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ON THE ROLE OF STEROID SULFATES IN HORMONE BIOSYNTHESIS

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SUMMARY

The conversion of steroid sulfates to active steroid hormones has been studied. This conversion is limited in the adrenal by the low concentration of steroid sulfatase (SS) present. In contrast, free 5-ene-3β-hydroxy-steroids are effectively converted to active hormones. ACTH stimulates corticosteroidogenesis through a mechanism of action located in the scheme of biosynthesis, somewhere before free pregnenolone is formed. Since ACTH stimulates SS-activity and increases its concentration, the participation of SS and steroid sulfates in corticosteroidogenesis could be important. Pregnenolone-sulfate (PS) is desulfated more effectively than DHEA-sulfate (DS). When both are incubated together, a reciprocal competitive type of inhibition was observed, PS being more potent as inhibitor than DS, since PS as substrate and as inhibitor has greater affinity than DS for SS. Regarding cofactor requirements for SS, its performance was significantly improved by the addition of ATP and ADP; a smaller increment in SS-activity was achieved with DPN. However, neither c-AMP nor TPNH, added in comparable molar concentrations, seem to increase SS-activity. It is probable that ACTH, whose accepted mechanism of action involves c-AMP, could have a separate mechanism to increase corticosteroidogenesis in which SS-activity is enhanced without c-AMP participation. Stressed rat adrenals exhibited higher SS-activity than control rat adrenals.

INTRODUCTION

As is shown in the top line of Fig. 1, various 5-ene-3β-hydroxy steroids, conjugated as sulfates, such as cholesterol–sulfate, pregnenolone–sulfate, 17α-hydroxy-pregnenolone–sulfate and DHEA–sulfate, are products involved in steroid hormone biosynthesis [1–5] and they have been isolated from circulating blood as well as from adrenal tissue [6–11]. DHEA–sulfate is one of the major steroids secreted by the human adrenal [6, 7, 12, 13] and probably in other species [11]. This fact gives to the steroid sulfates a very intriguing and particular interest. Besides, it has been demonstrated by several researchers that the sequence

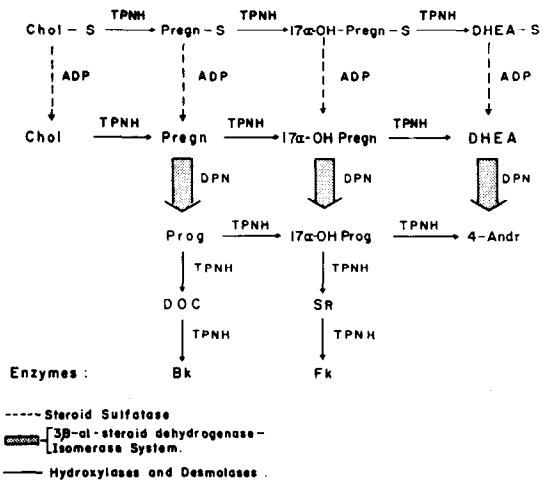

Fig. 1. General scheme of steroid hormone biosynthesis in the adrenal gland, indicating the cofactor requirements for the various enzymatic steps and a relative appreciation of enzyme concentrations involved.

of conversions from cholesterol to DHEA, *via* pregnenolone and 17α-hydroxypregnenolone, takes place both for the free 5-ene-3β-hydroxy-ol-steroids [14–16] as well as for the corresponding steroid sulfates [1, 3, 7], in which case the sulfate group remains in the molecule throughout the whole conversion. The enzymes involved in these consecutive transformations are hydroxylases and desmolases requiring TPNH as cofactor.

The participation and regulation of steroid sulfatase activity [17–30], as well as the role of steroid sulfates as precursors in the biosynthesis of active steroid hormones [1–4, 7] has also been extensively studied. This participation has been observed in the adrenals, gonads and placenta.

In order to transform any 5-en-3β-ol-steroid sulfate into its corresponding 4-ene-3-keto-steroid, it is necessary, first of all, to remove the sulfate group by the action of steroid sulfatase, which releases a free 5-ene-3β-ol-steroid as an obligatory intermediate. See Fig. 2.

Once the free steroid is formed, the free 5-en-3β-ol structure is quickly and efficiently converted to the corresponding 4-ene-3-keto structure by the action of the 5-ene-3β-hydroxy steroid dehydrogenase-Isomerase system. This enzyme system is very active and probably the highest in concentration with respect to all the other steroid biosynthesizing enzymes present in steroid producing tissues [20, 21].

The 5-en-3β-ol-steroid dehydrogenase requires DPN as a cofactor [31, 32], in contrast to most of the other enzymes involved in steroid biosynthesis which require TPNH, such as with hydroxylases [33–35], desmolases [33, 36, 37] and several dehydrogenases [38, 39].

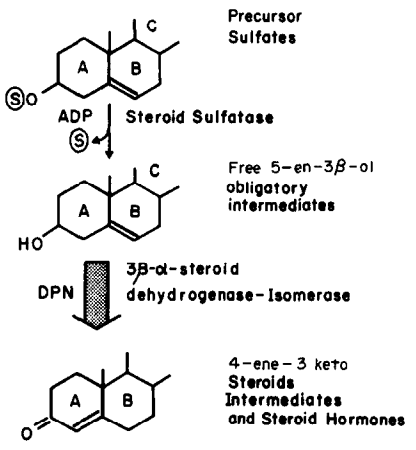

Fig. 2. Steps involved in the conversion of a 5-ene-3β-ol steroid sulfate as a precursor of an active steroid hormone via free 5-en-3β-ol steroid.

It is important to point out the peculiar fact that the 3β-hydroxy-dehydrogenase-Isomerase system, which exhibits the greatest activity and highest concentration in various tissues, acts directly on the very limited amount of substrate released by the steroid sulfatase, which has one of the lowest concentrations among the steroid biosynthesizing enzymes [20, 26, 30]. This gives to the steroid sulfatase a very strategic situation in the scheme of biosynthesis, with the possibility to act as a gate which determines and limits, quantitatively, the portion of precursor which, from the top line (Fig. 1), is desulfated and transferred to the second line (Fig. 1), from where they are effectively transformed thereafter into an active 4-ene-3-keto steroid.

None of the enzymes involved in steroid hormone biosynthesis after pregnenolone seems to be a limiting factor in the quantitative production of the final corticoid [20, 21].

It has also been demonstrated that none of the enzymes after pregnenolone seems to be stimulated by ACTH [20, 30, 40]. Koritz and other researchers suggest, actually, that the mechanism of action of ACTH on the adrenal may occur somewhere before pregnenolone is formed [21, 23, 30, 40].

While Burstein and others have studied the limiting conversion of free cholesterol to free pregnenolone [37–41] and others the conversion of cholesterol–sulfate to pregnenolone–sulfate. [41, 42], our group became interested in the limiting conversion of steroid sulfates to their corresponding free steroids [22–30].

In contrast to several investigators interested in the best precursors, the most efficient conversion, the predominant pathway, etc., our group became interested in one limiting factor, in a restricted reaction, in the gate which could determine the limited availability of intermediary substrates in the scheme of biosynthesis. If this limiting adrenal enzyme, namely steroid sulfatase can respond to the action of ACTH, one could have a gate whose slit could be regulated and the flow through which could be controlled by the existing feedback mechanisms.

METHODS AND EXPERIMENTS

The steroid sulfatase activity was measured by two different methods

A. The first method was called the "indirect method" [22–24, 30] and required double isotope techniques. It was based on the relatively small steroid sulfatase concentration and activity with respect to a very high concentration of the 3β-ol-steroid-dehydrogenase-Isomerase system present both in the homogenized rat adrenal tissue. Both [7α-^{3}H]-pregnenolone–sulfate and free [4-^{14}C]-pregnenolone were added together in equimolar amounts and incubated in the presence of homogenized adrenals from normal rats and from rats stimulated *in vivo* with ACTH. The ratio [^{3}H]-d.p.m./[^{14}C]-d.p.m. in the progesterone formed from both substrates could indicate the relative conversion of [7α-^{3}H]-pregnenolone–sulfate to progesterone *via* free pregnenolone, with respect to the direct conversion of free [4-^{14}C]-pregnenolone to progesterone, taken as reference. See Fig. 3. Since in preliminary experiments ACTH did not change the conversion rate of free pregnenolone to progesterone [20, 22, 24, 30], any increment observed in the ratio [^{3}H]/[^{14}C] in progesterone after ACTH stimulation could be interpreted as an increase in the steroid sulfatase activity.

In order to avoid further transformation of progesterone to corticoids, the incubations were carried out under nitrogen atmosphere and without the addition of exogenous TPNH. The only product formed under these experimental conditions was essentially progesterone and its formation was favored by the addition of ADP (tentative cofactor for steroid sulfatase) and DPN, cofactor required by the 3β-ol-steroid-dehydrogenase–Isomerase system.

At the end of the incubation period, the media was extracted with hexane and the progesterone

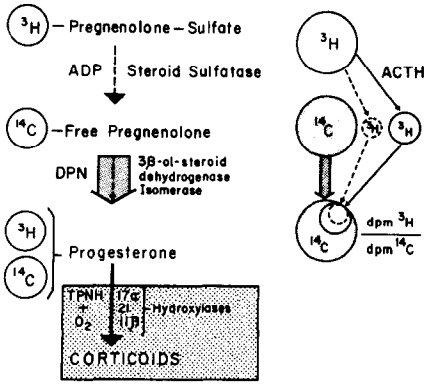

Fig. 3. Scheme to illustrate the "Indirect Method" to determine the steroid sulfatase activity with respect to that of 3β-hydroxy-steroid-dehydrogenase-isomerase, by measuring the relative conversion of [7-^{3}H]-pregnenolone-sulfate and [4-^{14}C]-free pregnenolone to progesterone. ACTH seems to stimulate the first without affecting the second enzyme reaction. To minimize further conversion to corticoids and to favor progesterone accumulation, TPNH was not added to the media and the atmospheric air was substituted with N_2.

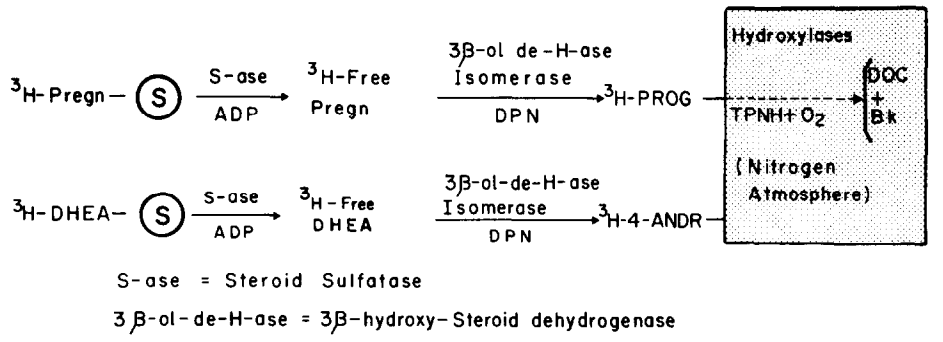

Fig. 4. Scheme to illustrate the "Direct Method" to determine steroid sulfatase activity, comparing the desulfation of ^{3}H-pregnenolone-sulfate and ^{3}H-DHEA-sulfate used as substrates in the presence of ADP and DPN as cofactors as well as in the absence of TPNH and O_2, required by hydroxylations and to favor the accumulation of progesterone and 4-androstenedione, respectively, as final products of the incubation.

formed was purified and isolated by paper chromatography in a Hexane:Propyleneglycol solvent system [43, 44]. The progesterone was eluted and crystallized after the addition of 10 mg of pure progesterone until a constant ratio [^{3}H]-d.p.m./[^{14}C]-d.p.m. and constant specific activities, [^{3}H]-d.p.m./mg and [^{14}C]-d.p.m./mg, were achieved. Double isotope counting was done by applying the channel ratio technique [45, 46] using a Nuclear Chicago Liquid Scintillation spectrometer.

In all the experiments, the amounts of free pregnenolone isolated were very small. In some instances, it was not present in detectable amounts, however, the main product formed and isolated, under the experimental conditions used, was progesterone.

B. The second method used was basically that described by Burstein [47] to measure the steroid sulfatase activity but it was slightly modified in our laboratory and was called the "direct method" [25, 26, 29, 30].

The modifications made to the original method are as follows: see Fig. 4.

1. [7-^{3}H]-pregnenolone-sulfate was used as substrate, besides [7-^{3}H]-DHEA-sulfate, in parallel incubations, instead of using only [7-^{3}H]-DHEA-sulfate as described in the original method. This modification was done because pregnenolone-sulfate is the logical precursor for corticoid biosynthesis. The DHEA-S, we think, is a side product by the adrenal rather than a precursor of steroidogenesis.

2. The second modification consisted in carrying out the incubations under nitrogen atmosphere and without the addition of exogenous TPNH to avoid the action of hydroxylases present in the media which require both oxygen and TPNH. We assure in this way that progesterone will be the main product when pregnenole-sulfate is used as substrate and 4-androstenedione will be the final product when DHEA-sulfate is used as substrate.

3. The third modification consisted in the addition of 1·0 mM ADP and 3·0 mM DPN to the incubation media. The ADP seems to facilitate the action of steroid sulfatase as described later in this paper. The addition of DPN permits the conversion of the intermediate free 5-en-3β-ol steroid formed to the corresponding 4-ene-3-keto compound (progesterone and 4-androstenedione, respectively).

In this way, the final products formed, proportional to the steroid sulfatase present, were easily and quantitatively extracted by the scintillation solvent (toluene–PPO–POPOP) in the single partition made with the aqueous incubation media which retained in solution the remaining steroid sulfate, as described in the original method.

The direct method, therefore, consisted of: (see Fig. 5).

Incubation of various concentrations of [7-^{3}H]-

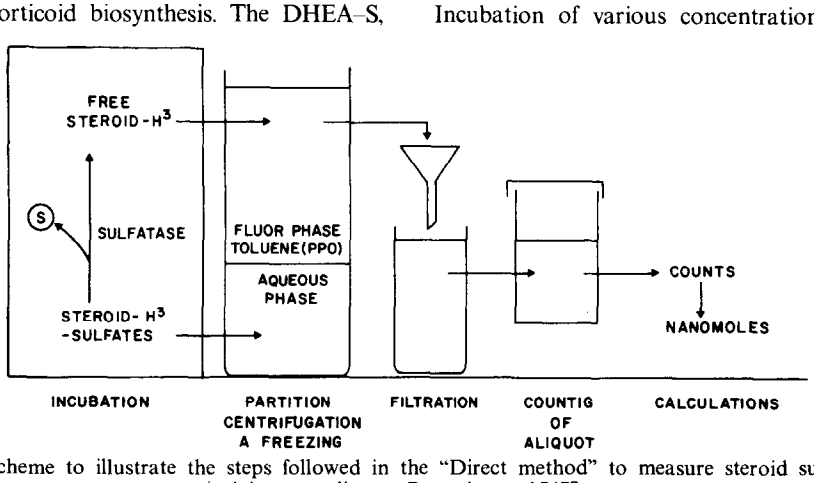

Fig. 5. Scheme to illustrate the steps followed in the "Direct method" to measure steroid sulfatase activity according to Burstein *et al.*[47].

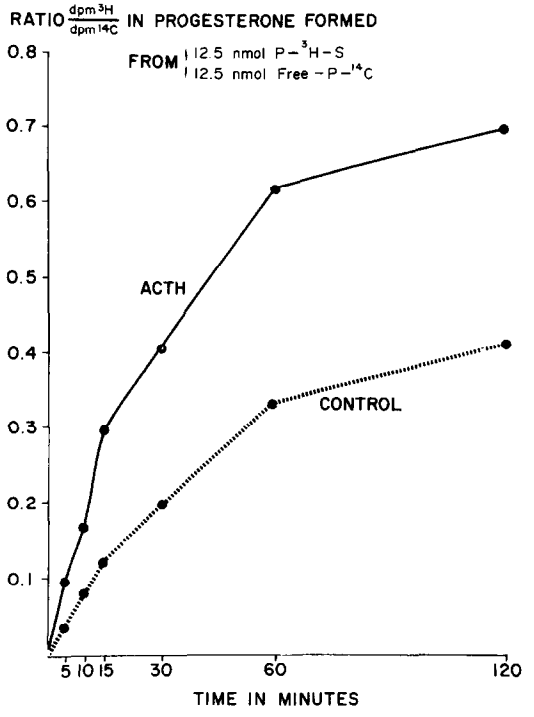

Fig. 6. Relative incorporation of ^{3}H (from [7-^{3}H]-pregnenolone-sulfate) and ^{14}C (from [4-^{14}C]-free pregnenolone) in progesterone formed at various times of incubation, using equimolar amounts of both initial substrates by rat adrenals and the effect of *in vivo* stimulation with ACTH.

steroid sulfate in a Krebs–Bicarbonate nicotinamide buffer at pH 7·4, in the presence of homogenized tissue containing the steroid sulfatase to be measured, plus the addition of 1·0 mM ADP and 3·0 mM DPN. After substituting the air with nitrogen, the tubes were stoppered and incubated for either 5 or 10 min at 37°C in a Dubnoff incubator.

At the end of the incubation period, the partition, centrifugation and freezing, the filtration, counting and calculations were performed as in the original method described by Burstein[47].

RESULTS AND DISCUSSION

Applying the "indirect method", the following results were obtained when 12·5 nmol of each substrate ([7-^{3}H]-pregnenolone-sulfate and [4-^{14}C]-free pregnenolone) were incubated for different periods of time with one homogenized adrenal from either normal control rats or from rats stimulated with 0·6 mU i.v./day of ACTH during a three week period (see Fig. 6).

One can see that the [^{3}H]-d.p.m./[^{14}C]-d.p.m. ratios, in the progesterone formed are greater (about double) for the ACTH-treated adrenals than for the control group, at all incubation times, suggesting that the *in vivo* stimulation with ACTH increased the steroid sulfatase activity in the adrenals.

Since in the present experiment the [^{3}H]-d.p.m./[^{14}C]-d.p.m. ratios were low in the final progesterone formed due to the small concentration of steroid sulfatase with respect to that of 3β-ol-steroid-dehydrogenase–Isomerase and although the differences shown between the ACTH-treated and control groups were significant, it was decided to increase the [^{3}H]-d.p.m./[^{14}C]-d.p.m. ratios to 10/1 in the initial substrates. Their specific activities were adjusted, in such a way that both [7-^{3}H]-pregnenolone-sulfate and [4-^{14}C]-free pregnenolone were added to the incubations in equimolar amounts.

Aliquots equivalent to one homogenized adrenal either from normal control rats or from ACTH treated rats were incubated in the presence of various concentrations of each of the two substrates, for 10 min. The results are summarized in Table 1. It is possible to compare in the two adrenal groups (control and ACTH-treated), the percent conversion of the two individual substrates to progesterone, the nmol amounts of [7-^{3}H]- or [4-^{14}C]-progesterone formed from the various initial concentrations of pregnenolone–sulfate or from free pregnenolone, respectively and the [^{3}H]-d.p.m./[^{14}C]-d.p.m. ratios found in the identified progesterone formed.

Starting with the [^{3}H]-d.p.m./[^{14}C]-d.p.m. ratios found in the isolated progesterone, one can see that

Table 1. Relative conversion of [7-^{3}H]-pregnenolone-sulfate and [4-^{14}C]-free pregnenolone to progesterone by homogenized rat adrenals from control rats and from ACTH treated rats

Adrenal group	Initial amount of substrates nm [7-^{3}H]-PS (1)	[4-^{14}C]-P (2)	Product formed per adrenal/10 min: [7-^{3}H]-Progesterone % Conversion from [7-^{3}H]-PS (1)	[7-^{3}H]-Progesterone nm formed	[4-^{14}C]-Progesterone % Conversion from [4-^{14}C]-P (2)	[4-^{14}C]-Progesterone nm formed	Ratio [^{3}H]-d.p.m. / [^{14}C]-d.p.m. in progesterone
Control group	2·5	2·5	36·4	0·9	85·0	2·1	7·4
	5·0	5·0	21·6	1·1	84·6	4·2	3·3
	12·5	12·5	9·8	1·2	82·0	10·3	2·4
	25·0	25·0	5·5	1·4	88·3	22·1	0·9
	50·0	50·0	2·8	1·4	87·6	43·8	0·6
ACTH group	2·5	2·5	88·0	2·2	90·3	2·3	13·1
	5·0	5·0	66·8	3·2	90·0	4·5	9·0
	12·5	12·5	26·6	3·4	89·8	11·3	3·8
	25·0	25·0	12·6	3·3	87·3	21·8	1·8
	50·0	50·0	5·7	3·2	86·7	43·4	1·1

(1) [7-^{3}H]-PS = [7-^{3}H]-pregnenolone-sulfate.
(2) [4-^{14}C]-P = [4-^{14}C]-free pregnenolone.

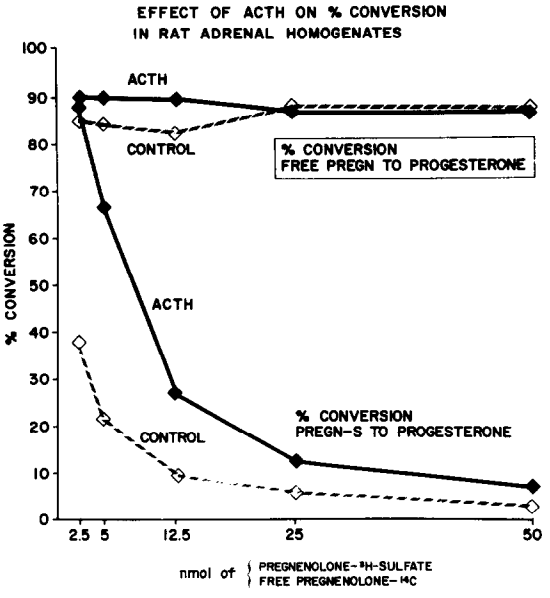

Fig. 7. *In vivo* effect of ACTH on the percent conversion of various mμmolar concentrations of either free pregnenolone or pregnenolone-sulfate to progesterone by one homogenized rat adrenal (control group vs ACTH-treated group).

those observed in the progesterone formed by the ACTH-treated adrenals were significantly higher than those observed in the progesterone formed by the control adrenals for each of the individual substrate concentrations used. Since ACTH does not stimulate the conversion of free-[4-^{14}C] pregnenolone to progesterone, the increment observed in the [^{3}H]-d.p.m./[^{14}C]-d.p.m. ratio in the progesterone formed could be interpreted as the stimulation that ACTH has on the steroid sulfatase. These ratios diminished in both groups as the concentration of the substrates increases due to the low and limited concentration of steroid sulfatase, which was saturated by the [7-^{3}H]-pregnenolone-sulfate, while the 3β-ol-steroid-dehydrogenase-Isomerase because of its high concentration in the tissue converted almost all the free pregnenolone present.

If one plots the substrate concentration on the abscissa against the percent conversions of free pregnenolone and pregnenolone-sulfate to progesterone on the ordinate axis and one compares the results obtained for both control adrenals and ACTH-treated adrenals (see Fig. 7), one can see that the efficiency of conversion of pregnenolone-sulfate to progesterone is greater in the ACTH group than in the control group for all the substrate concentrations used. However, as the substrate (pregnenolone-sulfate) increases, there is a diminution in the percent conversion to progesterone because the steroid-sulfatase concentration becomes limiting and the enzyme is saturated. On the other hand, the percent conversion of free pregnenolone to progesterone was about the same for all the substrate concentrations used and practically all the free pregnenolone was converted to progesterone, indicating that the 3β-ol-steroid-dehydrogenase was not limiting the reaction within the range of substrate concentration used. The nm amounts of progesterone formed from free pregnenolone by both the control and the ACTH group are almost identical, as shown in Table 1, and both are proportional to the substrate concentration, which indicates that the 3β-ol-steroid-dehydrogenase operated as a first order kinetic reaction and was not affected by the action of ACTH.

In contrast, when one plots the nm amounts of [7-^{3}H]-progesterone formed against the amount of substrate (pregnenolone-sulfate) and the curves obtained for the control adrenals and ACTH-treated adrenals are compared, it is possible to observe that both curves attain a definite plateau at a level which is proportional to the steroid-sulfatase concentration, which becomes a limiting factor in the conversion of pregnenolone-sulfate to progesterone.

The adrenal steroid-sulfatase concentration seems to be about double in the ACTH-treated adrenals than in the control adrenals and in both groups of adrenals, the steroid-sulfatase seems to operate as a zero order kinetic reaction in which the product formation (progesterone) is limited by the enzyme concentration, although enough substrate pregnenolone-sulfate is still available but not converted. When ACTH is added *in vitro* to human adrenal slices, stimulation of the steroid-sulfatase is also observed [23].

When the "direct method" was used to measure steroid-sulfatase activity by Burstein's method, the results obtained were as follows:

In Fig. 8, the comparative degree of desulfation of pregnenolone-sulfate, with respect to DHEA-sulfate, is illustrated when the substrates were incubated at various concentrations during a 10 min period with normal homogenized rat adrenal. It is shown that the desulfation of pregnenolone-sulfate was significantly greater than that of DHEA-sulfate by the rat adrenal steroid-sulfatase.

When 10 nm of either pregnenolone-sulfate (PS) or DHEA-S (DS) were incubated for 5 or 10 min with one homogenized rat adrenal (as shown in Fig. 9) from control animals (white bars) or from rats stimulated *in vivo* with ACTH (shaded bars), one can see that pregnenolone-sulfate was hydrolyzed by the steroid-sulfatase more effectively than DHEA-sulfate for both adrenal groups. Besides, one can see that the ACTH stimulated adrenals seem to exhibit a greater steroid sulfatase activity than the control rat adrenals, the stimulation being more noticeable for pregnenolone-sulfate than for DHEA-sulfate. The desulfation reaction follows a straight line at 5 and 10 min for both steroid sulfates used as substrates and for both the control and for the ACTH-stimulated adrenals.

When the results are plotted according to Lineweaver-Burk as previously reported [26, 29, 30], the steroid sulfatase seems to have the same maximum velocity for both substrates, $9{\cdot}6 \times 10^{-6}$ M/l/h, but the K_m values for the two substrates seem to be quite different, indicating that the steroid sulfatase has a

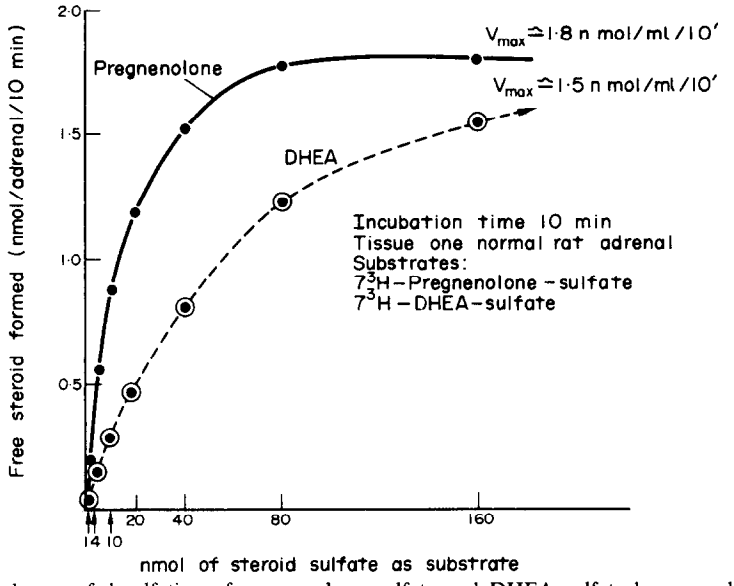

Fig. 8. Relative degree of desulfation of pregnenolone–sulfate and DHEA–sulfate by normal adrenal steroid sulfatase at various equivalent substrate concentrations.

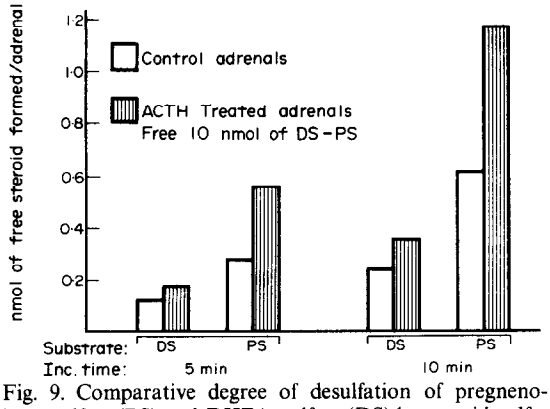

Fig. 9. Comparative degree of desulfation of pregnenolone–sulfate (PS) and DHEA–sulfate (DS) by steroid sulfatase from control rat adrenals and from rat adrenals stimulated *in vivo* with ACTH. Results obtained at 5 min and 10 min of incubation.

greater affinity for pregnenolone–sulfate $K_m = 7{\cdot}5 \times 10^{-6}$ M/l than for DHEA–sulfate $K_m = 36{\cdot}6 \times 10^{-6}$ M/l [26, 29, 30].

In order to study the possible interaction that could exist between the two substrates and the steroid sulfatase, the following experiments were conducted. See Table 2.

In experiment A, 10 nm of radioactive pregnenolone–sulfate were incubated in the absence as well as in the presence of various concentrations of cold DHEA–sulfate, acting as inhibitor. In experiment B, we reverse the role of the two steroids. Now 10 nm of radioactive DHEA–S were incubated in the absence as well as in the presence of various concentrations of cold pregnenolone–sulfate. The degree of desulfation of both radioactive substrates, as shown in the columns of free radioactive steroid formed, decreases as the concentration of the cold steroid sulfate, acting as inhibitor, increases. The result indicated that there is a reciprocal inhibitory effect which means that both steroid sulfates inhibit the desulfation of the other. Under identical conditions and in molar basis, pregnenolone–sulfate seems to be a more potent inhibitor than DHEA–S on the desulfation of the other.

Similar, but more complete experiments than the ones previously described were carried out to find

Table 2. Reciprocal inhibitory effect of cold pregnenolone-sulfate and DHEA-sulfate on the desulfation of the other radioactive steroid sulfate used as substrate for the rat adrenal steroid sulfatase

Experiment A			Experiment B		
Substrate [7-³H]-P-S (nm)	Inhibitor cold DHEA-S (nm)	Free [7-³H]-steroid formed (nm)	Substrate [7-³H]-DHEA-S (nm)	Inhibitor cold Pregn-S (nm)	Free [7-³H]-steroid formed (nm)
10	—	1·30	10	—	0·30
10	2·5	1·12	10	2·5	0·24
10	5	1·01	10	5	0·21
10	10	0·92	10	10	0·16
10	20	0·86	10	20	0·14
10	40	0·76	10	40	0·10

[7-³H]-P-S = [7-³H]-pregnenolone-sulfate.
DHEA-S = Dehydroepiandrosterone-sulfate.
[7-³H]-DHEA-S = [7-³H]-dehydroepiandrosterone-sulfate.
Pregn-S = Pregnenolone-sulfate.

out the type of inhibition through which both steroid sulfates seem to interact. Both the substrate concentration as well as the inhibitor concentration were varied. In Experiment A, [7-^{3}H]-pregnenolone–sulfate acted as substrate and cold DHEA–sulfate as inhibitor, while in Experiment B, the role of the two sulfates were inverted, using [7-^{3}H]-DHEA–sulfate as substrate and cold pregnenolone–sulfate as inhibitor. All the curves obtained cross the ordinate axis at the same point, exhibiting essentially the same V_{max} value for all the concentrations of substrate and inhibitor studied. However, a significant change in their corresponding K_m values was observed as the concentration of the steroid acting as inhibitor changed. The K_i values calculated according to Dixon's method for DHEA–sulfate and pregnenolone–sulfate were essentially the same as their corresponding K_m values calculated according to Lineweaver–Burk's method. The results suggest that both substrates are hydrolized by the same sulfatase which exhibits greater affinity for pregnenolone–sulfate than for DHEA–S either when used as substrate as well as when acting as inhibitor. The reciprocal inhibition observed between pregnenolone–sulfate and DHEA–S seems to be of the competitive type [30].

Since pregnenolone–sulfate has a greater affinity for the steroid sulfatase than DHEA–sulfate, the inhibitory effect of pregnenolone–sulfate on the desulfation of DHEA–S is even greater. On the other hand, DHEA–S inhibited very slightly the desulfation of pregnenolone–sulfate. Therefore, when both substrates are together in the adrenal, pregnenolone–sulfate is effectively hydrolyzed and the free pregnenolone formed could be available for further transformation to corticosteroid hormones, while DHEA–sulfate remains essentially intact as a sulfate.

Regarding the cofactor requirements for the rat adrenal steroid sulfatase, ATP, ADP, c-AMP and DPN were added in equimolar amounts in parallel incubation tubes. The amounts of free pregnenolone formed from 5 nm of initial pregnenolone–sulfate by one half homogenized rat adrenal were measured. The results are illustrated in Table 3 and show that ATP and ADP increase significantly the sulfatase activity with respect to that observed in the control incubation without cofactors (underlined figure). In contrast, c-AMP, does not seem to increase the sulfatase activity.

The addition of DPN to the control or in addition to each of the other cofactors seems to increase the degree of desulfation at about the same degree in all cases. Since DPN is a cofactor required by the 3β-ol-steroid–dehydrogenase [31, 32], most of the free pregnenolone formed could be transformed to progesterone and, therefore, the disappearance of free pregnenolone from the media could result in a better performance of the steroid sulfatase. On the other hand, since progesterone is more easily extracted than pregnenolone by toluene from water, the final product formed in the presence of DPN may give better yields in the extraction procedure.

The effect of stress on the activity of adrenal sulfatase was studied next and the results summarized in Fig. 10. Two groups of normal rats were compared. In experiment 1, the animals were kept without stress as a control group, while in experiment 2, the animals were kept under stress conditions (noise, irregularities in temperature, environment, light and times of feeding) as a control stress group. One can see that the base line in the control group (top graph) showed less sulfatase activity than the one observed in the stressed group (lower graph). The stress itself seems to increase the sulfatase activity to about 50%, with respect to the control group.

Again, the addition of either ATP or ADP seems to increase significantly the efficiency of steroid sulfatase in both groups of rat adrenals, with a larger increment in the stressed adrenal group than in the control adrenal group. c-AMP alone, did not affect the sulfatase activity in the control group and the increment observed in the stressed adrenals was very small.

Once more, the addition of DPN seems to increase the efficiency of steroid sulfatase by a similar magnitude. TPNH and butyryl-c-AMP were also studied but they did not seem to stimulate the adrenal steroid sulfatase.

Table 3. Effect of ATP, ADP, c-AMP and DPN on the adrenal steroid sulfatase activity *in vitro*

Cofactor added*	Amount of free steroid formed from P-S†		Ratio Cofactor / Control
	Cofactor alone	Cofactor + DPN	
ATP	0·40		1·5
ATP + DPN		0·46	
ADP	0·36		1·4
ADP + DPN		0·43	
c-AMP	0·27		1·0
c-AMP + DPN		0·35	
Control	0·26		
Control + DPN		0·33	

* All the cofactors were present in equimolar amounts.
† nm of free steroid formed/0·5 adr/10′ from 5 nm of pregnenolone-sulfate.

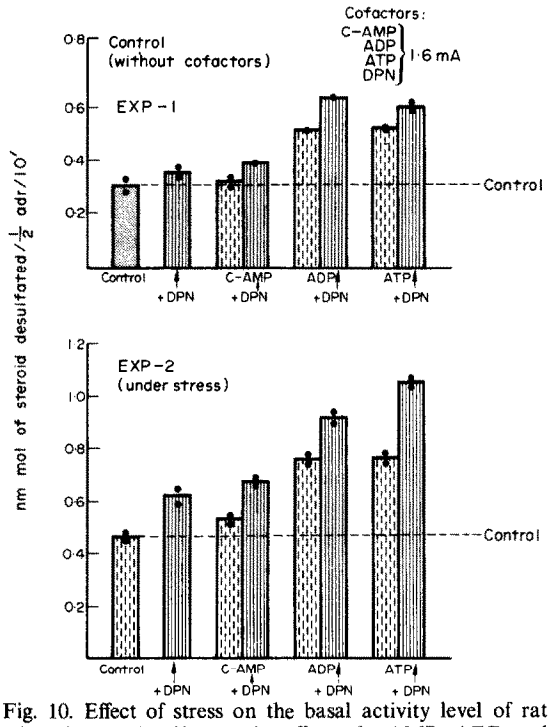

Fig. 10. Effect of stress on the basal activity level of rat adrenal steroid sulfatase, the effect of c-AMP, ADP and ATP when present in the media (1·6 mM) and the additive effect of DPN on the steroid sulfatase efficiency.

CONCLUSIONS

It has been generally accepted that the mechanism of action of ACTH always involves an initial release of c-AMP through adenyl cyclase activation [40, 48, 49] which, among many other things, increases adrenal phosphorylation, release of TPNH required for a good performance of steroid hydroxylases and a final increment in corticoid biosynthesis [50, 51].

The correlation between the accumulation of c-AMP and corticosterone in response to ACTH stimulation on dispersed adrenal cell preparations has been studied [52] and c-AMP seems to mimic the effect that ACTH exerts on the isolated adrenal cells regarding corticosterone production [48, 52, 53]. This correlation between c-AMP and corticosterone accumulation has been shown for natural and synthetic ACTH and has been studied for several ACTH analogues and polypeptides containing portions of the 39 ACTH aminoacid sequences [54] with the development of agonists and antagonists of ACTH.

Sayers [52] reported a very interesting observation. When dispersed adrenal cell preparations are stimulated with various doses of ACTH and both c-AMP and corticosterone accumulation are measured simultaneously, the response seems to vary in pattern, depending upon the amount of ACTH added. Sayers described three distinct phases in the rat adrenal cell response when the amount of ACTH, in the abscissa, is plotted against the accumulation of corticosterone and c-AMP, in the ordinate. With 5 to 25 μU of ACTH, the adrenal cells respond to the stimulation by increasing the accumulation of corticosterone without apparent formation or accumulation of c-AMP. With 25 to 250 μU of ACTH, the accumulation of c-AMP and corticosterone have a good correlation. Finally, with more than 250 μU of ACTH, c-AMP continues rising while corticosterone production seems to reach a plateau due to the fact that the adrenal cell had reached its maximum capacity for corticosterone biosynthesis.

Based on the recent results reported here, it is possible to suggest that ACTH probably has more than one mechanism of action through which corticosteroidogenesis could be stimulated. The accepted mechanism of action of ACTH through c-AMP formation may occur in stress conditions when large and prolonged stimulation with ACTH activates the whole adrenal machinery in response to an emergency. However, since ACTH stimulates the adrenal steroid sulfatase and, therefore, enhances production of active hormones, within a limited range, through an unknown but apparently existing mechanism in which c-AMP does not seem to participate, the steroid sulfatase may play a role in the regulatory feedback mechanisms as well as in mild increments in circulating ACTH. The equilibrium and regulation between the sulfatase and sulfoquinase activities in the adrenal and their relative response to ACTH deserve investigation. May be, the interaction between sulfatase and sulfokinase in releasing free steroids and conjugating them also participates in the steroid hormone mechanism of action in various target organs. This hypothesis requires, naturally, further support and therefore may deserve further investigation.

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