Stimulatory Action of Adrenal Medulla and Catecholamines upon Hydroxylation of Steroids by Adrenocortical Homogenates. (25724)

DAVID Y. COOPER,* OTTO ROSENTHAL, V. J. PILEGGI AND WILLIAM S. BLAKEMORE
(With technical assistance of Olga Foroff)
Harrison Dept. of Surgical Research, Schools of Medicine, University of Pennsylvania

During comparative study of steroid synthesis by slices of human, canine and bovine adrenal glands incubated in autologous plasma, it was observed that slices containing cortical and medullary tissue produced more 17α,21-dihydroxycorticosteroids than slices containing cortical tissue alone. Medullary slices by themselves, when incubated under identical conditions, did not produce detectable amounts of dihydroxycorticosteroids. However, addition of such slices or of homogenized medullary tissue to incubates of adrenal cortex slices resulted in markedly increased synthesis of corticosteroids. To study the mechanism of accelerating effect of adrenal medulla, a simpler and better reproducible assay system than the slice-plasma system was needed. This report deals with effect of medullary homogenates on conversion of progesterone to 17α, 21-dihydroxycorticosteroids by bovine adrenal cortical homogenates in a Ringer-bicarbonate bovine albumin medium. Comparative studies of the effect of catecholamines on corticosteroid production by cortical homogenates are also reported.

Method. Steer adrenals, removed from carcass within 15 to 20 minutes after animal's death, were packed in cracked ice and brought to cold room of laboratory. The capsules were dissected, glands sliced and slices carefully freed of medullary tissue. Cortical and medullary tissue samples were then separately homogenized in double-distilled water in glass homogenizers equipped with Teflon pestles. 0.5 ml of cortical homogenate (equivalent to 100 to 125 mg of cortical tissue) was added to 125 ml Erlenmeyer flasks containing following ingredients:† (1) 0.5 mg (1.64 μmole) progesterone dissolved in alcohol and evaporated to dryness; (2) 7 ml of Krebs Ringer-bicarbonate medium containing 2% albumin; (3) 8 mg (24.2 μmole) of glucose-6-phosphate (disodium salt) and varying amounts of triphosphopyridine nucleotide (TPN). To selected flasks medullary homogenate or D (−) norepinephrine or D (−) epinephrine was added. Quantities studied are listed in Table I and Figures. Reaction volumes were equalized by addition of H_2O and usually were 8.5 ml. Flasks were gassed with 5% CO_2 in O_2 and shaken 4 hours at 37°C in Brunswick shaker. Contents of each flask were then extracted with 25 ml of chloroform and 17α,21-dihydroxycorticosteroids were estimated by method of Silber and Porter(1). Results of duplicate experiments agreed within ±5%. In some experiments chloroform extracts were subjected to paper chromatography in system of Burton, Zaffaroni and Keutman(2). Steroids separating in cortisol region were quantitated by reaction with blue tetrazolium(3). These results were in good agreement with those obtained with procedure of Silber and Porter.

Results. Rates of formation of 17α,21-dihydroxycorticosteroids by homogenates of adrenal cortex and increments of rate due to addition of medullary homogenates are recorded in Table I. It is important to point out that there was no detectable steroid formation in absence of added progesterone. Likewise addition of glucose-6-phosphate and TPN was indispensable. The system represents thus hydroxylation of progesterone with the aid of TPNH regenerated by glucose-6-phosphate dehydrogenase of the homogenates.

Preliminary experiments had shown that concentrations of progesterone and glucose-6-

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† Organic compounds were obtained as follows: crystalline bovine albumin from Nutritional Biochemical; 1-norepinephrine bitartrate (levophed), 1-epinephrine bitartrate and 1-isoproterenol HCl from Winthrop-Stearns; L-Dopa and 3-OH tyramine HCl from Mann Research Labs, 3-OH tyramine from California Corp. for Biochem. Research. All others from Sigma Chem. Corp.

TABLE I. Effect of Medullary Homogenate on Production of 17α,21-Dihydroxycorticosteroids by Adrenocortical Homogenates, in 13 Experiments.

	Cortex alone†	Cortex + medulla	
TPN,* mg	Rate,‡ μg/g/4 hr	Medulla added, mg	Δ Rate,‡ μg/g/4 hr
16	99	34	+182
8	111	34	+153
4	111	34	+153
8	157	34	+121
8	222	38	+234
8	73	41	+185
8	370	41	+130
8	830	50	+172
4	349 ± 25	30	+116 ± 5
4	171 ± 1	40	+ 94
4	83 ± 2	60	+223 ± 10
4	191 ± 7.5	68	+304 ± 5
2	28 ± 2.3	31	+ 75 ± 0
2	19 ± 1.0	40	+ 61 ± 3
2	601 ± 13	50	+654 ± 50

* Sodium salt MW 837.

† Cortex homogenate equivalent to 115-125 mg of tissue.

‡ Basal rates, increments of rate (Δ rate) due to addition of medulla, and deviations of duplicates from their mean are recorded.

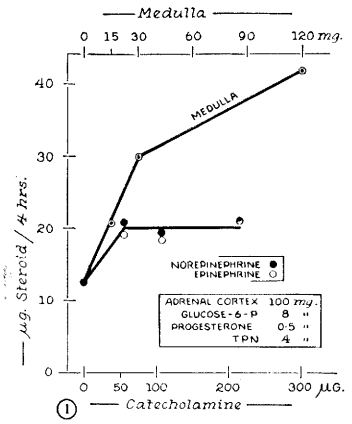
phosphate used provided maximal rate of steroid formation. Data in Table I show that TPN at concentration levels employed was not rate-limiting since there was no correlation between rate of steroid formation and amount of TPN added to the system. The great differences in rate of steroid formation of individual cortical homogenates were thus probably due to variation in enzymatic activity of adrenal glands obtained from abbatoir. Regardless of activity of the cortical homogenate, however, addition of medullary homogenate markedly increased rate of corticoid formation.

To explore the mechanism of accelerating action of medullary homogenates, the effect of catecholamines on corticoid production of cortical homogenates was studied. Fig. 1 shows results of representative experiment in which effects of various concentrations of D-epinephrine, D-norepinephrine and medullary homogenate were compared.

The lower curve in Fig. 1 shows that addition of either of 2 catecholamines greatly accelerated corticosteroid formation of adrenocortical homogenates. Amounts required for maximum stimulation varied somewhat among individual glands studied but never exceeded 100 μg of either D-norepinephrine or D-epinephrine.

Stimulation of steroid formation of adrenocortical homogenate by increasing amounts of medullary homogenate (upper curve of Fig. 1) far exceeded maximum obtainable with catecholamines. The shape of response curve indicated that the medullary preparation contained at least 2 active agents responsible for the steep initial slope and slower, almost linear, rise beyond inclination point of curve. This point lay between 15 and 30 mg of medullary tissue added.

To evaluate the contribution of medullary



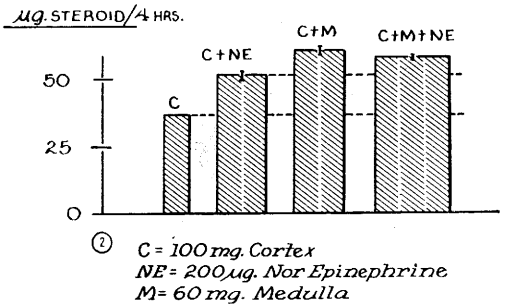
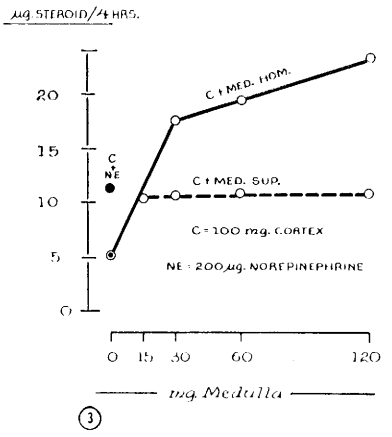
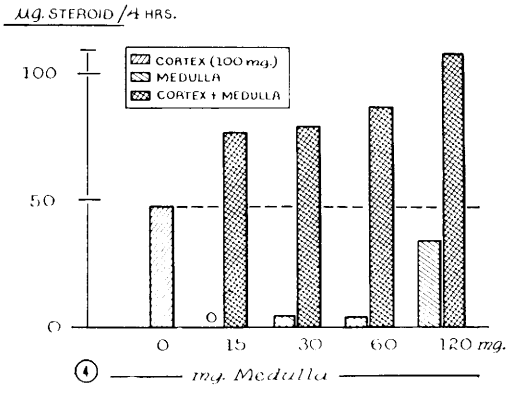


FIG. 1. Effect of increasing additions of medullary homogenates or catecholamines upon formation of dihydroxycorticosteroids by cortical homogenates. All flasks contained adrenocortical homogenate equivalent to 100 mg of cortical tissue and 4 mg (4.8 μmole) of TPN. Additions of medullary homogenate expressed as equivalent amounts of medullary tissue and of epinephrine or norepinephrine in terms of free amines (100 μg = 0.57 μmole) are indicated on top and bottom ordinates. All other additions as described under *Methods*. Total reaction volume = 8 ml.

FIG. 2. Effect of medullary homogenate and norepinephrine singly and combined on dihydroxycorticosteroid production. Designations as in legend to Fig. 1.





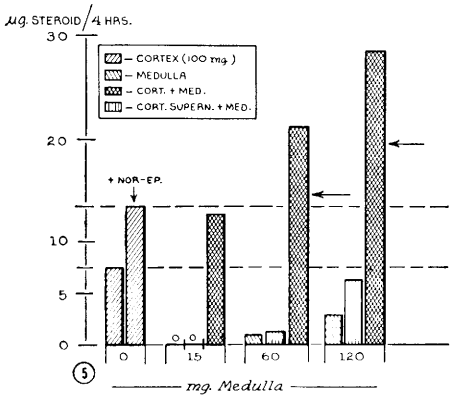


FIG. 3. Effects of increasing amounts of medullary homogenate and its supernatant fraction on steroid formation by cortical homogenates.

FIG. 4. Steroid formation by medullary homogenates and stimulatory action of medulla on cortical homogenates.

FIG. 5. Effect of supernatant fraction of cortical homogenate on steroid formation by medullary homogenate relationship to stimulatory activity of medulla.

catecholamines to the response curve, 2 types of experiments were performed (Fig. 2, 3). Fig. 2 shows that norepinephrine failed to accelerate steroid formation of cortical homogenate already stimulated by medullary homogenate; an indication that the system was saturated with respect to catecholamines. Since it could be expected that the catecholamines would be present largely in the soluble fraction of distilled water homogenates of adrenal medulla, the effect of supernatant liquid obtained by high-speed centrifugation (100,000 × g, 60 min) was compared with that of whole homogenate (Fig. 3). The response curve to increasing amounts of supernatant liquid was undistinguishable from that to increasing amounts of catecholamines (Fig. 1). Rate of steroid formation virtually coincided with that produced by saturating concentration of norepinephrine used as control (C + N E in Fig. 3). Catecholamine assays (method of Euler and Orwen(4)) upon some medullary homogenates used, gave values of 80-115 μg/15 mg of medulla, sufficient to produce maximum stimulation of corticosteroid synthesis obtainable with catecholamines. Hence the steep initial rise of response curves to medullary homogenates (Fig. 1 and 3), can be ascribed mainly to action of medullary catecholamines. The slower rise beyond the inclination point of curves was due in part to contamination of the medulla with cortical tissue derived from folds of the cortex which penetrate into the medulla.

The contribution of cortical remnants to stimulating action of medullary homogenates on steroid synthesis of cortical preparations was not readily noticeable since biosynthetic activity of homogenates ceased to be proportional to quantity of tissue used when concentration of tissue became low. Fig. 4 shows that at concentrations up to 60 mg of tissue/8.5 ml of medium, medullary homogenates by themselves produced negligible amounts of corticosteroids whereas twice this concentration synthesized about 70% as much steroid as 100 mg of cortical tissue in absence of added catecholamines. A similar, though less striking, dilution effect was found with cortical homogenates.

In experiment Fig. 5 special care was taken

to remove particles of cortex-like color from dissected and minced adrenal medulla. Steroid formation of this medullary homogenate was lower than that of previous preparations of unselected medullary tissue. Yet its stimulatory action on steroid formation of cortical homogenate was far greater than could be accounted for by activity of cortical remnants introduced with the medulla and stimulation of cortical homogenate by medullary catecholamines.

Since it appeared possible that activity of cortical remnants in medullary homogenates was limited by lack of soluble enzymes and cofactors present in the more concentrated cortical homogenates, one concentration series of medullary homogenates was supplemented with the soluble fraction of 100 mg of cortical homogenate obtained by high-speed centrifugation. Fig. 5 shows that this procedure failed to abolish the dilution effect although it did augment steroid formation. Arrows in Fig. 5 serve to indicate that 70% of total steroid formation can be accounted for by additive action of medullary cortical remnants and catecholamines. It thus appears that the stimulatory effect of medullary homogenates of corticosteroid formation of cortical homogenates is due to 3 factors: 1) medullary catecholamines, 2) cortical remnants, and 3) an interaction of unknown nature between particulate components of medullary and cortical homogenates.

Discussion. Hayano and Dorfman(5,6) reported that 11β-hydroxylating activity of bovine adrenal medulla was almost as high as that of cortex. Kahnt(7), on the other hand, failed to detect medullary activity. Microscopic examination of serial sections of bovine adrenal glands reveals that medulla invariably contains islands or strands of cortical tissue. Our experiments showed that hydroxylating activity of such cortical remnants becomes manifest only when comparatively high concentrations of medullary homogenates are assayed since activity/unit weight of homogenized cortical tissue decreases with increasing dilution of the assay system. Conflicting results regarding hydroxylating activity of medullary preparations can thus be explained as due to varying degrees of contamination with cortical tissue and operation of the dilution effect.

Our most interesting finding is the pronounced acceleration of conversion of progesterone to 17α,21-dihydroxycorticosteroids when small amounts of medullary homogenates were added to adrenocortical homogenates. Under these conditions hydroxylating activity of cortical remnants in medullary homogenates can be disregarded since the supernatant fraction which is free of such activity produced as high stimulations as whole homogenate. Significantly, epinephrine or norepinephrine effected accelerations of rate of hydroxylation of a similar order of magnitude as small amounts of whole medullary homogenates or their supernatant fractions. The quantity of catecholamines required matched satisfactorily total catecholamine content of medullary samples of equal stimulatory activity. Unpublished data indicate that isopropylnorepinephrine (isoproterenol), the third medullary catecholamine, and catecholamine precursor, dihydroxyphenylalanine were equally effective. In contrast, dihydroxyphenylethylamine (3-hydroxytyramine) considered the immediate precursor of norepinephrine(8), did not stimulate. No explanation of this discrepancy can now be offered. Dihydroxyphenylserine, an alternate potential precursor of norepinephrine, has not been tested. Adrenochrome is ineffective at low concentrations and inhibitory at higher concentrations.

While in majority of experiments only conversion of progesterone to 17α,21-dihydroxycorticosteroids was determined by the Silber-Porter reaction, paper-chromatographic separations of conversion products have been performed in exploratory experiments. Progesterone as well as derivatives hydroxylated at the C-17 and/or C-21 position were employed as substrates. With progesterone main reaction products were cortisol and corticosterone, the amount of the latter being about 3 times that of the former. 17α-progesterone yielded mainly cortisol. With either substrate, quantity of all reaction products was markedly increased by medullary homogenates, epinephrine or norepinephrine. There was no detectable change in relative

amounts of reaction products. Conversion of 11-deoxycorticosterone was not significantly accelerated by norepinephrine. It thus appears that catecholamines accelerate hydroxylations at C-21 and, perhaps, C-17 but not at C-11. This may explain Kahnt's(7) failure to detect an effect of medullary homogenates or epinephrine on 11β-hydroxylation of substance S by bovine adrenocortical homogenates. Firmer information on site and mechanism of action of catecholamines will require separation of the 3 hydroxylase systems.

Summary. 1. Conversion of progesterone to 17α,21-dihydroxycorticosteroids by homogenates of bovine adrenal cortex is markedly stimulated by addition of small amounts (15 mg/100 mg cortex) of medullary homogenate. Larger amounts produce additional, though relatively smaller increments. 2. The soluble fraction of medullary homogenates equivalent to 15 mg of medulla stimulates as much as the corresponding amount of whole homogenate. Larger amounts of this supernatant fraction do not produce additional increments. 3. Increments similar to those obtainable with 15 mg of medullary homogenate or medullary supernates are produced with 100 μg or less of D(−)epinephrine, D(−)norepinephrine, or D(−)isopropyl-norepinephrine. No further increment results from larger additions. 4. Total catecholamine content of 15 mg of medullary homogenate or corresponding supernatant fraction ranged from 80-115 μg, indicating that the stimulatory action of small amounts of medulla can be ascribed mainly to catecholamines. Additional increments produced by larger amounts of medulla are apparently due to contamination with cortical remnants and, possibly, an additional factor of unknown nature. 5. Exploratory experiments suggest that catecholamines accelerate C-21 and, perhaps, C-17, but not C-11 hydroxylations.

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Parainfluenza 3 — Assay and Growth in Tissue Culture.* (25725)

R. Q. MARSTON AND E. R. VAUGHAN
Dept. of Medicine, Medical College of Virginia, Richmond, and Dept. of Bacteriology and Immunology, University of Minnesota, Minneapolis

Parainfluenza 3 (HA1) causes human(1) and possibly bovine disease(2,3), and is a virus which may produce persistent infection in tissue cultures(4). The process of formation of multinucleated giant cells(5), and a plaque assay technic dependent on these cytopathic changes have been reported(6). We describe a plaque assay system based on hemadsorption characteristics of HA1. Data are presented on thermal stability, rate of adsorption to cells, growth characteristics, and variation in cytopathic response of different cell systems to HA1.

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Materials and methods. Virus. Mill's strain of HA1 was obtained from Dr. Wallace Rowe. Virus pools of passages 11 through 13 in our laboratory were used. Hemadsorption tests were performed by exposing washed tissue cultures to 0.4% suspension of thrice washed guinea pig red cells in phosphate buffered saline, pH 7.3. In hemagglutination