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Exposure of bovine adrenal cortex microsomes to phospholipase A impairs the rate of the NAD-dependent 3β-hydroxypregn-5-en-3-one dehydrogenation as well as the rates of the 5-androstene-3,17-dione and 5-pregnene-3,20-dione isomerization. None of the enzymatic activities is released into the supernatant upon digestion with phospholipase. Addition of Asolectin (a mixture of soybean phosphatides) to the treated membrane fraction does not restore the dehydrogenase activity whether the products of the phospholipase A action are present or not. In contrast to the 3β-hydroxysteroid dehydrogenase the 3-oxosteroid-Δ5-4-isomerase activities are restored to the original levels in the presence of Asolectin. The maximal activity which can be restored (minimum 70%) depends on the extent of the phospholipase A digestion. This methodology has not been able to show any significant difference whenever the 5-androstene-3,17-dione or the 5-pregnene-3,20-dione is used as substrate. The data suggest the phospholipid dependence of the isomerase(s). They would agree with the existence of only one C_{19} and C_{21} 3-oxosteroid-Δ5-4-isomerase. The results of our experiments indicate that the dehydrogenase and the isomerase behave differently towards phospholipids.

3B 2. Steroid biosynthesis: Adrenal Cortex—II

63. Mammalian adrenal cells in monolayer culture: Biogenesis of steroid hormones

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Adrenocortical cells from different mammalian sources (beef, rabbit and human fetus) were dispersed by enzyme hydrolysis (trypsin 0·25%) and grown for short- and long-term periods under conditions of monolayer culture in Ham's F-10 supplemented with 10% fetal calf serum, in the presence or absence of ACTH. The biogenesis of corticosteroids was analyzed using labelled precursors, the products of which were purified and characterized by chromatography. Beef adrenal cells kept under such conditions were capable of responding to ACTH stimulation with accumulation of cyclic AMP (quantified by adrenal cytosol protein binding assay), increased production of corticosteroids (measured by CBG assay), and, in studies performed with either labelled progesterone or pregnenolone, changes in the specific activity of either cortisol or corticosterone. Responsiveness to ACTH was consistently observed for at least five days following implantation. From day five on there was an appreciable loss of 11β-hydroxylase activity in both control and ACTH treated cells (100 μU/ml), resulting in a sharp decrease in cortisol and corticosterone production, while 11-deoxycortisol and 11-deoxycorticosterone were formed in increasing ampunts. In fetal rabbit adrenal cultures, using progesterone as precursor, a sequential appearance of steroid hydroxylases was observed as a function of gestational age. This was characterized by the exclusive formation of 11-deoxycorticosterone during early gestation, followed by that of corticosterone and finally of aldosterone near term. The presence of ACTH, while enhancing total corticosteroid production, did not affect enzyme ontogenesis. Similar cell preparations from midterm human fetuses actively metabolize pregnenolone to cortisol and corticosterone and to the sulfates of corticosterone and 11-deoxycorticosterone. Total corticoidogenesis was enhanced several fold in the presence of ACTH (500 μU/ml).

64. Isolation of subcellular fractions from the zona glomerulosa of the bovine adrenal cortex

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A method has been established to obtain uncontaminated mitochondrial or microsomal fraction from the zona glomerulosa of the bovine adrenal cortex with respect to the zona fasciculata-reticularis. Mitochondria thus obtained showed a different configuration from those of the mitochondria of the zona fasciculata-reticularis. They showed good controls in the presence of albumin; RCI = 7·8, ADP/O = 1·8, succinate as the oxidizable substrate. Distribution of cytochrome P-450 which might be concerned with steroid hydroxylase reactions in the zona glomerulosa was studied by CO-difference spectra using subcellular fractions; homogenates, 0·15, mitochondria, 0·70 and microsomes, 0·39 nmoles/mg protein. Amount of cytochrome P-450 in the microsomal fraction presented here is less than half that reported by others for the microsomal fraction of the zona fasciculata-reticularis. It was confirmed by electron microscopy that the microsomal fraction obtained in the present study was not contaminated with mitochondria. The microsomal fraction was further subfractionated into smooth- and rough-surfaced vesicles by sucrose gradient. Thus, it becomes possible to explore the roles of subcellular organelles in the biosynthesis of aldosterone in the zona glomerulosa of the adrenal cortex using uncontaminated materials.

65. Interaction between the zona glomerulosa and the inner zones of the rat adrenal cortex incubated *in vitro*

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In conventional incubations of rat adrenal tissue, the rates of corticosterone (B) formation from endogenous precursors reach a maximum only after 60–90 min. This is at variance with results from superfusion or suspended cell preparations in which the maximum rate of steroid release occurs immediately, and also with the pattern of ^{3}H-B formation from ^{3}H-pregnenolone. It suggests that in conventional incubations unknown factors inhibit the early release of steroid from endogenous precursors. Further experiments show that inhibition only occurs when whole tissue is used; when capsule and inner zones are incubated separately, the rate of steroid formation is maximal immediately. When inner zone tissue was incubated (i) in capsule preincubation medium (ii) with a lipid extract of capsule preincubation medium (iii) with added aldosterone, significant inhibition of corticosterone was observed. This was related to aldosterone concentration, and could be observed with the addition of only 100 ng aldosterone per ml. B was affected specifically, and deoxycorticosterone and 18-hydroxycorticosterone were less affected: Also the conversion of ^{3}H-pregnenolone to ^{3}H-corticosterone was unaffected. The results are of special significance in relation to (i) consideration of the site of action of sodium depletion in the biosynthetic pathway for aldosterone production, since reduced corticosterone could indicate not increased 18-hydroxylase activity, but simply depressed corticosterone (ii) interpretation of the functional zonation of the rat adrenal cortex.

66. Vinblastine-induced ultrastructural changes in the rat adrenal cortex

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Electron microscopy has revealed the presence of microtubules in the adrenocortical cells of rats. They are randomly distributed in the cytoplasm and fail to accumulate around the mitochondria or adjacent to the plasmalemma, nucleus or cytoplasmic organelles. Vinblastine (5 mg/100 g, i.v.) in 450 g Sprague–Dawley male rats, caused disruption of microtubules, and paracrystalline inclusions were found within two hours in the cytoplasm. No marked alterations were seen in other cellular constituents. The paracrystalline inclusions, which were evident in the cells of the zona glomerulosa and fasciculata, consisted of closely packed tubular structures in longitudinal sections and resembled a honeycomb pattern in cross sections. The functional significance of microtubules in the adrenal cortex is not clear. Temple and Wolff (1973) showed that vinblastine stimulates steroid production by mouse adrenal tumour cells *in vitro* and proposed that in normal adrenal cortex, microtubules restrict the access of cholesterol to the mitochondria. Antimicrotubular agents, such as vinblastine, by removing this physiologic inhibition, enhance the rate of steroid production above the normal level. The fine structural changes detected during the present study may represent the morphologic manifestations of this functional abnormality.

67. Effect of ACTH on the steroid metabolic pools in the rat adrenal gland

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To study *in vivo* the mechanism of ACTH action on steroid biosynthesis in the rat adrenal gland, we determined in the subcellular fractions by radioimmunoassay the metabolic pool changes of pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone and their corresponding sulfates, as well as progesterone, 17-hydroxyprogesterone, corticosterone and cortisol, establishing a correlation with the serum concentrations. To achieve this purpose we utilized 44 Sprague–Dawley strain male rats; 26 received 0·1 I.U./g weight of ACTH I.P. and the control group (18) received isotonic saline solution; both groups were decapitated at 0, 5, 10 and 20 min after injection. Our results showed at zero time the highest serum concentrations in corticosterone, pregnenolone-sulfate and dehydroepiandrosterone-sulfate, while in the mitochondria pregnenolone and also corticosterone were in greater quantity and progesterone was the highest in the microsomal fraction. The ACTH activity depleted the pools of pregnenolone and progesterone, increasing the one of corticosterone with minor modifications in the others; its maximal effect was obtained at 10 min. In contrast with previous reports, we found 17-hydroxylated compounds such as cortisol, revealing 17-hydroxylase enzyme activity. We conclude that the ACTH not only stimulates pregnenolone synthesis but also increases the enzymatic activity of other systems which utilized this compound as a sustrate to produce corticosterone selectively.

3C. Steroid biosynthesis: Ovary

68. Influence of hFSH and hFSH + hLH on steroidogenic enzymes in immature mouse ovaries

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Most evidence indicates that FSH alone does not stimulate estrogen biosynthesis in immature ovaries in spite of increased follicular growth, but the influence of "biologically pure" FSH on enzymes of steroid biosynthesis has not been determined. We have studied this problem. Female mice 21–22 days old were injected for 3 days with either saline preparations having both FSH + LH activities, or the same preparations in which the LH activity had been neutralized with 2 × the neutralizing dose of anti-hCG. In one series endogenous mouse LH was neutralized by administration of anti-rLH. Each major step between cholesterol and estrogens was studied by incubating appropriate substrates with aliquots of ovarian homogenates. The major effect of FSH was to increase the enzyme activities in proportion to the general increase in protein, a growth effect. FSH + LH caused marked differential increases in cholesterol side chain splitting, 20α-hydroxysteroid dehydrogenase, and aromatizing activities per mg protein. These enzyme activities per mg protein were slightly increased in the FSH groups, even with twice the dose of anti-hCG needed to inactivate the LH contamination. This would seem to indicate a slight intrinsic LH effect of FSH analogous to the MSH activity of pure ACTH. (Supported by USPHS Grant CA-10935 and a grant from Mr. S. Lunenfeld).

69. Steroidogenesis in dispersed, superfused corpora luteal cells

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Enzymic digestion has been used to disperse pregnant rat corpora luteal cells. The quality of dispersed cell preparations has been monitored by electron microscopy (EM), dye exclusion, and measurement of progestin secretion during superfusion. Prolonged incubation (> 1 h) with trypsin and collagenase reduced the yield of viable cells. Reduction of exposure time to 30 min with 0·12% trypsin, 0·25% collagenase and 0·2% hyaluronidase followed by 30 min exposure to 0·2% lima bean trypsin inhibitor yielded $0{\cdot}511 \times 10^6$ viable cells per corpus lutem (25%). EM examination demonstrated intact microvilli. Mitochondria appeared normal, and smooth endoplasmic reticulum predominated over rough. Such characteristics appear compatible with active steroid secretion. Superfusion of dispersed cells, using Dulbecco's modified Eagle medium, provided evidence of *de novo* steroidogenesis. Progesterone (P) was secreted at an average rate of 26 ng/h/10^6 cells, representing a 14-fold higher secretion rate than that obtained with intact corpora lutea. P exceeded 20α-dihydro-P in the effluent medium by 1·8-fold. Replenishment time of the P content of the average luteal cell was estimated at about 1 h.

70. Effect of estradiol-17β on progesterone biosynthesis in rhesus corpus luteum

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There are no data on the utilization of sodium acetate as a substrate for progesterone biosynthesis in the rhesus corpus luteum. Estradiol-17β has been shown to decrease peripheral progesterone levels in this species. This study was designed to assess the effect of estradiol-17β on two indices of progesterone biosynthesis: (a) The *de novo* incorporation of sodium acetate and (b) total progesterone content, in rhesus corpora lutea of menstruation. Corpora lutea were excised,