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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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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