% ethanol–water into adrenalectomized rats (6 per sample) which had been maintained for 24 hours on a low sodium diet. Urines of groups of three rats were collected over 4.5 hours; before collections, each rat received 5 ml. of saline intraperitoneally. Sodium estimations were run spectrometrically. Sodium levels in "control" animals (vehicle alone) were usually about 20 meq./liter; in "comparison" animals which received 60 μg. of DOCA, about 4 meq./liter.

Preparation of Crude Extract.—Frozen beef adrenal glands were extracted and preliminary purification carried out by a modification of the method of Cartland and Kuizenga.[10] Hundred-kilogram lots of glands were ground with Dry Ice and stirred for 24 hours at −15° with 70-gallon volumes of acetone. The slurries were filtered and the solid residues extracted twice more with 70-gallon quantities of 80% aqueous acetone, each time for 24 hours. The combined acetone extracts were concentrated *in vacuo* to *ca.* 15-gallon aqueous sludges. These were extracted with three portions of *ca.* 5 gallons each of petroleum ether to remove fatty materials. The aqueous residues were next extracted three times with 10-gallon quantities of ethylene chloride. The ethylene chloride extracts, which still contained varying amounts of fatty materials in addition to the adrenal steroids, were concentrated to about one liter. Operations up to this point were done in pilot plant equipment; subsequent steps were carried out in the laboratory.

Preliminary Purification.—The steroid concentrates from five 100-kilogram lots of glands were combined and filtered, the ethylene chloride was removed completely *in vacuo*, and the residual dark oil (50–400 g.) taken up in 300 ml. of hexane and 150 ml. of methanol. A 150-ml. volume of water was added and the mixture well shaken. After separation of layers, the aqueous phase was extracted four more times with 300-ml. portions of hexane, and the hexane extracts back-washed with two 200-ml. volumes of 50% aqueous methanol. The hexane phase was discarded, the combined aqueous alcoholic solutions were concentrated to about 200 ml. *in vacuo*, and the residual aqueous sludge was extracted with six 200-ml. volumes of ethyl acetate.

The ethyl acetate extract was washed with three 50-ml. portions of saturated aqueous sodium bicarbonate solution, three 50-ml. portions of 0.1 *N* hydrochloric acid and three 50-ml. volumes of water. The resulting base, acid and water washes were then back-extracted, in that order, with three 100-ml. portions of ethyl acetate, and the aqueous residues discarded. All solvent was removed from the combined ethyl acetate solutions by distillation *in vacuo* with use of a heating bath held below 50°. The resulting neutral steroid concentrate was a dark amber sirup which weighed about 10 g.

First Partition Chromatogram.—Supercel, the inert support for the stationary phase, was purified[11] by digestion overnight with hot concd. hydrochloric acid, washing with water until neutral and then exhaustive extraction with methanol and benzene. The clean Supercel was finally dried in an oven at 100°. All solvents used were of reagent grade, and were redistilled. The solvent system[12] was prepared by equilibrating two volumes of benzene, one volume of methanol and one volume of water.

The first partition column, 9.5 × 100 cm., required two kilograms of Supercel. The support was equilibrated with the solvents by slurrying 100-g. portions with excess upper phase and 100 ml. of lower phase in a Waring Blendor. The resulting slurries were combined and slowly poured into the column, which had been about half-filled with upper phase. Excess solvent was allowed to drain off, and the column packed by brief application of vacuum at the bottom and manual tamping at the top.

To load the column, the steroid concentrate (*ca.* 10 g.) was dissolved in 40 ml. each of benzene and methanol 40 ml. of water added, and the mixture shaken and centrifuged. The upper phase was removed and put on the column. The aqueous phase was transferred to a beaker, and any insoluble residue re-extracted with a further 20 ml. of each solvent. The total aqueous phase was then mixed with slightly more than the amount of Supercel required to bring the ratio to 1 ml./g., the mixture stirred well manually and extracted,

(1) S. A. Simpson, J. F. Tait, A. Wettstein, R. Neher, J. v. Euw and T. Reichstein, *Experientia*, **9**, 333 (1953).

(2) (a) V. R. Mattox, H. L. Mason, A. Albert and C. F. Code, THIS JOURNAL, **75**, 4869 (1953); (b) V. R. Mattox, H. L. Mason and A. Albert, *Proc. Staff Meetings Mayo Clinic*, **28**, 569 (1953).

(3) S. A. Simpson, J. F. Tait, A. Wettstein, R. Neher, J. v. Euw, O. Schindler and T. Reichstein, *Experientia*, **10**, 132 (1954).

(4) R. E. Knauff, E. D. Nielson and W. J. Haines, THIS JOURNAL, **75**, 4868 (1953).

(5) G. L. Farrell and J. B. Richards, *Proc. Soc. Exptl. Biol. Med.*, **83**, 628 (1953).

(6) S. A. Simpson, J. F. Tait and I. E. Bush, *Lancet*, **263**, 226 (1952).

(7) J. A. Luetscher and B. B. Johnson, *J. Clin. Investigation*, **32**, 585 (1953).

(8) J. J. Majnarich and R. N. Dillon, *Arch. Biochem. Biophys.*, **49**, 247 (1954).

(9) R. I. Dorfman, A. M. Potts and M. L. Fell, *Endocrin.*, **41**, 464 (1947).

(10) G. F. Cartland and M. H. Kuizenga, *J. Biol. Chem.*, **116**, 57 (1936).

(11) W. R. Butt, Peggy Morris, C. J. O. R. Morris and D. C. Williams, *Biochem. J.*, **49**, 434 (1951).

(12) A paper chromatography system investigated by I. E. Bush, *ibid.*, **50**, 370 (1952).

and finally poured on top of the prepared column. It was slurried well in upper phase, care being taken that the prepared column was not disturbed; the load was carefully and gently packed, overlaid with a disc of coarse filter paper, and finally a glass wool mat. A constant-level solvent trough was placed in position and effluent collected (140 ml./hour) in a large flask.

The first 3 l. of eluate was discarded; the next 7 l. was collected and put aside. This portion of the eluate contained corticosterone and steroids of similar polarity, as well as much of the cortisone. One-hour fractions were then collected until a total of 20 l. of solvent had passed through the column. At this point, the eluate was analyzed spectrometrically for Δ^4-3-ketosteroid content. One-quarter-ml. aliquots of every third fraction were taken to dryness, and the residues dissolved in 3 ml. each of ethanol. Optical density at 240 mμ was plotted against eluate volume. Aldosterone could not be definitely located thus at this stage, since it moved in the trailing shoulder of a much larger cortisone peak. However, the analysis gave some assurance that the column had operated properly, and was useful in estimating quantities of Δ^4-3-ketosteroid for the bioassay. Finally, the column was flushed with methanol, and steroids more polar than aldosterone collected.

For positive location of aldosterone, samples of effluent in the range 12 to 18 liters (total) were bioassayed. The hormone was generally found between 13.0 and 16.5 l. It may be noted that while initially some half-dozen assays were required to delineate the aldosterone zones in partition column eluates, increasing experience with the columns and solvent systems permitted a reduction in the number of assays needed. On the basis of bioassay and spectrometric analysis, those active fractions which were judged to be nearly free of cortisone were combined and the resulting 75–150 mg. of amber sirup resolved further in a second partition column. Experiments in which only the center fractions from active zones were chosen were most successful in later yielding crystalline aldosterone.

Repartition.—Selected active material from the first column was subjected to further resolution in a 6 × 150-cm. Preparation of the column, which required 1 kg. of Supercel, and loading technique, were as described above except that benzene containing 10% (v./v.) of ethyl acetate was substituted for pure benzene in the solvent system. Effluent was collected at about 60 ml./hour. Active material was found generally between 4.0 and 5.5 liters of total effluent. The spectrometric analysis (1-ml. aliquots) was now useful in location of aldosterone, since the quantities of cortisone and the new hormone were now comparable; most second partitions had two ultraviolet peaks, the early one cortisone and the later, aldosterone. Assays in the range 3.5–6.0 liters covered the active area completely.

The new hormone was frequently obtained crystalline from peak fractions of the aldosterone region of the second partition chromatogram. The solvent for crystallization was ethyl acetate. When crystallization could not be induced, a third partition was carried out in the 6-cm. column with the same solvent system.

Final Purification, Yield.—One or two crystallizations of crude tan-colored aldosterone from ethyl acetate generally yielded colorless material which melted above 160°. The procedure as described has consistently yielded 10–25 mg. of pure aldosterone from each 500-kilogram lot of glands. Amorphous mother liquors, and side fractions of low activity from both columns, have been successfully used in preparation of aldosterone degradation products.

Physical Properties of Aldosterone.—The pure crystalline hormone had m.p. (micro hot-stage) 164–169° (Simpson, *et al.*,[1] record 153–158°, Mattox, *et al.*,[2] 163–164°). Some samples, possibly hydrated, melted at *ca.* 105°, then partially resolidified on further heating and finally melted in the range 155–165°. Aldosterone had $[\alpha]^{25}D$ +161 ± 10° (*c* 0.1, chloroform); Simpson, *et al.*,[1] give $[\alpha]^{23}D$ +145 ± 2° (*c* 0.9896, acetone). The R_f of aldosterone when chromatographed on Whatman #1 filter paper in the solvent system benzene–50% aqueous methanol was 0.35, while cortisone had R_f 0.40.

The compound had an absorption maximum in ethanol solution at 240 mμ, ϵ_{mol} 15,000 ± 500 (Simpson, *et al.*,[1] give 15,850; Mattox, *et al.*,[2] 15,500). The sulfuric acid chromogen was characterized by a minimum at 235, a maximum at 287, a plateau of low intensity centered at about 420 and very low absorption above 450 mμ. The absorption maxima of principal interest in the infrared spectrum of aldosterone in the solid state were the hydroxyl band at 2.92 μ, the Δ^4-3-keto bands at 6.05 and 6.18 μ, and in some samples a weak absorption band ascribable to saturated carbonyl at 5.87 μ. The compound in chloroform solution had bands at 2.9 (broad), 3.39, 3.49, 5.84, 6.0, 6.17, 6.83, 6.93, 7.02, 7.21, 7.33, 7.44, 7.80, 8.04, 8.38, 9.30, 9.58, 9.88, 10.12, 10.39, 10.68, 10.95, 11.15 and 11.51 μ.

Minimum observable activity of the pure hormone in the urinary sodium retention assay occurred at between 0.05 and 0.01 μg. per rat, while at least 16 μg. of desoxycorticosterone acetate was required for the same level of activity; activity of the same order has been recorded for aldosterone by Simpson, *et al.*,[1] and by Mattox, *et al.*[2]

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