20α-OH-cholesterol (20α) and 22R-OH-cholesterol (22R) yielded pregnenolone + isocaproaldehyde. 25-OH-cholesterol (25-OH) formed pregnenolone + malonic dialdehyde + acetone. AG (40 μg/ml) fully blocked pregnenolone formation from cholesterol and 25-OH, while side-chain cleavage of Δ^{20-22}, 20α, and 22R was only partially inhibited. AG therefore exerts its main action on the reaction cholesterol → Δ^{20-22}. It is highly probable, that in CLAH *this* step is blocked. 25-OH in the presence of AG yields mainly 3β-OH-cholenic aldehyde (CA) + acetone while 20α partially yielded 20α,25-di-OH-cholesterol which was slowly converted into 20-hydroxylated CA. Isolated rat adrenal cells (stimulated with 1 mU ACTH/ml) were incubated with AG (20 μg/ml). Addition of 25-OH partially inhibited corticosterone production. Without AG,25-OH has a stimulating effect. We propose the hypothesis, that abnormal compounds like CA are responsible for the severity of CLAH.

55. On the unique status of cholesterol 20α-hydroperoxide in steroid metabolism

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Our prior demonstration of the rearrangement of cholesterol 20α-hydroperoxide to cholest-5-ene-3β,20α,22R-triol by bovine adrenal cortex mitochondria suggested the intermediacy of the 20α-hydroperoxide in pregnenolone biosynthesis from cholesterol. Additional studies of C_{27}-, C_{21}-, and C_{18}-hydroperoxide metabolism in mammalian, plant, and microbial systems failed to provide other examples of the hydroperoxide-diol rearrangement, reduction to the corresponding alcohol being commonly encountered. Formation of the 20α-hydroperoxide by rat adrenals and of cholesterol 7α- and 7β-hydroperoxides by rat liver has been observed, but enzymic hydroperoxide formation is not readily distinguished from nonenzymic peroxidation. Ethyl linoleate appears to stimulate 20α-hydroperoxide formation in rat adrenal incubations and markedly stimulates 7α- and 7β-hydroperoxide formation in incubations of soybean lipoxygenase or rat liver microsomes. The status of cholesterol 20α-hydroperoxide is unique as regards its metabolic rearrangement to a vicinal diol implicated in steroid hormone biosynthesis. (Supported by Robert A. Welch Foundation and U.S. Public Health Service Grant HL-10160).

56. Cholesterol side chain cleavage in microsomes and mitochondria from corpora lutea

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Adrenals effect cholestrol side chain cleavage (SCSS) only in mitochondria. This is not true in corpora lutea (CL) but luteal microsomal fractions have been little investigated. CL from pigs, sheep or cows were homogenized; nuclear, mitochondrial, microsomal and cytosol fractions were prepared by ultracentrifugation. Fractions were incubated for up to one hour in the presence of malate or succinate and an NADPH generating system, and cholesterol, pregnenolone and progesterone were determined by gas-liquid chromatography. CSCC activity was confined to microsomal and mitochondrial fractions and the specific activities (μg progesterone/mg protein) of the CSCC complex did not differ significantly between mitochondria and microsomes for any species. Under our incubation conditions, progesterone was produced rather than pregnenolone, regardless of cell fraction or species. Some mitochondrial preparations were examined in a 10–55% sucrose gradient using an MSE HS zonal rotor. Mitochondria were homogeneous in size; protein concentration, cytochrome C oxidase activity and CSCC activity were well correlated. We conclude that, in luteal cells, mitchondria and endoplasmic reticulum are equally important in CSCC and, if LH controls progesterone biosynthesis, both fractions should be responsive to the ultimate effector of the gonadotrophin.

3B 1. Steroid biosynthesis: Adrenal Cortex—I

57. Alternative pathways of corticosteroid synthesis in rat adrenals

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After incubation of rat adrenal quarters with ^{3}H-acetate, specific radioactivities of cholesterol, pregnenolone and progesterone were 10–100 times lower than those of 11-desoxycorticosterone (DOC) and corticosterone (B). ACTH decreased specific radioactivities of cholesterol by factors of 3–8, but it did not alter those of B and it increased those of DOC 2–3 fold. It seems to be unlikely, therefore, that ^{3}H-acetate had been incorporated into DOC and B *via* cholesterol, pregnenolone and progesterone. Specific radioactivities of cholesterol analyzed separately in mitochondria and in the remaining cell fraction were identical. This does not support the hypothesis that only a small pool of highly labelled cholesterol (which should be expected within the mitochondria) serves as steroid precursor. 21-OH-pregnenolone, the only alternative to progesterone as direct precursor of DOC, was 30–50 (control) and 3–9 (ACTH) times higher in specific radioactivity than DOC and B. Under the influence of "triparanol" (1-(p-diethylaminoethoxyphenyl)-1-(p-tolyl)-2-(p-chlorophenyl)-ethanol) which is known to inhibit the step "desmosterol-cholesterol", specific radioactivities of cholesterol decreased to $\frac{1}{10}$ of the control values. In contrast, there were only slight alterations in the specific radioactivities of 21-OH-pregnenolone, DOC and B. These data strongly suggest that in rat adrenals DOC and B can be synthesized from acetate *via* alternative pathways not including cholesterol, pregnenolone and progesterone as intermediates, in which 21-OH-pregnenolone may be the direct precursor of DOC.

58. Reciprocal interactions of progesterone and 17α-hydroxyprogesterone as exogenous substrates of rat adrenal 21-hydroxylase

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Due to the small concentration and activity of 17α-hydroxylase present in the rat adrenal, the main corticoids secreated in the rat are DOC, B_k, A_k, 18-OH-DOC and aldosterone, formed directly from progesterone(I). Because of the limited amounts of 17α-OH-progesterone (II) available, the biosynthesis of S_R, F_k and E_k is restricted. Since

21-OH steroid hydroxylase (21-OH-ase) uses both I and II in corticoid biosynthesis in other species, it was considered of interest to study the comparative interactions which could exist between these two precursors and the rat adrenal 21-OH-ase, determining enzymatic constants for I and II (usual and unusual substrates, respectively). Homogenized adrenals from normal rats were incubated with various combinations of concentrations of I-7^{3}H and/or II^{14}C, acting as substrates and/or inhibitors of 21-OH-ase. The results showed that 21-OH-ase uses II almost as efficiently as I. The K_m values were about the same for both I and II (13·9 and $14{\cdot}2 \times 10^{-6}$ M/L), respectively, however, the V_{max} values were 54·6 and $26{\cdot}0 \times 10^{-7}$ M/L/h for I and II, respectively. The amounts of I required to saturate the 21-OH-ase was double than that of II. Further kinetic studies showed that both I and II inhibit the 21-hydroxylation of the other in a reciprocal fashion. While II inhibits the 21-hydroxylation of I by competitive inhibition, I inhibits the 21-hydroxylation of II through a mixed type of inhibition. The results suggest that, rather than the existence of two different specific enzymes (one for I and another for II) as it has been postulated by others, it seems that we are dealing with a 21-hydroxylating system with two active sites. One site uses only I and the other site uses I and/or II indistinctively.

59. The 11β-hydroxylase activity of cell-free adrenal preparations from Echidnas (Tachyglossus aculeatus) in various physiological states

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The echidna has a much lower rate of corticosteroid secretion than eutherian mammals and it survives adrenalectomy. Adrenal 11β-hydroxylase activity was found to be extremely low, although 17α- and 21-hydroxylase activities were comparable to eutherians. In echidnas treated for 1 week with frusemide (Lasix), which caused Na^+ depletion and dehydration, there was a marked increase in 11β-hydroxylation of the adrenal homogenates, 60% of the end products from progesterone being 11β-hydroxylated, compared with less than 1% in controls. This activity was also enhanced by dehydration or treatment of the animal with ACTH. Using purified mitochondrial preparations and deoxycorticosterone substrate the yield of corticosterone was 8·3% in an echidna treated with frusemide, less than 0·1% in a control echidna and 25·5% in a normal rat. The K_m values from Lineweaver–Burke plots for 11β-hydroxylase for the treated echidna and the rat in these experiments were 0·24 and 0·30 μM and the V_{max} values $8{\cdot}2 \times 10^{-6}$ and $6{\cdot}7 \times 10^{-4}$ μM min^{-1} mg^{-1}, respectively. Thus the efficiencies of 11β-hydroxylase in the two animals were comparable, but the amount of active enzyme in the echidna was approximately 1% of that in the normal rat. The low corticosteroid secretion rate in the echidna may be a consequence of a relatively meagre mitochondrial enzyme system.

60. A lasting effect of ACTH on adrenal 11β-hydroxylation in guinea-pig

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The day following i.v. ACTH administration to guinea-pigs, the steroidogenic response to further ACTH stimulation is enhanced, as evidenced by plasma and adrenal tissue levels of cortisol (F), while 11-deoxycortisol (S) is not modified. This suggests a prolonged ACTH effect on late steps of F biosynthesis. This hypothesis was evaluated on isolated guinea-pig adrenal cells by studying ACTH action on F, S, cyclic-AMP production and on 11β-hydroxylation of ^{3}H-S. Adrenal cells were harvested from 26 control adult male guinea-pigs and from 18 animals treated with ACTH (75 μg $ACTH_{1-24}$ i.v. over 3 h, 24 h previously). The 11β-hydroxylation index [(F/F + S) × 100] determined from F and S adrenal tissue content before cell dispersion, was 82·7 ± 1.1 in controls and 99·5 ± 0·2 24 h after ACTH ($p < 0{\cdot}001$). Incubation of isolated cells with tracer amounts of ^{3}H-S resulted in ^{3}H-F formation, itself undergoing conversion into cortisone (E). The 11β-hydroxylation index, calculated therefore as (F + E/F + E + S) × 100, increased in cells from ACTH-treated animals, averaging after 30 min incubation 88 ± 1 *vs.* 77 ± 2 in controls ($p < 0{\cdot}01$). The cells from both groups were then challenged with ACTH *in vitro* for 2 h, at doses ranging from 1 to 1000 pg/ml cell suspension. The F secretory response of isolated adrenal cells from ACTH-treated animals was enhanced when compared to controls, maximal F production (at 1000 pg ACTH/ml) averaging 1236 and 836 ng F/10^5 cells, respectively ($p < 0{\cdot}05$), while net S and cyclic-AMP production did not differ in both groups. An acute exposure of cells to ACTH during incubation does not influence any further the activity of the 11β-hydroxylation enzyme system, as judged from ^{3}H-S conversion into ^{3}H-F and ^{3}H-E. In conclusion: (1) No change in 11β-hydroxylase activity of guinea-pig adrenocortical cells results from acute exposure to ACTH; (2) Activity of this enzyme system increases as a delayed effect of ACTH stimulation, demonstrable the day after infusion with this hormone; (3) The increase in 11β-hydroxylase activity could account, at least in part, for the enhanced F secretory response obtained upon repeated ACTH stimulation. In contrast, generation of cyclic-AMP does not seem to be modified under these circumstances.

61. Regulation of androgen synthesis in the human adrenal gland *in vitro*

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The possibility that local factors at a cellular level might influence androgen synthesis by the human adrenal gland was investigated in an incubation system *in vitro*. The synthesis of DHA was controlled by the availability of its major precursor 17α-hydroxypregnenolone, the obligatory cofactor for this reaction NADPH, and by the ratio of $NADP^+$ to NADPH. 17α-hydroxyprogesterone and DHA itself exerted non-competitive inhibition on the conversion. The synthesis of androstenedione from its two immediate precursors, 17α-hydroxyprogesterone and DHA, was also investigated. Both reactions were dependent upon the availability of the substrate and of the obligatory cofactors, NADP and NAD^+, respectively. The conversion of 17α-hydroxyprogesterone to androstenedione was competitively inhibited by pregnenolone, progesterone and 17α-hydroxypregnenolone, while the synthesis of androstenedione from DHA was found to be non-competitively inhibited by oestrone and oestradiol-17β. It is suggested that androgen synthesis is regulated by feedback inhibition at the cellular level.

62. Bovine adrenal cortex 3β-hydroxysteroid dehydrogenase and 3-oxosteroid-Δ5-4-isomerase: phospholipid requirement?

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