PAPEL DE LOS ESTEROIDES SULFATOS EN LA BIO-
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MAESTRIA BIOQUIMICA

RESUMEN DE LOS TRABAJOS QUE COMO TESIS PARA OPTAR AL GRADO DE MAESTRIA EN CIENCIAS QUIMICAS (BIOQUIMICA), HAN SIDO AUTORIZADOS POR LA DIVISION DE ESTUDIOS SUPERIORES DE LA FACULTAD DE QUIMICA DE LA UNAM.

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ABREVIACIONES

C = Colesterol = 3β-hidroxi-colest-5-eno

P = Pregnenolona = 3β-hidroxi-pregn-5-en-20-ona

17OHP = 17α-hidroxipregnenolona = 3β,17-dihidroxi-pregn-5-en-20-ona

CS = Colesterol Sulfato = 3β-hidroxi-colest-5-en-sulfato

PS = Pregnenolona Sulfato = 3β-hidroxi-pregn-5-en-20-ona-sulfato

17OHPS = 17α-hidroxipregnenolona sulfato = 3β,17α-dihidroxi-pregn-5-en-20-ona-sulfato

Sulfatasa = Δ^5-3β-ol-esteroide sulfatasa = esterol sulfato sulfohidrolasa

INTRODUCCION

Entre la gran variedad de esteroides conjugados que se han aislado de tejidos y fluidos biológicos de animales superiores y humanos, se encuentran dos formas importantes.

Los esteroides conjugados con el ácido glucorónico para formar los glucosiduronatos y los esteroides conjugados con el ácido sulfúrico y que dan origen a los esteroides sulfatos.

Los glucosidurónidos son en general productos del catabolismo de hormonas esteroides, éstos son solubles en agua y difunden fácilmente a través del glomérulo de Malpighi en el riñón, en esta forma es como se excretan en la orina los glucosidurónidos de la androsterona, etiocolanolona y en general, los tetrahidro derivados de las hormonas activas.

Los esteroides sulfatos comprenden dos grupos principales: los alquil sulfatos y los aril sulfatos.

El primer grupo lo constituye una serie de compuestos esteroidales con estructura Δ^5-3β-hidroxi, en los que el hidroxilo en 3β se encuentra conjugado con el ácido sulfúrico; por ejemplo CS, PS, 17-OH-PS, etc.

Se sabe que estos compuestos participan en la biosíntesis de hormonas esteroidales.

El grupo sulfato de estos compuestos, al igual que el glucorónido, confieren a la molécula esteroide una gran polaridad, presentando la característica de ser hidrosolubles, a diferencia de los esteroides libres que son liposolubles. La alta polaridad de los esteroides conjugados se debe, en el caso del ácido glucurónico, a que se trata de una molécula con varios hidroxilos y un grupo carboxilo capaz de formar sales que a su vez ionizan. En el caso de los conjugados sulfatos, la polaridad se debe a que el grupo sulfato puede formar sales monosustituídas de sodio, potasio o amonio, que también ionizan.

El segundo grupo de esteroides sulfatados está constituído por los esteroides ectrógenos que exhiben un grupo fenólico en el anillo A, éstos son principalmente productos de degradación de otros precursores, que al conservar el grupo sulfato unido a su molécula, permiten ser también excretados en la orina.

En algunos casos se ha reportado la existencia de multiconjugados.

Ha sido generalmente aceptado que los esteroides Δ^5-3β-ol conjugados como sulfatos, no son productos exclusivamente de desecho o finales del catabolismo ni que la única función del grupo sulfato es la de conferir una mayor solubilidad al esteroide al que se encuentra unido para hacerlo más fácilmente excretable.

Wang (1) y otros autores han establecido que la sulfatación de los esteroides hace que se incremente su vida media en circulación. Por una parte, presentan una mayor afinidad por algunas proteínas plasmáticas, disminuyendo en esta forma su fácil accesibilidad a la acción de enzimas catabólicas y por otra parte, por ser eficientemente reabsorbidas en el riñón.

Notation y Ungar (2) han sugerido que la fracción de esteroides sulfatados, además de los esteroides sulfatos que se sintetizan de novo dentro de las glándulas (3-5), representan una forma de reserva para la esteroidogénesis. Debido a este reservorio, los esteroides monosulfatados proveen a los diversos tejidos productores de hormonas esteroides de los precursores que pueden ser utilizados en los procesos metabólicos que dan lugar a la formación

de hormonas activas. Se ha descrito que varios esteroides conjugados, tales como el colesterol sulfato (CS), pregnenolona sulfato (PS), 17α-hidroxipregnenolona sulfato (17-OH-PS) y dehidroepiandrosterona sulfato (DS), son productos que intervienen en la biosíntesis de esteroides activos (6-10). Varios de estos monosulfatos existen en cantidades apreciables, tanto en circulación (11) como en diversos tejidos (11-14), habiéndose encontrado que la DS es uno de los principales esteroides secretados por las suprarrenales humanas (13,15,16).

A la fecha se conocen varias de las reacciones enzimáticas en el esquema de biosíntesis que utilizan a los esteroides de la serie Δ^5-3β-ol, tanto en su forma libre como esterificados con sulfato. Así la serie de biotransformaciones reportadas de colesterol (C) a pregnenolona (P), 17-OH-pregnenolona (17-OH-P) y dehidroepiandrosterona (D), puede ocurrir tanto para sustratos libres como cuando los precursores sulfatos se utilizan como sustratos. Es de especial interés el mencionar que en las reacciones enzimáticas mencionadas, cuando el precursor inicial está conjugado como sulfato, el éster se conserva a lo largo

de las diversas transformaciones que ocurren. De tal manera, tanto los sustratos (CS y PS, por ejemplo), los intermediarios (17α-OH-PS) como el producto final (DS) permanecen conjugados, explicándose de esta manera la elevada secreción de la DS por la adrenal y los altos niveles de este esteroide conjugado en circulación.

Se ha reportado que el CS sirve de precursor de la PS (17) y de la DS secretada por la suprarrenal (8); también se ha visto que la pregnenolona-^{3}H-sulfato-^{35}S se convierte tanto in vitro como in vivo en 17-OH-P-S (7) y en DS (6) conservando idéntica su relación $^{3}H/^{35}S$. Por otra parte es bien conocida la ruta metabólica que utiliza a los precursores libres de la serie Δ^{5}-3β-ol como sustratos para la biosíntesis de hormonas esteroides (18-20).

La participación "in vivo" de los esteroides monosulfatados y la utilización de los precursores libres endógenos siguen siendo hasta el momento tema de controversia, ya que no se han podido establecer los factores que intervienen y regulan las rutas metabólicas de esteroides libres y sulfatos.

En condiciones "in vitro", cuando los esteroides que exhiben la estructura Δ^{5}-3β -ol libre son exogenamente

agregados al medio de incubación y en presencia de tejidos endocrinos productores de hormonas esteroides, independientemente de su concentración y al estar en contacto con diversas enzimas biotransformadoras presentes en esos tejidos, son transformadas eficientemente en productos intermedios, así como en una gran variedad de hormonas activas con estructura Δ^4-3 cetona.

La elevada concentración en estos tejidos del sistema enzimático denominado Δ^5-3β-hidroxideshidrogenasa-$\Delta^{4,5}$-Isomerasa, responsable de la conversión de esteroides de la serie Δ^5-3β-ol a sus correspondientes congéneres de la serie Δ^4-3 cetona, hace que cuando el precursor exogenamente añadido está en forma libre, éste se convierta eficientemente en el esteroide Δ^4-3 cetona correspondiente.

La presencia del grupo sulfato en estos esteroides impide la acción del sistema enzimático antes mencionado, por lo que los precursores Δ^5-3β-ol-sulfatos no son convertidos directamente a esteroides Δ^4-3 cetona.

Para que el tejido pueda transformar los esteroides sulfatos a productos activos, es necesario eliminar de la molécula, el grupo sulfato, lo cual tiene lugar mediante la acción enzimática de la esteroide sulfatasa.

Se ha reportado que esta enzima está presente en diversos tejidos, entre ellos adrenales, aunque en muy baja concentración (21-23), en comparación con otras enzimas biosintetizantes de hormonas esteroides (24).

Una vez que el grupo sulfato es eliminado, el esteroide libre Δ^5-3β-ol puede ser eficientemente transformado a sus correspondientes productos esteroides con estructura Δ^4-3 cetona.

Debido a la baja concentración de la Δ^5-3β-ol esteroide sulfatasa, resulta limitada la cantidad de precursor libre que se forma y que queda a disposición de las enzimas que posteriormente participan en la biosíntesis de hormonas activas. De esta manera, la producción de hormonas finales puede estar regulada o determinada por la concentración y/o actividad de la esteroide sulfatasa presente, constituyendo así un paso limitante en la conversión de precursores presentes en altas concentraciones (precursores sulfatos) a hormonas esteroides finales, secretadas en bajas concentraciones.

Ninguna de las reacciones ulteriores a la formación de pregnenolona en el cuadro de biosíntesis de corticoides, parecen ser etapas limitantes.

En trabajos realizados tanto in vitro como in vivo, se ha observado que el ACTH aumenta la producción de corticoides a partir de precursores endógenos (26,27). Se han efectuado varios estudios para precisar en que etapa de la esteroidogénesis actúa directamente el ACTH (24,28). Los resultados parecen indicar que la acción del ACTH en adrenal tiene lugar en etapas previas a la formación de pregnenolona libre.

Burstein (29) encontró que el ACTH incrementa la conversión de C→P, Hall (11) y Ragatt (30), midiendo la conversión de CS→PS, encontraron que ésta era más efectiva que la conversión de los esteroides libres correspondientes C→P.

OBJETIVOS Y RESULTADOS

Basándose en los antecedentes expuestos, se elaboró un programa de investigación dividido en varias etapas, que fueron ajustándose conforme se obtuvieron resultados progresivos.

Los experimentos realizados durante el desarrollo de los trabajos que se presentan, se sumarizan a continuación. Los objetivos y resultados fueron los siguientes:

1.- Estudio de la conversión de esteroides sulfatos ($PS-^{3}H$) a corticoides en suprarrenal de rata.

Resultado.- Se obtuvieron diversos intermediarios y corticoides radiactivos.

2.- Estudio comparativo de la conversión de esteroides sulfatos $7-^{3}H$ y sus homólogos libres $4-^{14}C$, empleando técnicas de doble isótopo.

Resultado.- Se aislaron e identificaron varios esteroides doblemente marcados, principalmente corticosterona, desoxicorticosterona y progesterona.

3.- Establecimiento de las condiciones de incubación que permiten la conversión de $PS-^{3}H$ y $P-^{14}C$ a progesterona, tratando de evitar su ulterior conversión a DOC y corticosterona, en las que participan la 21 y 11β hidroxilasas, que requieren oxígeno atmosférico y TPNH como cofactor.

Resultado.- Al incubar por diversos tiempos y variando las concentraciones de los precursores (sulfato y libre), específicamente marcados, en atmósfera de N_2 y sin agregar TPNH exógeno, se logró acumular progesterona-$7-^{3}H-4-^{14}C$, originada de los precursores, con mínima conversión a esteroides más polares.

4.- Comparación de la cinética enzimática de las dos reacciones involucradas: esteroide sulfatasa y Δ^5-3β-ol deshidrogenasa-Isomerasa.

Resultado.- Las curvas obtenidas en la formación de progesterona-4-^{14}C a partir de pregnenolona libre-4-^{14}C y de progesterona-7-^{3}H a partir de pregnenolona sulfato-7-^{3}H, empleando diferentes concentraciones equimoleculares para ambos sustratos, incubados simultaneamente, indican que la Δ^5-3β-ol deshidrogenasa-Isomerasa dentro del rango de concentraciones empleadas, presenta una cinética de primer orden, en tanto que en idénticas condiciones, la esteroide sulfatasa presenta una cinética de orden cero. Esto permitió establecer que la concentración de la esteroide sulfatasa es fácilmente saturable a muy bajas concentraciones de sustrato, en tanto que la Δ^5-3β-ol deshidrogenasa-Isomerasa no llega a presentar el menor indicio de saturación a las más elevadas concentraciones del sustrato ^{14}C empleado.

Conclusión.- La concentración de la esteroide sulfatasa es menor que la de la Δ^5-3β-ol deshidrogenasa-Isomerasa.

5.- Efecto del ACTH sobre la actividad y/o concentración de la esteroide sulfatasa.

Dado que la Δ^5-3β-ol deshidrogenasa-Isomerasa no es estimulada por ACTH (24) y habiendo comprobado por los resultados obtenidos que las curvas de conversión de pregnenolona libre a progesterona son idénticas, tanto para las adrenales de los animales tratados con ACTH como para las adrenales de los animales control. Se corrobora que efectivamente el ACTH no afecta el comportamiento de dicho sistema enzimático. Por este motivo, el contenido de 4-^{14}C en la progesterona formada, fue utilizado como referencia, respecto a cualquier cambio observado por estímulo de ACTH sobre el contenido de ^{3}H en la progesterona formada, proveniente del sustrato PS-^{3}H.

Resultado.- Los resultados obtenidos en estos experimentos demuestran que la relación ^{3}H/^{14}C en la progesterona formada en adrenal de ratas estimuladas in vivo con ACTH, fue mayor para todos los tiempos de incubación, a la relación ^{3}H/^{14}C presente en la progesterona formada en adrenales de ratas control.

Conclusión.- El ACTH administrado in vivo y en forma crónica durante tres semanas, estimula la actividad de la esteroide sulfatasa adrenal.

6.- Comprobación del estímulo que el ACTH ejerce sobre la esteroide sulfatasa adrenal, empleando el método directo de Burstein y Dorfman (23) y modificación de este método.

En el método original se emplea como sustrato la DS, midiendo el esteroide liberado por acción de la sulfatasa presente en el tejido incubado. Debido a que uno de los objetivos de los trabajos realizados en este laboratorio era el estudiar la participación de precursores sulfatos en la biosíntesis de corticoides y dado que éstos tienen el núcleo pregnano de 21 átomos de carbono, se consideró esencial emplear a la PS-^{3}H como sustrato, ya que sería el precursor adecuado para estudiar su conversión a corticoides. Para fines comparativos, se incubaron en paralelo, tanto PS-^{3}H como DS-^{3}H.

Resultado.- a) Se observó que la PS-^{3}H fue más eficientemente desulfatada que la DS-^{3}H; b) La afinidad de la enzima por PS es mayor que la de la enzima por DS; c) Las curvas obtenidas empleando diferentes concentraciones sugieren que probablemente una sola sulfatasa sea la responsable de la desulfatación de los dos sustratos; d) Se comprobó que el ACTH aumenta la actividad de la sulfatasa adrenal para ambos sustratos, habiendo sido más notable el estímulo sobre la desulfatación de la PS que sobre la desulfatación de la DS.

Conclusión.- Además de los resultados antes mencionados y tomando en cuenta la peculiar diferencia en el estímulo

observado que el ACTH ejerce sobre la desulfatación de los dos sustratos (d), los resultados indicados (c) sugieren, por el valor de Vmax, que se trata de una sola enzima sulfatasa para ambos sustratos.

7.- Interacción entre PS y DS en la esteroide sulfatasa adrenal.

Con el objeto de estudiar la interacción que pudiera existir entre ambos sustratos, se incubaron simultaneamente alícuotas de un homogeneizado de varias adrenales de rata, en presencia de los dos sustratos, de tal manera que:

a) En una serie PS-^{3}H actuaba como sustrato en presencia de DS no radiactiva actuando como inhibidor.

b) En otra serie DS-^{3}H actuaba como sustrato en presencia de PS no radiactivo actuando como inhibidor.

En ambas series, se incubaron cantidades conocidas y diferentes, tanto de sustrato como de inhibidor, para establecer en caso de existir alguna inhibición, el tipo de inhibición que tuviera lugar.

Resultado.- a) Se demostró que existe un efecto inhibitorio recíproco de tipo competitivo de un sulfato en la desulfatación del otro; b) Las constantes enzimáticas

indican un mismo valor de Vmax para ambos sustratos, tanto en ausencia como en presencia del otro, actuando como inhibidor; sin embargo, los valores de Km diferentes para cada sustrato fueron modificados al ester presente el otro sulfato, que actuaba como inhibidor.

8.- Estudio sobre el requerimiento de cofactores para la actividad de la esteroide sulfatasa.

En estudios preliminares se había observado que en presencia de ADP, se aumentaba la eficiencia de la desulfatación, tanto de PS como la de DS. Por otra parte, previamente había sido reportado que la Δ^5-3β-ol deshidrogenasa-Isomerasa, es un sistema enzimático dependiente de DPN (25). Por estos motivos, en todos los estudios realizados y aquí reportados, se agregó ADP (1mM) y DPN (3.0 mM) al medio de incubación.

Se estudiaron, por lo tanto, en forma comparativa, varios cofactores: TPNH, AMP-c, ATP, ADP y otros, todos ellos en ausencia o presencia de DPN.

Resultado.- La actividad de la esteroide sulfatasa no se modificó con TPNH ni con AMP-c, habiéndose encontrado un aumento significativo en presencia de ATP o ADP.

Conclusión.- Los resultados presentados permiten sugerir que el ACTH tiene probablemente dos mecanismos de acción diferente. Uno, el oficialmente aceptado, estimulando la adenil ciclasa, consencuente liberación de AMP-c, lo cual, finalmente, resulta en una elevación de TPNH requerido para multitud de enzimas biotransformadoras de esteroides, particularmente hidroxilasas y desmolasas. Por este mismo mecanismo se favorecen además, los sistemas de fosforilación oxidativa y síntesis de proteínas, entre ellas, enzimas biotransformadoras de esteroides.

Los resultados obtenidos en esta fase del proyecto sugieren que al no haber aumento en la actividad de la sulfatasa, al agregar TPNH ni al agregar AMP-c, el ACTH probablemente estimula la desulfatación de precursores por un mecanismo en el que no se involucra la participación de AMP-c.

Sayers demostró (31) que al estimular células suspendidas de adrenal de rata con diferentes cantidades de ACTH, obtenía una respuesta diferente en la producción de AMP-c y de corticosterona. En concentraciones bajas de ACTH obtuvo biosíntesis de corticosterona, sin que se acumulara AMP-c, en cambio a concentraciones mayores de

ACTH, demostró una correlación de AMP-c con biogénesis de corticoides.

Los trabajos de Sayers sugieren, en combinación con los resultados generales del presente trabajo, que el ACTH, a bajas concentraciones, puede favorecer la conversión de precursores endógenos en corticoides sin la intervención de AMP-c, siendo tal vez este efecto por un mecanismo aún no establecido, en el que se involucre al ATP y al ADP, sugiriéndose la futura demostración de este nuevo mecanismo propuesto para la acción del ACTH.

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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

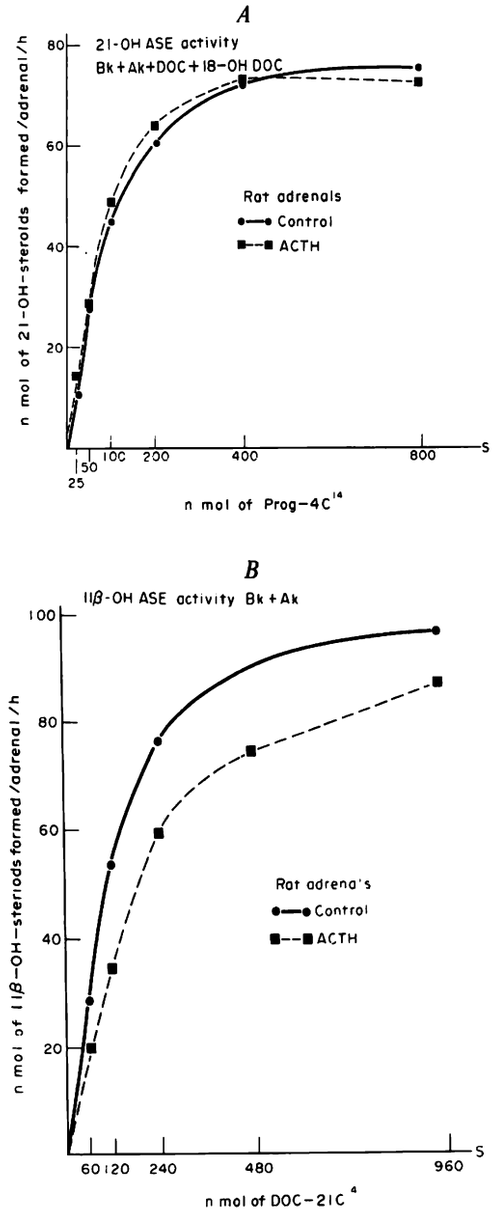

Fig. 1. Effect of *in vivo* chronic stimulation with ACTH (0·6 mU I.V./day, during 3 weeks) on rat adrenal 21 and 11β-hydroxylases. *A* (top) 21-hydroxylase activity = nmol of 21-hydroxylated steroids formed from [4-^{14}C]-progesterone. *B* (bottom) 11β-hydroxylase activity = nmol of 11β-hydroxylated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

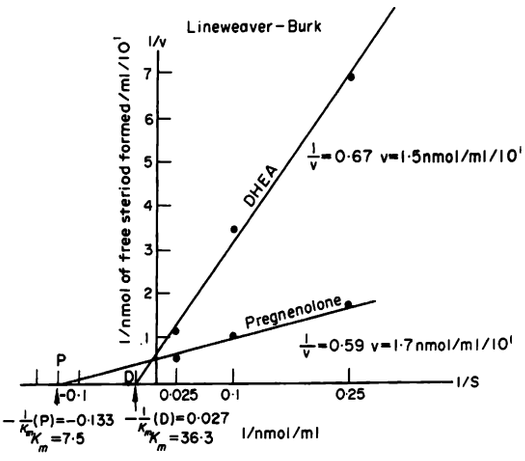

Fig. 2. Comparative desulfation of pregnenolone-sulfate and DHEA-sulfate as substrates of adrenal steroid sulfatase. V_{max} is essentially the same for both substrates but K_m values are very different indicating that pregnenolone-sulfate is more efficiently desulfated than DHEA-sulfate.

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

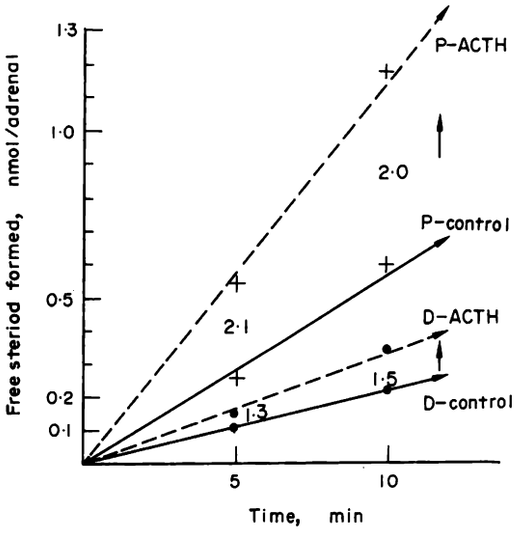

Fig. 3. Comparative degree of desulfation of pregnenolone-sulfate and DHEA-S by the action of steroid sulfatase in rat adrenals. Increase in steroid sulfatase activity in the adrenals from rats stimulated *in vivo* with ACTH (0·6 mU I.V./day, during 3 weeks) when compared to that observed in adrenals 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. 44 and 4*B* to emphasize the tremendous difference in the concentrations of steroid sulfatase and the 3β-ol steroid dehydrogenase-Isomerase system.

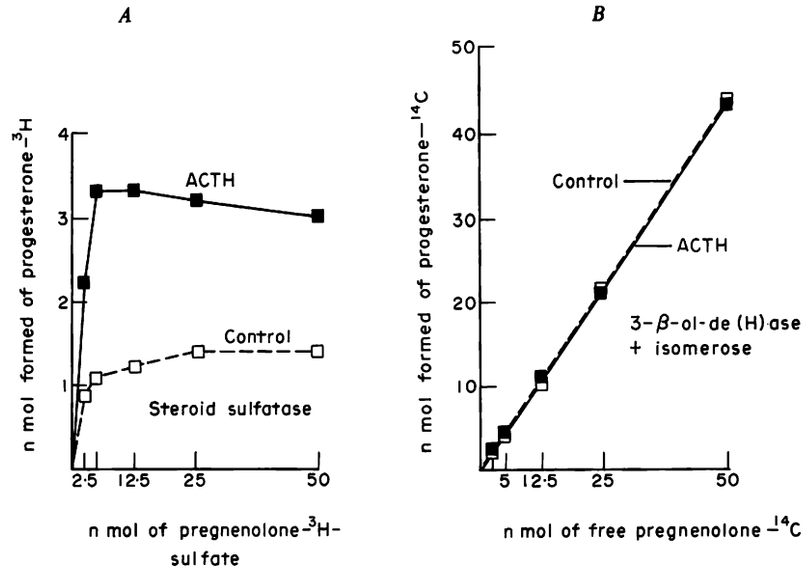

Fig. 4. Effect of ACTH on adrenal steroid sulfatase and on adrenal 3β-hydroxy-steroid dehydrogenase-Isomerase activities. 4*A*, represents the conversion of [^{3}H]-pregnenolone-sulfate to progesterone (*via* free pregnenolone) and 4*B*, represents the conversion of free [4-^{14}C]-pregnenolone to progesterone, 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-

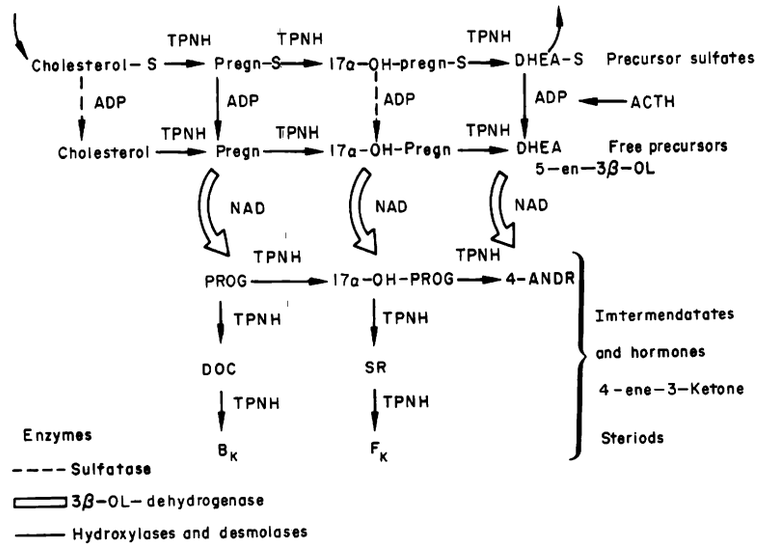

Fig. 5. Scheme of steroid hormone biosynthesis in the adrenal indicating cofactor requirements, participating enzymes and limiting reactions. The conversion of precursor sulfates (top line) to their corresponding free precursors (second line) is limited by the steroid sulfatase activity and probably 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.

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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

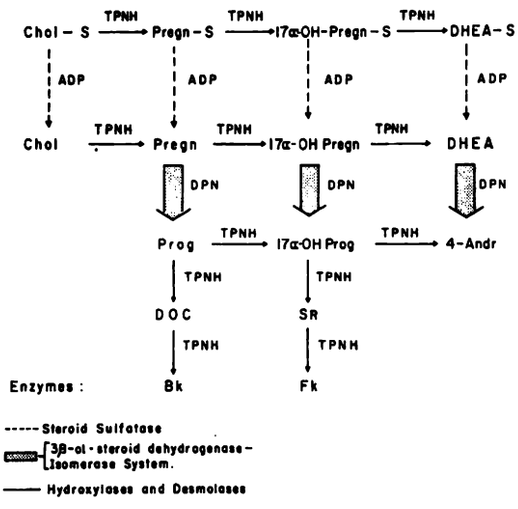

Fig. 1. General scheme of steroid hormone biosynthesis in the adrenal gland, indicating the cofactor requirements for the various enzymatic steps and a relative appreciation of enzyme concentrations involved.

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].

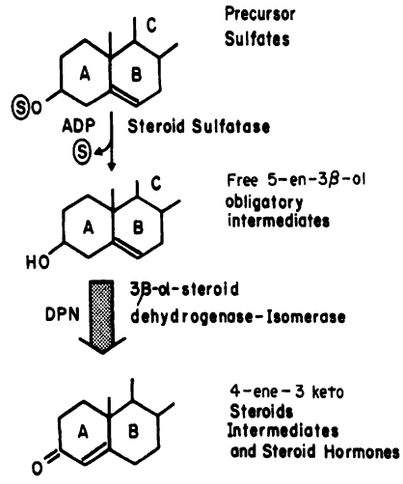

Fig. 2. Steps involved in the conversion of a 5-ene-3β-ol steroid sulfate as a precursor of an active steroid hormone via free 5-en-3β-ol steroid.

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

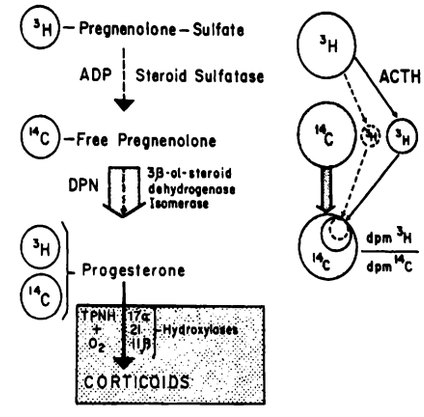

Fig. 3. Scheme to illustrate the "Indirect Method" to determine the steroid sulfatase activity with respect to that of 3β-hydroxy-steroid-dehydrogenase-isomerase, by measuring the relative conversion of [7-^{3}H]-pregnenolone-sulfate and [4-^{14}C]-free pregnenolone to progesterone. ACTH seems to stimulate 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.

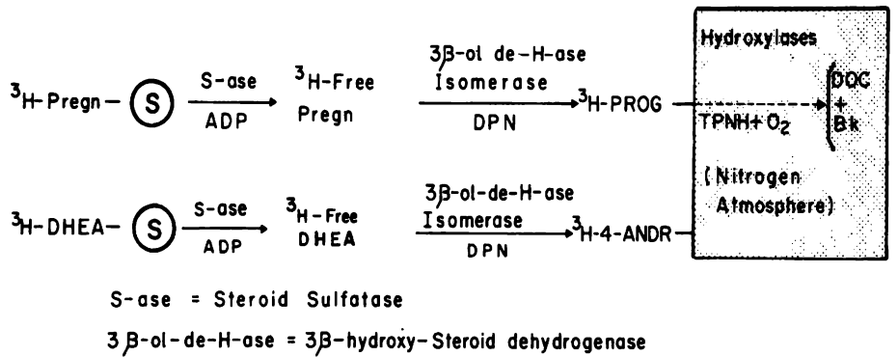

Fig. 4. Scheme to illustrate the "Direct Method" to determine steroid sulfatase activity, comparing the desulfation of ^{3}H-pregnenolone–sulfate and ^{3}H-DHEA–sulfate used as substrates in the presence of ADP and DPN as cofactors as well as in the absence of TPNH and O_2, required by hydroxylations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

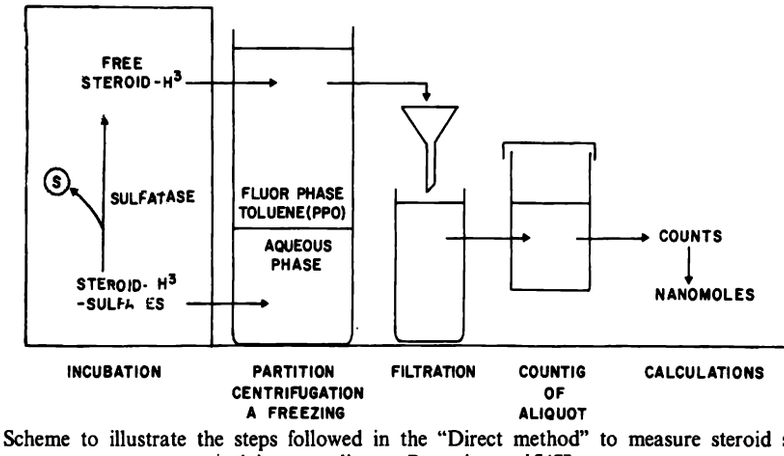

Fig. 5. Scheme to illustrate the steps followed in the "Direct method" to measure steroid sulfatase activity according to Burstein *et al.*[47].

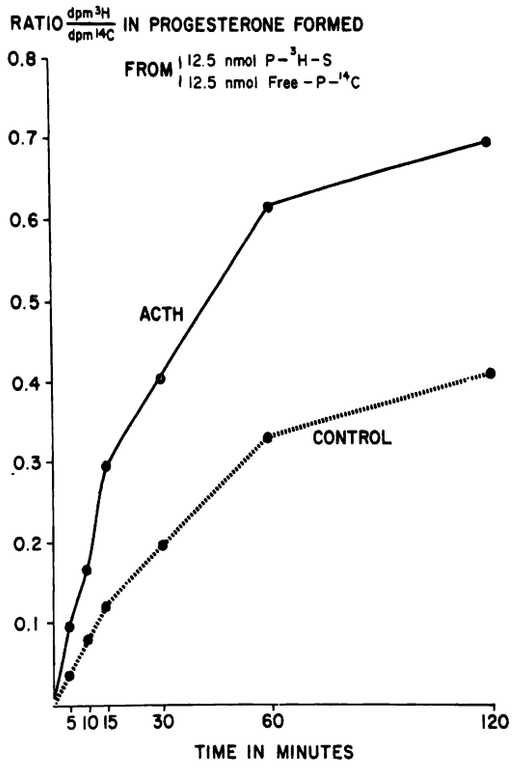

Fig. 6. Relative incorporation of ^{3}H (from [7-^{3}H]-pregnenolone-sulfate) and ^{14}C (from [4-^{14}C]-free pregnenolone) in progesterone formed at various times of incubation, using equimolar amounts of both initial substrates by rat adrenals and the effect of *in vivo* stimulation with ACTH.

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)	nm formed	[4-^{14}C]-Progesterone % Conversion from [4-^{14}C]-P (2)	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.

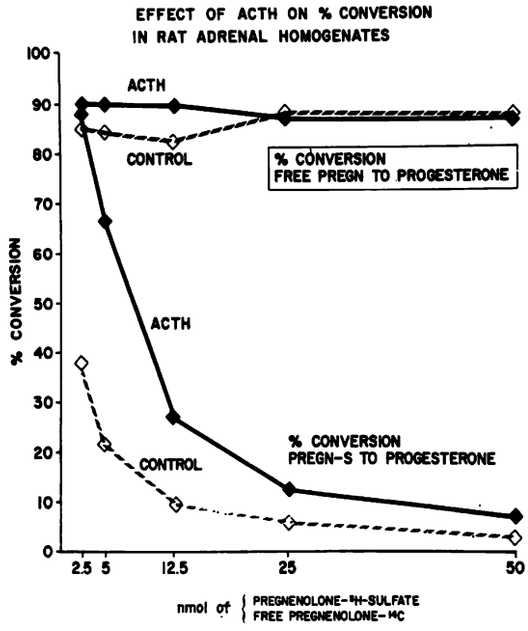

Fig. 7. *In vivo* effect of ACTH on the percent conversion of various mμmolar concentrations of either free pregnenolone or pregnenolone-sulfate to progesterone by one homogenized rat adrenal (control group vs ACTH-treated group).

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/1/h, but the K_m values for the two substrates seem to be quite different, indicating that the steroid sulfatase has a

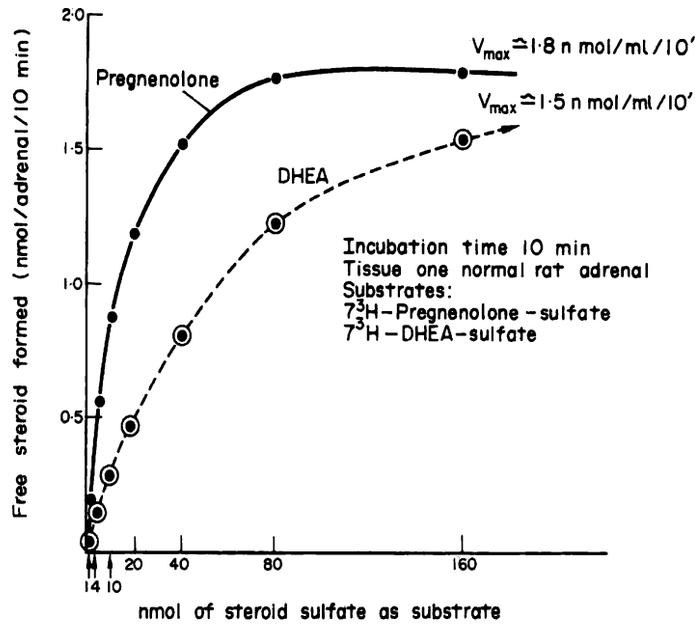

Fig. 8. Relative degree of desulfation of pregnenolone-sulfate and DHEA-sulfate by normal adrenal steroid sulfatase at various equivalent substrate concentrations.

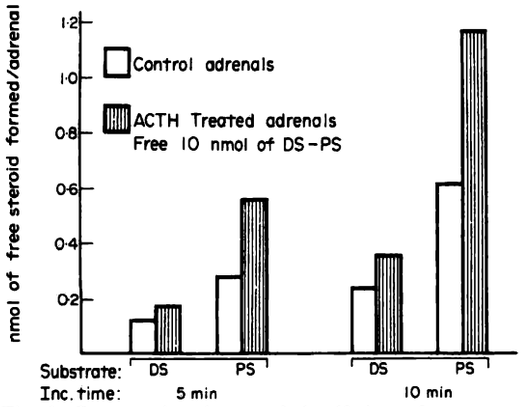

Fig. 9. Comparative degree of desulfation of pregnenolone-sulfate (PS) and DHEA-sulfate (DS) by steroid sulfatase from control rat adrenals and from rat adrenals stimulated *in vivo* with ACTH. Results obtained at 5 min and 10 min of incubation.

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 alone	Cofactor + DPN	Cofactor / Control
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.

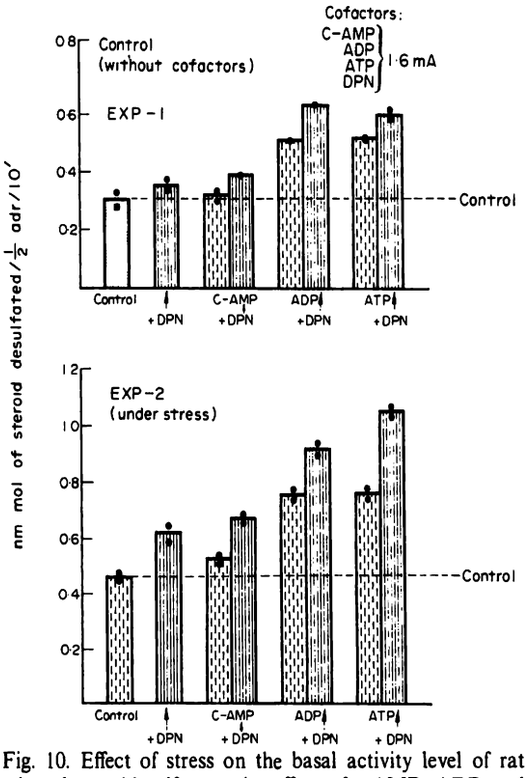

Fig. 10. Effect of stress on the basal activity level of rat adrenal steroid sulfatase, the effect of c-AMP, ADP and ATP when present in the media (1·6 mM) and the additive effect of DPN on the steroid sulfatase efficiency.

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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