Determination of Mammalian Steroid Sulfatase with 7α-H3-3β-Hydroxyandrost-5-en-17-one Sulfate*

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Mammalian steroid sulfatase which cleaves dehydroisoandrosterone sulfate and certain other steroid sulfates with planar rings A and B, such as sulfates of the 3β-hydroxy-5α-androstan-17-one, or 5α-pregnane series, was first discovered by Gibian and Bratfisch (1) in ox and rat liver and confirmed by Roy (2). The occurrence and intracellular distribution of the enzyme have been further studied by Roy (3) and by Ney and Ammon (4). The extreme insolubility of this microsomal enzyme and its resistance to solubilization have not permitted extensive purification and separation from the many other enzymes present in the microsomal particle. Investigations by nonisotopic methods into the presence and properties of steroid sulfatase in endocrine tissues were further complicated by the existence of side reactions (3β-ol dehydrogenase and the 17α-dehydrogenase) that yielded products not measurable by the Pettenkoffer and Zimmermann reactions. In a preliminary report (5) a method for the determination of steroid sulfatase was described which uses 7α-H3-dehydroisoandrosterone sulfate as substrate and measures the H3 extracted into toluene as a function of time. The purpose of this paper is to report on the development of the method and on some kinetic data obtained with enzyme from mammalian liver, testis, adrenal, and ovary.

EXPERIMENTAL PROCEDURE

7α-H3-Dehydroisoandrosterone Sulfate—Chromatographically homogeneous 7α-H3-dehydroisoandrosterone (2.8 mc, 1.4 mg), kindly supplied by Dr. Marcel Gut, was mixed with 1 g of non-labeled steroid and sulfated as previously described (6). The potassium salt was crystallized several times from methanol to obtain a constant specific activity, and the material used as the substrate had a H3 content of 1520 c.p.m. per pg.

H3 Counting—H3 was determined in an automatic Packard Tri-Carb liquid scintillation spectrometer with low potassium glass vials from the Wheaton Glass Company. The scintillation mixture was a solution of 0.4% of 2,5 diphenyloxazol-c and 0.015% of 1,4-bis-(5-phenyloxazolyl)benzene, obtained from Pilot Chemicals, in toluene, scintillation grade, obtained from Matheson, Coleman, and Bell, Inc. The counts per minute reported were corrected for an efficiency of 17.2%, which was the efficiency most frequently obtained.

7α-H3-Dehydroisoandrosterone Sulfate Stock Solution—The substrate could not be kept in aqueous buffers even at 4–5° without a progressive, significant formation of free steroid, presumably because of the i-steroid rearrangement (7). In methanol, at 4–5°, no significant amount of free steroid was formed for at least 1 month. Aliquots of the methanolic solution were evaporated in 50-ml Erlenmeyer flasks before enzyme assay.

Enzyme Assay—The substrate was dissolved in 1 ml of buffer and allowed to reach 37°, the incubation temperature. The enzyme solution, 9 ml, at this temperature, was then quickly added to the substrate, and the Erlenmeyer flasks were shaken. One-milliliter aliquots were delivered at appropriate intervals into glass-stoppered tubes (culture tubes equipped with Teflon-lined screw caps are also suitable) that contained 3 ml of aqueous alkali and 15 ml of scintillation mixture, and the tubes were shaken immediately. In the early experiments 4 m NaOH was used, but this concentration was replaced with 0.1 m NaOH later. Unless otherwise specified, 0.1 m NaOH was the concentration employed. The tubes were then immersed in a bath containing a saturated solution of solid carbon dioxide in methyl Cellosolve to freeze the aqueous phase which remained in the bottom of the tubes. The toluene layer is filtered immediately through a fluid filter paper (Whatman No. 12), and 10-ml aliquots of the filtrate, withdrawn at room temperature, were counted for H3. The enzyme activity was determined from the slope of the curve of H3 released with time. The enzyme activity was expressed as counts per minute of toluene-extractable H3 formed during 10 minutes of incubation. The substrate concentration used was in most cases 10-5 M, which is approximately 10 times the Ks values found in the uninhibited preparations. With some preparations, especially at higher tissue concentrations, increased substrate concentrations, such as 10-3 M, may be necessary to achieve Vmax.

Since in this study an over-all picture of the sulfatase distribution was desired in respect to conditions in vivo, enzyme concentration was determined only relative to wet tissue weight. Because the enzyme has not as yet been freed from the crude particulates, enzyme activity relative to protein content was not determined.

Tissue Preparations—Laboratory animals were decapitated and the tissue removed and placed on ice. Tissues of larger animals were removed after anesthesia and kept frozen at -20° until worked up. Freezing of rat testis and liver and pig testis tissues did not have any significant effect on the total steroid sulfatase activity or on the intracellular distribution. Homogenization of tissues was done with Potter-Elvehjem type homogenizers equipped with Teflon pestles that are driven by a motor.

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† The full designation for dehydroisoandrosterone is 3β-hydroxyandrost-5-en-17-one.

We wish to thank Dr. J. F. Wagner of Eli Lilly for providing the testis tissue of bull, ram, and boar.
Subcellular fractions were prepared by the method of Hogeboom (8). When only microsomes were desired, the procedure of Keller and Zamecnik (9) was followed except that the salts were omitted from their sucrose medium.

RESULTS

Separation of H\textsuperscript{3}-Dehydroisoandrosterone from Its Sulfate—By the technique described above, the free steroid can be efficiently separated from unreacted sulfate. Thus, H\textsuperscript{3}-sulfate containing 58,000 c.p.m. yielded in a blank experiment 40 c.p.m. in the toluene phase. In Fig. 1, a typical blank experiment (substrate without tissue) is presented. As can be seen, incubation with buffer alone caused only a negligible release of free steroid, and this blank H\textsuperscript{3} activity released, because of the i-steroid rearrangement, can be neglected in most enzyme assays. The recovery of the H\textsuperscript{3} in the toluene after the addition of the end product H\textsuperscript{3}-dehydroisoandrosterone to rat testis homogenates ranged from 89 to 96\% (10 experiments; mean = 93 ± 3\% (s.d.)) (Fig. 1). Similar results were obtained with rat liver homogenates (88 to 95\%, 20 experiments; mean = 91 ± 3\% (s.d.)). The losses incurred could not be explained by quenching effects, which were below 2\%. As is apparent from Fig. 1, the extractability of the dehydroisoandrosterone did not change significantly during the first 30 minutes of incubation. Similar results were obtained with rat liver.

Solubilization of H\textsuperscript{3}-Dehydroisoandrosterone Sulfate by Tissue Preparations—A typical reaction progress curve obtained with rat testis whole homogenates and the 105,000 x g sediment is shown in Fig. 1. Inspection of Fig. 1 will reveal that the progress curves with the testis whole homogenate and 105,000 x g sediment did not pass through the origin but cut the ordinate at 250 and 500 c.p.m., respectively. This is more apparent from Fig. 4. This extractable H\textsuperscript{3} was found to contain mostly substrate which is extracted into the toluene in the presence of tissue preparations. This was shown by washing toluene extracts of tissue preparations with distilled water. Approximately 80 to 90\% of the H\textsuperscript{3} was removed into the aqueous phase of high ionic strength. Catalysts with high solubilizing activity was obtained with the phosphatide (L-a-lecithin (P, y-dipalmitoyl)) complexes previously studied were form-methanol mixture (2:1). However, it is of interest that the phosphatide (L-a-lecithin (P, y-dipalmitoyl) and L-a-cepahalin (P, y-dipalmitoyl)) complexes previously studied were more strongly adsorbed to the filter paper than the naturally occurring materials, possibly indicating the presence of hitherto unknown substances. These compounds are also undoubtedly associated with proteins in lipoprotein complexes and make their appearance when these aggregates are denatured.

Distribution of Steroid Sulfatase—The data obtained with several tissues from different species are given in Table I. Although freezing did not affect significantly the sulfatase activity of rat liver and testis or pig testis tissues, it is not possible to infer from this that no adverse effects may have been obtained with other tissues from other species because of tissue freezing.

Steroid Sulfatase in Rat Liver—The pH optimum of this enzyme from rat liver homogenates was quite broad, ranging from approximately 6.6 to 7.5. The activity completely disappeared on heating the preparation at 96° for 3 minutes. The subcellular
Determination of Mammalian Steroid Sulfatase

**Table I**

Distribution of mammalian steroid sulfatase from various sources

Incubations were done in 0.1 M Tris-acetate buffer at pH 7.2, 37°. Substrate concentration was 1.0 ± 0.1 x 10⁻⁴ M.

<table>
<thead>
<tr>
<th>Tissue preparation</th>
<th>Sulfatase specific activity (mole (as dehydroisoandrosterone) formed per min per g of wet tissue (X 10⁻¹))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver (m.)</td>
<td>506</td>
</tr>
<tr>
<td>Rat testis (m.)</td>
<td>36</td>
</tr>
<tr>
<td>Rat adrenal (w.h.)</td>
<td>356</td>
</tr>
<tr>
<td>Guinea pig liver (w.h.)</td>
<td>13</td>
</tr>
<tr>
<td>Guinea pig testis (m.)</td>
<td>228</td>
</tr>
<tr>
<td>Guinea pig adrenal (w.h.)</td>
<td>116</td>
</tr>
<tr>
<td>Bull testis (w.h.)</td>
<td>2</td>
</tr>
<tr>
<td>Ram testis (w.h.)</td>
<td>2</td>
</tr>
<tr>
<td>Boar testis (w.h.)</td>
<td>26</td>
</tr>
<tr>
<td>Human testis (w.h.)</td>
<td>26</td>
</tr>
<tr>
<td>Human adrenal (w.h.)</td>
<td>22</td>
</tr>
<tr>
<td>Human ovary (w.h.)</td>
<td>10</td>
</tr>
</tbody>
</table>

* These values were calculated from the total toluene-extractable H² formed.

m.—microsomes.

w.h.—whole homogenate.

From a Stein-Leventhal syndrome patient.

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Steroid Sulfatase in Rat and Guinea Pig Testis—The pH optimum for this enzyme in rat testis homogenates was also quite broad, ranging from 6.8 to 7.7, and the activity disappeared upon heating at 96° for 3 minutes. The progress of substrate cleaving with rat testis subcellular fractions is shown in Fig. 4.

**Fig. 5** presents a typical [S]/v versus [S] plot for rat testis microsomes for which Kₐ equals 1.2 x 10⁻⁵ M. Phosphate ions were inhibitory and in the presence of 0.1 to 0.2 M phosphate buffer, distribution of the enzyme in rat liver is shown in Fig. 2. There was no sulfatase activity in the 105,000 x g supernatant solution. A typical [S]/v versus [S] plot for fresh rat liver microsomes is shown in Fig. 3 from which a Kₐ value of 1.0 x 10⁻⁴ M was obtained. The apparent Kₐ values were found to increase with the tissue concentration used in the determination. Thus, an apparent Kₐ value of 8 x 10⁻⁵ M was obtained at a liver microsome concentration equivalent to 100% liver. The Kₐ values at 11 to 12.5% tissue concentrations varied from 0.8 to 1.0 x 10⁻³ M. The reason for this is not entirely clear at present. The possibility of the existence of an inhibitor in these preparations is under further study.

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**FIG. 2.** Subcellular distribution of steroid sulfatase in rat liver. Incubations were done in 0.1 M Tris-acetate buffer, pH 7.2, at 37°. Tissue concentration was approximately 50% of whole rat liver; substrate concentration was 10⁻⁴ M.

**FIG. 3.** Effect of substrate concentration ([S]) on initial rate (v) of toluene-extractable H² formation catalyzed by rat liver microsomes. Incubations were done in 10 ml of 0.1 M Tris-acetate buffer, pH 7.2, at 37°. Tissue concentration was 11% of rat whole liver. The rate was expressed as the H² c.p.m. released during a 10-minute incubation. One-milliliter aliquots were taken for each determination.

**FIG. 4.** Subcellular distribution of steroid sulfatase in rat testis. Same conditions as in Fig. 2. Tissue concentration was 30% of whole rat testis. In the partitions, 4 M NaOH was used.
unless steroids of a much higher polarity, sufficient to affect their
the liberated dehydroisoandrosterone would not be observed
traction of free steroid; the further chemical transformation of
rat liver (Table I).

Guinea pig testis contained roughly 50% of the enzyme concen-
was a rich source of the enzyme and contained roughly 10 to 15
times as much sulfatase per unit weight as rat testis, guinea pig
were determined by Dr. Eliahu Boger.


d DISCUSSION

A sensitive and simple method has been presented for the
the distribution of the enzyme was studied in liver and endocrine
tissues of several species. Its presence in human testicular
tissue extracts which renders the sulfate extractable into toluene,
first 30 minutes of incubation. Because of the nonenzymatic
in the presence of aqueous solutions of high ionic strength.
shown to solubilize dehydroisoandrosterone sulfate into toluene
similar to those described by Oertel (13) would, of course, lead to
erroreous results. Such a possibility cannot entirely be excluded
from the results reported here. Although (unpublished results)
roughly 50% of the material produced in testis or liver homoge-
could be accounted for as dehydroisoandrosterone by column
and paper chromatography, there were in these extracts other
other as yet unidentified components. The use of 7α-H-dehy-
dehydroisoandrosterone sulfate labeled also with S35 would provide
information on the enzymatic formation of lipid-soluble com-
the sulfate into less polar derivatives such as long chain fatty
acid ceto or to covalently linked phosphatide derivatives simi-
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the principle of the method described may also be used for
A sensitive and simple method has been presented for the
assay of steroid sulfatase with 7α-H3-dehydroisoandrosterone sulfate
and measuring the radioactivity extracted
to measure the activity as a function of time. With the use of this method,
the distribution of the enzyme was studied in liver and endocrine
tissues of several species. Its presence in human testicular
Guinea pig testis microsomes exhibited a Km value of 1.6 ×
Tris buffer, pH 7.2, 37°, and at a tissue concentration of 25%.

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SUMMARY

A method is described for the determination of steroid sul-
fatase with 7α-H3-3β-hydroxyandrost-5-ene-17-one sulfate as
substrate and measurement of the radioactivity of the material
extracted into the toluene as a function of time. Steroid sul-
fatase activity was found in mammalian liver, testis, ovary, and
adrenal. The intracellular distribution of the enzyme was
studied in rat liver and testis, and the highest concentration was
found in the microsomes. Naturally occurring materials which
solubilize the substrate in toluene have been found in tissue
preparations, especially after denaturation. Km values have
been determined for rat liver and rat and guinea pig testis mi-
crosomes.

Acknowledgments—The able assistance of Richard L. Brunelle
is gratefully acknowledged. Some of the Km values reported
were determined by Dr. Eliahu Boger.

REFERENCES

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