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ACTH AND SULFATASE ACTIVITY

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SUMMARY

The conversion of 5-en-3β-ol steroid sulfates to active hormones has been extensively studied. A preliminary desulfation of the precursors is required to allow their structure 5-ene-3β-ol to be transformed to the configuration 4-ene-3-keto present in active hormones. Steroid sulfatase is a very active enzyme but present at a very low concentration with respect to other steroid biosynthesizing enzymes in the adrenal tissue. It represents a limiting enzyme reaction in corticoid production from early precursors. Steroid sulfatase hydrolyzes pregnenolone-sulfate (P–S) much more effectively than DHEA-sulfate (D–S). Its activity and concentration in the adrenal is increased by *in vivo* stimulation with ACTH, however, ACTH does not seem to stimulate other enzyme reactions after free pregnenolone. Both P–S and D–S act on the steroid sulfatase inhibiting reciprocally the desulfation of the other, in such a way that when both sulfates are present simultaneously, P–S is efficiently hydrolyzed to release free P which is quickly converted to progesterone and corticoids, while most of D–S remains as sulfate and is excreted as such. Measurements of the endogeneous pools of steroid precursors in the adrenal show that, ACTH stimulation decreases the levels of P–S, while free P increases first and then decreases as it is converted to progesterone and corticoids. ACTH may have two parallel but distinct mechanisms of action on corticoid biosynthesis. One, already established, is through the activation of adenylcyclase, c-AMP release and a final increment of TPNH to favor adrenal hydroxylations and side chain cleavages. The other, to be demonstrated as yet, suggests a stimulation of steroid sulfatase without c-AMP participation and regulates the amount of free 5-en-3β-ol steroid available for active hormone biosynthesis.

INTRODUCTION

The isolation of cholesterol-sulfate from bovine adrenals[1] and from human blood[2] as well as the isolation of various steroid sulfates such as pregnenolone-sulfate from rat plasma[3], 17α-hydroxypregnenolone-sulfate from human adrenal venous blood[4] and DHEA-sulfate as one of the major steroids secreted by the human adrenal[5] give to the steroid sulfates of the 5-en-3β-ol series an intriguing interest. It has been shown that cholesterol-sulfate serves as precursor of DHEA-sulfate secreted by the adrenal[6] and that pregnenolone-^{3}H-sulfate-^{35}S is converted *in vitro* to 17α-hydroxypregnenolone-sulfate[7] and to DHEA-sulfate[8], with identical ^{3}H/^{35}S ratios.

The conversion of cholesterol-sulfate to pregnenolone-sulfate has been also found[9].

The conversion of steroid sulfates to active steroid hormones has been extensively studied[5, 6, 10–13] and their actual role on adrenocortical hormone biosynthesis and production has been discussed[10, 14–16]. However, in order to transform any 5-en-3β-ol steroid sulfate into the corresponding 4-ene-3-keto steroid, the removal of the sulfate group, by enzymic hydrolysis of the ester, and the release of a free 5-en-3β-ol steroid

STEROID NOMENCLATURE

Cholesterol = 3β-hydroxy-cholest-5-ene
Cholesterol-sulfate = cholesteryl-sulfate = 3β-hydroxy-cholest-5-ene-3-sulfate
Pregnenolone = 3β-hydroxy-5-pregnen-20-one
Pregnenolone-sulfate = 3β-hydroxy-5-pregnen-20-one-3-sulfate
17α-hydroxypregnenolone = 3β,17α-dihydroxy-5-pregnen-20-one
17α-hydroxypregnenolone-sulfate = 3β,17α-dihydroxy-5-pregnen-20-one-3-sulfate
DHEA = Dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one
DHEA-sulfate = 3β-hydroxy-5-androsten-17-one-3-sulfate
Progesterone = 4-Pregnene-3,20-dione
DOC-11-deoxycorticosterone = 21-hydroxy-4-pregnene-3,20-dione
B_k-corticosterone = 11β,21-dihydroxy-4-pregnene-3,20-dione
A_k = 21-hydroxy-4-pregnene-3,11,20-trione
18-hydroxy-DOC = 18,21-dihydroxy-4-pregnene-3,20-dione

are required. The sulfate group is removed from the esterified steroid by the action of steroid sulfatase, an enzyme present in rat adrenals in a very low concentration[10, 16, 17] in comparison with that of other steroid biosynthetic enzymes[14]. Once the free steroid is released, the free 5-en-3β-ol structure is very quickly converted to the corresponding 4-ene-3-keto structure by the action of the 3β-hydroxysteroid dehydrogenase-isomerase system[18]. The latter enzyme is very active and probably the highest in concentration in all the steroid hormone-producing tissues. The 5-ene-3β-hydroxy-dehydrogenase requires DPN as a cofactor[18], in contrast to most of the other steroid hormone biosynthesizing enzymes, which require TPNH, such as the case of hydroxylases[19, 20], desmolases[19, 21, 22] and several dehydrogenases. The actual cofactor requirements for the steroid sulfatase have not been established as yet, except that its activity is enhanced by the addition of ADP[10, 15, 23] and, apparently, c-AMP does not seem to be involved[24].

Animals and treatment

Sprague–Dawley female rats, four months old, were divided into two groups. The first group received a daily I.V. injection of 0·6 nM of ACTH for a period of three weeks. The second group received a daily injection of saline solution. Otherwise, they were treated and fed in an identical manner. At the end of the treatment period, the animals were sacrificed by decapitation and the adrenals removed, decapsulated and homogenized in a Krebs-Bicarbonate buffer pH 7·4, containing 30 mM Nicotinamide. The homogenate was prepared in such a way that 0·8 ml contained one rat adrenal.

Substrates

[7-^{3}H]-pregnenolone-sulfate, [7-^{3}H]-DHEA-sulfate, free [4-^{14}C]-pregnenolone, [4-^{14}C]-progesterone and [21-^{14}C]-11-deoxycorticosterone (from New England Nuclear Corp.) were used as substrates in the various experiments, adjusting their specific activities according to the experiment, with the addition of the corresponding cold steroid (Steraloids) which had been previously purified through crystallization. The amount of the radioactive substrate to be incubated was placed in the bottom of incubating tubes, the solvent evaporated to dryness under nitrogen stream, and redissolved in one drop of ethyl alcohol.

Incubation

For sulfatase activity, 0·8 ml of homogenized adrenal (one rat adrenal) were added to the tubes containing the prepared substrate followed by the addition of 0·2 ml of Krebs-Bicarbonate buffer containing ADP and DPN in such amount that the final media contained 1·0 and 3·0 mM concentration of each cofactor, respectively. The air inside the tubes was removed by passing a nitrogen stream for 60 s. The incubation tubes were firmly stoppered and the tube placed in a Dubnoff incubator at 37°C for 5, 10 or 30 min, according to the experiment. All the manipulations preceding the incubation were done at 0°C.

For the study on 21 and 11β-hydroxylases, the cofactors added were 0·4 mM TPNH, and 0·4 Fumaric

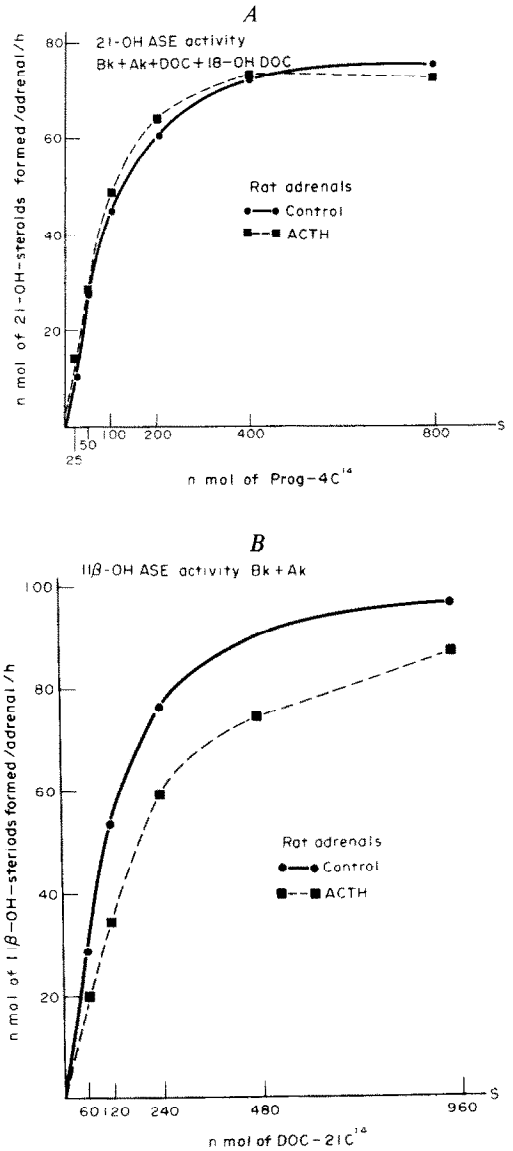

Fig. 1. Effect of *in vivo* chronic stimulation with ACTH (0·6 mU I.V./day, during 3 weeks) on rat adrenal 21 and 11β-hydroxylases. *A* (top) 21-hydroxylase activity = nmol of 21-hydroxylated steroids formed from [4-^{14}C]-progesterone. *B* (bottom) 11β-hydroxylase activity = nmol of 11β-hydroxylated steroids formed from [21-^{14}C]-DOC.

acid and the incubations were carried out in air for one hour.

Extraction

Different procedures were used in the various experiments:

(1) 21 *and* 11*β-hydroxylase activities.* At the end of the incubation period, the reactions were stopped by adding 10 ml of cold 0·1 N HCl and the radioactive steroids extracted five times with ether–chloroform (4:1, v:v). The dried extract was chromatographed on paper using various solvent systems[25] to accomplish the separation of B_k, A_k, 11β-oxi-steroids formed from [21-^{14}C]-DOC or B_k, A_k, DOC and 18-hydroxy-DOC, 21-hydroxylated steroids formed from [4-^{14}C]-progesterone. The 21-hydroxylase activity was indicated by the total nmol of 21-hydroxylated steroids formed per rat adrenal per hour from the various concentrations of [4-^{14}C]-progesterone incubated. The 11β-hydroxylase activity was calculated from the total nmol of 11-oxi-steroids formed per adrenal per hour from the various concentrations of radioactive DOC used as substrate. The results were plotted as shown in Figs. 1*A* and 1*B*, respectively.

(2) *Steroid sulfatase activity.* For the measurement of the steroid sulfatase activity and to observe the effect of the *in vivo* stimulation of ACTH on the adrenal steroid sulfatase, two main approaches were used.

(A) The first approach used was called "direct method", which consists of measuring the sulfatase activity by using the Burstein method[17] which was slightly modified in two ways[23, 26, 27]:

(a) Pregnenolone-sulfate as well as DHEA-S were used as substrates in parallel incubations, instead of incubating only DHEA-S as described in the original method[17]. This modification was done because a 21-carbon steroid is a logical substrate for corticoid biosynthesis and besides, as will be shown in the results, the desulfation of pregnenolone-sulfate was significantly more effective than that of DHEA-S.

(b) The incubations were carried out under nitrogen atmosphere and without the addition of TPNH, to avoid further transformation of progesterone and 4-androstendione formed from pregnenolone-sulfate and DHEA-sulfate, respectively. Since both TPNH and oxygen are required for hydroxylase, desmolase and dehydrogenase activities, the conditions used permitted the accumulation of progesterone, formed from pregnenolone-sulfate and from free pregnenolone, and 4-androstendione formed from DHEA-sulfate.

The 3β-hydroxy-dehydrogenase activity was not altered but was actually favored by the addition of DPN, cofactor required by this enzyme[18].

Both progesterone and 4-androstenedione were very

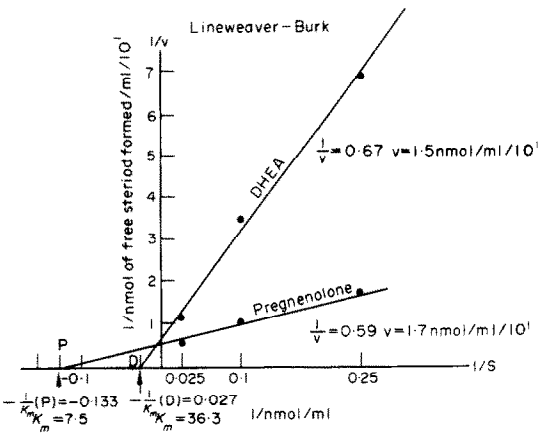

Fig. 2. Comparative desulfation of pregnenolone-sulfate and DHEA-sulfate as substrates of adrenal steroid sulfatase. V_{max} is essentially the same for both substrates but K_m values are very different indicating that pregnenolone-sulfate is more efficiently desulfated than DHEA-sulfate.

easily and quantitatively extracted by a simple partition between the aqueous media and the scintillating solvent (toluene-POPOP-PPO). Essentially all the unconverted steroid sulfates remained in the water phase, as shown in the original method[17]. The sulfatase activities were plotted as shown in Figs. 2 and 3.

(B) The second approach used was called the "indirect method" and it required double isotope

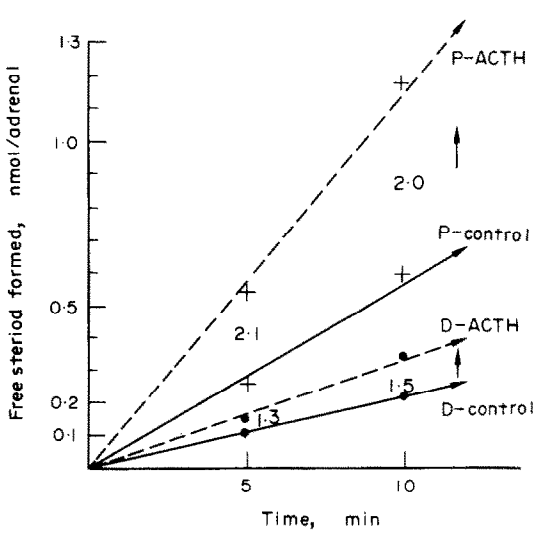

Fig. 3. Comparative degree of desulfation of pregnenolone-sulfate and DHEA-S by the action of steroid sulfatase in rat adrenals. Increase in steroid sulfatase activity in the adrenals from rats stimulated *in vivo* with ACTH (0·6 mU I.V./day, during 3 weeks) when compared to that observed in adrenals from control rats. P = free pregnenolone and D = free DHEA released in control (—) and in ACTH treated rats (– – –) from 10 nmol of either P-sulfate or DHEA-sulfate.

techniques[10, 15]. It was based on the fact that the steroid sulfatase concentration and activity are very small, in contrast with those for the 3β-hydroxysteroid dehydrogenase-Isomerase system, which is present simultaneously in the homogenized rat adrenal tissue incubated. Both [7-^{3}H]-pregnenolone-sulfate and free [4-^{14}C]-pregnenolone were incubated together in equimolar amounts, at ^{3}H/^{14}C ratios of 10:1 in the initial substrates. The incubations were carried out varying both times of incubation and substrate concentrations in the presence of homogenized adrenals from normal control rats and from rats stimulated with ACTH.

Since ACTH treatment did not seem to change the conversion rate of free pregnenolone to progesterone, as was shown in preliminary experiments, the ^{3}H/^{14}C ratio in the progesterone formed could indicate the relative conversions of free [4-^{14}C]-pregnenolone to progesterone [7-^{3}H]-pregnenolone-sulfate to free pregnenolone (by the action of steroid sulfatase) which is almost immediately converted to progesterone. An increase in the ^{3}H/^{14}C ratio in the final progesterone could be attributed to the stimulation or increase in the steroid sulfatase activity by the effect of ACTH.

Besides the ^{3}H/^{14}C ratio determined in the progesterone formed from the two substrates incubated, the nmolar amounts of progesterone originating from either substrate were determined, in order to compare more clearly the differences of the ACTH action. The nmol of [7-^{3}H]-progesterone formed were proportion to the steroid sulfatase activity and the nmol of [4-^{14}C]-progesterone formed were the index for the 3β-ol-dehydrogenase activity in the tissues studied, in the control vs the ACTH-treated adrenals.

The radioactive progesterone formed was extracted with hexane, after the addition of 1 M NaOH to the aqueous incubation media.

Progesterone was the main product formed in all instances and it was isolated from pregnenolone and pregnenolone-sulfate by paper chromatography in a hexane/propyleneglycol solvent system which was allowed to migrate for six hours[25]. The progesterone area was eluted and crystallized to constant d.p.m. ^{3}H/d.p.m. ^{14}C ratio and to constant specific activity d.p.m. ^{3}H/mg and d.p.m. ^{14}C/mg.

RESULTS

As it is shown in Figs. 1*A* and 1*B*, neither the 21-hydroxylase nor the 11β-hydroxylase activity of the rat adrenal was increased by *in vivo* stimulation with ACTH; actually it seemed that the 11β-hydroxylase activity was slightly diminished by ACTH. This result is in agreement with a previous report[14].

The maximum velocity for rat adrenal 11β-hydroxylase seems to be slightly higher than that for 21-hydroxylase under identical experimental conditions which indicates a similar but slightly greater concentration of 11β-hydroxylase per rat adrenal.

When the sulfatase activity in the adrenal was measured by a "direct method"[17, 26] and comparing the behavior of two substrates, pregnenolone-sulfate and DHEA-sulfate, it was observed that although the initial velocity values were essentially the same for both substrates, 1·7 and 1·5 nmol/ml/10′, respectively, the affinity between substrate and sulfatase was greater for pregnenolone-sulfate than for DHEA-sulfate, their K_m values being $7{\cdot}5 \times 10^{-6}$ and $36{\cdot}3 \times 10^{-6}$ M/l, respectively, as it is shown in Fig. 2.

In the next experiments, 10 nmol of either pregnenolone-sulfate or DHEA-sulfate were incubated with one homogenized rat adrenal for 5 and 10 minutes (a straight line is obtained up to 15 minutes incubation and their slopes start to decline between 15 to 30 minutes of incubation). Both control adrenals from normal rats as well as adrenals from rats chronically stimulated with ACTH, were incubated in parallel. The nmol of desulfated steroid per adrenal were plotted against time of incubation, as shown in Fig. 3. One can see that, again, pregnenolone-sulfate was more effectively desulfated than DHEA-sulfate by the action of adrenal steroid sulfatase in control adrenals (P-control and D-control solid lines). The adrenals stimulated with ACTH seem to have a higher sulfatase activity, since both pregnenolone-sulfate and DHEA-sulfate were desulfated more effectively by the ACTH group than by their corresponding control adrenals (P-ACTH > P-control and D-ACTH > D-control). The sulfatase activity in the ACTH adrenals was double that in control adrenals using pregnenolone-sulfate as substrate and the stimulation in the desulfation of DHEA-sulfate was somewhat less but significant.

When both sulfates were incubated simultaneously, one labeled with ^{3}H, as substrate, and the other one cold, acting as inhibitor, and varying their relative concentrations in the media, it was observed that both inhibited the desulfation of the other. The inhibition was therefore reciprocal indicating that both substrates are desulfated by the same enzyme. Besides, the results indicated that the reciprocal inhibition was of the competitive type (no change in V_{max} but a significant change in their K_m values as the concentration of the steroid acting as inhibitor was increased). The K_i values calculated according to Dixon's method for pregnenolone-sulfate and for DHEA-sulfate were essentially the same as their K_m values.

Since pregnenolone-sulfate has a greater affinity than DHEA-sulfate for the steroid sulfatase, the inhibitory effect of pregnenolone-sulfate was much

greater on the desulfation of DHEA-sulfate. On the other hand, DHEA-sulfate inhibited only very slightly the desulfation of pregnenolone-sulfate. Therefore, when both substrates are incubated together, pregnenolone is effectively desulfated while DHEA-sulfate remains essentially intact.

In the following experiments, the "indirect method" was used to measure the effect of ACTH on the adrenal steroid sulfatase, based on the relative conversion of [7-^{3}H]-pregnenolone-sulfate to progesterone (passing through free pregnenolone), as compared to the conversion of free [4-^{14}C]-prenenolone to progesterone.

While the d.p.m. of ^{3}H present in the progesterone formed depends on the limited activity of steroid sulfatase, the d.p.m. of ^{14}C present in the progesterone formed depends on the activity of the 3β-hydroxy-steroid dehydrogenase-Isomerase system.

Figure 4*A* shows the nmol of progesterone formed from pregnenolone-sulfate used as substrate at various concentrations. It is clearly shown that steroid sulfatase in control rat adrenals was apparently saturated with a very small amount of substrate and the maximum level of desulfation was limited to about 1·3 nmol of free pregnenolone, this intermediary steroid being quickly converted to progesterone (no radioactive pregnenolone was detected in the chromatographic separation of progesterone), and progesterone accumulated as the major product formed because of the experimental conditions used in which further conversions were avoided.

The ACTH-stimulated adrenals show a significant increase in steroid sulfatase activity, indicated by the elevation of the ^{3}H incorporation into progesterone, coming from pregnenolone-sulfate.

Due to the small concentration of steroid sulfatase in the rat adrenal and the range of substrate concentrations used (2·5 to 5·nmol), this enzyme reaction operated as a zero order kinetic reaction and, therefore, the plateau shown becomes proportional to the enzyme concentration. The chronic *in vivo* stimulation with ACTH seems to increase the steroid sulfatase concentration in the rat adrenal.

In Fig. 4*B*, the progesterone formed directly from free [4-^{14}C]-pregnenolone is plotted against the substrate concentration. In this instance what we see is the activity of the 3β-hydroxy-steroid dehydrogenase for various concentrations of substrate (identical range of those used in Fig. 4*A* for pregnenolone-sulfate). It can be seen that the control curve and the ACTH curve are identical and the curve is a perfect straight line. This reaction operated as a first order kinetic reaction which means that the product formed is proportional to the substrate concentration and the point of saturation is so far away that there is no indication of deflection in the curves. It is important to point out the difference in the scales used in the ordinates of both Figs. 4 4 and 4*B* to emphasize the tremendous difference in the concentrations of steroid sulfatase and the 3β-ol steroid dehydrogenase-Isomerase system.

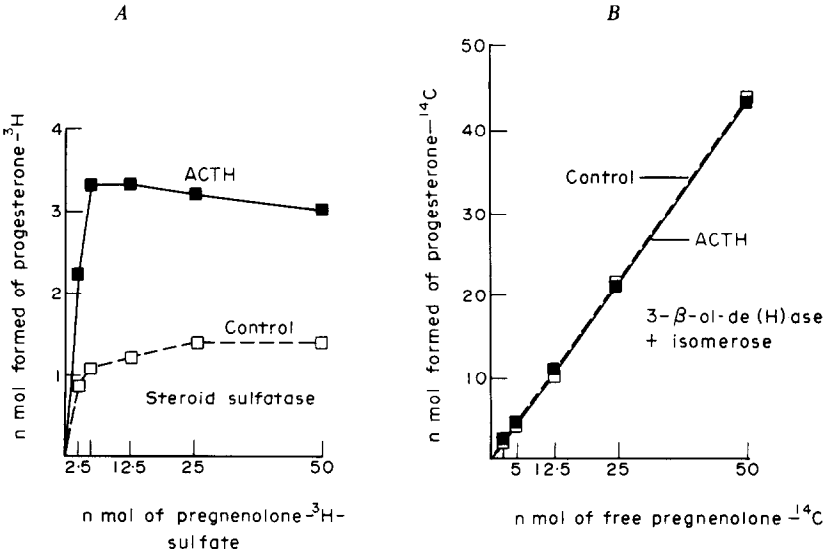

Fig. 4. Effect of ACTH on adrenal steroid sulfatase and on adrenal 3β-hydroxy-steroid dehydrogenase-Isomerase activities. 4*A*, represents the conversion of [^{3}H]-pregnenolone-sulfate to progesterone (*via* free pregnenolone) and 4*B*, represents the conversion of free [4-^{14}C]-pregnenolone to progesterone, in both instances by one adrenal from control rats and by one adrenal from rats chronically stimulated *in vivo* with ACTH.

The pool sizes of free pregnenolone and pregnenolone-sulfate were measured through radioimmunoassay in homogenized adrenals as well as in subcellular fractions obtained from both control rats as well as from rats stimulated with ACTH (5 min after I.V. injection of 10 I.U. of ACTH per gram of body weight).

The free pregnenolone pool was essentially the same in the control and in the ACTH treated adrenals when it was measured in the total homogenates and in the microsomal fraction. A small but insignificant increase in the free pregnenolone pool was observed in the mitochondrial and soluble fraction after ACTH stimulation. In contrast, the pregnenolone-sulfate pool decreased significantly after the stimulation with ACTH, from 17·7 to 10·3 ng/mg of protein in total homogenate and from 67·6 to 32 ng/mg protein in the microsomal fraction. No change was observed in the pregnenolone-sulfate pool in the mitochondrial fraction or soluble fraction. The pool size of free pregnenolone and pregnenolone-sulfate in the microsomal fraction of control rats was 74·4 and 67·6 ng/mg protein, respectively.

DISCUSSION

The sequence of the enzyme reactions cholesterol → 5-pregnenolone → 17α-hydroxypregnenolone → DHEA seems to occur in the free 5-en-3β-ol series as well as in the corresponding sulfated series[5, 12]. See Fig. 5.

The origin of plasma cholesterol-sulfate, which is very low in concentration[2] in comparison to that of free cholesterol, 300 μg/100 ml and 150 mg/100 ml, respectively, is unknown (it is probably formed mainly in the liver). Comparing these values with the plasma concentration of DHEA-sulfate, 250 μg/100 ml [28], and free DHEA, 1–5 μg/100 ml[25], one can see that both cholesterol-sulfate and DHEA-sulfate have similar concentrations, suggesting that perhaps there exists a metabolic stream which enters, passes through and leaves the adrenal gland and which is represented by the chain of enzymic reactions starting from cholesterol-sulfate, passing through pregnenolone-sulfate, 17α-hydroxy-pregnenolone-sulfate and DHEA-sulfate, which leaves the adrenal gland as a final product secreted into the circulation and, eventually, excreted in the urine.

Furthermore, it has been considered that the main source of precursors in corticoid biosynthesis may come from the blood and from esterified cholesterol[21], more than from endogenous free cholesterol, without eliminating the participation of *de novo* synthesis within the adrenal itself[29, 30].

If the adrenal gland utilizes either cholesterol-sulfate or pregnenolone-sulfate ditectly from what is available in the adrenal blood stream (although this has not yet been clearly established), preliminary studies suggest that pregnenolone-sulfate enters the adrenal cells more readily than free pregnenolone[10].

In the rat, the plasma concentrations of pregnenolone-sulfate and DHEA-sulfate are greater than those of their corresponding free steroids[3]. This fact suggests that perhaps the role of steroid sulfates in adrenal biosynthesis might be similar in both rat and human, without pretending the extrapolation of find-

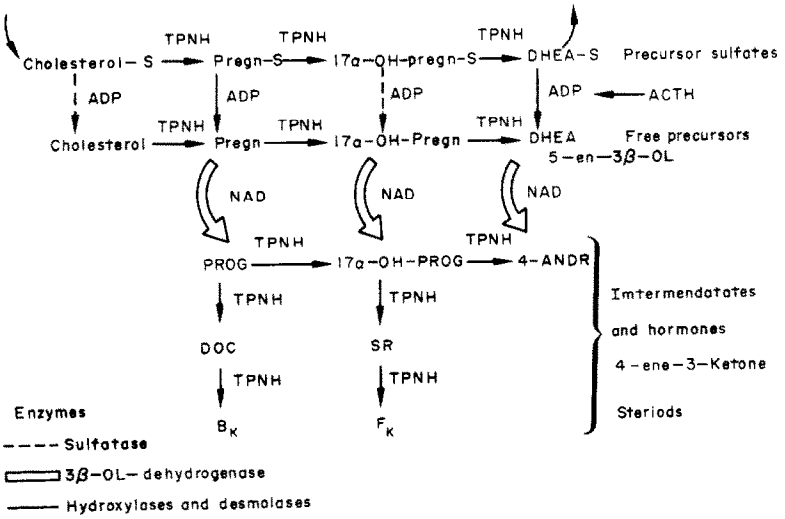

Fig. 5. Scheme of steroid hormone biosynthesis in the adrenal indicating cofactor requirements, participating enzymes and limiting reactions. The conversion of precursor sulfates (top line) to their corresponding free precursors (second line) is limited by the steroid sulfatase activity and probably regulated by ACTH stimulation. The following steps are not limiting factors in corticoid hormone biosynthesis.

ings and experimental results obtained in one species to what could actually occur in the other.

Steroid sulfatase may act on various intermediates of the chain of reactions already mentioned (see Fig. 5) from cholesterol-sulfate to DHEA-sulfate, the degree of desulfation being limited by the relative affinities and relative concentrations of the various steroid sulfates which are substrates of the steroid sulfatase. In all instances, however, the corresponding desulfated steroid (free pregnenolone, for instance) is almost immediately and very efficiently converted to progesterone by the action of the 3β-hydroxy-steroid dehydrogenase-Isomerase system. None of the reactions following pregnenolone in the process of corticosteroid biosynthesis seem to be limiting steps (neither for their corresponding enzyme concentration in the tissue nor if one considers their enzyme constants V_{max} and K_m values).

Although ACTH stimulates the corticoid production in the adrenal from endogenous precursors[31, 32], when specific enzyme steps were studied, Koritz[14] demonstrated that neither the 3β-hydroxy-steroid dehydrogenase-Isomerase system nor the 21 and 11β-hydroxylases was stimulated by ACTH. The results shown in Figs. 1*A* (for 21-OH-ase) and 1*B* (for 11β-OH-ase) are in complete agreement with Koritz's findings.

Apparently, ACTH seems to act somewhere prior to the formation of pregnenolone[14]. While Burstein studied the limited conversion of free cholesterol to free pregnenolone[21] and found stimulation by ACTH in the overall conversion, Hall[9] and Raggatt[33] studied the conversion of cholesterol-sulfate to pregnenolone-sulfate.

The present report involves some of the experiments that have been carried out to study the conversion of steroid sulfates to their corresponding free steroids by the action of steroid sulfatase, a limiting enzymic step in the adrenal steroid hormone biosynthesis which determines the amount of free pregnenolone which could be available for further conversion to steroid hormones in the adrenal.

An attempt has been made to demonstrate that this limiting gate, the steroid sulfatase, is stimulated by ACTH and that the concentration of this enzyme increases by the action of ACTH to constitute a regulatory mechanism in the corticoid production by the adrenal.

Experimental results related to the possible effect of ACTH on the human adrenal sulfatase are difficult to obtain: However, it is of interest to mention the recent findings by Mathur[34] who studied the conversion of [^{14}C]-acetate to free and sulfated steroids in two different human adrenal tissues. One was a feminizing adrenocortical carcinoma and the other, a bilateral diffused adrenocortical hyperplasia (Cushing) in which the stimulation by endogenous ACTH could be considered significantly different. The results showed that the radioactive 5-ene-3β-ol steroids isolated and identified were 13·9% free and 71·4% sulfated, in the first case, while they were 85·0% free and only 15% sulfated steroids in the second case.

How ACTH could stimulate the adrenal steroid sulfatase is still unknown. However, an interesting observation was made by Sayers[35] while measuring the response to increasing concentrations of ACTH of corticosterone and c-AMP production in dispersed rat adrenal cell preparations. At low doses of ACTH (5 to 25 μU), corticosteroidogenesis is stimulated without causing detectable changes in the c-AMP release. In doses of ACTH (between 50 and 250 μU) parallel increases in c-AMP and corticosterone were observed. Finally, larger doses of ACTH (250 to 10,000 μU), caused an additional increase in the c-AMP concentration without causing further increase in corticosterone accumulation. Probably, c-AMP is not, under all circumstances, an obligatory intermediary in the mechanism of action through which ACTH stimulates corticosterone biosynthesis. Perhaps, ACTH stimulates corticosterone biosynthesis through a mechanism not involving c-AMP when present at low concentrations or during small fluctuations of ACTH concentration and could act by increasing the steroid sulfatase activity. However, when high amounts of ACTH stimulate the adrenal tissue, the participation of other mechanisms would be required in response to the need, such as the complete enzymic and metabolic machinery of the adrenal cell involved in massive corticoid production in response to stress.

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DISCUSSION

Neher:

Am I correct in interpreting that all the effect seen by ACTH was from ACTH supplied *in vivo* to your rats? This was after about three weeks treatment? Have you any kinetic data on an early effect of ACTH on this sulfatase activity?

Domínguez:

In early studies, using human as well as rat adrenal slices (ref.) we reported an increase in the adrenal steroid sulfatase activity by the addition, *in vitro*, of ACTH. We plan to repeat those experiments using adrenal cell suspension preparations.

Regarding your second question, rather than adrenal sulfatase activity, we had measured the pool sizes of steroid sulfates and free steroids in the rat adrenals, after 5 min of ACTH injection. The pool of pregnenolone sulfate decreased in the rat adrenal after the ACTH injection. Fig. 4, in the text, summarizes the effect of ACTH on the endogenous pools of both free pregnenolone and its sulfate. Both steroids were measured in control rat adrenals (white bars) and in rat adrenals removed after 5 min of ACTH administration (dark bars). One can see that, in the total homogenate, pregnenolone sulfate decreases significantly by the action of ACTH, this diminution being more noticeable in the microsomal fraction. Although the levels of free pregnenolone and pregnenolone sulfate were about the same in the control microsomal fraction, the ACTH decreased to less than half the amount of pregnenolone sulfate without modifying significantly the amount of free pregnenolone. I do not consider significant the other changes shown, except the slight rise observed in mitochondrial free pregnenolone, perhaps due to the conversion of cholesterol to pregnenolone.

Neher:

This was after 5 min?

Domínguez:

This was after 5 min of ACTH injection intravenously. It was a massive amount, 10 units of ACTH.

Vihko:

In your abstract you state that ACTH increases the sulfatase activity without cyclic AMP participation, would you like to comment on that?

Domínguez:

In preliminary experiments which I did not include in my talk, we observed that addition of cAMP did not increase the sulfatase activity, while ATP or ADP added to the media definitely increase the sulfatase activity. Doctor Sayers has shown that by increasing the amounts of ACTH added to dispersed adrenal cell preparations, the response to various doses of ACTH can be divided in three stages as far as the correlation of corticosterone and *c*-AMP production. At very low doses of ACTH, there is no increase of *c*-AMP while corticosterone increases. In a second range of ACTH doses, there seems to be a direct correlation of *c*-AMP and corticosterone formation. Finally, at higher doses of ACTH, although *c*-AMP continues to rise, corticosterone apparently reaches a maximum production by the cell preparation.

Now, in the first stage (low concentration of ACTH), perhaps, the sulfatase is being stimulated by ACTH and an increment in corticosterone production occurs without the participation of *c*-AMP. Probably, ACTH stimulates an ATP-ase that releases ADP, which enhances sulfatase activity. Perhaps, what happens is that variations of ACTH within the low range, such as is observed during the diurnal variations and in the normal feedback, the response of the adrenal to ACTH may occur via activation of steroid sulfatase; however, in excess of ACTH, stress, the whole machinery of the adrenal requires *c*-AMP participation and it may come into the picture, because of the need for TPNH, protein synthesis and many other reactions.

One interesting finding reported recently by Sayers is the following: when he compared different synthetic ACTH analogues (portions of the natural polypeptide in which certain amino acids are missing), he observed that some of them behave exactly as the natural ACTH, however, other analogues, exhibiting activity for both corticosterone and *c*-AMP production, similar to that of natural ACTH, seem to change their whole behaviour. The first of the three distinct types of response described above for varying ACTH concentration disappears. There is no longer any corticosterone production without an increase of *c*-AMP. I was wondering if some of the amino acid sequences absent in those ACTH analogues could be responsible for the stimulation of the sulfatase, perhaps through the stimulation of a ATP-ase that releases ADP. Naturally, this is just hypothetical but probable.

I would like to mention something which is very important. Everyone has a tendency to try to find the *best* substrate, the *best* yield, etc. I am looking for the contrary, a limiting reaction, the one that can be controlled to determine the quantitative gate. Actually, if one considers free pregnenolone, it is a very good precursor of progesterone. As I said before, you can add a lot of free pregnenolone in homogenized tissue and every little bit goes into progesterone. Moreover, if the reactions after pregnenolone are not limiting reactions, why does the adrenal produce such small amounts of corticosterone and cortisol when we have huge amounts of precursors. It has to be a precursor whose production is limiting the final hormone output in one of the steps. It may be the cleavage of cholesterol's side chain, it may be the desulfation of precursor sulfates. In general, I am not looking for the *best* substrate but for the most probable limiting reaction that could determine the quantitative output and production of corticoids.